

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

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21 **Abstract**

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

33

34 **Keywords:**

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

37 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

38

1. INTRODUCTION

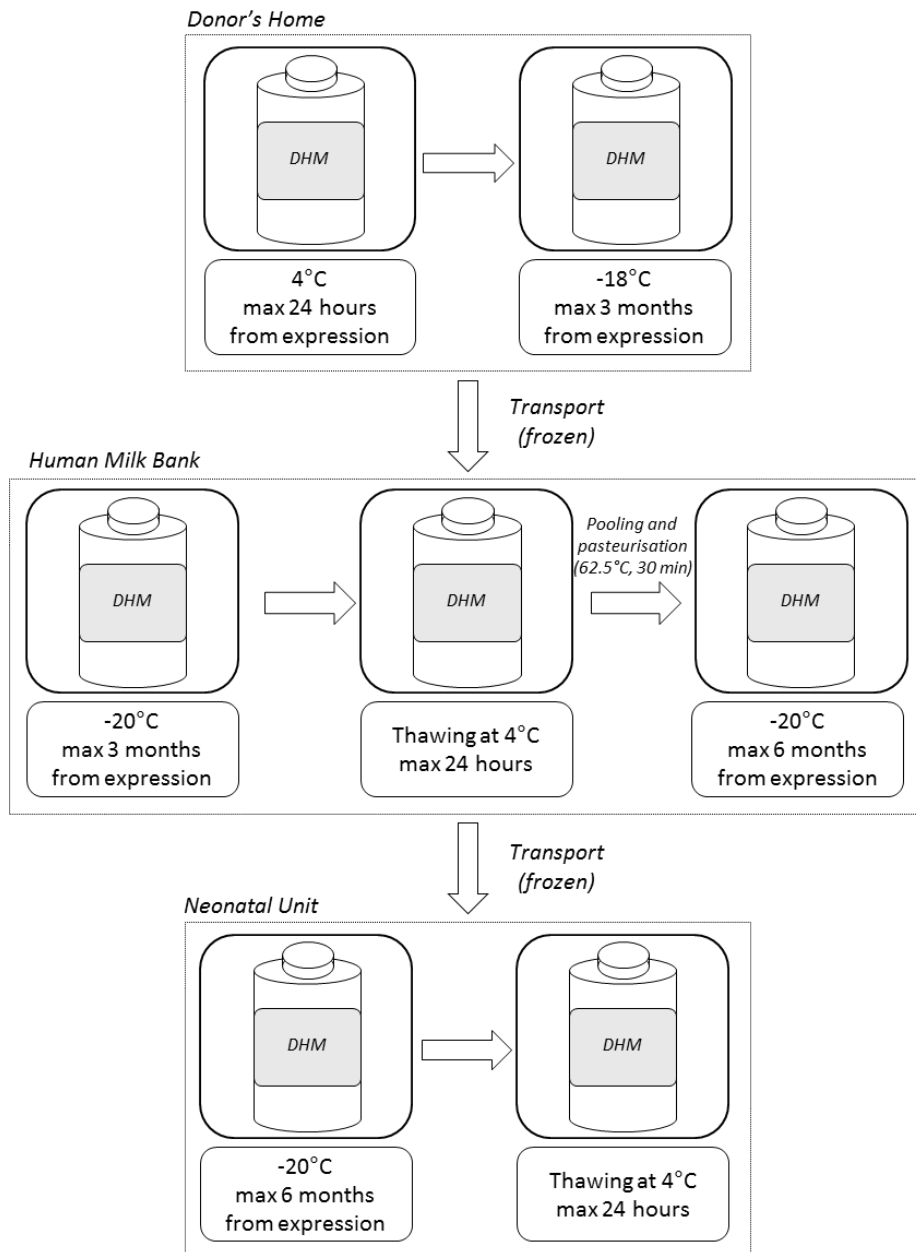
39 Mother's own breast milk is the accepted best practice for feeding neonates [1] and exclusive breast
40 feeding for the first six months of life is recommended [2] for term infants. For preterm infants as
41 well, mother's own breast milk is the favoured feeding choice. However, it may need to be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

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

63 DHM undergoes prolonged cold storage, freeze-thaw cycles, and processing, before it is fed to
64 infants, which may negatively affect the breast milk composition. For example, in the U.K., expressed
65 breast milk for donation can be stored for up to 24 hours at 4°C, before transferring to a -18°C (or
66 below) freezer for up to three months [15]. Breast milk is then thawed, Holder pasteurised (62.5°C,
67 30 minutes) and refrozen for up to three months. Before feeding, thawed pasteurised DHM can be
68 stored at 4°C for up to 24 hours. These conditions have been summarised in Figure 1. Similar
69 guidelines are followed widely, including in Australia, North America, Sweden, Italy, Spain, and India
70 [16-21]. Additionally, in Italy, breast milk undergoing direct pasteurisation after expression, can be

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

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76 *Figure 1: Donor human milk storage and processing conditions*

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

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

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2. SEARCH METHODOLOGY

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

112

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

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

140 Storing breast milk at 4°C for 48 hours has been shown to not significantly change the absolute or
141 relative fatty acid content [46], or triacylglycerol content [47]. The latter was also not affected by
142 refrigeration at 4°C for up to three days [48]. Total lipid content was also unchanged by refrigeration
143 at 4°C for 24 hours [49], or up to 96 hours [50]. Similarly, polyunsaturated fatty acid (PUFA) content
144 (including linoleic acid (LA, 18:2n-6), α -linolenic acid (ALA, 18:3n-3), ARA and DHA), as well as
145 saturated and monounsaturated fatty acid content was not significantly altered when stored for
146 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

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

184 In conclusion, the available evidence suggests that storage at 4°C is sufficient to minimise decreases
185 in total fat and LCPUFAs in breast milk for up to 96 hours. However, for longer-term storage the data
186 for storage at -20°C is highly discordant, especially pre-pasteurisation. These differences may be
187 related to variations in analytical methods used [69, 70], or methodological variations, such as not
188 sufficiently homogenising the breast milk after storage [71], differences in fat adherences to the
189 container walls [54, 61], particularly to polyethylene [72], and variations in fat loss due to different
190 thawing methods [55], which are not specifically defined in the literature. Furthermore, breast milk
191 is a complex biological matrix, and variations in unmeasured endogenous antioxidant levels or other
192 components, may influence fat content stability during storage, discussed further in Section 7 below.
193 However, overall the evidence suggests storage at -80°C is the best option for longer-term storage to
194 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 -20°C for 30 days	Significant fat decrease after frozen storage 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) -20°C or -80°C for 4, 12, 24 weeks	Fat content was consistently higher in breast milk stored at -80°C than at -20°C (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 -20°C for 4, 8 days	Significant total lipid decrease in breast milk stored at -20°C 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) Analysed directly after 1, 2, or 3 freeze-thaw cycles (dry ice and	Maximum 5% decrease in triacylglycerols after freeze-thawing of fresh breast milk Breast milk storage at -20 °C resulted in a 10% decrease of triacylglycerols, or a

	acetone-cold water for thawing)	13% decrease after 3 freeze-thaw cycles
	Storage at -20°C or -70°C for 5 months after 0, 1, 2, or 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

196

4. FREE FATTY ACID LEVELS

197 The lipid portion of breast milk consists of approximately 98% triacylglycerols, 1% phospholipids, and
198 0.4% cholesterol and cholesterol esters [73]. Breast milk also contains the bile salt-dependent lipase,
199 which aids in the digestion of milk fat and compensates for the immature digestive system in new-
200 borns [74, 75]. However, the bile salt-dependent lipase loses its bile salt specificity during two weeks
201 frozen storage at -10°C [76], potentially resulting in lipolysis of triacylglycerols and an increase in
202 free fatty acid levels. Freezing and thawing also damages the fat globule membrane, allowing the
203 lipases greater access to triacylglycerols, thereby increasing free fatty acid levels [59, 77]. LCPUFAs
204 appear more susceptible to hydrolysis than shorter-chain fatty acids and the degree of hydrolysis is
205 temperature and time dependent [78]. Additionally, elevated levels of free fatty acids have the
206 potential to increase lipid peroxidation [79, 80], discussed in Section 6.

207 Storing breast milk at 4°C increases the free fatty acid content significantly from 51% to 454% after
208 24 hours [51, 78, 81], from 76% to 502% after 48 hours [51, 78], from 85% to 101% after 72 hours
209 [82] and by 265% after 96 hours [50], although breast milk samples in the latter study were stored
210 at -80°C after refrigeration, until subsequent free fatty acid analysis [50]. In a time course
211 experiment, higher free fatty acid levels were seen at 48 hours compared to 24 hours, although not
212 statistically significant, whereas levels were significantly higher at 72 hours, with the greatest
213 increases seen with omega-3 PUFAs [51]. However, pasteurised, frozen, and thawed DHM samples
214 stored at 4°C for up to 96 hours show no change in free fatty acid levels following thawing [50].

215 Free fatty acids in breast milk increased significantly (+589% vs baseline) after eight weeks storage
216 at -11°C [78], also, an accumulation of free fatty acids has been seen after 24 hours at -20°C,
217 increasing by 167% after 30 days, and 833% after 180 days [83]. This is supported by other studies
218 showing significant increases in free fatty acids after storage at -20°C for two to five months [84],
219 four months [77], five months [59], and nine months [52]. No free fatty acids were detected after
220 breast milk storage at -80°C for four months [77]. Storage for two months [78], two to five months
221 [84], or five months [59] at -70°C did also not increase free fatty acid concentrations significantly.
222 Storage of Holder pasteurised breast milk for one month at -25°C [64], or for three months at -20°C
223 [62] did not significantly alter the free fatty acid content, and heating for 1.5 minutes at 80°C
224 prevented the formation of free fatty acids in breast milk samples stored for four months at -20°C
225 [77].

226 Thoroughly thawing DHM and keeping it in the fridge for a maximum of 24 hours is recommended
227 by the U.K. guidelines [15]; however, thawing breast milk at 4°C for 24 hours resulted in 10% and
228 29% higher free fatty acid concentrations than thawing at room temperature for 2.5 to 4.25 hours,

229 or thawing in a water-bath (50°C, 12 to 30 minutes), respectively [85]. A significant increase in free
230 fatty acids was found after thawing (tepid water or waterless dry heater) breast milk [68].
231 Furthermore, refrigeration of thawed breast milk for up to 24 hours before warming and feeding
232 further increased free fatty acids compared to only warming. Thawing and storing breast milk for
233 24 hours at 4°C, after 30 days storage at -20°C, further increased the free fatty acid concentration by
234 approximately 288%, compared to storage at -20°C for 30 days alone [83].

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

242

243

5. LIPID MEDIATORS

244

5.1 Eicosanoids

245 Eicosanoids include the eicosapentaenoic acid (EPA, 20:5n-3) and ARA-derived thromboxanes,
246 prostaglandins and leukotrienes, which are important mediators of the inflammatory response [87].
247 Prostaglandins also modulate gastrointestinal function and may protect against gastrointestinal
248 injuries [88]. Eicosanoids (leukotriene E₄, prostaglandin E₂, cysteinyl leukotrienes, prostaglandins E
249 and F, as well as the inactive thromboxane A₂, prostacyclin, and prostaglandin F metabolites
250 thromboxane B₂, 6-keto-prostaglandin F_{1α}, and 13,14-dihydro-15-ketoprostaglandin) are secreted
251 into breast milk [89-91]. To the authors' knowledge, there have to date been no published studies
252 looking at the effects of storage conditions or DHM processing on eicosanoid levels in breast milk.
253 However, Lucas and Mitchell hypothesize that a low 13,14-dihydro-15-
254 ketoprostaglandin F:prostaglandin F ratio in breast milk suggests that prostaglandins are not rapidly
255 metabolised in breast milk and may persist long enough to have an effect in the infant [89], and
256 tritiated prostaglandins show minimal degradation after incubation in breast milk for 30 minutes at
257 37°C [92], suggesting that further work should seek to explore this area.

258

259 **5.2 Specialised pro-resolving mediators**

260 Specialised pro-resolving mediators (SPMs) facilitate the resolution of inflammation, are anti-
261 inflammatory, reduce pain, and facilitate wound healing [93, 94]. They include the ARA-derived
262 lipoxins, EPA-derived resolvins, and docosapentaenoic acid (22:5n-3) and DHA-derived resolvins,
263 (neuro)protectins and maresins [95]. Breast milk contains the SPMs resolvin D1, resolvin D2, resolvin
264 D3, resolving D4, resolvin D5, resolvin D6, protectin 1, maresin 1, resolvin E2, resolvion E3, lipoxin A₄
265 and lipoxin B₄ in biologically relevant concentrations, which have shown to reduce the maximum
266 neutrophil number and to shorten the resolution interval *in vivo* and to stimulate efferocytosis *in*
267 *vitro* [96]. Resolution of inflammation is especially important for extremely preterm infants, in which
268 sustained elevated inflammation in the first month of life is associated with cognitive impairment at
269 ten years of age [97]. To the authors' knowledge, there are currently no studies that have
270 investigated the effects of storage conditions on specialised pro-resolving mediator levels in breast
271 milk. Interestingly, the breast milk samples in the above study [96] were obtained from a commercial
272 supplier, who stores breast milk at -20°C , and therefore, it is likely that specialised pro-resolving
273 mediators tolerate some frozen storage; however further work should seek to extend these
274 observations and investigate the effects of different storage and processing conditions on SPM
275 levels.

276

277 **5.3 Endocannabinoids**

278 Endocannabinoids include the ARA derived compounds arachidonoyl ethanolamide (anandamide,
279 AEA), 2-arachidonoylglycerol (2-AG), and the DHA-derived docosahexaenoyl ethanolamide (DHEA)
280 [87], which have been identified in breast milk [98, 99]. The endocannabinoid system plays an
281 important role in neuronal development and neuroprotection early in life [100, 101]. Animal studies
282 showed that 2-AG and activation of the Cannabinoid Receptor 1 plays a critical role in milk suckling,
283 holding on to the nipple, and therefore, growth and survival in the first week of life [98, 100]. An
284 analysis of breast milk found a non-significant increase of 503% in 2-AG levels after storage at 4°C for
285 24 hours and a significant increase (1166%) after storage at -20°C for three months [99]. Storage at -
286 80°C for three months did not affect AEA and 2-AG concentrations. DHEA was no longer detectable
287 after storage at 4°C for one day, or storage at -20°C or -80°C for three months. The authors
288 suggested that the concentrations of 12 endocannabinoid related compounds (2-AG, AEA,
289 oleoylethanolamide, palmitoylethanolamide, *N*-arachidonoyl glycine, eicosapentaenoyl
290 ethanoamide, DHEA, *N*-palmitoleoyl-ethanolamine, dihomo- γ -linolenylethanolamine,
291 *N*-stearoylethanolamine, prostaglandin F_{2 α} ethanolamide, prostaglandin E₂ ethanolamide) in breast

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

298

299

6. LIPID PEROXIDATION PRODUCTS

300 Omega-3 and omega-6 LCPUFAs are highly susceptible to peroxidation by oxygen radicals [104].
301 There is a linear dependency between the number of double bonds and the oxidisability of PUFAs
302 [105]. Lipid hydroperoxides are unstable primary lipid peroxidation products, which react further to
303 form secondary lipid peroxidation products [106]. For example, malondialdehyde (MDA) is produced
304 from the unspecific peroxidation of PUFAs with more than two double bonds. At high levels, lipid
305 peroxidation products can bind to DNA and proteins, which can lead to cell and tissue damage, and
306 may thereby increase inflammation [107]. Repeated intake of lipid peroxidation products has been
307 shown to induce growth retardation, intestinal irritation, cardiovascular diseases, and to be
308 carcinogenic in animal studies [108]. Direct activation of inflammatory pathways such as nuclear
309 factor κ B has also been shown after feeding lipid peroxidation products to mice [109]. More
310 importantly, lipid peroxidation products do not only act locally in the intestine, but can also be
311 absorbed and act elsewhere in the body [109].

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

323 No significant increases in breast milk MDA levels were seen after storage at -20°C for ten days
324 [110], or 15 or 30 days, although increases were found after 60 days [116]. Similarly, no increases in

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

335

336

7. ANTIOXIDANTS

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

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

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

365

366 **7.1 Vitamin C**

367 Term breast milk contains around 34.7 ± 1.33 mg/L vitamin C (ascorbic acid + dehydroascorbic acid)
368 [23]. Significant reductions in the vitamin C content of term breast milk have been reported after
369 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
370 well as after two months at -16°C [24]. Interestingly, significant decreases in vitamin C were seen
371 after three months storage at -20°C in term, but not preterm breast milk [134], although, in another
372 study significant decreases were seen in preterm breast milk after seven and 30 day storage at the
373 same temperature [135]. However, others have reported that vitamin C levels are stable at -20°C in
374 pooled breast milk for four week [136], and up to three months, but significantly decrease after
375 eight months [23]. Vitamin C content appears stable with storage at -80°C for eight months,
376 although a significant decrease of 12% was seen at 12 months [23]. Overall, although the results are
377 somewhat mixed, the evidence supports breast milk storage at lower temperatures to protect
378 Vitamin C content, with storage at both 4°C and -20°C leading to decreases, and storage at -80°C
379 preferable, for the maximum recommended storage time of six months, although this is based on
380 one publication.

381

382 **7.2 Vitamin E**

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

390 did not affect vitamin E levels of breast milk stored for 30 days [46], 16 weeks [138], six months
391 [139], or 12 months [23], and no changes in vitamin E levels were seen after storage for 16 weeks
392 [138], or six months at -70°C [139], or 12 months at -80°C [23]. Overall, the evidence suggests that
393 current human milk banking storage processes are safe to protect the vitamin E content in DHM.

394

395 **7.3 Superoxide dismutase, glutathione, and glutathione peroxidase**

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

409

410 **7.4 Total antioxidant capacity**

411 Total antioxidant capacity (TAC) measures the additive effects of antioxidants and may provide a
412 more useful measure than the assessment of individual antioxidants [142]. However, the different
413 analytical methods for TAC have a weak or no correlations [143], making it difficult to compare
414 results between studies. Significant reductions in the TAC of preterm and term breast milk have
415 been reported after storage at 4°C for 48 [144], and 72 hours [145], although, others have shown
416 that storing pooled preterm breast milk at 6.8°C for up to 96 hours not affect TAC [51]. Freezing
417 breast milk at -20°C shows significantly reduced antioxidant capacity after 48 hours, which further
418 decreased after one week [146], and a significant decrease after one week with further decrease
419 after one month [147], with similar effects seen at -8°C [144]. However, others report no changes in
420 TAC after storing preterm milk for 30 days at -20°C [135], or storing DHM at -20°C for two months
421 [80]. Preterm colostrum stored for up to three months at -80°C did not show any change in TAC

422 [148], whereas term mature breast milk stored at -80°C showed significantly lower TAC after two
423 months [149]. Thawing breast milk at 4°C for 24 hours (as recommended by the U.K. guideline [15]),
424 as well as thawing at room temperature for 2.5 to 4.25 hours did not change TAC [85], whereas using
425 a water-bath for thawing (50°C, 12 to 30 minutes) resulted in a significant decrease in TAC. Overall,
426 the evidence of different storage conditions on TAC is equivocal, potentially due to differences in
427 analytical techniques, or it may be an indicator of potential variations in the antioxidant
428 requirements of the different samples.

429

430

8. CONCLUSION AND RECOMMENDATIONS

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

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

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

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

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473

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481

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