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Changes to breast milk fatty acid composition during storage, handling and processing: A systematic review

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ABSTRACT

This review evaluated the effect of various storage and handling conditions on the fat composition of expressed breast milk (EBM). Three databases PubMed, Embase and Scopus were searched in April 2019 with words from the three key components: human milk, handling process (i.e. storage and/or pasteurization), and fatty acid composition. The comparisons were EBM subjected to handling processes versus fresh EBM or versus EBM subjected to another handling processes. Both intervention and observational studies were included, and the outcomes measured included total fat and lipid classes of the EBM. We included 42 studies (43 reports), 41 of which were assessed to be of good quality. Relative changes to the fat composition of EBM subjected to handling processes were calculated based on the data provided in the included studies, and the results were synthesized narratively. The total fat content and total fatty acid composition of EBM was not generally influenced by storage and handling process, with most changes less than 10%, which is likely a result of methodological variation. A reduction in EBM triglyceride concentration and concomitant increase in free fatty acid concentration were seen after exposing to various conditions, probably due to endogenous lipase.

1. Introduction

Breast milk is the gold standard for infant feeding due to the nutrients and other non-nutritive components essential for the growth and development of infants [1]. When not possible to feed directly from the breast, expressed breast milk, either from the mother, or from a donor, is often used and is preferable to formula [2,3]. The use of donor human milk is also becoming more widely used for premature infants. Donated expressed breast milk (EBM) from a milk bank undergoes storage processes, which may involve pasteurization and repeated freeze and thaw cycles, and this may affect the integrity of the EBM.

The impact of various storage and handling processes on EBM has been studied from many perspectives including the preservation of bacteriostatic properties [4–9]; the impact of storage on immunological factors [10], digestive enzymes [11], and the antioxidant capacity of breast milk [12–14]. However, the impact of storage and handling on fat composition of EBM has been less studied.

Fat in breast milk is the major source of energy for infants, contributing more than 50% of the total energy [15]. There are four main lipid classes in breast milk: triglycerides (TG), free fatty acids (FFA), cholesterol esters (CE) and phospholipids (PL). In freshly expressed breast milk, most fats (≥ 98%) are in the form of TG while FFA, CE and PL are only present in small amounts, which makes breast milk energy dense but low in osmotic load. There are lipases that naturally presented in breast milk that can convert TG into FFA, which may assist in the digestion of fats when consumed fresh by infants. However, the effect of lipase activity on the storage of breast milk fats is not clear and can be seen as an indication of milk fat degradation. This degradation in fat could potentially increase the osmolality of breast milk, which may be related to slowed gastric emptying and other feeding intolerance

Abbreviations: CE, cholesterol esters; EBM, expressed breast milk; FFA, free fatty acids; GC, gas chromatography; HP, Holder pasteurization; HPP, high pressure processing; PL, phospholipids; PRISMA, referred Reporting Items for Systematic Reviews and Meta-Analysis; TG, triglycerides; TLC, thin layer chromatography

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events commonly seen in preterm infants [16,17].

While two systematic reviews have been published on the effects of Holder Pasteurization (HP) [18] and short-term refrigeration storage [19] on fat composition, there has been no systematic review encompassing all EBM handling processes conducted to date. This review assesses the impact of handling processes of EBM on the breast milk fat composition, including storage (room temperature, refrigerator and frozen storage), pasteurization (HP and other available techniques), and freeze and thaw methods and repetition of freeze and thaw cycles.

2. Methods

This review has been conducted according to Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) [20] and the PRISMA checklist can be found in **Supplementary** Table 1. The protocol of this systematic review was registered on PROSPERO international prospective register of systematic reviews [21].

2.1. Search strategy

The electronic databases PubMed/Medline, Embase and Scopus were searched during April 2019 using the combination of Mesh/Emtree terms and free words in three main components: breast milk, handling process (i.e. storage and pasteurization), and fat composition (full search strategies are available in **Supplementary** Table 2). References of identified studies were also checked to capture all potential studies. Searches were restricted to humans only, and studies published in English but no date restriction was applied.

2.2. Inclusion and exclusion criteria

Both intervention and observational studies were included in this review. Reviews, case reports and commentaries were excluded but were checked for component studies. Studies that determined the impact of any one, or a combination of storage, pasteurization, and freeze and thaw methods and repeated freeze and thaw cycles were eligible for this review. Outcomes of interest were total fat content (and its total fatty acid composition) and the fat composition (lipid classes: the concentration and composition of TG and FFA). Studies needed to report a change from baseline (fresh EBM) and after being subjected to a handling process or comparison of two different handling process.

Two authors independently screened the studies against the inclusion and exclusion criteria using Covidence [22]. Any conflicts raised during the process were resolved in consultation with the primary author.

2.3. Data extraction and quality appraisal

Data were extracted into two structured tables: general study characteristics and detailed outcomes. Study characteristics included authors, publication year, milk collection method, intervention, methods and outcomes.

Study quality of was assessed by two reviewers independently using the quality appraisal tool retrieved from Academy of Nutrition and Dietetics [23] for primary research papers. Studies were rated positive, neutral or negative based on ten questions related to the validity and four questions related to the rationale and significance of the study.

2.4. Data synthesis

Due to the heterogeneity of both the interventions and outcomes reported, it was not possible to undertake a meta-analysis. Studies were grouped according to the outcome they reported and synthesised narratively. For comparison between studies, the authors calculated the relative percentage change to the fat composition from the baseline to the post-handling procedure, where relevant. Changes were considered minimal if they were less than the expected accuracy or precision (10%) of the techniques used to measure the variable [24].

3. Results

The study selection process is detailed in the PRISMA diagram (Fig. 1). A total of 42 unique studies (with 43 reports), including 40 observational studies, one non-randomized study [25] and two randomized trials [26,27] were included in this review. Characteristics, general information and the quality rating of these studies are available in Table 1. These included studies were conducted in 15 countries, and the sample size ranged between 1 and 90, with most studies having <30 samples. A total of 20 included studies were conducted between 2010 and 2019, and the rest were published prior to 2009 with the earliest records published in 1978 [25,28]. Most included studies were rated as positive quality and reliable, except one rated as neutral due to misreporting of the data [29] and another rated as negative as no clear description of methodology was given [30].

Storage conditions were classified as: room temperature ($\geq 15\,^{\circ}C$), fridge (≤ 0–$7\,^{\circ}C$), domestic freezer (<0 to $-20\,^{\circ}C$) and deep freezer (-70 to $-80\,^{\circ}C$). Changes to breast milk fat composition after exposing to different treatments are classified into two major categories: total fat content and the total fatty acid composition of the total fat, presented in Table 2; and fat composition (lipid classes) including concentration of TGs and FFAs, presented in Table 3.

3.1. Total fat content

A total of 21 studies (22 reports), reported total fat content and are detailed in Table 2. Eight measured fat by gravimetrical method following a modified Folch [31–34] or Rose-Gottlieb [30,35–37] method, four by creamatocrit [38–41], two titration [25,26], two by Gerber butyrometer [42,43] method, and another five a human milk analyzer [27,44–47] and one by lipid test kit [28]. We acknowledge that there are variations within and between these methods [24]. Therefore, studies reporting less than 10% statistically significant changes in total fat content are considered to be within the error of the methodology and will be referred as minimal changes in the following section.

Studies that investigated the impacts of different storage conditions on breast milk total fat content reported changes that were either not statistically significant [30,31,40] or minimal [39,42,45,47], with the exception of Silprasert et al. [39] who found a 18.7% reduction in total fat content of breast milk in samples that were frozen and thawed twice in a domestic freezer for 28 days [39]. Despite a significant reduction in total fat content of breast milk after sterilisation reported by Fidler et al. [35,36] and Legge et al. [28], changes to the total fat content of breast milk after exposing to various other pasteurization technologies (alone or followed by storage) or different freezing and thawing method are either not statistically significant [25–27,32–38,41] or minimal [34,43,44,46].

In conclusion, it appears that storage and pasteurization produce only small changes to breast milk total fat content, most less than 10%, which is potentially a result of methodological variations. The gravimetric method that used in most studies for measuring total fat content of breast milk is particularly likely to produce variations in the results, which may not reflect a true fat loss in the samples. Fat globules can adhere to the surface of containers during storage. Moreover, it is common for the fat of breast milk to separate and float to the surface after defrosting, and accurate measurements of the fat content will not occur unless sufficient homogenisation of the breast milk has been achieved before sampling.

3.1.1. Total fatty acid composition of total fat

Fifteen studies measured total fatty acid composition using gas chromatography (GC) [27,29,33,35–37,48–56] and are detailed in Table 2.

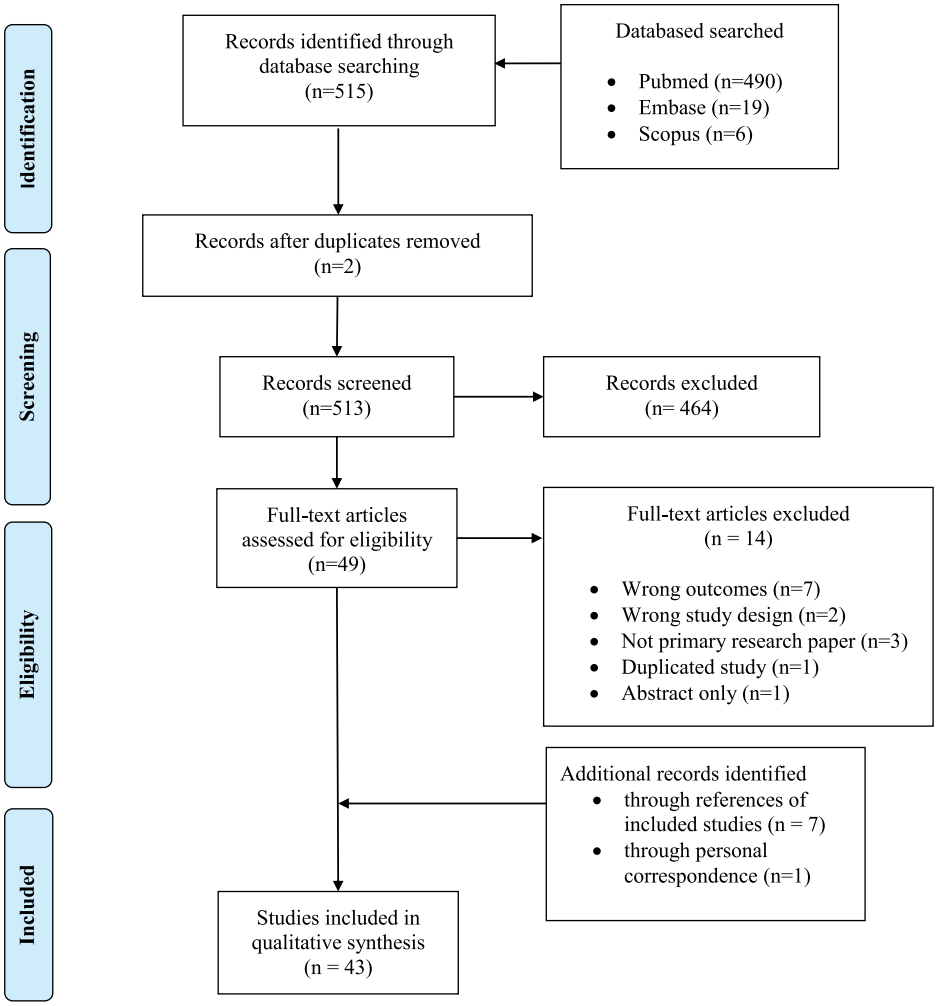

Fig. 1. PRISMA diagram describing the study selection process and reasons for exclusion.

Overall, there were no important changes to the total fatty acid composition of breast milk stored at different temperature regardless of the length of storage with or without HP [29,52,57]. A majority of the studies that determined the effect of HP on total fatty acid composition of breast milk reported no changes after the treatment [33,35,36,48,50,53,55,56], with the exception of a slight reduction in the percentage of C18:0 accompanied by a small increase in the percentage of C22:2 n-6 fatty acid reported by de Oliveira et al. [27]. The total fatty acid composition of breast milk remained stable after being pasteurized by high pressure processing (HPP) [50,56], sterilisation [35,36,53], flash heat treatment [56] or ultraviolet irradiation [49,56], and the concentration of C18:2 n-6 and C18:3 n-3 was not affected by microwave heating [58]. In addition, the combination of HP and freeze storage had minimal effects on the fatty acid composition of breast milk [29]. Nevertheless, Delgado et al. [48] reported a reduction in n-3 PUFA of breast milk after HPP, which correlated with time and pressure of the treatment. The method of freezing (quick vs. slow) did not alter the total fatty acid composition of breast milk [37].

To summarize, we conclude that regardless of any changes in the type of fats in milk (see below), the total fatty acid composition of breast milk is relatively stable and independent of storage and pasteurization conditions.

3.2. Fat composition (lipid classes)

This section reports on the changes in the lipid classes of fat in breast milk, specifically the concentration of TGs and the FFAs in the breast milk fat. The concentration of TG and FFA can vary significantly depending on different storage and handling processes due to the influence of lipases that are naturally present in human breast milk. In general, variations in FFA levels in breast milk are due to lipase activity on TG, but there has been no available data on the variations of FFA level in breast milk from different mothers. Although most of the included studies measured only the concentration of TG or FFA, reduction in TG concentration essentially means a rise in FFA concentration, and vice versa. Caution is required in interpreting FFA data since the concentration of FFA is very low in fresh EBM ($\leq$ 1% of total fat); so that any increase in FFA concentration can result in large proportional changes in the relative percentage increase in FFA levels.

Studies which separated and/or quantified TGs and/or FFAs used several methods including: five by thin layer chromatography (TLC) equipped with scanner [34,59,60] or following a GC analysis [27,61], one by high performance liquid chromatography [62], or gas chromatography-mass spectrometry [63], or mass spectrometry [64] or solid phase extraction [57], or colorimetric [65], or titration [25], or automated TGs analyser [66], three by assay kit [31,32,41] and two by titration method [37,67].

3.2.1. Storage

Ten studies determined the effect of different storage conditions on the concentration of TGs [59,60,62,65,66] and FFAs [31,40,54,57,59,60,62,63,67] in breast milk, and the results are shown in Table 3.

Table 1
Characteristics of included studies.

Study year, Country and Design	Preterm/term	Phase of lactation	Expression method	Status of sample (at receiving)	Pre-treatment storage	Intervention	Sample Size	Analytical method	Quality*
Ahrabi et al. [31] 2016 U.S	N/S	N/S	Electric pump	Fresh	N/S	Freeze storage	40	Gravimetric method (Folch extraction): total fat content Assay kit: concentration of FFA (g/L)	Positive
Andersson et al. [26] 2007 Sweden	Preterm	colostrum	Electric pump	Fresh	Stored at −20°C	HP	5	Titration: total fat content (g/ml)	Positive
Baack et al. [55] 2012 U.S	N/S	N/S	N/S	Frozen	Stored at −20°C	HP	1 pool (31 samples)	GC: FA composition (weight% of total FA)	Positive
Berkow et al. [59] 1984 U.S	N/S	Colostrum and mature	Mechanical pump	Fresh	Stored at +4°C	Freeze/thaw cycle Freeze storage	3	TLC scanner: TG and FFA concentration (% of total fat)	Positive
Bertino et al. [57] 2013 Italy	Preterm	N/S	Electric pump	Fresh	N/S	Fridge storage	3 pools (each with 4, 8 and 5 samples)	GC: FA composition (g/100 g lipid) SPE: FFA concentration (mg/L)	Positive
Bitman et al. [60] 1983 U.S	Preterm & term	N/S	Mechanical pump	Frozen (1st part) Fresh (2nd part)	N/S	Freeze storage + freeze/thaw Freeze storage	25 (1st part) 6 (2nd part)	TLC scanner: lipid classes (% of total fat)	Positive
Borgo et al. [29] 2015 Brazil	N/S	N/S	N/S	N/S	N/S	HP followed by freeze storage	1	GC: FA composition (% mg)	Neutral
Chan et al. [41] 2011 U.S	Preterm NS (milk bank sample)	N/S	N/S	N/S	Stored at −20°C	Thawing methods	17	Creamatocrit: total fat content (%) Assay kit: FFA concentration (nmol/μl)	Positive
Christen et al. [49] 2013 Australia	N/S	N/S	N/S	Frozen	Stored at −20°C	Pasteurisation (UV irradiation)	10	GC: FA composition (log_{10} peak area)	Positive
de Oliveira et al. [27] 2017 France	Preterm	N/S	N/S	Fresh (raw milk group) Frozen (pasteurised milk group)	Stored at +4°C Stored at −20°C	HP	64 (raw milk group) 12 pools (pasteurised milk group)	Human milk analyser: total fat content (g/L) TLC + GC: lipid classes and FFA (%lipolysis); FA composition (weight% of total FA);	Positive
Delgado et al. [48] 2014, Spain	N/S	Mature	N/S	Fresh	Stored in fridge (temperature not specified)	HP Pasteurisation (high pressure processing)	1 pool (6 samples)	GC: FA composition: (weight% of total FA)	Positive
Ezz El Din et al. [30] 2004 Egypt	N/S	Colostrum and mature	Manual expression OR Mechanical pump	Fresh	N/S	Fridge storage Freeze storage	61	Gravimetric method (Roese-Gottlieb method): total fat content (g/L)	Negative
Fidler et al. [35] 1998 Germany and **Fidler et al.** [36] 2001 Germany	Preterm and Term	Colostrum	Electric pump	Fresh	N/S	HP Pasteurisation (sterilisation)	12	Gravimetric method (modified Roese-Gottlieb method): total fat content (g/dl) GC: FA composition (weight% of total FA)	Positive
Friend et al. [37] 1983 U.S	N/S	Mature	Manual expression	Fresh	Stored in fridge	Freezing methods	30 (individual) 20 pools (each pool with 8 ~ 12 samples)	Gravimetric method (Roese-Gottlieb method): total fat content (g/100 ml) GC: FA composition (weight% of total FA) Titration: FFA (g/100 g Lipid)	Positive
Garcia-Lara et al. [45] 2013 Spain	N/S	N/S	Manual expression OR Electric/ Mechanical pump	Frozen	Stored at −20°C	HP followed by freeze storage	34 (from 28 donors)	Human milk analyser: total fat content (g/dl)	Positive
Garcia-Lara et al. [46] 2012 Spain	N/S	N/S	Manual expression OR Electric/ Mechanical pump	Fresh	Stored at < 5°C	Freeze storage	61 (from 59 donors)	Human milk analyser: total fat content (g/dl)	Positive
Goes et al. [38] 2002 Brazil	N/S	Mature	Manual expression OR Electric/ Mechanical pump	Frozen	N/S	HP followed by freeze storage	15	Creamatocrit method : total fat content (g/L)	Positive

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Table 1 (*continued*)

Study year, Country and Design	Preterm/term	Phase of lactation	Expression method	Status of sample (at receiving)	Pre-treatment storage	Intervention	Sample Size	Analytical method	Quality*
Hamosh et al. [67] 1996 U.S	N/S	Mature	Breast milk pump (N/S)	Fresh	N/S	Room temperature storage	11	Titration: FFA concentration (% of total fat)	Positive
Handa et al. [32] 2014 U.S	N/S	N/S	Electric pump	Fresh	N/S	Thawing and warming methods	40	Gravimetric method (Folch extraction) total fat content Assay kit: FFA concentration (% of total fat)	Positive
Henderson et al. [33] 1998 U.S	N/S	Mature	N/S	Frozen	N/S	HP	3 pools	Gravimetric method (Folch extraction): total fat content (g/L) GC: total fat content; FA composition (weight% of total FA)	Positive
Janjindamai et al. [42] **2013 Thailand**	N/S	Mature	Electric pump	Fresh	N/S	Freeze storage	90	Gerber butyrometer: total fat content (g/100 ml)	Positive
Lavine et al. [54] 1987 U.S	N/S	Mature	Electric pump	N/S	N/S	Room temperature and freeze storage	8 (each participants assigned to half treatment)	GC: FFA concentration (mg/ml)	Positive
Legge et al. [28] 1978 New Zealand	N/S	Colostrum	N/S	N/S	N/S	Pasteurisation (sterilisation)	5 pools (each with 2–3 samples)	Lipid test kit: total fat content (g/L)	Positive
Lepri et al. [34] 1997 Italy	N/S	N/S	Manual expression	Fresh	N/S	HP followed by freeze storage	1 pool (16 samples)	Modified Folch extraction: total fat content (mg/mL) TLC scanner/TLC-GC: FFA concentration (weight% of total FA)	Positive
Lev et al. [47] 2014 U.S	Preterm	N/S	Electric pump	Fresh	Stored at −5°C	Freeze storage	60 samples (3 samples from each of 20 mothers)	Human milk analyser: total fat content (g/100 ml)	Positive
Molto-Puigmarti et al. [50] 2011 Spain	N/S	Mature	Mechanical pump	fresh	N/S	HP Pasteurisation (high pressure processing)	10	GC: FA composition (weight% of total fat)	Positive
Morera Pons et al. [62] 1998 Spain	term	Mature	Manual expression	N/S	N/S	Freeze storage Freeze/thaw cycle	30 samples (from 6 mothers)	HPLC: lipid classes	Positive
Ovesen et al. [58] 1996 Demark	N/S	Mature	N/S	Frozen (either pasteurised or unpasteurised)	N/S	Warming methods	1 pool (25 samples)	GC: concentration of LA and ALA (mg/100 g)	Positive
Pitino et al. [56] 2019 Canada	N/S	N/S	N/S	N/S	Stored at −20°C	Pasteurization (HP, HPP, UV irradiation, flash heat)	8	GC: FA composition (weight% of total FA)	Positive
Reynolds et al. [40] 1982 Australia	N/S	Colostrum	Mechanical pump	Fresh	N/S	Freeze storage	20	Creamatocrit: total fat content (%) Copper soap method: FFA concentration (mequiv./L)	Positive
Romeu-Nadal et al. [53] 2008 Spain	N/S	Mature	Mechanical pump	N/S	N/S	HP Pasteurization (sterilization)	10	GC: FA composition (weight% of total FA)	Positive
Romeu-Nadal et al. [52] 2008 Spain	Term	Mature	Mechanical pump	N/S	N/S	Fridge and freeze storage	2 pools from 10 samples	GC: FA composition (weight% of total FA)	Positive
Silprasert et al. [39] 1986 Thailand	N/S	Colostrum	Mechanical pump	N/S	N/S	Room temperature, fridge and freeze storage	72 pools	Creamatocrit: total fat content (%)	Positive
Slutzah et al. [51] 2010 U.S	Term and preterm	N/S	Electric pump	Fresh	N/S	Fridge storage	36	Direct transmethylation of FFA– GC: FFA concentration (g/L)	Positive
Spitzer et al. [64] 2013 Germany	N/S	Mature	Electric/ Mechanical pump	Fresh	N/S	Fridge storage	7 pools from 33 samples	MS: short- to medium-chain FFA composition (μg/kg)	Positive
Tacken et al. [65] 2009 Netherland	N/S	Mature	Electric/ Mechanical pump	Fresh	–	Fridge and freeze storage Warming method	30	Colorimetric test: TG concentration (mmol/L)	Positive

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Table 1 (*continued*)

Study year, Country and Design	Preterm/term	Phase of lactation	Expression method	Status of sample (at receiving)	Pre-treatment storage	Intervention	Sample Size	Analytical method	Quality*
Thatrimontrichai et al. [59] 2012 Thailand	Term and preterm	N/S	Electric pump	N/S	Stored at −20°C	Thawing methods	90 samples	Gerber butyrometer: total fat content (g/100 ml)	Positive
Van Zoeren-Grobben et al. [63] 1996 Netherland	N/S	Colostrum and mature	Mechanical pump	Fresh	N/S	Fridge storage	10	GCMS: free LA (μM)	Positive
Vieira et al. [44] 2011 Brazil	N/S	N/S	Manual expression OR Electric/ Mechanical pump	Fresh	N/S	HP Thawing methods	57 samples	Human milk analyser: total fat content (mg%)	Positive
Wardell et al. [61] 1981 U.K	N/S	Colostrum	N/S	N/S	N/S	HP Freeze/thaw cycle	8 samples	TLC-GC: TG composition (weight% of total FA)	Positive
Williamson et al. [25] 1978 U.K	Preterm	Mature	N/S	N/S	N/S	HP Pasteurisation (sterilisation)	7 pools (each pool made of several samples)	Titration: total fat content (g/L) and FFA concentration (%)	Positive
Yuen et al. [66] 2012 China	Term	Colostrum and mature	Manual expression	Fresh	N/S	Fridge storage	25 colostrum samples 11 mature milk samples	Chemistry analyser: TG content (mmol/L)	Positive

Abbreviations: FA: fatty acid; FFA: free fatty acid; TG: triglyceride; TLC: thin layer chromatography; LA: linoleic acid; ALA: α-linolenic acid; HPLC: high performance liquid chromatography; GC: gas chromatography; GCMS: gas chromatography mass spectrometry; SPE: solid phase extraction; MS: mass spectrometry; NS: non-specified; UV: ultraviolet.
The temperature of storage conditions are classified as following: room temperature room temperature (≥15°C), fridge (≥0–7°C), domestic (< 0 to −20°C) and deep freezer (−70 to 80°C).
* Quality of these studies were rated using the quality appraisal tool retrieved from Academy of Nutrition and Dietetics for primary research papers.

3.2.1.1. Room temperature. Two studies concur that storing breast milk at room temperature leads to an increase up to 686% in the concentration of FFAs as the result of lipase activity [54,67], and the rates of lipolysis varies slightly across temperatures [67].

3.2.1.2. Refrigeration. Both Tacken et al. [65] and Yuen et al. [66] stored breast milk at 4 °C, for 2 and 3 days, respectively, and detected no changes in the concentration of TGs at either time [64,65]. In contrast, another five studies reported a significant increase in overall [51,54,57] or individual [63,64] FFA of breast milk stored at refrigerator for up to 4 days. Combined, these data may be explained by the fact that small changes in TG levels can result in large changes in FFA level as each TG molecule yield three FFAs.

3.2.1.3. Freezing. The concentration of TG in breast milk stored in a domestic freezer remained stable as reported by Yuen et al. [66] and Tacken et al. [65] for 3 and 28 days, respectively. A reduction in TG concentration was seen in two other studies [59,60] as a result of prolonged storage between 2 and 5 months in domestic freezer [60]. Similarly, one study reported that the FFA concentration after storage of breast milk in a domestic freezer for up to 4 weeks did not result in any changes [40]. However, continuing storage at this condition may lead to increase in the FFA concentration as found by several studies [31,54,59,60,62]. Three studies found the TG concentration of breast milk stored in a deep freezer remained unchanged even after prolonged storage [59,60,62].

3.2.2. Pasteurization

Pasteurization is designed to inactivate pathogens and viruses but can also denature enzymes such as lipases. The effect of HP on the concentration of TGs [61] and FFAs [25,34] was determined by three studies. Lepri et al. [34] reported an 83% increase in the concentration of FFA due to HP, while there was 21% reduction in FFA concentration reported in Williamson et al. [25]. Wardell and colleagues [61] analyzed the fatty acid composition of TG and found only C18:3 n-3 was reduced by 22% [60]. When combined with post pasteurization freeze storage, the TG concentration was found unchanged [62]. In contrast, two studies reported that FFAs concentration in Holder pasteurized and freeze stored breast milk increased significantly through the process [27,34].

3.2.3. Freeze and thaw

Breast milk is often stored frozen in the home and in milk banks. The potential changes in milk lipids as a result of freezing and thawing is therefore of interest.

The concentration of TGs in breast milk samples warmed by microwave remained unchanged [65]. However, the FFA concentration in breast milk increased significantly to different extents (between 35% and 253%) after thawing and warming with either water or waterless method [32]. Chan and colleagues [41] determined changes to the FFA concentration of breast milk that was thawed using different methods. Breast milk that was thawed in a refrigerator at 4°C was used as the reference value, and a reduction was seen in breast milk thawed in a water bath and microwave, but not those thawed at room temperature [41].

Three authors studied [59,61,62] repeated freezing and thawing of breast milk and its impact on the concentration of TGs and/or FFAs. FFAs and other hydrolysis products of TGs were found in breast milk subjected to a single freeze and thaw cycle with domestic freezer storage in Morera Pons et al. [62]. Breast milk that was frozen and thawed three times and then stored in a domestic freezer showed a reduction in overall TG concentration in one study [59], and a reduction selected fatty acid in TG fraction in another study [61]. Significant reduction appeared after the second freeze and thaw cycle, but it was only seen in the relative percentage of C18:3 n-3, and not other fatty acids in TG fraction [61]; and there was a concomitant increase in the FFA

Table 2
Effect of various interventions on the total fat content of breast milk.

Component	Intervention	Effect
Total fat	Room temperature storage	**Reduction** (4.28–7.18%) [39]
	Fridge storage	
	• 24 h	**No statistically significant change** [30]
	• Up to 28 days	**Reduction** (4.48%) [39]
	Domestic freezer storage	
	• Up to 90 days	**Reduction** (up to 18.7%) [39,42,45]
	• Up to 9 months	**No statistically significant change** [30,31,40]
	Deep freezer storage	**Reduction** (9.7%) [47]
	Holder pasteurisation	**Reduction** (3.5–5.5%) [44,46]
		No statistically significant change [25–27,33–36]
	Sterilisation	**Reduction** (13–28%) [28,35–36]
		No statistically significant change [25]
	Holder pasteurisation + domestic freezer storage (up to 6 months)	**Reduction** (5.8–6.2%) [34,46]
		No statistically significant change [38]
	Quick/slow freeze	**No statistically significant change** [37]
	Thawing & Warming	
	• Water bath thawing	**Reduction** (8.5–18%) [41,44,59]
		No statistically significant change [32]
	• Room temperature	**No statistically significant change** [41]
	• Fridge thawing	**Reduction** (7.4%) [43]
	• Microwave	**Reduction** (8.5–31%) [41,44]
Total fatty acid composition	Refrigerator storage	**No statistically significant change** [52,57]
	Domestic freezer storage	**No statistically significant change** [52]
	Deep freezer storage	**No statistically significant change** [52]
	Holder pasteurisation	**Increase in C22:2 n-6 and reduction in C18:0** [27]
		No statistically significant change [33–36,48,50,53,55,56]
	Holder pasteurisation + freeze storage	**No statistically significant change** [29]
	High pressure processing	**Reduction in n-3 PUFA** [48]
		No statistically significant change [50,56]
	Sterilisation	**Reduction in C18:2 n-6 and C20:4 n-6** [35,36]
		No statistically significant change [53]
	Ultraviolet irradiation	**No statistically significant change** [49,56]
	Flash heat treatment	**No statistically significant change** [56]
	Quick/slow freeze	**No statistically significant change** [37]

Table 3
Effect of various interventions on the fat composition (lipid classes) of breast milk.

Component	Intervention	Effect
Triglycerides concentration	Fridge storage	**No statistically significant change** [65,66]
	Domestic freezer storage	
	• Up to 28 days	**No statistically significant change** [65,66]
	• Up to 5 months	**Reduction** (4.5–8.7%) [59,60,62]
	Deep freezer storage	**No statistically significant change** [59,60,62]
	Holder pasteurisation	**Reduction in C18:3 n-3** (22%) [61]
	Pasteurisation + domestic freezer storage for 4 months	**No statistically significant change** [62]
	Warming (microwave)	**No statistically significant change** [65]
	Freeze/thaw cycles + domestic freezer storage	
	• 1 time	**Reduction** [62]
	• 3 times	**Reduction** (13%) [59]
		Reduction in 18:3 n-3 (61%) [61]
	Freeze/thaw cycles + deep freezer storage (1 & 3 times)	**No statistically significant change** [59,62]
Free fatty acids concentration	Room temperature storage	**Increase** (up to 686%) [54,67]
	Fridge storage	**Increase** (up to 502%) [51,54,57,63,64]
	Domestic freezer storage	
	• Up to 4 weeks	**No statistically significant change** [40]
	• Up to 9 months	**Increase** (up to 1002%) [54,59–62]
	Deep freezer storage	**No statistically significant change** [54,59,60,62]
	Holder pasteurisation	**Increase** (83%) [34]
		Reduction (21%) [25]
	Sterilisation	**Reduction** (21%) [25]
	Pasteurisation + domestic freezer storage	**Increase** [27,34]
		No statistically significant change [62]
	Thawing	
	• Water bath thawing	**Reduction** (29%) [41]
	• Room temperature	**No statistically significant change** [41]
	• Microwave	**Reduction (39%)** [41]
	Thawing + warming	**Increase (35–235%)** [32]
	Freeze/thaw cycles + domestic freezer storage (1 & 3 times)	**Increase (353%)** [59,62]
	Freeze/thaw cycles + deep freezer storage (1 & 3 times)	**No statistically significant change** [59,62]

concentration [59,62]. The same authors also found no changes to neither TG nor FFA concentration in breast milk that was freeze-thawed three times but stored at deep freezer [59,62].

In conclusion, inconsistent results are seen in studies reporting the changes to the lipid classes of breast milk subjected to various storage conditions. The concentration of TGs and/or FFAs are affected by both storage temperature and duration. Storing breast milk at lower temperatures suppressed the release of FFAs, however, the concentration increases slowly with time. However, prolonged storage in a deep freezer, but not domestic freezer, seem to preserve the lipid classes of breast milk for at least up to 5 months. It is likely that lipases in breast milk are completely destroyed during pasteurization and therefore no changes to the concentrations of TG or FFAs were seen afterwards during storage. However, during the phase of pasteurization where the temperature is rising, there may be lipolysis of triglyceride. This assumption is in agreement with the findings discovered by Wardell et al. who found C18:3 n-3 in TG fraction decreased significantly while temperature reached 62.5 °C during HP, and no further reduction was seen from this point beyond till the HP process completed [61]. Moreover, there seems to be differences in concentration of FFAs/TGs after various thawing or warming process, which may be due to differing in rate of temperature raise throughout the process.

4. Discussion and conclusion

This systematic attempted to cover all possible procedures involved in EBM storage and handling, including storage under different conditions (room temperature, refrigerator, domestic and deep freezer), various pasteurization methods (HP, HPP, sterilisation and UV irradiation), various freeze and thaw methods (quick and slow freeze, microwave, warm water and refrigerator thawing) and repeated freeze and thaw cycles. The total fat content of breast milk appears to decrease after prolonged storage, pasteurization and thawing. However, we suspect this might be due to poor homogenisation of breast milk samples or other methodological variations as the reduction seen was mostly less than 10%. Total fatty acid composition of breast milk seems likely not be affected by most procedures. Subtle changes to the relative percentage of some fatty acids were only seen after various pasteurization treatments. The TG and FFA concentration of breast milk are sensitive to different conditions and changes are seen after prolonged storage at different temperatures (4.5–8.7% reduction in overall TG concentration) and pasteurization (22% reduction for selected TG fraction). Since FFA are only present in fresh breast milk in trace amounts, small actual changes can result in much larger relative changes in FFA (up to 1000% increase after storage, 83% increase after HP and 35–235% increase after thawing and warming, respectively).

The findings of the current systematic review are in agreement with past reports. Authors from a systematic review of safe management of EBM also reported that short-term refrigeration storage of EBM does not alter the fat content and the total fatty acid composition of such EBM [19]. In another systematic review investigating the impact of HP on various nutritional and immunological component of EBM, the authors concluded that the total fat content and total fatty acid composition is unlikely to be affected, but the FFA concentration increased significantly after HP [18].

This review combined published literature relating to all storage conditions and treatments seen in milk banks, making this the most comprehensive of its kind. However, we were not able to perform meta-analysis due to the high level of heterogeneity of the data, which was a result of different sampling methods, various technologies used to measure the components, different components of fat composition reported with various expressions of results. The fat content of breast milk is highly dynamic, varies between individuals, among the day, before, during and after feeding, and may potentially be different due to collection methods [1,68]. In addition, breast milk samples varied (fresh vs. frozen) at study baseline, which may have already received different treatments beforehand. More than half of the included studies were published dated prior to 2009, and sixteen of which were published before 2000, the technologies used in these studies may be out-dated.

4.1. Implication for practice

The reduction of total fat content seen in some of the studies were likely due to fat adheres to the surface of containers, although methodological errors cannot be ruled out. Therefore, we could expect this to happen clinically as EBM is transferred from container to syringe for delivery to the infant, or transferred between different containers for pasteurization, storage and distribution in milk banks. if we assume that EBM has an energy density of 700 kcal/L [69,70] and fat concentration of 40 g/L, but there is a 20% loss of fat during handling processes, then a 2 kg preterm infant, fed at 150 ml/kg could lose 10% of energy intake. Though the addition of human milk fortifier to EBM may compensate some of the energy loss (mainly derived from proteins), this loss of fat and energy supply from EBM may be clinically significant to preterm infants.

The lipases that are naturally presented in breast milk to assist the digestion and absorption of the fats may be destroyed by HP or other thermal treatments. However, these lipases continue to function in the early part of the process when the temperature is gradually raised. This conversion of milk fat from complex structured molecule TG to the digested form FFA may be beneficial to the infants, and aid fat absorption. However, a study conducted by Andersson et al. [26] revealed a lower coefficient of fat absorption in preterm infant fed with pasteurized milk than those fed with raw unpasteurized milk. In contrast, de Oliveira et al. [27] found pasteurization does not affect the gastric digestion of preterm infants. At this stage, there is not enough evidence to conclude the relationship between the FFA concentration of breast milk and the digestion and absorption in either preterm or term infants, therefore the best practice would be allowing minimal changes to the composition as comparing to the fresh milk.

4.2. Implications for research

Inconsistent results were seen in the studies included in this review are partially due to the techniques used in these studies. The technique for measuring TG are more reliable than the methods available for FFA. Given that the bulk of milk fat is in the form of TG, even a minute (0.1%) conversion of TG to FFA could result in large increase in reported FFA level, whereas a change in TG level could not be detected. TLC was used in most included studies that separated and quantified FFA, which is considered the optimal parameter to measure fat degradation, as it is sensitive to different conditions. However, the use of TLC for separating FFA is very likely to be contaminated by the dominant TG trace (due to tailing of TG on the TLC plate) and this could result in large errors. Development of new technologies for accurately separating and measuring FFA in breast milk fats is required.

While fatty acid metabolites are beyond the scope of this study, new emerging evidence shows that pasteurization increases the concentration of fatty acid metabolites of EBM, but has no effect on fatty acid composition of EBM [56]. Changes to these fatty acids metabolites were also seen under different storage conditions [71]. The effects of various handling processes on the fatty acid metabolites of EBM and its clinical impact on infants receiving these EBM needs to be further studied.

We also recommend that the collection and storage of breast milk, methods for analysis and items and units for reporting breast milk fat composition should be standardised in order to allow direct comparisons between studies and ease of combining data for systematic reviews.

CRediT authorship contribution statement

Chang Gao: Conceptualization, Writing - original draft,

Visualization, Data curation, Writing - review & editing, Validation. **Jacqueline Miller:** Writing - original draft, Visualization, Data curation, Writing - review & editing, Validation. **Philippa F. Middleton:** Writing - original draft, Visualization, Data curation, Writing - review & editing, Validation. **Yi-Chao Huang:** Data curation, Writing - review & editing. **Andrew J. McPhee:** Conceptualization, Writing - review & editing. **Robert A. Gibson:** Conceptualization, Writing - original draft, Writing - review & editing, Validation.

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Supplementary materials

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