The effects of storage conditions on long-chain polyunsaturated fatty acids, lipid mediators, and antioxidants in donor human milk – a review

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Abstract

Donor human milk (DHM) is the recommended alternative, if maternal milk is unavailable. However, current human milk banking practices may negatively affect the nutritional quality of DHM. This review summarises the effects of these practices on polyunsaturated fatty acids, lipid mediators and antioxidants of human milk. Overall, there is considerable variation in the reported effects, and further research is needed, particularly with lipid mediators and antioxidants. However, to preserve nutritional quality, DHM should be protected from light exposure and storage at 4°C minimised, to prevent decreases in vitamin C and endocannabinoids and increases in free fatty acids and lipid peroxidation products. Storage at -20°C prior to pasteurisation should also be minimised, to prevent free fatty increases and total fat and endocannabinoid decreases. Storage ≤-70°C is preferable wherever possible, although post-pasteurisation storage at -20°C for three months appears safe for free fatty acids, lipid peroxidation products, and total fat content.

Keywords:

Donor human milk, omega-3 fatty acids, docosahexaenoic acid, lipid mediators, antioxidants, preterm

[1]

[1] Abbreviations:
2-AG: 2-arachidonoylglycerol
4-HHE: 4-hydroxy-2-nonenal
4-HNE: 4-hydroxy-2-hexanal
AEA: arachidonoyl ethanolamide (anandamide)
ALA: α-linolenic acid
ARA: arachidonic acid
DHA: docosahexaenoic acid
DHM: donor human milk
DHEA: docosahexaenoyl ethanolamide
EPA: eicosapentaenoic acid
LA: linoleic acid
LCPUFA: long-chain polyunsaturated fatty acid
MDA: malondialdehyde
PUFA: polyunsaturated fatty acid
SPM: specialised pro-resolving mediator
TAC: total antioxidant capacity

1. INTRODUCTION

Mother's own breast milk is the accepted best practice for feeding neonates [1] and exclusive breast feeding for the first six months of life is recommended [2] for term infants. For preterm infants as well, mother's own breast milk is the favoured feeding choice. However, it may need to be fortified to accommodate the preterm infant's requirements [3]. Producing an inadequate milk supply is nearly three times more likely in preterm mothers than in term mothers [4]. Underlying reasons can be physiological, such as incomplete development of the mammary glands, or poor hormonal response, as well as psychological [5]. In some cases, maternal breast milk might not be appropriate, due to illness or medication. In these instances, donor human milk (DHM) from a human milk bank is the best alternative [2, 6, 7]. Although, at least in the U.K., there are no clear guidelines regulating the use of DHM to a specific preterm gestation, most clinicians agree that extremely preterm infants (born at less than 28 weeks gestational age) should receive DHM [8]. Similarly the Human Milk Banking Association of North America recommends the use of DHM for preterm infants or infants with a birth weight of less than 1750 g [9]. The American Academy of Pediatrics recommends use of DHM for all preterm infants, especially those weighing <1500 g, when mother's own milk is not available or sufficient [10].

Breast milk is generally the only food infants receive for the first few months of life. It provides macro- and micronutrients, immunological factors, hormones, enzymes, growths factors, essential fatty acids and other biologically active compounds, essential for the infant's development [11]. Adequate dietary nutrient supply is especially important for preterm infants since their maternal nutrient supply has been interrupted prematurely. For example, during the last trimester, the brain weight increases approximately five-times and at the same time around 80% of the brain docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (ARA, 20:4n-6) are accumulated [12]. Preterm birth also deprives the infant of enzymatic and non-enzymatic antioxidants that would have been matured or gained through maternal transfer in the third trimester, respectively [13, 14].

DHM undergoes prolonged cold storage, freeze-thaw cycles, and processing, before it is fed to infants, which may negatively affect the breast milk composition. For example, in the U.K., expressed breast milk for donation can be stored for up to 24 hours at 4°C, before transferring to a -18°C (or below) freezer for up to three months [15]. Breast milk is then thawed, Holder pasteurised (62.5°C, 30 minutes) and refrozen for up to three months. Before feeding, thawed pasteurised DHM can be stored at 4°C for up to 24 hours. These conditions have been summarised in Figure 1. Similar guidelines are followed widely, including in Australia, North America, Sweden, Italy, Spain, and India [16-21]. Additionally, in Italy, breast milk undergoing direct pasteurisation after expression, can be

stored for up to 72 hours at 4°C [18]. In Sweden, fresh and pasteurised DHM can be stored for 48 hours at 4°C and can be kept for a maximum of two hours at room temperature [19]. Lack of evidence is one reason for the different practices used for some aspects of human milk banking [22].

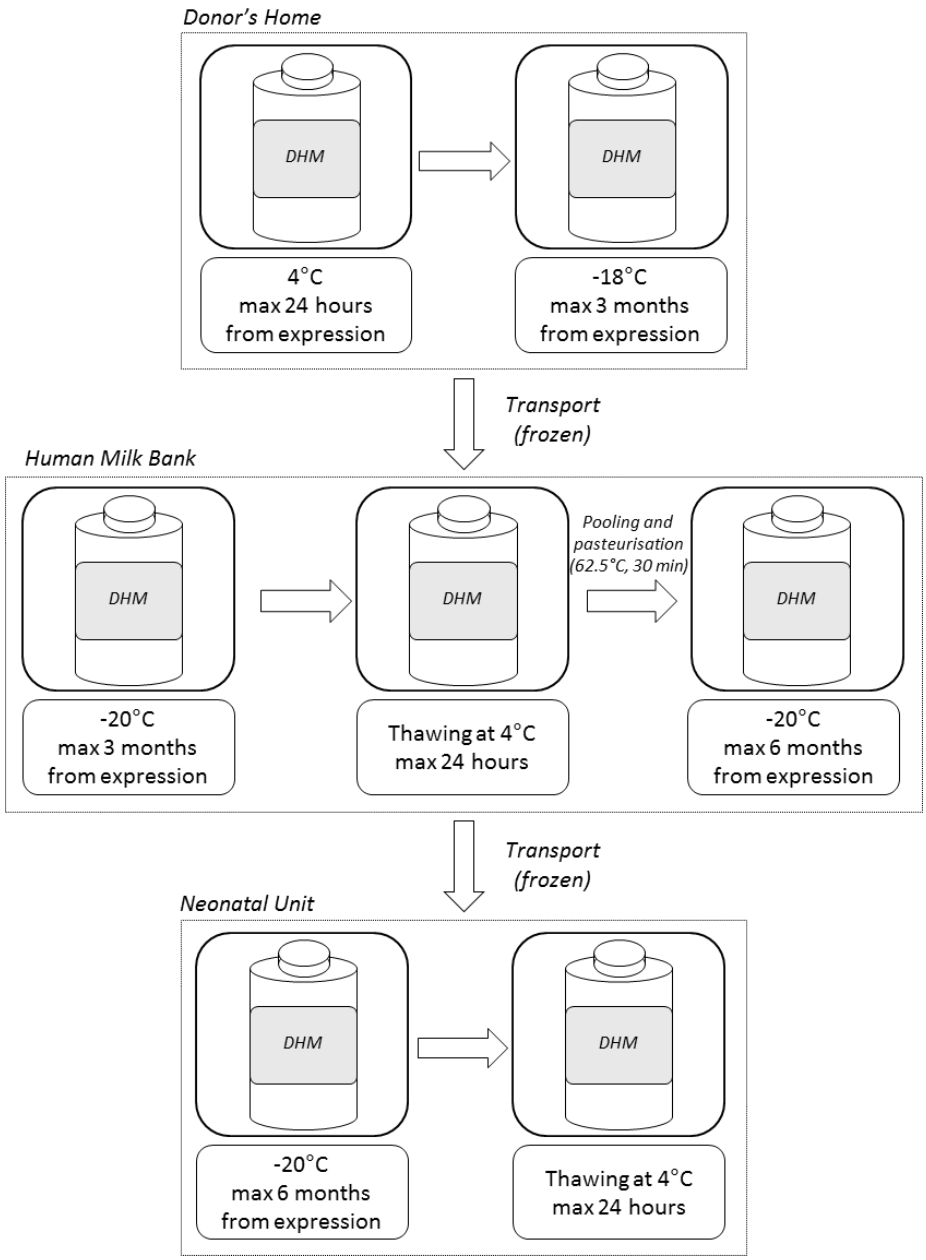

Figure 1: Donor human milk storage and processing conditions

Donor human milk (DHM) is exposed to various storage conditions at donors' homes, the human milk bank, and the neonatal unit. This figure displays the storage conditions allowable under the U.K. National Institute of Health and Care Excellence (NICE) guidelines [15]. Similar processes are used worldwide.

Recommendations for human breast milk storage conditions were predominantly developed to minimise bacterial growth, rather than to preserve nutritional components [23, 24]. However, with the increasing demand for DHM, and improvements in neonatal care leading to even younger infants surviving, it is now imperative that the nutritional quality of DHM is prioritised. Therefore, this article reviews the effects of current storage and processing conditions on long-chain polyunsaturated fatty acid (LCPUFA) content, bioactive lipid mediators, and antioxidants in human breast milk. Gao and colleagues recently published a systematic review on the effects of storage, handling and processing on breast milk fatty acid composition [25]. This present article compliments and extends these observations by also reviewing lipid peroxidation, bioactive lipid mediators and endogenous antioxidants. The effects of Holder pasteurisation on nutrients have been described elsewhere [26], and are not part of this review.

2. SEARCH METHODOLOGY

A search and discovery tool was used to search 80 databases, including Scopus, Web of Science, Medline, and Cinahl, using the following search terms: ((human milk) OR (donor milk) OR (donor human milk) OR (breast milk)) AND ((thaw* OR freeze* OR storage OR processing OR (cold storage) OR (-20 degree C) OR (-80 degree C) OR refrigeration) OR stability) AND ((fat OR lipid* OR triacylglycerol* OR triglyceride* OR (fatty acid) OR (long chain polyunsaturated fatty acid*) OR (polyunsaturated fatty acid*) OR (docosahexaenoic acid) OR (arachidonic acid) OR (free fatty acid*) OR lipolysis OR macronutrient* OR eicosanoid* OR leukotriene OR prostaglandin OR thromboxane OR (specialized pro resolving mediator*) OR (specialized pro-resolving mediator*) OR lipoxin* OR resolvin* OR protectin* OR maresin* OR endocannabinoid* OR (arachidonoyl ethanolamide) OR (docosahexaenoyl ethanolamide) OR arachidonylglycerol OR (lipid peroxidation) OR hexanal OR alkenal OR (lipid hydroperoxide*) OR malonyldialdehyde OR TBARS OR MDA OR hydroxynonenal OR hydroxyhexenal OR antioxidant* OR (vitamin C OR ascorbic acid) OR (vitamin E) OR tocopherol* OR (superoxide dismutase) OR catalase OR glutathione OR (glutathione peroxidase) OR (total antioxidant capacity)) OR (antioxidant capacity) OR (antioxidant status)). Furthermore, ‘snowballing’, searching the reference lists of the identified literature, was used [27], as well as searching google scholar. Studies were included when full text was available, the language of the publication was English and the publication date was before April 2019. Studies describing solely the effect of pasteurisation on nutrients were excluded.

3. TOTAL FAT CONTENT AND LONG-CHAIN POLYUNSATURATED FATTY ACIDS

Breast milk contains DHA and ARA, LCPUFAs of the omega-3 and omega-6 series, respectively [28]. DHA levels in breast milk are highly variable, ranging from 0.17% to 0.99% of total fatty acids, whereas ARA levels are more constant (0.36% to 0.49% of total fatty acids) [29]. Intrauterine accretion rates for DHA and ARA peak in the last trimester [30], a time when DHA is also selectively favoured for placental transport to the foetal circulation [31]. This leads to a bio-magnification of LCPUFAs in the foetus, providing it with substrates for the developing brain [32]. In preterm infants, maternal supply has been interrupted prematurely, and they therefore have an elevated requirement for enteral LCPUFA intake. Indeed, preterm infants have significantly lower DHA and ARA blood levels than term infants [33]. Term infants fed with formula milk devoid of DHA will rapidly exhaust their adipose tissue DHA stores [34]. This is also reflected by significant decreased erythrocyte DHA levels at day five of feeding formula milk devoid of DHA to term infants [35]. Importantly, erythrocyte DHA status has been correlated with brain DHA status [36]. Preterm infants have in contrast to term infants very low adipose tissue stores [30, 37], which makes them even more dependent on adequate enteral LCPUFA intake. Inefficient conversion rates from precursor fatty acids [38, 39], as well as an enteral LCPUFA absorption rate of only 80% [40], and the prolonged period it may take until full enteral feeding is achieved, further limit the LCPUFA availability for preterm infants. However, it is critical to provide preterm babies with sufficient amounts of LCPUFAs optimal for brain and visual development, as well as cell and immune system function [41, 42]. Although preterm breast milk may contain higher DHA levels than term breast milk, [43], we have previously shown that extremely preterm infants under standard care receive very low levels of DHA and ARA, which are reflected in low blood fatty acid levels [44]. Importantly, DHM is provided generally by mothers of term infants and consequently lower in LCPUFA levels [45]. It is therefore imperative that all appropriate steps are taken to maintain LCPUFA levels on the journey from donor to recipient. Since the total fat and LCPUFA content of DHM may be sensitive to human milk banking practices, the following section provides an overview of the literature investigating the effects of different storage conditions on human breast milk lipids, and is summarised in Table 1.

Storing breast milk at 4°C for 48 hours has been shown to not significantly change the absolute or relative fatty acid content [46], or triacylglycerol content [47]. The latter was also not affected by refrigeration at 4°C for up to three days [48]. Total lipid content was also unchanged by refrigeration at 4°C for 24 hours [49], or up to 96 hours [50]. Similarly, polyunsaturated fatty acid (PUFA) content (including linoleic acid (LA, 18:2n-6), α-linolenic acid (ALA, 18:3n-3), ARA and DHA), as well as saturated and monounsaturated fatty acid content was not significantly altered when stored for 96 hours at 4°C [23] or 6.8°C [51].

147 Several studies show that total fat content is not significantly altered following storage at -20°C for
148 nine months [52], nor does absolute fatty acid and relative fatty acid content change significantly in
149 studies ranging from storage for 30 days [46] to 12 months [23]. Furthermore, storage at -20°C for
150 3 days, or -18°C for 28 days does not change total triacylglycerols levels [47, 48]. Total fat and
151 relative fatty acid levels were unaffected by storage at -25°C for three months, although these
152 samples were refrigerated for up to 48 hours before baseline analysis [53]. Consistent with these
153 observations, storing breast milk for one week at -4 to -8°C did not change the fat content [49].
154 However, others have found that storage at -20°C significantly decreases total fat after 48 hours
155 [54], 30 days [55, 56], and up to 24 weeks [57]. Similarly, total lipid concentrations [58], and
156 triacylglycerols [59], significant decrease after eight days, and five months, at -20°C respectively.
157 Significant reductions in fat content after storage at -20°C were also seen after seven days and up to
158 90 days, with the biggest decreases in the first week (-0.027 g/dL/day) [60]. Freezing at -80°C for five
159 months did not affect saturated or monounsaturated fatty acids, or PUFAs [23], or triacylglycerols
160 for 12 months [59]. Although -80°C storage was shown to result in a significant decrease of fat, this
161 was lower than the decreases seen at -20°C [57]. In contrast, significant decreases in fat content of
162 91% were seen after 44 days of breast milk storage at -80°C, which led to the conclusion of the
163 authors that storage at -80°C should not be the gold standard as recommended by other researchers
164 [61]. This result is unexpected and since no comparison was undertaken with storage at -20°C, the
165 results should be considered within the context of the wider literature. Discordant observations
166 have also been seen post-pasteurisation, with storage at -20°C for 90 and 180 days resulting in 5.7%
167 and 2.9% decreases in total fat content, respectively [62, 63], whereas, no differences in total fat
168 content [64, 65], or relative fatty acid concentrations [64] of pasteurised breast milk stored for
169 1 month at -25°C or up to 12 months at -20°C, respectively were seen.

170 During human milk banking breast milk is thawed and then refrozen, which has the potential to
171 affect the milk fat quality. Three-times freezing and thawing has been shown to lead to reductions in
172 fresh breast milk triacylglycerols of up to 5% [59]. Freezing and thawing breast milk for three-cycles
173 before storing it at -20 °C for five months resulted in an additional 3% triacylglycerol loss (13% in
174 total, compared to 10% after freezing only). Relative amounts of saturated, and monounsaturated
175 fatty acids of breast milk triacylglycerols did not change significantly after two freeze thaw cycles,
176 whereas the relative LA content decreased by -65% [66]. It is noteworthy that thawing in the fridge
177 (4°C) for 24 hours, as recommended in the U.K. [15], significantly reduced total fat loss compared to
178 thawing in a water-bath (37°C, 30 minutes) [55]. Vieira and colleagues found no significant
179 difference in total fat content when pasteurised breast milk was thawed in a water-bath (40°C,
180 10 minutes) or thawed in a microwave (45 seconds) [67]. No difference in fat content was found

when thawing under tepid water and thawing by a waterless dry heat warmer were compared [68]. Chang and colleagues also found that storage (-20°C, 48 hours) in light brown coloured polyethersulfone bottles resulted in the least fat decreases [54].

In conclusion, the available evidence suggests that storage at 4°C is sufficient to minimise decreases in total fat and LCPUFAs in breast milk for up to 96 hours. However, for longer-term storage the data for storage at -20°C is highly discordant, especially pre-pasteurisation. These differences may be related to variations in analytical methods used [69, 70], or methodological variations, such as not sufficiently homogenising the breast milk after storage [71], differences in fat adherences to the container walls [54, 61], particularly to polyethylene [72], and variations in fat loss due to different thawing methods [55], which are not specifically defined in the literature. Furthermore, breast milk is a complex biological matrix, and variations in unmeasured endogenous antioxidant levels or other components, may influence fat content stability during storage, discussed further in Section 7 below. However, overall the evidence suggests storage at -80°C is the best option for longer-term storage to maintain total fat and CLPUFA levels.

195 Table 1: Summary of studies investigating the effects of storage conditions on total fat and LCPUFA content

	Breast milk samples, storage temperature and duration	Study outcome
[46]	Fresh 4°C for 48 hours, or -20°C for 30 days	No significant differences in absolute or relative fatty acid content between fresh, refrigerated, or frozen breast milk
[47]	Fresh 4°C for 48 hours, or -18°C for 28 days	No significant differences in triacylglycerol content between fresh, refrigerated, or frozen breast milk
[48]	Fresh 4°C for 72 hours, or -20°C for 72 hours	No significant differences in triacylglycerol content between fresh, refrigerated or frozen breast milk
[49]	Fresh 4-6°C for 24 hours, or -4 to -8°C for 1 week	No significant differences in fat content between fresh, refrigerated or frozen breast milk
[50]	Fresh 4°C for 24, 48, 96 hours All samples were stored at -80°C until analysis	No significant differences in total lipid content between fresh, or refrigerated breast milk
[23]	Fresh 4°C for 3, 6, 9, 12, 24, 48, 72, 96 hours -20°C or -80°C for 3, 5, 8, 12 months	No significant differences in relative LA, ALA, ARA, DHA, saturated, monounsaturated, or polyunsaturated fatty acid content between the different storage times and temperatures

[51]	Fresh (within 3 hours of collection) 6.8°C for 24, 48, 72, 96 hours	No significant differences in absolute saturated or monounsaturated fatty acids, PUFAs, LCPUFAs, or the saturated to unsaturated fatty acid ratio between fresh and refrigerated breast milk at any storage time
[52]	Fresh 4°C for 72 hours and then stored at -20°C for 1, 3, 6, 9 months All samples stored at -80°C after the initial storage time until analysis	No significant differences in total fat content between fresh, refrigerated and frozen, or directly frozen breast milk at the different storage conditions
[53]	Fresh (up to 48 hours at 4°C) -25°C for 1 week, 1, or 3 months	No significant differences in total lipid or relative fatty acid concentration between fresh or frozen breast milk
[54]	Samples stored in 9 different commercial milk containers for maximum 3 days at 4°C, before transfer to -20°C for 2 days	No significant differences in total fat content of breast milk stored in different containers Least fat loss in light brown coloured polyethersulfone bottles Significant total fat decrease in breast milk after freezing, storing and thawing (-0.27 to -0.30 g/dL, p = 0.02)
[55]	Fresh -20°C for 30 days, then thawed at 4°C for 24 h, or at 37°C for 30	Total lipids (g/100 mL): Fresh: 2.98 vs thawing at 4°C: 2.76 vs thawing at 37°C: 2.66 Significant less mean fat loss in frozen breast milk thawed at 4°C compared to

	minutes	thawing at 37°C (p = 0.02)
[56]	Fresh	Significant fat decrease after frozen storage
	-20°C for 30 days	Fat (g/100 mL): Fresh: 2.98 vs 30 days frozen: 2.66 (p < 0.001)
[57]	Fresh (up to 24 hours at 4°C)	Fat content was consistently higher in breast milk stored at -80°C than at -20°C
	-20°C or -80°C for 4, 12, 24 weeks	(p < 0.0005)
		Difference in fat content between 4 and 24 weeks was 0.3 g/100 mL at -20°C
		(p = 0.001) and 0.14 g/100 mL at -80°C (p = 0.009)
[58]	Fresh	Significant total lipid decrease in breast milk stored at -20°C
	-20°C for 4, 8 days	Lipids (g/100 mL): Group 1 (no bacterial growth): fresh: 3.92 vs frozen for 4 days:
		3.61 vs frozen for 8 days: 3.54, no significant differences
		Group 2 (containing saprophytes): fresh: 3.84 vs frozen for 4 days: 3.8 vs frozen for
		8 days: 3.61 (p = 0.003)
		Group 3 (containing potential pathogens): fresh: 4.75 vs frozen for 4 days: 4.76 vs
		frozen for 8 days: 4.65, (p = 0.002)
[59]	Fresh (up to 3 hours before analysis)	Maximum 5% decrease in triacylglycerols after freeze-thawing of fresh breast milk
	Analysed directly after 1, 2, or 3 freeze-thaw cycles (dry ice and	Breast milk storage at -20 °C resulted in a 10% decrease of triacylglycerols, or a

	acetone-cold water for thawing) Storage at -20°C or -70°C for 5 months after 0, 1, 2, or 3 freeze-thaw cycles	13% decrease after 3 freeze-thaw cycles Storage at -70°C resulted in no significant changes
[60]	Fresh -20°C for 7, 15, 30, 60, 90 days	Significant total fat reduction in breast milk at each day after storage Fat (g/dL): Fresh: 4.88 vs 7 days frozen: 4.69 ($p = 0.002$) vs 15 days frozen: 4.54 ($p = 0.001$) vs 30 days frozen: 4.54 ($p < 0.001$) vs 60 days frozen: 4.37 ($p < 0.001$) vs 90 days frozen: 4.19 ($p < 0.001$)
[62]	Fresh (4°C during same day transport to the laboratory) Pasteurised, then stored at -20°C for 35, 70, 90 days	Post-pasteurisation frozen storage for 90 days decreased total fat concentration by 5.7%, which was above the relative standard deviation of fresh breast milk
[63]	Storage in donors' freezers until transfer to the hospital Storage at -20°C at the hospital Thawing, heating to 40°C and homogenisation, Holder pasteurisation, heating to 40°C and homogenisation Analysis day 0 (post-pasteurisation)	Significant total fat decrease over time ($p = 0.001$) in frozen breast milk Mean difference between 0 and 180 days was -0.13 g/dL

	Storage at -20°C for 30, 60, 90, 120, 150, 180 days	
[64]	Fresh (up to 48 hours at 4°C) Holder pasteurised, then stored at -25°C for 1 month	No significant differences in total lipid or relative fatty acid concentration between fresh or pasteurised and frozen breast milk
[65]	Pasteurised, then stored at -20°C for 1, 2, 3, 4, 5, 6, 8, 10, 12 months	No significant differences in total fat between any of the storage times
[61]	Fresh (up to 24 hours at 4°C) -80°C for mean of 43.8 days (range 8-83 days)	Significant decrease of total fat concentration after frozen storage Total fat (g/100 mL): Fresh: 37.2 vs frozen: 3.36 ($p < 0.001$)
[66]	Fresh (up to 3 hours at 18-20°C) Frozen at -20°C, thawed at room temperature once, twice or three times	Freezing and thawing resulted in a loss of absolute milk triacylglycerols Relative amounts of saturated and monounsaturated fatty acids of breast milk triacylglycerols did not change significantly after two freeze-thaw cycles Relative LA concentration (%) changed significantly: Control: 6.64 vs freeze thaw 1: 4.71 vs freeze thaw 2: 2.35 ($p < 0.01$) vs freeze thaw 3: 2.60 ($p < 0.002$) ALA could not be measured accurately
[67]	Frozen at -20°C, thawed in a water-bath (40°C, 10 minutes) or in a microwave (45 seconds)	No significant difference in fat content between the two thawing methods
[68]	Frozen at -20°C, thawed under tepid water or by waterless dry	No significant differences in fat content between the two thawing methods

heat warmer

4. FREE FATTY ACID LEVELS

The lipid portion of breast milk consists of approximately 98% triacylglycerols, 1% phospholipids, and 0.4% cholesterol and cholesterol esters [73]. Breast milk also contains the bile salt-dependent lipase, which aids in the digestion of milk fat and compensates for the immature digestive system in new-borns [74, 75]. However, the bile salt-dependent lipase loses its bile salt specificity during two weeks frozen storage at -10°C [76], potentially resulting in lipolysis of triacylglycerols and an increase in free fatty acid levels. Freezing and thawing also damages the fat globule membrane, allowing the lipases greater access to triacylglycerols, thereby increasing free fatty acid levels [59, 77]. LCPUFAs appear more susceptible to hydrolysis than shorter-chain fatty acids and the degree of hydrolysis is temperature and time dependent [78]. Additionally, elevated levels of free fatty acids have the potential to increase lipid peroxidation [79, 80], discussed in Section 6.

Storing breast milk at 4°C increases the free fatty acid content significantly from 51% to 454% after 24 hours [51, 78, 81], from 76% to 502% after 48 hours [51, 78], from 85% to 101% after 72 hours [82] and by 265% after 96 hours [50], although breast milk samples in the latter study were stored at -80°C after refrigeration, until subsequent free fatty acid analysis [50]. In a time course experiment, higher free fatty acid levels were seen at 48 hours compared to 24 hours, although not statistically significant, whereas levels were significantly higher at 72 hours, with the greatest increases seen with omega-3 PUFAs [51]. However, pasteurised, frozen, and thawed DHM samples stored at 4°C for up to 96 hours show no change in free fatty acid levels following thawing [50].

Free fatty acids in breast milk increased significantly (+589% vs baseline) after eight weeks storage at -11°C [78], also, an accumulation of free fatty acids has been seen after 24 hours at -20°C, increasing by 167% after 30 days, and 833% after 180 days [83]. This is supported by other studies showing significant increases in free fatty acids after storage at -20°C for two to five months [84], four months [77], five months [59], and nine months [52]. No free fatty acids were detected after breast milk storage at -80°C for four months [77]. Storage for two months [78], two to five months [84], or five months [59] at -70°C did also not increase free fatty acid concentrations significantly. Storage of Holder pasteurised breast milk for one month at -25°C [64], or for three months at -20°C [62] did not significantly alter the free fatty acid content, and heating for 1.5 minutes at 80°C prevented the formation of free fatty acids in breast milk samples stored for four months at -20°C [77].

Thoroughly thawing DHM and keeping it in the fridge for a maximum of 24 hours is recommended by the U.K. guidelines [15]; however, thawing breast milk at 4°C for 24 hours resulted in 10% and 29% higher free fatty acid concentrations than thawing at room temperature for 2.5 to 4.25 hours,

or thawing in a water-bath (50°C, 12 to 30 minutes), respectively [85]. A significant increase in free fatty acids was found after thawing (tepid water or waterless dry heater) breast milk [68]. Furthermore, refrigeration of thawed breast milk for up to 24 hours before warming and feeding further increased free fatty acids compared to only warming. Thawing and storing breast milk for 24 hours at 4°C, after 30 days storage at -20°C, further increased the free fatty acid concentration by approximately 288%, compared to storage at -20°C for 30 days alone [83].

Overall, the evidence strongly suggests that storing breast milk at 4°C prior to pasteurisation significantly increases the free fatty acid levels, although these changes are not observed post-pasteurisation. These differences are potentially due to inactivation of the breast milk lipases [86]. Similarly, pre-pasteurisation storage at -20°C has been shown to increases free fatty acid levels, which are also not seen in post-pasteurisation breast milk. Therefore, in order to minimise increases in free fatty acids levels, it is recommended expressed breast milk should be frozen immediately and stored at the lowest possible temperature (ideally -70°C or below) prior to pasteurisation.

5. LIPID MEDIATORS

5.1 Eicosanoids

Eicosanoids include the eicosapentaenoic acid (EPA, 20:5n-3) and ARA-derived thromboxanes, prostaglandins and leukotrienes, which are important mediators of the inflammatory response [87]. Prostaglandins also modulate gastrointestinal function and may protect against gastrointestinal injuries [88]. Eicosanoids (leukotriene E_4, prostaglandin E_2, cysteinyl leukotrienes, prostaglandins E and F, as well as the inactive thromboxane A2, prostacyclin, and prostaglandin F metabolites thromboxane B_2, 6-keto-prostaglandin $F_1\alpha$, and 13,14-dihydro-15-ketoprostaglandin) are secreted into breast milk [89-91]. To the authors' knowledge, there have to date been no published studies looking at the effects of storage conditions or DHM processing on eicosanoid levels in breast milk. However, Lucas and Mitchel hypothesize that a low 13,14-dihydro-15-ketoprostaglandin F:prostaglandin F ratio in breast milk suggests that prostaglandins are not rapidly metabolised in breast milk and may persist long enough to have an effect in the infant [89], and tritiated prostaglandins show minimal degradation after incubation in breast milk for 30 minutes at 37°C [92], suggesting that further work should seek to explore this area.

5.2 Specialised pro-resolving mediators

Specialised pro-resolving mediators (SPMs) facilitate the resolution of inflammation, are anti-inflammatory, reduce pain, and facilitate wound healing [93, 94]. They include the ARA-derived lipoxins, EPA-derived resolvins, and docosapentaenoic acid (22:5n-3) and DHA-derived resolvins, (neuro)protectins and maresins [95]. Breast milk contains the SPMs resolvin D1, resolvin D2, resolvin D3, resolving D4, resolvin D5, resolvin D6, protectin 1, maresin 1, resolvin E2, resolvion E3, lipoxin A_4 and lipoxin B_4 in biologically relevant concentrations, which have shown to reduce the maximum neutrophil number and to shorten the resolution interval *in vivo* and to stimulate efferocytosis *in vitro* [96]. Resolution of inflammation is especially important for extremely preterm infants, in which sustained elevated inflammation in the first month of life is associated with cognitive impairment at ten years of age [97]. To the authors' knowledge, there are currently no studies that have investigated the effects of storage conditions on specialised pro-resolving mediator levels in breast milk. Interestingly, the breast milk samples in the above study [96] were obtained from a commercial supplier, who stores breast milk at -20°C , and therefore, it is likely that specialised pro-resolving mediators tolerate some frozen storage; however further work should seek to extend these observations and investigate the effects of different storage and processing conditions on SPM levels.

5.3 Endocannabinoids

Endocannabinoids include the ARA derived compounds arachidonoyl ethanolamide (anandamide, AEA), 2-arachidonoylglycerol (2-AG), and the DHA-derived docosahexaenoyl ethanolamide (DHEA) [87], which have been identified in breast milk [98, 99]. The endocannabinoid system plays an important role in neuronal development and neuroprotection early in life [100, 101]. Animal studies showed that 2-AG and activation of the Cannabinoid Receptor 1 plays a critical role in milk suckling, holding on to the nipple, and therefore, growth and survival in the first week of life [98, 100]. An analysis of breast milk found a non-significant increase of 503% in 2-AG levels after storage at 4°C for 24 hours and a significant increase (1166%) after storage at -20°C for three months [99]. Storage at -80°C for three months did not affect AEA and 2-AG concentrations. DHEA was no longer detectable after storage at 4°C for one day, or storage at -20°C or -80°C for three months. The authors suggested that the concentrations of 12 endocannabinoid related compounds (2-AG, AEA, oleoylethanolamide, palmitoylethanolamide, *N*-arachidonoyl glycine, eicosapentaenoyl ethanoalmide, DHEA, *N*-palmitoleoyl-ethanolamine, dihomo-γ-linolenoylethanolamine, *N*-stearoylethanolamine, prostaglandin $F_{2\alpha}$ ethanolamide, prostaglandin E_2 ethanolamide) in breast

milk are stable for a maximum of 24 hours at 4°C, maximum one week at -20°C and that longer term storage requires temperatures of -80°C. The same group also demonstrated that two freeze-thaw cycles, as used in human milk banking, resulted in losses of 37% AEA, 49% 2-AG, and 36% DHEA in bovine milk [102]. Additionally, it has been shown that 2-AG in culture medium and biological buffers adheres to glass and plastic surfaces [103], which could impact on their availability for the infant.

6. LIPID PEROXIDATION PRODUCTS

Omega-3 and omega-6 LCPUFAs are highly susceptible to peroxidation by oxygen radicals [104]. There is a linear dependency between the number of double bonds and the oxidisability of PUFAs [105]. Lipid hydroperoxides are unstable primary lipid peroxidation products, which react further to form secondary lipid peroxidation products [106]. For example, malondialdehyde (MDA) is produced from the unspecific peroxidation of PUFAs with more than two double bonds. At high levels, lipid peroxidation products can bind to DNA and proteins, which can lead to cell and tissue damage, and may thereby increase inflammation [107]. Repeated intake of lipid peroxidation products has been shown to induce growth retardation, intestinal irritation, cardiovascular diseases, and to be carcinogenic in animal studies [108]. Direct activation of inflammatory pathways such as nuclear factor κ B has also been shown after feeding lipid peroxidation products to mice [109]. More importantly, lipid peroxidation products do not only act locally in the intestine, but can also be absorbed and act elsewhere in the body [109].

Various lipid peroxidation products have been detected in breast milk, including MDA [79, 110], the omega-6 and omega-3 PUFA derived 4-hydroxy-2-hexanal (4-HNE) and 4-hydroxy-2-nonenal (4-HHE), respectively [111], lipid hydroperoxides [80, 112], isoprostanes [113], alkanals including pentanal, hexanal, octanal, nonanal, and 2-octanal [114], as well as conjugated dienes [80]. Storage of fresh breast milk for 24 hours at room temperature significantly increases the 4-HNE:omega-6 fatty acid ratio [111]. Storage at 4°C for 48 hours was shown to significantly increase MDA content of breast milk [110], whereas others found storage at 4°C for 96 hours has no effect on MDA content of preterm milk [51] potentially due to higher antioxidant capacity in the latter [115]. Although thiobarbituric acid reactive substances increased by 66% and conjugated dienes by 31% in the same samples, this was not statistically significant [51]. Storage at 4°C for four days increases LA hydroperoxides significantly [112].

No significant increases in breast milk MDA levels were seen after storage at -20°C for ten days [110], or 15 or 30 days, although increases were found after 60 days [116]. Similarly, no increases in

thiobarbituric acid reactive substances or conjugated dienes were seen after storage at -20°C for two months, although significant increases in precursor lipid hydroperoxides were found [80]. However, it should be noted that the fresh breast milk samples were from different donors than the frozen samples. MDA levels also significantly increased in term breast milk stored at -80°C for 60 days [116]. Hexanal levels significantly increased after three months storage at -18°C, with further increases after five and six months [117]. In this study, four months storage of breast milk in amber glass bottles also reduced the hexanal increase significantly compared clear glass bottles or low density polyethylene bags. Overall, the literature suggests an increase in lipid peroxidation when breast milk is stored at 4°C, with short-term storage at -20°C for maximal one month preferable, although there needs to be more research to clarify this, as well as whether storage at -80°C would be beneficial.

7. ANTIOXIDANTS

Preterm infants have immature antioxidant systems and inadequate antioxidant capacity [118] and there is a frequent requirement for blood transfusions, which increases oxidative stress [119]. Furthermore, the foetal to neonatal transition rapidly increases tissue oxygenation, thereby abruptly increasing the generation of reactive oxygen species [120], and oxygen therapy as well as total parenteral nutrition expose the premature infant to further sources of oxidative stress [121]. As a consequence, there is great potential for peroxidation of endogenous lipids and subsequent tissue damage. Bronchopulmonary dysplasia, retinopathy of prematurity, necrotising enterocolitis and peri-ventricular leukomalacia are common comorbidities in preterm infants, which are classified as oxygen radical associated diseases [122]. Moreover, extremely and very preterm infants are not routinely supplemented with dietary antioxidants, as there has been limited research in this area and the outcomes of some trials have been equivocal [123]. Therefore, breast milk, which includes enzymatic (e.g. superoxide dismutase, glutathione peroxidase) and non-enzymatic (e.g. vitamin C, vitamin E, glutathione) antioxidants, is the only enteral source of antioxidants for preterm infants. However, DHM, compared to breast milk, has significantly lower concentrations of several antioxidants [124].

Antioxidants are not only beneficial to the infant directly, but they also serve to protect PUFAs in the breast milk from lipid peroxidation and may subsequently decrease the levels of potentially toxic compounds [125]. For example, vitamin C can directly prevent lipid peroxidation by scavenging free radicals, and thereby preventing the initiation stage of lipid peroxidation [126], and vitamin E can scavenge lipid peroxyl radicals and is then regenerated by vitamin C [127], which in turn is regenerated by glutathione [128]. Glutathione and the glutathione peroxidase can form more stable

lipid alcohols from lipid hydroperoxides [129], and glutathione is also involved in the detoxification of MDA [130]. Although evidence for the prevention of lipid peroxidation in human milk by antioxidants is limited, evidence suggests that the vitamin E content of formula milk is inversely related to thiobarbituric acid reactive substances and conjugated dienes [80, 131], and lower glutathione peroxidase activity is associated with higher MDA concentrations in breast milk following refrigeration [110]. Due to the interplay and synergistic effects between antioxidants, antioxidant capacity should also be considered an appropriate measure of the antioxidant status of breast milk.

7.1 Vitamin C

Term breast milk contains around 34.7 ± 1.33 mg/L vitamin C (ascorbic acid + dehydroascorbic acid) [23]. Significant reductions in the vitamin C content of term breast milk have been reported after storage at 4°C for six hours, and 24 hours [24, 49, 132-134], and after one week at -4 to -8°C [49], as well as after two months at -16°C [24]. Interestingly, significant decreases in vitamin C were seen after three months storage at -20°C in term, but not preterm breast milk [134], although, in another study significant decreases were seen in preterm breast milk after seven and 30 day storage at the same temperature [135]. However, others have reported that vitamin C levels are stable at -20°C in pooled breast milk for four week [136], and up to three months, but significantly decrease after eight months [23]. Vitamin C content appears stable with storage at -80°C for eight months, although a significant decrease of 12% was seen at 12 months [23]. Overall, although the results are somewhat mixed, the evidence supports breast milk storage at lower temperatures to protect Vitamin C content, with storage at both 4°C and -20°C leading to decreases, and storage at -80°C preferable, for the maximum recommended storage time of six months, although this is based on one publication.

7.2 Vitamin E

Vitamin E is a class of compounds including α-, β-, γ- and δ-tocopherol, with α-tocopherol being the main isomer in term mature breast milk, and one of the main contributors to antioxidant capacity of breast milk, which is found at concentrations of 2.32 ± 0.11 mg/L [137]. Storage of breast milk at 4°C for 24 hours did not affect α- and γ-tocopherol levels in several studies [23, 49, 138], likewise, no significant changes were found after 48 hours for α-, β-, γ-, and δ-tocopherol [46], although others have reported significant reductions in α- and γ-tocopherol levels after 48 hours [23]. Storing breast milk at -4 to -8°C for one week resulted in a significant decrease in vitamin E [49]. Storage at -20°C

did not affect vitamin E levels of breast milk stored for 30 days [46], 16 weeks [138], six months [139], or 12 months [23], and no changes in vitamin E levels were seen after storage for 16 weeks [138], or six months at -70°C [139], or 12 months at -80°C [23]. Overall, the evidence suggests that current human milk banking storage processes are safe to protect the vitamin E content in DHM.

7.3 Superoxide dismutase, glutathione, and glutathione peroxidase

Superoxide dismutase is an enzyme involved in the dismutation of the superoxide radical. Its activity has been reported to be 36 U/mL in term mature breast milk [140]. Although there is a paucity of research in this area, superoxide dismutase activity was reported to be significantly reduced after preterm breast milk was stored at -20°C for seven and 30 days [135]. Glutathione content of mature breast milk is approximately 163.9 µmol/L [130]. A significant 79% loss of glutathione was noted after two hours storage at 4°C as well as at -20°C (-81% vs baseline) [130]. Glutathione peroxidase activity in mature term breast milk was reported as 38.8 U/mL [141]. Significant reductions in activity were seen in term milk after 48 hours at 4°C [110], although these were not reported following storage of preterm breast milk for 30 days at -20°C [135]. Activity decreases were reported with increased storage time at -20°C, with activity completely lost after one week [141], and significant reductions in activity after 15, 30 and 60 days in another study [116]. However, significant reductions were only shown after 60 days at -80°C, where the activity was not significantly different between the -20°C and -80°C conditions.

7.4 Total antioxidant capacity

Total antioxidant capacity (TAC) measures the additive effects of antioxidants and may provide a more useful measure than the assessment of individual antioxidants [142]. However, the different analytical methods for TAC have a weak or no correlations [143], making it difficult to compare results between studies. Significant reductions in the TAC of preterm and term breast milk have been reported after storage at 4°C for 48 [144], and 72 hours [145], although, others have shown that storing pooled preterm breast milk at 6.8°C for up to 96 hours not affect TAC [51]. Freezing breast milk at -20°C shows significantly reduced antioxidant capacity after 48 hours, which further decreased after one week [146], and a significant decrease after one week with further decrease after one month [147], with similar effects seen at -8°C [144]. However, others report no changes in TAC after storing preterm milk for 30 days at -20°C [135], or storing DHM at -20°C for two months [80]. Preterm colostrum stored for up to three months at -80°C did not show any change in TAC

[148], whereas term mature breast milk stored at -80°C showed significantly lower TAC after two months [149]. Thawing breast milk at 4°C for 24 hours (as recommended by the U.K. guideline [15]), as well as thawing at room temperature for 2.5 to 4.25 hours did not change TAC [85], whereas using a water-bath for thawing (50°C, 12 to 30 minutes) resulted in a significant decrease in TAC. Overall, the evidence of different storage conditions on TAC is equivocal, potentially due to differences in analytical techniques, or it may be an indicator of potential variations in the antioxidant requirements of the different samples.

8. CONCLUSION AND RECOMMENDATIONS

Current human milk banking practices have been developed to provide microbiological safe DHM, with limited emphasis on the nutritional quality of DHM. There are currently no globally accepted guidelines for human milk banking practices, with wide variations in practices, regulations, and organization in each country, in part due to a lack of robust evidence [22]. However, more consideration must be given to the nutritional quality of DHM to ensure optimum nutritional intake for the infants. Specific focus should be given to components such as LCPUFAs, bioactive lipid mediators, and their supporting antioxidants, as their levels are essential for the health and development of preterm infants. The literature reviewed within this article clearly demonstrates that the quality of DHM can be influenced by the various storage and processing conditions used in human milk banking. The observations of minimal changes in fat composition are consistent with a recent systematic review [25]; however, levels of lipid peroxidation products, and endogenous antioxidants appear more sensitive to the storage conditions, and when considering the effects of human milking banking practices on overall DHM lipid nutritional quality these aspects should also be considered, although further research is needed to understand these effects.

Due to the diversity of methodological approaches, and biological variability of the human breast milk samples, there remain many uncertainties and a general lack of consistency in the current literature around the optimal DHM storage conditions. Indeed, this is even more apparent when considering a range of different nutritional components, where there are different sensitivities to the storage conditions and processing. It is clear that further research is needed to improve the evidence base for human milk banking practices, particularly on the effects of storage conditions on bioactive compounds such as eicosanoids, SPMs and the TAC of DHM. However, in the interim, in order to maximise the LCPUFA content, and to ensure maintenance of supporting antioxidants we must accept a certain degree of uncertainty and adopt a precautionary approach. Therefore we

suggest considering the following recommendations where possible, to supplement current local and national guidelines:

- Breast milk containers should protect the milk from exposure to light, either through the use of amber containers, or if unavailable, other approaches should be put in place, such as wrapping containers in aluminium foil, and putting covers over fridges and freezers with glass doors.
- DHM should be frozen at -20°C directly after expression, instead of pooling over 24 hours in the fridge.
- Storage at 4°C at the human milk bank should be minimized wherever possible, and every effort should be made to transport DHM to the human milk bank as soon as possible after expression.
- At the human milk bank the DHM should ideally be frozen at -70°C or below, particularly prior to pasteurisation, although more research is needed to explore the effects of long-term storage of post-pasteurised DHM.
- Using different thawing methods (at room temperature, in the fridge or using a water-bath), affects breast milk components differently, and currently, the evidence suggests that thawing at 4°C is not detrimental to the fat content or TAC of the DHM.

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