REVIEW ARTICLE

Lyophilized (freeze-dried) human milk for preterm infants: a scoping review

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Freeze-drying (FD), or lyophilization, is commonly used to preserve foods. FD offers potential to create a human milk-derived human milk fortifier, and an alternative to freeze-storing human milk. However, processing human milk is known to affect its components. This scoping review explores the effect of FD on the; macronutrient, micronutrient, vitamin, bioactive components, microbes and anti-microbial factors in human milk, and studies where lyophilized human milk has been given to newborn infants. 48 articles were identified after full text review. FD human milk reduces the fat globule size and as well as the quantity of enzymes, vitamin C and immunoglobulin. Common serum electrolyte disturbances have been reported when preterm infants' are fed FD human milk however it appears a promising method to avoid exposure of preterm infants' to cows' milk. Due to limited data, further studies exploring the safety and efficacy of FD human milk in preterm infants are needed.

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INTRODUCTION

Freeze-drying (FD), or lyophilization, has been long described for preserving food products [1]. FD involves freezing a substance and then using low pressure to sublime the frozen water into vapor, leaving a dry product [2] The amount of water extracted during the process can be adjusted [1].

FD human milk as a concept is appealing to organizations who care for newborn infants; both as a possible means to reduce storage challenges of human milk and as an additive to human milk (fortification) in order to offer additional nutrients to preterm infants (commonly met by fortifiers derived from bovine milk). Human milk is the preferred diet for newborn infants for multiple reasons including associations with improved long-term maternal and infant health and reduced major neonatal morbidities, such as necrotizing enterocolitis and bronchopulmonary dysplasia [3–5]. However, human milk alone does not meet the high nutritional needs of preterm infants. Concentrations of protein, sodium, calcium and phosphorous often do not meet the needs of preterm infants [6].

There remain concerns regarding the effect that FD could have on what is a complex biological fluid. Alterations to human milk could lead to excessive or inadequate macronutrients, micronutrients or vitamins. FD could lead to bacteria growth or loss of antimicrobials and bioactive components. Furthermore, there could be functional changes to any of these elements.

The aim of this review is to examine the effect of FD on the nutrients and bioactive composition of human milk and review the clinical effects of feeding FD human milk to preterm infants.

METHODS

We followed the guidelines of PRISMA.

Inclusion criteria

We included pre-clinical and clinical studies. Studies published in non-English language or as abstracts were not included. The search was limited to human milk only. Criteria for inclusion and exclusion clinical studies were based on PICOD format—(1) target population: preterm infants; (2) intervention: any FD supplement; (3) comparison: bovine-based supplementation or no intervention; (4) outcome: growth as defined in each study or any other changes to milk content in pre-clinical studies; and (5) study design: randomized-controlled and quasi-randomized trials. Search was performed electronically. All retrieved articles were reviewed to determine studies that comprised preterm infants. The differences in opinion encountered during the process of review were resolved by consensus.

Search strategy

The literature search was performed in December 2022. Medline, Pubmed, Scopus, Web of Science, Cochrane Library and Embase were searched individually with the following terms ["freeze dried" OR "freeze dry*" OR "lyophili*" OR "freez*" OR "Freeze Drying" OR "frozen"] AND ["human milk" OR "breast milk"]. All publication types from 1980-2022 were then reviewed electronically using a systematic review management tool (Covidence systematic review software, Veritas Health Innovation, Melbourne, Australia. Available at www.covidence.org). We used 1980 as a cut-off as there were multiple steps to standardize the FD process which were undertaken from 1980 [7]. The combination of text words and exploded medical subject headings were used to maximize the quantity of the data and articles retrieved.

Two study members screened each abstract. Any abstract that was screened as potentially eligible then had a full text review by two study members. Articles were deemed eligible if they

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described the effect of FD on either the composition of human milk, or clinical effect of FD human milk in newborn infants. Articles were excluded if they were not readily available in English, did not provide new data or offer a comparator.

Data extraction and synthesis

Data charting was completed independently in a standardized format as described by Arksey and O'Malley [8]. Data items sought were year of publication, country of publication, type of milk used (mothers own milk (MOM) or donor human milk (DHM), MOM was defined as freshly expressed milk for analysis without freezing or pasteurization), participants (mothers and/or infants), sample size, setting (in vitro, in vivo), comparison used, FD process (device, pressure, temperature (freezing and for heating), length), and outcomes measured.

RESULTS

We identified 2073 studies using our selection criteria with two additional articles discovered on citation searching. After abstract review and removal of duplicates, 63 full texts remained. Forty-eight articles remained after full text review (Fig. 1). Articles covered all areas of lyophilization of human milk which we have separated into the effect of FD on the macronutrient, micronutrient & vitamins, bioactive component and microbial/antimicrobial composition of human milk. We have separately described articles exploring the clinical effect of feeding FD human milk to newborn infants. The results are presented based on the primary focus of each study (Tables 1–5). Figure 2 summarizes the key study findings.

Macronutrients

Fourteen articles were identified that describe the effect of FD on the macronutrient composition of human milk (Table 1). Two studies described using MOM whilst the others reported using donor human milk (DHM). The study design varied with 4 studies exploring the macronutrient composition of human milk fortified with FD human milk, and the remaining 10 studies exploring the effect of FD on human milk composition.

Lipids

Two studies report that the total lipid content of human milk does not reduce following FD [9, 10]. However, both an increased concentration of lipids in FD DHM (compared to raw DHM) [11, 12], and an assumed decreased concentration of fats (concentration of fat did not double when adding FD DHM to DHM suggesting a loss in fats) have been reported [13]. Several studies explored the effect of FD on the free fatty acid (FFA) composition or lipid profiles of human milk. The majority of these studies did not report any significant change in either FFA composition or lipid profiles over time [10, 11, 14, 15]. However, Blackshaw et al. did report an increase in the total FFA after FD [16]. They postulated that this could be related to preserved lipase activity or to damage or structural changes to the milk fat globule [16]. FD has been shown to significantly decrease the human milk fat globule size, therefore increasing its surface area which may improve its bioavailability [9].

Storage of FD human milk, for up to 4 months at a temperature of 4 °C (or less) has not been reported to change the composition of FFA [10, 17]. However, storage at 25°C or prolonged beyond 120 days led to accumulation of total FFA and monounsaturated fatty acids, respectively [10, 12, 17].

Protein

Five studies have explored the effect of FD on the protein content of human milk. All of these studies reported minimal total protein loss in human milk following FD [12, 13, 18–20]. Furthermore, there has been shown to be no difference in total protein content with up to 6 months storage (4 °C or −80 °C) of FD human milk [18] or in comparison to freezing human milk [20, 21]. FD fortified human milk has been shown to have an increased protein content in comparison to human milk fortified with cows' milk fortifier (CMF) and human milk fortified with evaporated human milk, however within recommended nutritional intakes [22]. Whilst total protein content does not seem to be affected by FD we have explored the effect of FD on specific proteins in the bioactive components section below.

Carbohydrate

The main carbohydrate in human milk is lactose. A lactose removal step before FD is often reported, making it difficult to interpret any effect of FD on carbohydrate content [19, 22, 23].

Variation in the lactose removal step may explain differences in the reported carbohydrate content of FD human milk. Thomaz et al. report less carbohydrate (7.25 ± 0.25 g/dl) in FD human milk combined with DHM than Grance et al. (9.22 ± 1 g/dl) despite both studies involving a lactose removal step [19, 22]. Human milk oligosaccharides (HMO's) are the other major human milk carbohydrate, with their composition not being affected by FD [24].

The evidence regarding whether FD human milk can be used as a human milk fortifier to achieve recommended macronutrients intakes for preterm infants' is unclear. Grance et al reported that the recommended (European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN)) macronutrient intake could be achieved by using FD DHM combined with DHM [19]. However, Fusch et al. report that there is no ideal lyophilization factor that leads to recommended (ESPGHAN) macronutrient intake when using FD MOM as a human milk fortifier [25]. Fusch et al. report that a lyophilization factor of 1.2 will lead to an acceptable fat content, however deficient protein, carbohydrate and energy content. Their results suggest that any increase in the lyophilization factor will lead to excessive macronutrients [25]. The different results of these studies could be related to variation in the processing (lactose and fat removal, pasteurization) of human milk, or to the use of DHM and MOM.

Osmolality

Osmolality is a measure of the number of solute particles in a solvent, whilst osmolarity is a measure of the number of solute particles in a solution.

Three studies have reported inconsistent results on the osmolality of FD fortified human milk as you would expect higher osmolality with increasing concentration of FD HM. Oliveira et al reported an osmolality of 452 mOsm/kg when combining 50 ml FD HM with 75 ml DHM [12]. The osmolality increased to 456 mOsm/kg following 6 months storage [12]. Grance reported an osmolality of 389.6 mOSm/kg when combining 45 ml FD DHM with 50 ml DHM [19]. Grance et al and Oliveira et al did report the mean osmolality of the human milk before addition of FD HM which was 22 mOSm/kg higher in the study by Oliveira et al which may explain some the discrepancy in the results likely due to variation in DHM [12, 19].

Micronutrients and vitamins

There have been concerns that FD would increase the concentration of micronutrients and vitamins to excessive levels. We identified six studies that explored the effect of FD on the micronutrient composition of human milk, and three studies that explored the effect of FD on vitamin composition.

Micronutrients

The micronutrient concentration of human milk fortified with FD human milk has been compared to the baseline micronutrient concentration of human milk. Using this comparison, there has been shown to be an increase in most micronutrients namely;

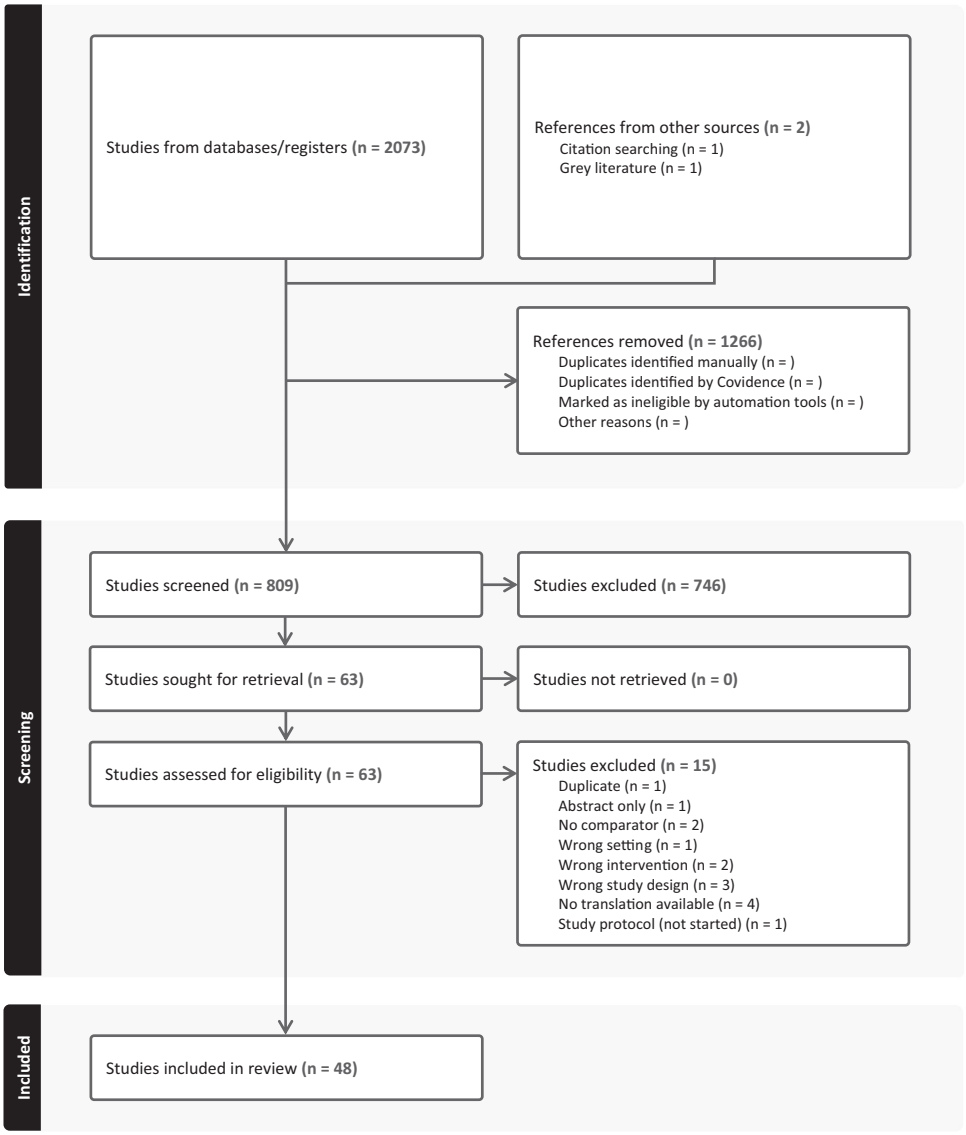

Fig. 1 PRISMA Flow diagram.

calcium, phosphorous [12, 13, 19, 22], magnesium [12, 13], sodium, potassium [12, 19], copper, zinc [12], manganese and selenium [26], however not to excessive levels. An outlier was the study by Lucas et al. which did not find an increase in sodium and potassium concentrations, potentially related to the FD method used in this study [13].

Comparing human milk fortified with FD human milk to human milk alone, there was no significant change in the concentration of potentially toxic elements (aluminum, arsenic, cadmium, chromium, mercury, iron, nickel, tin, thallium) [26]. This study did report a significant decrease in the concentration of lead [26]. Furthermore, lead and nickel concentrations do not seem to be affected by the geographical location or socio-economic status of the mother although due to changing diets and environmental exposures the reported concentration of these elements may not be representative of current human milk [27].

Lastly, it should be noted that human milk fortified with FD human milk has significantly less calcium and phosphorous than human milk fortified with CMF [22]. A diet using donor human milk fortified with FD human milk has been shown to meet the sodium and potassium requirements of preterm infants, but not the calcium or phosphorous requirements as defined by ESPGHAN [22].

Vitamins

Three studies have reported the effect of FD on the vitamin concentration of human milk. Whilst there appears to be no effect on the concentrations of B vitamins (Niacin, biotin, pantothenic acid) [15], a 31.5% reduction in vitamin C concentration has been reported following FD [28]. Interestingly, storage of FD human milk leads to increased retention of vitamin C (total and ascorbic acid) and vitamin E in comparison to human milk stored fresh or frozen [29].

Bioactive components

Human milk is a complicated biological fluid that contains a huge number of molecules that influence the developing immune system [30, 31]. Collectively these molecules have been termed bioactive components and include enzymes, immunoglobulin, immune cells (e.g. lymphocytes, stem cells), oligosaccharides, hormones and cytokines.

FD affects bioactive components to varying degrees. Human milk contains specific glycoproteins felt to be important for lipid breakdown and immune development. The glycoproteins, bile-salt stimulated lipase and esterase, have both been shown to reduce in quantity following FD and storage at −20 °C [32–34]. Similarly,

Table 1. Effect of FD on macro-nutrient composition in human milk.

Study	Country	DHM vs MOM	Participants/ samples (n/n)	Setting	Comparison	FD process 1. Pressure 2.Temperature (°C) (freezing and FD) 3. Length NA = not reported	Outcome
Lucas et al. [13]	UK	DHM	NA/8 Pooled	In vitro	HM vs FD fortified HM	1. NA 2. NA 3. NA	- Hypothesis that micro- and macro- nutrient concentration would double. - increase in fat by 184% and protein by 176%. - Significant increase in calcium and magnesium but not sodium, potassium and chloride - FD fortified HM made by first separating cream, then dialysis before FD to leave protein. Both FD protein and cream then re-added to HM
Dill et al. [17]	USA	DHM	60/10 Pooled	In vitro	Raw HM vs skin HM vs FD HM at −20 C and 25 C	1. NA 2. NA 3. NA	- Free fatty acid (FFA) accumulation did not occur in FD milk stored at −20 °C - FA accumulation in FD milk at 25 °C was rapid, which was accelerated in milk that had previously been rehydrated and stored at 4 °C
Dhar et al. [20]	Canada	MOM	NA/13 Pooled	In vitro	Freezing vs FD. FD + ultrasonification vs FD	1. NA 2. NA 3 days	- Ultrasonification then FD leads to loss of fat compared to ultrasonification and freezing - Less fat loss if process happens after storage - No significant difference in protein content
Thomaz et al. [22]	Brazil	DHM	Pooled samples	In vitro	HM with FD fortifier vs liquid fortifier vs formula	1. NA 2. NA 3. NA Fat and lactose removal before FD, 70 ml FD:100 ml HM. FD with Edwards© equipment	- Significantly more protein in FD fortified HM compared to other fortifiers - Significantly less carbohydrate, calcium and phosphorous in FD fortified HM
Cavazos-Garduño et al. [9]	Mexico	DHM	NA/60 Pooled	In vitro	FD vs spray-drying vs HoP (stored for up to 6 weeks)	1. 10 Pa 2. NA & 55 °C 3. NA Composition of lipids and effect of FD, HoP and spray-drying	- No difference in fat content - Fat globule size decreased considerably (result in an increase in surface area which could improve the bioavailability of the fat components)
Grance et al. [19]	Brazil	DHM	25/25	In vitro	FD fortified DHM vs DHM	1. NA 2. −22 °C & NA 3. 72 h	FD fortified DHM had Lactose, protein, lipids, osmolality, sodium, potassium within recommended limits Calcium and phosphorous did not meet recommended limits for VLBW infants
Cortez et al. [18]	Argentina	DHM	116/NA	In vitro	FD vs raw DHM (stored up to 60 days)	Thermovac 1. 1.33 Pa 2. −70 °C 3. 24 h	- No change for protein, triglyceride or glucose content in FD milk stored for 6 months in −80 °C or 4 °C
Bomfim et al. [11]	Brazil	DHM	50/NA	In vitro		Lyophiilizer L100 1. NA 2. NA 3. 72 h	- Higher lipid concentration in FD HM compared with human milk - No change in the concentration of total n3 and n6 LCPUFA or essential fatty acids (linoleic and α-linolenic acid) - Lower concentrations of Arachidonic acid, eicosapentaenoic acid and docosahexanoic acidNo evidence of peroxidation during storage

Table 1. continued

Study	Country	DHM vs MOM	Participants/ samples (n/n)	Setting	Comparison	FD process 1. Pressure 2.Temperature (°C) (freezing and FD) 3. Length NA = not reported	Outcome
Manin et al. [10]	Brazil	DHM	15/NA	In vivo	FD vs raw milk (colostrum, transitional and mature) (stored up to 6 months)	1. 2.1 Pa 2. -54 °C 3. 48 h	1. 4. No change in fat content and triacylglycerol(TAG) profile with FD process 5. A significant reduction in monounsaturated fatty acids content of transitional breastmilk after 180 days **of storage** compared to storage time of **1-120** days; however similar changes were not observed for colostrum and mature milk
Oliviera at al. [12]	Brazil	DHM	50/200	In vitro	Baseline vs human milk concentrate (FD fortified HM) vs storage (3 and 6 months)	1. NA 2. NA 3. 72 h 50 ml DHM used for FD DHM. Added to 75 ml DHM	Effect of FD: -Increase in all macro- and micro- nutrients between DHM and FD fortified DHM -Increase in calcium, magnesium, potassium and zinc compared to baseline Effect of storage: - Reduced energy, lipids and copper after 3 and 6 months storage but increased concentration of calcium and phosphorous - Increase in calcium, magnesium, potassium, zinc, phosphorous at 6 months compared to HMCI
Hahn et al. [21]	South Korea	MOM	3/6	In vitro	FD vs frozen raw milk	1. <1.33 Pa 2. −70 °C 3. 24 h	- No change in the quantity or functions of FD milk proteins when compared with frozen milk at -80 °C
Fusch et al. [25]	Canada/ Germany	MOM	103/3338	In vitro	Comparison of fortification methods	1. NA 2. NA 3. NA	- Compare macronutrient concentration to ESPGHAN recommendations - Suggest no lyophilization factor meets all requirements of macronutrients without excess nutrients Targeted fortification using human milk analysis possible solution
Blackshaw et al. [16]	Australia	DHM	NA/NA Pooled samples	In vitro	Effect of FD, HoP and gamma-irradiation	1. 0.1 Pa 2. −75 °C then −8 °C 3. 48 h Effect on volatiles and proteins	- 5-fold increase in total FFA after FD - Insignificant increase in lipid oxidation products - FD and HoP lead to loss of protein in 300-380 kDa (IgA) suggesting breakdown of this protein
Neia et al. [14]	Brazil	DHM	Pooled samples	In vitro	HoP vs FD vs spray-drying	1. 2.1 Pa 2. −54 °C 3. 48 h	- Effect on FFA, TAG profile in early, transitional and mature milk - FD not affect composition of fatty acids or the lipid profile (TAG profile)

Table 2. Effect of FD on the micronutrient and vitamin composition of human milk.

Study	Country	DHM vs MOM	Participants/ samples (n/n)	Setting	Comparison	FD process 1. Pressure 2.Temperature (°C) (freezing and FD) 3. Length NA = not reported	Outcome
Friend et al. [15]	USA	DHM	NA/8-12 samples/ pool	In vitro	Effect of FD, HoP and freezing on enzymes, B vitamins and lipids	1. NA 2. NA 3. NA	- FD decreased activity of toperoxidase and lysozyme but no effect on protease or lipase - 47% decrease in lysozyme and 77% decrease in lactoperoxidase - No change in vitamins (biotin, niacin, panthothenic acid) or FFA measured
Barnett et al. [27]	UK	DHM	NA/133	In vitro	Comparison of milk from 3 countries	1. NA 2. NA 3. NA	- Concentration of lead or nickel did not vary between country or socio-economic group
Lozano et al. [29]	Spain	DHM	8/72 (pooled)	In vitro	Effect of storage. FD HM (4 C) vs FD HM (40 C) vs FD HM (control)	1. 0.1 Pa 2. –80 °C & –46 °C 3. 24 h	- Total antioxidant capacity reduced with storage at 40 °C but not at fridge temperatures (5 °C) - Total vitamin C and ascorbic acid content was significantly less at each storage time (up to 90 days) - Total vitamin C and ascorbic acid reduced at a storage temperature of 40 °C to a greater extent than a storage temperature of 4 °C - Vitamin E content did not decrease in samples stored at 4 °C but decreased significantly at 40 °C after 60 days of storage
Martysiak-Żurowska et al. [28]	Poland	DHM	5/NA	In vitro	Raw vs FD HM	1. 94 Pa 2. –20 °C and –80 °C 3. 48 h	- Vitamin C content reduced by 31.5 ± 7.85% - Total antioxidant capacity decreased by 16.6 ± 2.05% - No impact on primary lipid peroxidation and TBARS (indicators of PUFA oxidation)
Oliviera et al. [26]	Brazil	DHM	NA/50	In vitro	HM vs Fortified (FD) HM 50 ml DHM FD: 75 ml DHM	1. NA 2. NA 3. 72 h	- Increase only in Manganese and Selenium - Decrease in lead in FD HM with DHM - No other difference in micronutrient or toxic element concentrations

Table 3. Effect of FD on the bioactive components of human milk.

Study	Country	DHM vs MOM	Participants/ samples (n/n)	Setting	Comparison	FD process 1. Pressure 2.Temperature (°C) (freezing and FD) 3. Length NA = not reported	Outcome
Dill et al. [33]	USA	DHM	60/10 (pooled)	In vitro	FD milk vs skim milk (<0.1% fat), stored at –20C or 25 C	1. NA 2. NA 3. NA	- Approx. 20% decrease in bile stimulated lipase activity after FD and 1 day storage at -20°C, stable thereafter for study period (180 days) - Little loss of bile stimulated lipase activity for 6 days after FD and storage at 25°C, however then 30–40% loss of activity during study period (total 30 days)
O'Connor et al. [34]	New Zealand	MOM (term)	1/NA	In vitro	Temperature, freezing and FD on esterase activity	1. NA 2. NA 3. NA	- Esterase and Bile-salt stimulated esterase activity minimally affected by FD (85% of total activity remained at 28 days storage at either -4°C or -19°C) - No significant difference between FD and freezing on esterase activities.
Cortez et al. [18]	Argentina	DHM	116/	In vitro	FD milk (stored for 6 months) vs raw HM	1. ≤1.33 Pa 2. ≤ –70 °C 3. 24 h	- No significant difference in polyphenols, lipoperoxides, hydroperoxides, superoxide anion, or nitrites.
Castro-Albarran et al. [35]	Canada/ Mexico	DHM	NA/ > 10	In vitro	Compared FD vs spray-drying with different heating plate temp	1. 4 Pa 2. –85 °C 3. Max 10 h	- FD led to approx. 80% retention of immunoglobulins IgG and IgM - FD led to approx. 80% retention at 30 C of IgA. Lower retention at the highest heating plate temperature (40 C) - FD led to improved Ig retention comparing to spray-drying - Sorption isotherm work suggest storage at humidity less than 20% or moisture protective package to avoid rehydration
Castro-Albarran et al. [65]	Mexico	MOM	4/NA	In vitro	MOM vs FD MOM (3 pasteurisation temp)	1. NA 2. –55 °C 3. 36 h	- Loss of immunoglobulin (A, G, M, C3) with increasing HoP temperature - FD led to decrease IgM and IgG with less loss at HOP of 72°C compared to 62°C or 82°C - No functional assessment of immunoglobulin
Hahn et al. [24]	South Korea	MOM	3/9	In vitro	Liquid vs pasteurized vs FD vs FD + pasteurized	1. ≤1.33 Pa 2. ≤ –70 °C 3. 24 h	- FD did not affect oligosaccharides' composition
Martysiak-Żurowska et al. [38]	Poland	DHM	7/pooled	In vitro	FD milk vs raw milk	1. NA 2. NA 3. ≤72 h	4. No impact on superoxide dismutase (SOD) and lactoferrin content 5. Bioactive substances including total antioxidant capacity (TAC) level, LF, FA and lysozyme remain stable even at room temperature **6. Storage of** FD milk decreased TAC by 22.1% and induced a minor increase in lysozyme activity by approximately 9.8%. TAC reduced more at higher storage temperatures (25 °C) but not in fridge temperature (4 °C)
Hahn et al. [37]	South Korea	DHM	3/6	In vitro	Pre- and post FD	1. ≤1.33 Pa 2. ≤ –70 °C 3. 24 h	- Freeze-drying did not negatively affect glycoprotein profile of DHM

Table 3. continued

Study	Country	DHM vs MOM	Participants/ samples (n/n)	Setting	Comparison	FD process 1. Pressure 2.Temperature (°C) (freezing and FD) 3. Length NA = not reported	Outcome
Jarzynka et al. [32]	Poland	DHM	3–4 donors per pool	In vitro	Effect of processing on hormones, enzymes and microbiological profile (below)	1. NA 2. 30–40 °C 3. 24–72 h	- FD alone had no impact on lipase, hepatocyte growth factors (HGF), insulin, leptin or adiponectin concentration compared to raw milk. - HoP then FD significantly decreased HGF, lipase and leptin concentrations - High-pressure processing (HPP) at 450 MPa then FD was more favorable except for adiponectin
Neia et al. [40]	Brazil	DHM	NA/NA (pooled)	In vitro	Effect of HoP, FD and spray-drying on cytokines and antioxidants	1. 2.1 Pa 2. −54 °C 3. ≤48 h	- FD did not alter cytokine levels or antioxidant capacity in all stages of milk (colostrum, transitional and mature)

FD leads to loss of approximately 20% of Immunoglobulin (Ig) G and IgM, whereas IgA appears more sensitive with greater losses which may be reduced depending on the heating plate temperature or holder pasteurization (HoP) temperature, if this is used in milk processing [16, 35, 36]. Whilst leptin, adiponectin, lysozyme and lactoferrin are not affected by FD [32, 37, 38], storage has been shown to increase the activity of lysozyme [38] and HoP decrease the concentration of leptin [32].

Human milk growth factors are thought to be important for the maturation and development of all organ systems [39]. Whilst FD alone has no impact on hepatocyte growth factor or insulin concentration, HoP then FD leads to a significant reduction in hepatocyte growth factor concentration [32].

FD does not affect the composition of human milk oligosaccharides (HMO's) which are important for gut microbial development and immune maturation

Anti-oxidant capacity is thought to be an important component of human milk especially in preterm infants [39]. FD does not appear to affect a range of human milk anti-oxidants including polyphenols, lipoperoxides, hydroperoxides, superoxide anion, or nitrites [18, 38, 40]. However, there is varying reports of the impact of storing FD milk with one study reporting a 20% reduction in total anti-oxidant capacity, which was exacerbated by higher storage temperatures [38], whilst a similar study reported no change to several antioxidants after 6 months of storage [18].

Human milk cytokines may be important for modulating any intestinal inflammatory response. Cytokine levels are not altered by FD [40].

Microbial and anti-microbial factors

Human milk contains bacteria that are beneficial for infants as well as factors that inhibit the growth of pathogenic bacteria.

FD has been shown to decrease the bacterial count of human milk, whilst the acidity (reflection of contamination) has been shown to remain unchanged [10, 41]. These studies did not explore the specific bacteria affected. FD significantly reduces the concentration of Staphylococcus epidermidis and other potentially pathogenic organisms (mesophilic aerobic microorganisms) in comparison to freezing milk [42]. Furthermore, two studies have explored the effect of FD (and processing) on milk contaminated with common milk contaminants. Blackshaw et al showed that FD initially reduced bacterial counts of Staphylococcus Aureus (S. Aureus), Escherichia Coli (E. Coli) and Salmonella typhimurium (S. typhimirium) however without either gamma irradiation or HoP there was bacteria growth [43]. Jarynzyska et al. performed a similar study however; human milk was contaminated with five bacteria (E. Coli, S. Aureus, Listeria monocytogenes, cronobacter sakazakii and bacillus cereus). This study suggested that FD reduced E. Coli and Listeria monocytogenes and bacillus cereus growth (with Bacillus Cereus removed by 6 months storage). However, there was no effect on S. Aureus or Cronobacter sakazakii. High-pressure processing then FD lead to complete irradication of bacteria [32].

Human milk contains molecules that inhibit bacterial growth. No study has found that FD affects the bacterial growth inhibition or bactericidal capacity of human milk [36, 42, 43].

Clinical

Clinical studies using FD human milk have focused on providing this to preterm infants to provide the additional protein and calories they require for growth. These studies have had small sample sizes with a maximum of 42 infants reported in one study [44] (Table 5). The studies have mainly been cohort or observational designs, frequently focusing on the metabolic effects of a diet containing FD human milk. Four randomised controlled trials have been reported with a maximum of 40 infants per trial. Boehm et al compared the use of CMF to FD HM (as source of HM fortifier) showing no difference in growth, nitrogen or fat balance [45]. The

Table 4. Effect of FD on the microbial/anti-microbial factors of human milk.

Study	Country	DHM vs MOM	Participants/ samples (n/n)	Setting	Comparison	FD process 1. Pressure 2.Temperature (°C) (freezing and FD) 3. Length NA – not reported	Outcome
Agel et al. [41]	USA	MOM	6/7 (and pooled samples)	In vitro	Pasteurisation vs FD vs slow freeze vs quick freeze	1. NA 2. NA 3. NA	- Freezing had little effect of bacterial count - FD had significant decrease in bacterial counts - Pre-study bacterial counts above recommended level (in 1982)
Carbonare et al. [36]	Brazil	DHM	46/66	In vitro	Effect of HoP, microwave radiation and FD	1. NA 2. –70 °C 3. <24 h	- Explored effect on pathogenic E.Coli adherence to HEp-2 cells - FD had no effect on Inhibitory levels of Enteropathogenic E. Coli(EPEC), - FD had no effect on total IgA or anti-EPEC specific IgA
Salcedo et al. [42]	Spain	DHM	65/125	In vitro	Effect of FD to freezing on microbial content and anti-microbial functionality	1. 1 Pa 2. –40 °C 3. 2–3 days	- FD lead to significant decrease in S. epidermidis and mesophilic aerobic microorganism compared to −20 °C - FD had no effect on bactericidal capacity (against E. Coli) compared to freezing at −20 C - Mature (>15 days postpartum) term milk had highest bactericidal capacity
Blackshaw et al. [43]	Australia	DHM	NA/NA	In vitro	Raw vs FD HM vs HoP FD HM vs formula Gamma irradiation vs HoP on anti-bacterial properties	1. 0.1 Pa 2. −8 °C 3. 48 h	- Contaminated FD samples with S. Aureus, E. Coli and S. typhimurium - FD then storage reduced bacterial colonies 1–2 fold. - HoP then removed S. Aureus, whilst gamma irradiation >2kGy removed all bacteria - FD did not affect bacterial growth inhibition of HM, but HoP does. FD HM then gamma radiation offers less affect on bacterial growth inhibition - Minimal (0.3%) live bacteria seen in HoP FD HM compared to raw milk, formula or HoP at 24 h - Pasteurising FD HM with 2-5kGy gamma radiation alternative to HoP and may preserve more antibactial properties
Jarzynka et al. [32]	Poland	DHM	NA/3–4 donors per pool	In vitro	Effect of processing (FD, HoP, high pressure processing (HPP)) on bacteria content	1. NA 2. 30–40 °C 3. 24–72 h	- Tested FD process on inoculated human milk samples with five bacteria (Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, and Cronobacter sakazakii, Bacillus cereus) - FD Reduced growth of E coli and removed strains and spores of Bacillus cereus at 6 months storage. - Slight reduction in listeria monocytogenes after FD and storage for upto 6 months - No change in the concentration of Staphylococcus aureus, and Cronobacter sakazakii - HPP at 450 MPa then FD lead to complete eradication of bacterial growth at all storage time points

Table 5. Clinical effect of feeding newborn infants FD human milk.

Study	Country	FD milk (DHM or MOM)	1.Participants (n) 2.GA mean or range (g) 3.BW (mean or range (g))	Study design	Comparison	FD process 1. Pressure 2.Temperature (°C) (freezing and FD) 3. Length (hours) NA – not reported	Outcome
Hagelberg et al. [48]	Sweden	DHM	1. 4 2. 30–33 3. 1390–1560	Observational	All fed lacto-engineered HM. Seperated then FD. FD HM then added to MOM	1. NA 2. –20 °C & –5 °C 3. 5 & 48 h	- Protein content of engineered HM matched expected protein requirement - Normal serum sodium, potassium, urea, albumin and protein - One infant had transient hypertyrosinaemia - Growth displayed but not reported - NB: infants possibly not representative, 2 ventilated for RDS and 2 had exchange transfusion for jaundice
Voyer et al. [52]	France/Belgium	DHM	1. 8 2. 28–32 3. 1270 ± 170 g	Observational	All fed HM + FD HM with additional MCT and electrolytes	1. NA 2. NA 3. NA	- Performed metabolic balance studies - Similar growth using to in-utero growth rates in infants fed FD HM - No electrolyte abnormalities reported
Boehm et al. [51]	Germany/ Czechoslovakia	DHM from preterm mothers	1. 24 2. 29–33 3. 1160–1460	Cohort	FD HM(AGA) vs FD HM (SGA) vs no fortifier	1. NA 2. NA 3. NA	- Infants studied for the first 8 days of life - Explored protein metabolism - SGA infants fed FD HM in the first week of life had increased amino acid excretion and serum bile acids
Schanler et al. [66]	USA	DHM	1. 18 2. 28–30 3. 1175 ± 146	Cohort	Fecal immune factors in infants fed FD HM vs CMF	1. NA 2. NA 3. NA	- Lactoferrin, lysozyme, IgA (total, secretory and E. Coli specific-) increased in feces of FD HM fed infants compared to CMF fed infants
Schanler et al. [50] & [49]	USA	DHM	1. 32 2. 29 3. 1200	Cohort	Comparison of cows milk formula to HM fortified with FD milk	1. NA 2. NA 3. NA	- FD process involved separation, dialysis of skim milk then FD of both cream and skim milk - Study explored micronutrient balance - Growth (weight, OFC, length) similar between groups - Lower micronutrients (phosphorous, calcium) in FD HM group with corresponding serum values - Expected retention levels of sodium, potassium and magnesium despite lower milk values

Table 5. continued

Study	Country	FD milk (DHM or MOM)	1.Participants (n) 2.GA mean or range (g) 3.BW (mean or range (g))	Study design	Comparison	FD process 1. Pressure 2.Temperature (°C) (freezing and FD) 3. Length (hours) NA – not reported	Outcome
Boehm et al. [58]	Germany/ Czechoslovakia	DHM	1. 21 2. 27-–31 3. 1400	Cohort	FD HM (redissolved) vs HM	1. NA 2. NA 3. NA	- FD HM was re-dissolved presumably in water, compared to HM diet (MOM/ DHM) - Less calories due to loss of fat in FD HM lyophilizate. - Weight gain 7.6 g/kg/day (FD HM lyophilizate) and 11 g/kg/ day (HM)
Boehm et al. [44]	Sweden/ Germany	DHM	1. 42 2. 29–36 3. 1350	Cohort	SGA vs AGA infants protein synthesis at DOL 8	1. NA 2. NA 3. NA	- No issues reported with use of FD HM but report only up to day of life 8 - Explored protein metabolism based on AGA/SGA
Boehm et al. [53]	Sweden/ Germany	DHM	1. 14 2. 29–36 3. 970–1475	Cohort	SGA vs AGA (all fed FD human milk)	1. NA 2. NA 3. NA	- Weight gain of 18.9 ± 2.9 g/kg/ day(SGA) and 21.3 ± 3.6 g/kg/ day (AGA) on 200 ml/kg/day human milk with 6 g/100 ml of FD human milk.
Boehm et al. [54] a	Sweden/ Germany	DHM	1. 14 2. 30 3. 1280	Observation	Exploring urea and ammonia excretion	1. NA 2. NA 3. NA	- Weight gain of 18.9 ± 3.4 g/kg/ day in these infants however fed up to 195 ml/kg/day
Boehm et al. [57] b	Germany/ Czechoslavakia	DHM	1. 14 2. 30 3. 1258	Observational	Exploring nitrogen metabolism	1. NA 2. NA 3. NA	- All infants fed MOM fortified with 6 g/100 ml FD human milk - Weight gain 17.1 ± 3.2 g/kg/ day - Suggest VLBW infants able to metabolise increased protein diet as early as day of life 8
Boehm et al. [55]	Sweden/ Germany	DHM	1. 26 2. 29–37 3. 980–1480	Cohort	SGA vs AGA (all fed human milk)	1. NA 2. NA 3. NA	- Study explored urea and ammonia excretion based on growth - Weight gain of 17.6 ± 3.1 g/kg/ day (SGA) and 19.3 ± 2.9 g/kg/ day (AGA)
Boehm et al. [45]	Sweden/ Germany	DHM	1. 28 2. 27–35 3. 720–1500	Observational	SGA vs AGA (similar BW but different GA)	1. NA 2. NA 3. NA	- Explored protein synthesis by measuring essential amino acids and urea - Weight gain of 18.6 ± 3.1 and 19.1 ± 3.1 g/kg/day - Plasma amino acids and urea correlated with postmenstrual age not weight - No significantly abnormal values

Table 5. continued

Study	Country	FD milk (DHM or MOM)	1.Participants (n) 2.GA mean or range (g) 3.BW (mean or range (g))	Study design	Comparison	FD process 1. Pressure 2.Temperature (°C) (freezing and FD) 3. Length (hours) NA – not reported	Outcome
Boehm et al. [56]	Germany/Italy	DHM	1. 24 2. 27–30 3. 920–1430	RCT	FD HM vs cows milk fortifier	1. NA 2. NA 3. NA	- No difference in weight gain or linear growth between study groups - No change in nitrogen or fat balance between study groups
Bechensteen et al. [46]	Norway	DHM	1. 32 2. 27–31 3. 920–1455	RCT (EPO and protein source)	FD HM vs CMF + HM	1. NA 2. NA 3. NA	- No difference in body growth between protein regimens - No data provided demonstrating this
Thomaz et al. [23]	Brazil	DHM	1. 24 2. 26.5 3. 990	RCT	DHM + CMF vs DHM + evaporised HM vs DHM + FD HM	1. NA 2. NA 3. NA	- FD HM using 70 ml DHM per 100 ml DHM, 70 ml was skimmed, evaporated (with lactose extracted) before lyophilized - Primary outcome change in phenylalanine - Significant increase in phenylalanine when given CMF but no increase with FD HM
Nogueira et al. [47]	Brazil	DHM	1. 40 2. 30 3. 1220	RCT (Phase 1)	FD DHM + MOM vs CMF + MOM	1. NA 2. NA (−30 °C before FD) 3. 72 h	- DHM frozen at −30 °C for 24 h then FD for 72 h (Liophilizer 108, Liobras, Brazil) - 50 ml FD DHM with 75 ml DHM versus MOM with CMF for 21 days - FD HM with DHM lower in protein, calcium, magnesium, sodium, copper, zinc, phosphorous and iron - No significant adverse effects in intervention group - Did not report g/kg/day weight but no obvious difference in growth - Did not report changes in electrolyte markers (considering decreased micronutrients above)

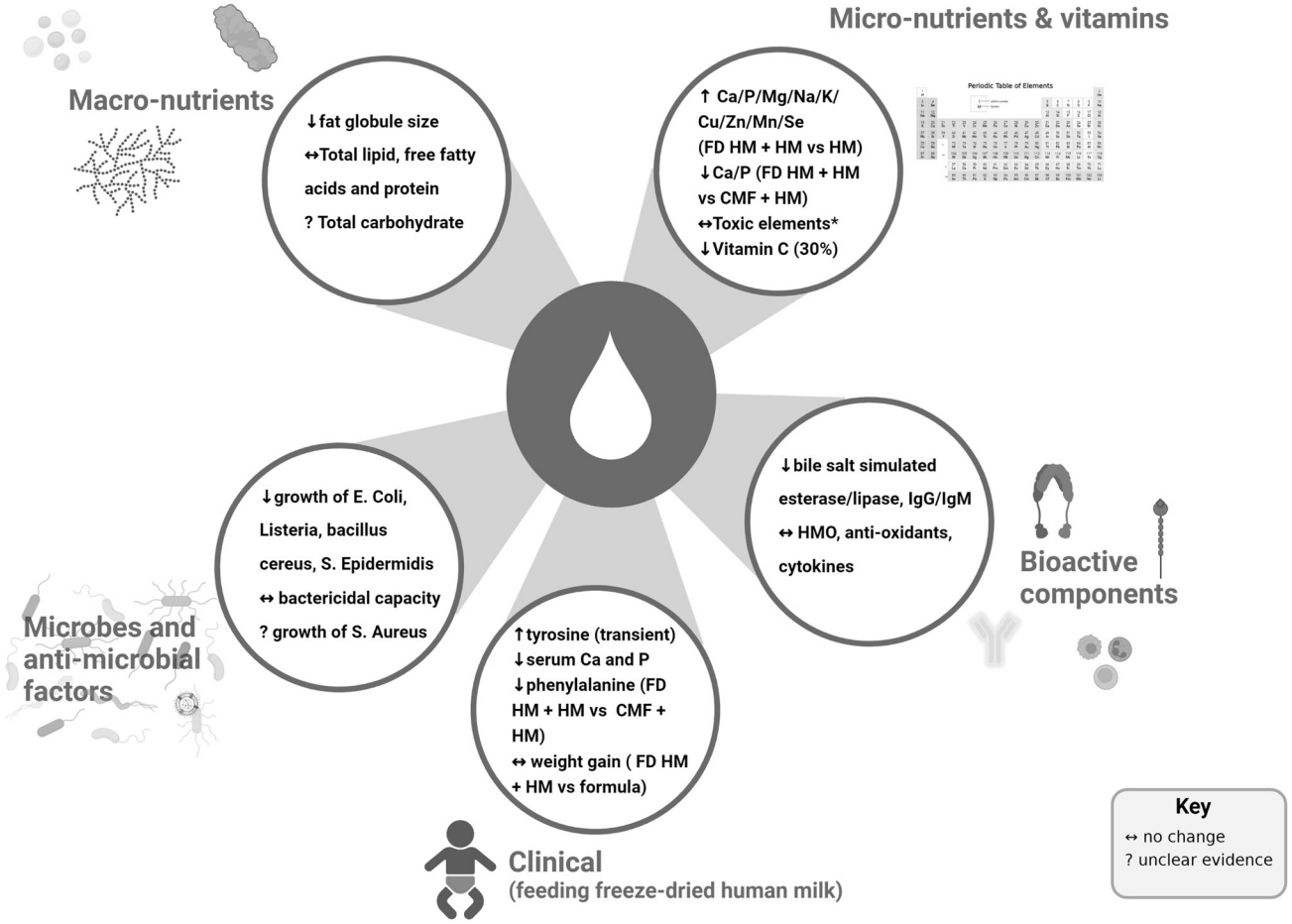

Fig. 2 Key study findings on the effect of freeze-drying (lyophilization) on human milk.

primary focus of Bechensteen et al was the effect of erythropoietin on erythropoesis with the infants also randomised to receive FD HM or CMF as the source of HM fortifier [46]. Bechensteen et al found no effect on growth or erythropoeisis comparing these different diets [46]. Thomaz et al compared three diets in 24 infants with a primary outcome of phenylalanine levels showing that using CMF lead to significant increase in phenylalanine levels in comparison to using FD HM (as the source of HM fortifier) [23]. Nogueira et al reported a phase 1 double blind randomised controlled trial comparing the source of fortification (CMF versus FD HM) in 40 infants with a combined primary outcome of feed tolerance and gastrointestinal complications (necrotising enterocolitis, sepsis, gastro-intestinal bleeding or perforation) reporting no difference in the primary outcome between groups [47].

There have been very few adverse events reported. Serum electrolyte disturbances have been the only adverse events reported namely; transient tyrosinaemia [48], hypocalcaemia, and hypophosphoraemia [49, 50]. Increased increased amino acid excretion and serum bile acids have also been reported but only in small for gestational age infants compared to appropriately grown infants [51].

Infants fed FD fortified human milk had similar weight gain when compared to cow's milk formula fed infants [46, 49, 50], or in comparison to intra-uterine growth rates [52].

Studies have reported weight gains between 16 g/kg/day and 21.3 g/kg/day in infants fed human milk fortified with FD human milk [45, 49, 53–57]. An outlier was a study reporting offering human milk lyophilizate that was re-dissolved to preterm infants, they found the infants had a weight gain of only 7.6 g/kg/day, which was likely due to not re-dissolving the FD human milk in human milk [58].

DISCUSSION

Summary of evidence

Our scoping review revealed that FD human milk results in a decrease in fat globule size, decreased growth of pathogenic bacteria, decreased bile salt-stimulated esterase and lipase, decreased vitamin C, decreased IgG and decreased IgM. When newborn infants' have been fed FD human milk serum electrolyte disturbances have been reported, namely tyrosinaemia, low phenylalanine, hypophosphataemia, and hypocalcaemia although no adverse clinical outcomes have been reported when compared with diets using CMF. Weight gain appears similar in comparison to when CMF is used (Fig. 2).

Macro-nutrients seem relatively unaffected by FD. The milk fat globule size is reduced which may be advantageous in improving its bioavailability. Similar to fortification with bovine based HMF, meeting the macronutrient needs of preterm infant's may be challenging when using FD HM fortified human milk [25]. This work by Fusch et al suggested that no lyophilization factor can be used to meet the macronutrient needs of preterm infants (without deficiency or excess in at least one macronutrient), it is plausible that the sane is true of the concentration of micronutrients [25]. Target fortification using human milk analysis may overcome this challenge and offers the possibility of providing an exclusive human milk diet or even an exclusive mothers' own milk diet to preterm infants. Alternatively, the addition of a fat and lactose removal step in the processing of FD HM may positively influence

these challenges [22]. There have been concerns regarding the osmolarity of feeds since a trial conducted in 1975 showed an association between very high osmolarity feeds (639 mOsm/l in this trial) and necrotising enterocolitis [59]. This led to the American Academy of Pediatrics (AAP) suggesting the osmolarity of milk feeds should not exceed 400 mOsm/l (approximate osmolality of 450 mOsm/kgH20) [60]. More recent publications by the AAP and ESPGHAN do not recommend an upper limit of osmolality or osmolarity for fortified milk, with ESPGHAN suggesting that there is not consistent evidence that feed osmolality between 300-500mOm/kg is unsafe [61, 62]. The reported osmolality of FD fortified HM of between 389 and 456 mOSm/kg is within these limits [12, 19].

The micronutrient composition of human milk is relatively unaffected with no reported increase in toxic elements. However, FD fortified HM has reduced calcium, phosphorous and vitamin C than CMF fortified HM which would need to be considered in the management of preterm infants.

The bioactive components of HM likely confer the health benefits of a human milk diet. A significant reduction in IgA, bile-stimulated lipase and enterase have been reported following FD. FD likely affects the immune cells (lymphocytes and stem cells) of HM however, this has not been investigated. These reductions are especially important if FD HM is used as a lyophilizate as it is unlikely to confer the same benefits as fresh MOM. However, if FD HM is used as a fortifier to MOM then the reduction is likely not as significant. A consideration is the effect of different FD conditions on all human milk components. Castro-Albarran et al. did explore different heating plate temperatures showing that the temperature effects the rate of sublimation as well as the immunoglobulin concentration in human milk [35].

FD appears to have a positive effect on reducing bacterial growth without affecting the natural bactericidal capacity of HM. While pasteurization of FD milk or using pasteurized DHM to produce FD fortifiers remains an option, current evidence support the safety of FD milk. Unpasteurized FD mother's own milk may promote healthy colonization of the neonatal gut microbiome, as unpasteurized MOM compared to pasteurized DHM has been shown to significantly affect gut microbial composition whilst pasteurized human milk derived human milk fortifier have little impact compared to bovine-derived fortifiers [63].

The clinical effect of using FD HM as a HM fortifier appear promising with acceptable weight gain and no significant adverse events reported. Electrolyte disturbances (particularly hypocalcaemia and hypophosphoraemia) are common in preterm infants however the reported disturbances require further exploration especially considering the work by Grance et al demonstrating that the calcium and phosphorous content of FD human milk (combined with DHM) does not meet the ESPGHAN recommendations for preterm infants [19]. However, there is limited clinical evidence available and reported studies may not be representative of preterm infants at all gestations. Additionally, the available studies are limited by the reporting of the FD method used which makes reproducibility challenging.

Whilst the aim of this review was to identify the effect of FD on HM, we have also described the effect of storage on FD HM. It has been shown using sorption isotherm that FD HM is easily rehydrated, however this means that storage should be in humidity of less than 20% or a moisture protective package [35].

Limitations

While our review summarizes the current knowledge and potential use of FD human milk in preterm infants, there are several limitations to acknowledge. We did not have access to translation services meaning that articles not in English were excluded. Furthermore, effects of FD process (temperatures, pressure, milk state (frozen, raw) prior to FD and length of process) were not available in most reported studies. 10 different lyophilization machines were mentioned in the articles reviewed making it difficult to ascertain similarities between the FD methods.. Defining and standardizing optimal conditions for FD are required for use in clinical practice. We acknowledge that FD human milk often does not happen in isolation as many studies use DHM that has undergone HoP or other processing before FD. Tables 1–5 gives detail of the comparisons used in the studies described and whether there have been other processes explored in the reported analysis.

CONCLUSION

Freeze-drying human milk has effects on the nutrient, microbial and bioactive components of human milk. Freeze-dried human milk appears a possible way to improve storage and potentially offer preterm infants' additional nutrients without exposure to cows' milk. Further clinical studies are required to prove the efficacy and safety of FD HM in preterm infants given the limited data and considering the potential benefit of delivering an exclusive human milk diet to preterm infants as highlighted by international pediatric societies [61, 64].

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AUTHOR CONTRIBUTIONS

BA & TDRS conceived and designed the work. TDRS/AG/BA reviewed the abstracts and full texts to identify suitable articles. TDRS charted and collated the data and drafted the initial manuscript. AG/BA reviewed and appraised the manuscript. All authors approved the final version.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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