

RESEARCH ARTICLE

Development of a human milk concentrate with human milk lyophilizate for feeding very low birth weight preterm infants: A preclinical experimental study

Mariana M. Oliveira¹, Davi C. Aragon¹, Vanessa S. Bomfim¹, Tânia M. B. Trevilato², Larissa G. Alves³, Anália R. Heck³, Francisco E. Martinez⁴, José S. Camelo, Jr.⁴

1 Department of Pediatrics, Children's Hospital, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil, **2** Laboratory of Pediatrics, Clinics Hospital of Ribeirão Preto, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil, **3** Human Milk Bank, Clinics Hospital of Ribeirão Preto, Ribeirão Preto, São Paulo, Brazil, **4** Department of Pediatrics, Neonatology, Children's Hospital, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

☞ These authors contributed equally to this work.

‡ These authors also contributed equally to this work.

* jscamelo@fmrp.usp.br



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Abstract

Breast milk is considered the gold standard nutritional resource for very low birth weight (VLBW) infants in terms of nutrients and protective factors. If mother's milk is not available, the second choice is donated and fortified human milk (HM) from the Human Milk Bank (HMB). This study hypothesized that HM could be lyophilized and used as an additive to increase the levels of macronutrients and micronutrients available to VLBW infants. This study aimed to constitute a lyophilized HM concentrate and determine the osmolality and the concentration of macronutrients and micronutrients in HM samples at "baseline" and in "HM concentrates", analyzed immediately (HMCI), and after 3 (HMC3m) and 6 (HMC6m) months of freezing. Osmolality was verified using the freezing point osmometric method. Macronutrient quantification was performed using the MIRIS Human Milk Analyzer. Micronutrients were determined by Flame Atomic Absorption Spectrophotometry and by the automated colorimetric method. Bayesian linear mixed effect models were adjusted using OpenBUGS to estimate mean differences and 95% credibility intervals (CrI) of osmolality and of macro- and micronutrients between the types of HM samples. A comparison of dosage values showed a significant increase between HM baseline and HMCI, HMC3m, and HMC6m. Comparing HM baseline and HMCI highlighted the increase in energy content and the concentration of carbohydrates and total lipids. The Ca and P contents increased and the levels of energy, total lipids, and Cu were reduced in HMC3m compared to HMCI. Ca, Mg, K, Zn, and P increased and the levels of energy, total lipids, and Cu were reduced in HMC6m, compared to HMCI. The present study confirms the possibility of formulation and utilization of the immediate concentrate. Partial stability of HM concentrates generated from freeze-drying of donated milk do not recommend storage.

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1. Introduction

Despite considerable advances, the optimal nutritional support for very low birth weight infants (<1500 g) has not yet been found. The current aims of neonatal care include promoting survival as well as mimicking fetal growth and proper neurodevelopment of these extremely immature infants. Providing for the nutritional needs of very low birth weight (VLBW) infants is a challenge since the preterm stage has a higher demand for energy, protein, and other nutrients than other development stages, as infants have low stores of key nutrients such as iron, zinc, and calcium. An adequate supply of energy and protein during the first week of life is associated with improved growth and development up to the post-conceptual age of 2 years. In addition, the physiological immaturity, diseases, and general stress of prematurity means that these infants require even more optimal nutritional support [1–6].

Breast milk is considered the gold standard nutritional resource due to the provision of nutrients and protective factors. Some advantages conferred to VLBW infants improved long-term outcomes of neurodevelopment, immune defenses, development of the microbiota and the gastrointestinal system, and reduced incidence of necrotizing enterocolitis (NEC) and sepsis [7–10]. In addition, early enteral nutrition with an exclusively human milk (HM) diet has proven to be an effective nutritional support strategy for VLBW infants because of its association with lower incidence of NEC, prevention of neonatal infant mortality, and reduction of time and cost of hospital admission with each preterm infant [11–13].

Current guidelines suggest that all preterm infants should receive HM and that the first choice should be the mother's own milk. Nonetheless, HM should be fortified to ensure optimal nutrient intake. If the own mother's milk is not available, the second choice is donated and fortified HM from the Human Milk Bank (HMB). The nutritional content of donated HM by itself may not satisfy the special nutritional needs of VLBW infants, especially for proteins, micronutrients, and energy, thereby jeopardizing the growth and development of these preterm infants [14–17]. A recent study reported that the adequacy of the quantity and quality of protein intake by VLBW infants influences the rate and relative quality of weight gain [18]. Moreover, an inadequate supply of the micronutrients calcium, phosphorus, and zinc can also culminate in deficient growth and development since these nutrients are responsible for maturation and for the functionality of several enzymatic systems [19–20]. Faced with the impossibility of increasing the volume of HM and thus the absorption of nutrients [21], the strategy to adapt this milk to the nutritional needs of VLBW infants is through the fortification of HM with commercial additives to be added to the mother's own milk (raw or pasteurized) or donated HM. However, widely used bovine milk protein-based products, despite providing growth and weight gain to preterm infants, partially alter the immunological quality of HM, increase osmolality and risk of sensitization by heterologous protein, as well as the occurrence of NEC [22–25]. More recently, human milk-based additives have been formulated and studies show the advantages of their use, such as improved morbidity and mortality rates, less incidence of NEC, decreased hospital stays, and improvement of infant growth and weight gain. Nevertheless, the cost of the product and the ethical questions related to its commercialization make it difficult to use it in neonatal intensive care units (ICUs) [26–29]. Thus, considering the recent scientific breakthroughs, the hypothesis of this study is that HM voluntarily donated to the HMB may be lyophilized, in a simple and effortless way, and used as an additive of HM to increase the levels of nutrients. The aims of this study were to constitute a concentrate with freeze-dried HM and determine the osmolality and the concentration of macronutrients and micronutrients in HM samples immediately and after 3 and 6 months in storage to evaluate the increase in concentration of nutrients followed by nutritional stability of the product. This study reports an innovative and simplified proposal of nutritional support for VLBW infants

with an exclusive HM diet to be implemented in the routine of the Human Milk Bank Network of Brazil (HMB Network Brazil) to ensure the nutrition of preterm infants and as an alternative to use of the artificial additives currently being used.

2. Materials and methods

This research project was carried out through a partnership with the HMB and the Laboratory of Metals and Rare Diseases–Pediatrics, at the Clinics Hospital of Ribeirão Preto, Ribeirão Preto Medical School, University of São Paulo (USP). The milk used in this study came from donations of surplus production of HM, that is, without compromising the child's own feeding. Since this study was carried out with human biological material, it was submitted to and approved by the Human Research Ethics Committee of the Clinics Hospital, Ribeirão Preto Medical School—USP (HREC Report No. 738.080). The donors were informed about the nature of the study and those who were willing to participate in the project signed a free and informed consent form; donors also underwent clinical and serological screening.

Material collection, processing, and quality control

The donors with a lactation period greater than 15 days were given instructions about massaging and milking their breasts, and about how to withdraw the milk into a sterile, inert glass bottle provided by the HMB. All the samples passed through the selection and classification processes recommended by the HMB Network Brazil (available at: <http://www.redeblh.fiocruz.br>). The selection process included packaging conditions, presence of dirt, color, off-flavor parameters, and Dornic acidity. The classification process included the verification of the lactation period, Dornic acidity, and energetic content—the crematocrit [30–33]. Considering a value of 0.36 for the standard deviation with respect to the expected average of protein concentration (2.20 g/dL) and an absolute error value of 0.1, with a confidence level of 95%, 50 samples were obtained. Additional data were collected to characterize the donors: age, weight, height, Body Mass Index (BMI), and gestational age. Inclusion criteria were surplus mature HM with a Dornic acidity value of up to 8°D.

Obtaining the HM concentrate

For lyophilization, 50 mL of donated HM was transferred to an inert, sterile glass container and frozen (-20°C for 24 hours). After this period, the frozen sample was placed in the vacuum chamber of a bench lyophilizer (Lyophilizer L108, LioTop, São Carlos—SP—Brazil). After 72 hours, the lyophilized sample was transferred from the lyophilizer to a cold chain, to be reconstituted with HM for use. The concentrate with the HM lyophilizate in the immediate period (HMCI) was composed from samples that were withdrawn from the lyophilizer and reconstituted with 75 mL of the donor's own HM baseline. These concentrates together with the HM baseline were passed through the processes of pasteurization and microbiological quality control. HM baseline and HMCI were pasteurized at 62.5°C for 30 minutes after a preheating period [34]. After 30 minutes of thermal treatment that is lethal to pathogenic bacteria, the vials were withdrawn from the bath and cooled until the HM reached a temperature $\leq 5^\circ\text{C}$. For the microbiological quality control check, the pasteurized HM baseline and HMCI samples were screened for total coliforms using bright green bile broth (50g/L; 5% w/v) contained within Durham tubes. The concentrate with the HM lyophilizate in the immediate period was subdivided in collection tubes and stored (-20°C) for 3 (HMC3m) and 6 (HMC6) months, to evaluate nutritional stability. Two hundred types of HM samples (50 of each type: HM baseline, HMCI, HMC3m, and HMC6m) were analyzed for osmolality, macronutrients, and

micronutrients. Osmolality verification was performed using the freezing point osmometric method in the PZL-1000 Microprocessed Osmometer (PZL).

Macronutrients

In the HMB, the 200 types of HM samples were homogenized using a Sonicator MIRIS (Miris, Uppsala, Sweden), and the macronutrient content (protein, carbohydrate, total lipids, total solids and true protein) was quantified in 2 mL aliquots of each sample type by using the MIRIS Human Milk Analyzer (Miris, Uppsala, Sweden). The MIRIS Human Milk Analyzer performs precise and accurate analyses based on infrared transmission spectroscopy. Sample types HMC3m and HMC6m were previously defrosted in a water bath at 37°C.

Micronutrients

After the pasteurization process, aliquots of each type of HM sample were immediately separated in collection tubes, frozen (-20°C for 24 hours), and sent to the Laboratory of Metals and Rare Diseases for immediate analysis of micronutrients and also 3 and 6 months post-freezing. The micronutrients Calcium (Ca), Magnesium (Mg), Sodium (Na), Potassium (K), Copper (Cu), Zinc (Zn) and Phosphorus (P) were determined “in natura”, after defrosting samples in a water bath at 37°C followed by homogenization of the material by ultrasound using Sonicator MIRIS. Proper dilutions were made within the concentration ranges of the calibration curves for readings in the Flame Atomic Absorption Spectrophotometry (EAA 55B VARIAN), in which each element uses a hollow-cathode lamp and specific wavelength, acetylene gas and compressed air. Phosphorus was dosed in the Metrolab equipment with a kit from Wiener Lab., Ref 1382321 by the automated colorimetric method.

Statistical analysis

The exploratory analysis of osmolality dosages and content of macronutrients and micronutrients was carried out through the mean values (standard deviation) and boxplots (S1 Fig). Bayesian linear mixed effects models were adjusted using OpenBUGS to estimate the mean difference and 95% credibility intervals when comparing the dosages of osmolality and of the macronutrients and micronutrients between the types of HM sample: HM baseline, HMCI, HMC3m, and HMC6m.

3. Results

Fifty HM donors participated in the study. After the selection and classification of the samples according to the inclusion criteria, the mean values (standard deviation) of the Dornic acid values were 4.34°D (1.59). The characteristics of the HM donors are summarized in Table 1, in which the mean (standard deviation) age, weight, height, BMI, and gestational age was, respectively, 30.45 (6.05) years, 67.52 (11.92) kg, 1.63 (0.59) m, 25.2 (4.39) kg/m², and 38.43 (2.27) weeks.

The descriptive dosage results of the mean (standard deviation) of osmolality and content of macronutrients and micronutrients in types of samples HM baseline, HMCI, HMC3m, and HMC6m presented in Table 2. An increase in the levels of the evaluated nutrients was observed in the concentrates of HMCI, HMC3m, and HMC6m, compared to the HM baseline.

The comparative values of dosages of osmolality and of macronutrients and micronutrients in the samples of the types (Table 3) show that there was a significant increase in all the parameters in HMCI, HMC3m, and HMC6m, compared to HM baseline. This highlights the

Table 1. Characteristics of the HM donors.

Mothers (n = 50)	Mean	Standard Deviation	Minimum	Maximum
Age (years)	30.45	6.05	17	44
Weight (kg)	67.52	11.92	47	95
Height (m)	1.63	0.59	1.52	1.77
BMI (kg/m ²)	25.2	4.39	18.1	38.1
Gestational age (weeks)	38.43	2.27	27	42

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increase in energy content (-23.680; 95% CrI [-25.910; -21.450]) and the concentration of carbohydrates (-2.095; 95% CrI [-2.286; -1.912]) and total lipids (-1.437; 95% CrI [-1.678; -1.193]). In HMC3m samples, a significant increase was observed in the levels of element Ca (-2.139; 95% CrI [-3.356; -0.971]) and P (-1.882; 95% CrI [-3.618; -0.209]), but a slight reduction in the levels of energy (2.988; 95% CrI [0.689; 5.193]) and total lipids (0.342; 95% CrI [0.099; 0.591]), while the osmolality and other nutrients remained stable, except for Cu (7.154; 95% CrI [3.840; 10.330]), compared to HMCI samples. In HMC6m samples, a significant increase was observed in the levels of Ca (-4.103; 95% CrI [-5.325; -2.904]), Mg (-0.357; 95% CrI [-0.515; -0.201]), K (-131.400; 95% CrI [-193.400; -70.690]), Zn (-47.290; 95% CrI [-72.040; -23.100]), and P (-2.022; 95% CrI [-3.738; -0.355]), but a slight reduction in the levels of energy (2.672; 95% CrI [0.417; 4.878]) and total lipids (0.335; 95% CrI [0.095; 0.579]), while the osmolality and other nutrients remained stable, except for Cu (9.040; 95% CrI [5.782; 12.203]), compared to HMCI samples. Finally, in HMC6m samples, stored for 6 months, a significant increase in the levels of Ca (-1.965; 95% CrI [-3.203; -0.770]), Mg (-0.216; 95% CrI [-0.377; -0.061]), K (-130.400; 95% CrI [-193.400; -69.810]), and Zn (-26.980; 95% CrI [-51.910; -2.026]) was observed, compared to HMC3m samples. The mean Cu levels presented a different pattern in relation to the rest. A significant decrease in Cu content in the comparison between HMCI and HMC3m and HMCI and HMC6m shows that the storage period reduced the content of this micronutrient.

Table 2. Descriptive statistics of dosage of osmolality and content of macronutrients and micronutrients in HM baseline and the concentrates HMCI, HMC3m, and HMC6m.

Dosages (units)	HM baseline	HMCI	HMC3m	HMC6m
Energy (Kcal/100 mL)	56.30 (10.51)	79.96 (13.75)	76.98 (13.91)	77.30 (13.78)
Protein (g/100 mL)	0.90 (0.49)	1.48 (0.58)	1.39 (0.61)	1.47 (0.54)
Carbohydrate (g/100 mL)	7.08 (0.67)	9.18 (0.68)	9.21 (0.63)	9.18 (0.64)
Total lipids (g/100 mL)	2.59 (1.08)	4.03 (1.44)	3.68 (1.34)	3.69 (1.35)
Total solids (g/100 mL)	10.76 (1.31)	14.77 (1.72)	14.48 (1.78)	14.53 (1.71)
True protein (g/100 mL)	0.75 (0.40)	1.20 (0.48)	1.13 (0.49)	1.19 (0.43)
Osmolality (mOsm/Kg H ₂ O)	289.48 (43.64)	452.12 (59.79)	456.16 (56.58)	458.14 (55.67)
Calcium (mg/100 mL)	23.24 (4.70)	36.52 (7.18)	38.67 (6.25)	40.63 (6.02)
Magnesium (mEq/L)	2.14 (0.46)	3.38 (0.65)	3.52 (0.83)	3.73 (0.90)
Sodium (mg/L)	135.04 (98.03)	222.52 (169.03)	244.87 (162.80)	233.19 (141.53)
Potassium (mg/L)	601.38 (147.98)	1013.85 (269.45)	1015.04 (222.82)	1152.31 (234.18)
Copper (µg/100 mL)	33.68 (14.60)	48.30 (19.99)	41.16 (15.12)	39.28 (13.71)
Zinc (µg/100 mL)	149.10 (128.15)	203.89 (126.20)	224.46 (152.50)	251.70 (158.76)
Phosphorus (mg/100 mL)	14.63 (6.09)	18.47 (5.97)	20.36 (7.52)	20.50 (7.35)

HM baseline: Human milk baseline; HMCI: HM concentrated for immediate analysis; HMC3m: HM concentrate for analysis after 3 months of storage; HMC6m: HM concentrate for analysis after 6 months of storage; Mean (standard deviation).

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Table 3. Mean difference of dosages of osmolality and content of macronutrients and micronutrients analyzed between HM baseline and concentrates HMCI, HMC3m, and HMC6m.

Variable	Comparisons	Mean difference	95% CrI lower limit	95% CrI upper limit
Energy (kcal/100 mL)	HM baseline—HMCI	-23.680	-25.910	-21.450
	HM baseline—HMC3m	-20.690	-22.930	-18.410
	HM baseline—HMC6m	-21.000	-23.240	-18.750
	HMCI—HMC3m	2.988	0.689	5.193
	HMCI—HMC6m	2.672	0.417	4.878
	HMC3m - HMC6m	-0.316	-2.598	1.951
Proteins (g/100 mL)	HM baseline—HMCI	-0.576	-0.668	-0.482
	HM baseline—HMC3m	-0.490	-0.584	-0.394
	HM baseline—HMC6m	-0.566	-0.660	-0.472
	HMCI—HMC3m	0.086	-0.010	0.179
	HMCI—HMC6m	0.010	-0.086	0.105
	HMC3m - HMC6m	-0.076	-0.174	0.018
Carbohydrates (g/100 mL)	HM baseline—HMCI	-2.095	-2.286	-1.912
	HM baseline—HMC3m	-2.126	-2.307	-1.934
	HM baseline—HMC6m	-2.099	-2.286	-1.912
	HMCI—HMC3m	-0.030	-0.220	0.151
	HMCI—HMC6m	-0.004	-0.189	0.176
	HMC3m - HMC6m	0.026	-0.161	0.209
Total lipids (g/100 mL)	HM baseline—HMCI	-1.437	-1.678	-1.193
	HM baseline—HMC3m	-1.095	-1.340	-0.845
	HM baseline—HMC6m	-1.102	-1.342	-0.863
	HMCI—HMC3m	0.342	0.099	0.591
	HMCI—HMC6m	0.335	0.095	0.579
	HMC3m - HMC6m	-0.006	-0.248	0.233
Total solids (g/100 mL)	HM baseline—HMCI	-4.004	-4.307	-3.703
	HM baseline—HMC3m	-3.715	-4.020	-3.406
	HM baseline—HMC6m	-3.769	-4.072	-3.463
	HMCI—HMC3m	0.289	-0.023	0.588
	HMCI—HMC6m	0.236	-0.070	0.535
	HMC3m - HMC6m	-0.053	-0.363	0.254
True protein (g/100 mL)	HM baseline—HMCI	-0.453	-0.531	-0.375
	HM baseline—HMC3m	-0.380	-0.458	-0.301
	HM baseline—HMC6m	-0.444	-0.522	-0.366
	HMCI—HMC3m	0.072	-0.008	0.149
	HMCI—HMC6m	0.008	-0.070	0.085
	HMC3m - HMC6m	-0.064	-0.143	0.015
Osmolality (mOsm/Kg H ₂ O)	HM baseline—HMCI	-162.200	-177.900	-146.600
	HM baseline—HMC3m	-166.200	-182.000	-150.300
	HM baseline—HMC6m	-168.100	-183.700	-152.300
	HMCI—HMC3m	-3.975	-20.040	11.420
	HMCI—HMC6m	-5.920	-21.630	9.556
	HMC3m - HMC6m	-1.945	-17.880	13.980

(Continued)

Table 3. (Continued)

Variable	Comparisons	Mean difference	95% CrI lower limit	95% CrI upper limit
Calcium (mg/100 mL)	HM baseline—HMCI	-13.290	-14.460	-12.100
	HM baseline—HMC3m	-15.420	-16.620	-14.210
	HM baseline—HMC6m	-17.390	-18.580	-16.200
	HMCI—HMC3m	-2.139	-3.356	-0.971
	HMCI—HMC6m	-4.103	-5.325	-2.904
	HMC3m - HMC6m	-1.965	-3.203	-0.770
Magnesium (mEq/L)	HM baseline—HMCI	-1.234	-1.386	-1.080
	HM baseline—HMC3m	-1.376	-1.532	-1.219
	HM baseline—HMC6m	-1.591	-1.747	-1.437
	HMCI—HMC3m	-0.141	-0.298	0.010
	HMCI—HMC6m	-0.357	-0.515	-0.201
	HMC3m - HMC6m	-0.216	-0.377	-0.061
Sodium (mg/L)	HM baseline—HMCI	-86.600	-113.500	-58.860
	HM baseline—HMC3m	-108.600	-136.600	-80.480
	HM baseline—HMC6m	-97.060	-124.900	-69.270
	HMCI—HMC3m	-22.020	-50.450	5.252
	HMCI—HMC6m	-10.460	-38.880	17.470
	HMC3m - HMC6m	11.570	-17.490	39.240
Potassium (mg/L)	HM baseline—HMCI	-392.100	-451.100	-331.600
	HM baseline—HMC3m	-393.000	-453.600	-330.500
	HM baseline—HMC6m	-523.400	-584.100	-462.800
	HMCI—HMC3m	-0.929	-62.620	58.190
	HMCI—HMC6m	-131.400	-193.500	-70.690
	HMC3m - HMC6m	-130.400	-193.400	-69.810
Copper (µg/100 mL)	HM baseline—HMCI	-14.950	-18.200	-11.740
	HM baseline—HMC3m	-7.799	-11.040	-4.511
	HM baseline—HMC6m	-5.914	-9.141	-2.656
	HMCI—HMC3m	7.154	3.840	10.330
	HMCI—HMC6m	9.040	5.782	12.230
	HMC3m - HMC6m	1.885	-1.388	5.166
Zinc (µg/100 mL)	HM baseline—HMCI	-54.530	-79.040	-30.160
	HM baseline—HMC3m	-74.840	-99.420	-49.910
	HM baseline—HMC6m	-101.800	-126.400	-77.040
	HMCI—HMC3m	-20.320	-45.420	3.872
	HMCI—HMC6m	-47.290	-72.040	-23.100
	HMC3m - HMC6m	-26.980	-51.910	-2.026
Phosphorus (mg/100 mL)	HM baseline—HMCI	-3.859	-5.557	-2.176
	HM baseline—HMC3m	-5.741	-7.437	-4.020
	HM baseline—HMC6m	-5.881	-7.575	-4.175
	HMCI—HMC3m	-1.882	-3.618	-0.209
	HMCI—HMC6m	-2.022	-3.738	-0.355
	HMC3m - HMC6m	-0.140	-1.865	1.570

HM baseline: Human milk baseline; HMCI: HM concentrated for immediate analysis; HMC3m: HM concentrate for analysis after 3 months of storage; HMC6m: HM concentrate for analysis after 6 months of storage. 95% CrI: Credibility interval 95%.

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4. Discussion

Considering the current need for the fortification of HM used in the nutritional support of VLBW infants, this study presents an innovative possibility of formulating a concentrated HM through a simplified method of direct lyophilization of milk donated to HMB. Thus, the merit of this study was to demonstrate the possibility of formulating a product with high nutritional quality components with low cost of production and without ethical bias.

Several authors emphasize the necessary addition of fortifiers to banked human milk in order to increase the nutrient content and thus to be able to meet the special nutritional needs of VLBW infants [2–4]. In the neonatal clinical practice of most public hospitals in Brazil, bovine milk protein-based products such as FM-85 (Nestlé) and Enfamil HMF (Mead Johnson) are used as fortifiers. However, studies report that such products delay gastric emptying and expose preterm infants to the risk of sensitization by heterologous protein and the occurrence of NEC [35–38].

The present study introduces an innovative proposal for the formulation of a human milk-based additive through a simplified direct freeze-drying method followed by a single pasteurization process, which minimizes the risk of contamination and nutrient loss. A similar study carried out in Brazil reports on the development of two human milk-based additives (liquid and powdered) from fat extraction, evaporation, lactose reduction, and lyophilization methods [37]. That same research group has recently improved the formulation method of the powder additive by minimizing the risks of the elaboration and handling processes of HM by simplifying the method to lactose reduction and lyophilization alone. The partial removal of lactose is justified by the control of osmolality of HM strengthened with the powdered additive, which increases tolerance by preterm infants, minimizing the risk of NEC [39–40]. In the present study, the proposed simplified method consists of lyophilization without lactose reduction, and it is important to highlight that the concentrated HM after the addition of the lyophilizate maintained an acceptable osmolality according to the values tolerated by VLBW infants.

The control of osmolality and the physical-chemical and microbiological qualities of HM is essential for its safe provision to VLBW infants. Recent studies show that the procedures carried out in this study, which were established as a protocol by the HMB Network in Brazil, do not change the osmolality or the levels of macronutrients and micronutrients in the milk [31,34,41]. In terms of the lyophilization process, studies report that lyophilizing HM before the freezing process allows for a better preservation of the nutritional properties of milk [42–43]. In this study, the presented values of osmolality and the levels of macronutrients and micronutrients refer to the moment post-lyophilization and pasteurization; therefore, they reflect the final composition of concentrated HM.

The concentrated milk produced in this study is a safe and viable nutritional support alternative since it presents acceptable osmolality and meets the nutritional needs of VLBW infants related to the recommended enteral intake of macronutrients and micronutrients, with few exceptions, and may allow for a reduction in the use of bovine milk protein-based additives. Consensus-based evidence regarding the optimization of nutritional support of VLBW infant reports that the first choice of enteral feeding is own mother's milk fortified with balanced osmolality to ensure safe supply of additional nutrients [4,15]. Studies show that osmolality of a preterm mother's milk is similar to our HM baseline, mature milk donated by "term" mothers in the later stage of lactation, and this allows our lyophilizate to be used as an additive to the milk of the VLBW infant's own mother, thus generating a concentrate with acceptable osmolality [22,40].

Regarding the recommendations of macronutrients and micronutrients expressed per 100 kcal of HMCI presented in [S1 Table](#), the mean and confidence interval (CI) of protein (1.85g;

90% CI [1.68–2.02]), Ca (45.70mg; 90% CI [43.56–47.81]), Mg (5.13mg; 90% CI [4.89–5.36]), Na (27.84mg; 90% CI [22.84–32.83]), Cu (60.42 μ g; 90% CI [54.51–66.33]), Zn (0.25mg; 90% CI [0.22–0.29]), and P (23.10mg; 90% CI [21.34–24.87]) content partially meet the recommendations of enteral intakes established by the Life Sciences Research Office (LRSO 2002) and the European Society of Pediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN 2010) for preterm infants. It is important to emphasize that the content of carbohydrate (11.48g; 90% CI [11.28–11.68]), total lipids (5.04g; 90% CI [4.61–5.46]), and K (126.83mg; 90% CI [118.87–134.79]), expressed per 100 kcal of HMCI (S1 Table) fully meet the established recommendations cited above [44–45].

The evolution of neonatal nutritional protocols over the years shows an improvement in the intake of energy and macronutrients through HM and, consequently, better neonatal growth and neurodevelopment [46–47]. Thus, given the updated literature cited and the adequacy of our concentrate on the recommendations of enteral intakes of LRSO 2002 and ESPGHAN 2010, the protein content of our concentrate could raise concerns. However, studies suggest that the quality of human milk protein and the adequate balance of energy and protein intake are associated advantages in rate and quality of growth as well as better clinical outcomes for VLBW infants [18,48–51]. Thus, we believe that our HM concentrate allows an optimization of the nutritional support of premature babies, as it guarantees the supply of high quality bioactive proteins and adequate content of carbohydrates, lipids and K.

The contents of Ca, Mg, Na, Cu, Zn, and P in the concentrated HM sample types also deserve special attention. According to the recommendations of LRSO 2002 and ESPGHAN 2010, these elements may merit isolated supplementation [44–45]. In addition, adequate early intake of protein and energy in the first week of life of VLBW infants improves the homeostasis of the electrolytes in question [52]. Preterm infants require high amounts of Ca and P elements and, due to low skeletal storages, are at increased risk of nutritional disorders such as growth and developmental deficits, hypophosphatemia, osteopenia in prematurity, and metabolic bone disease [53–54].

The essential micronutrients Mg, Zn and Cu are also related to child growth and development as well as immune function [55–57]. A retrospective study confirmed the association between serum levels of Cu and Zn, gestational age and anthropometric parameters of body weight, and body length and head circumference at birth in preterm infants [57]. Watson et al. warned that the most common cause of Zn deficiency is dietary because of the low micronutrient supply that can be generated by the intake of inadequate milk volumes and non-fortified HM [58]. In addition, Cu deficiency is also recurrent in VLBW infants and can cause anemia, neutropenia, failure to thrive, psychomotor retardation, and bone abnormalities [59]. In addition, it is important to note that chronic depletion of Na also negatively affects weight gain and the growth of preterm infants, especially VLBW infants, since they present higher losses of Na and thus require supplementation [60–61]. Therefore, considering the decision to use the HM concentrate produced in this study as a nutritional support strategy for VLBW infants, it will be necessary to monitor and eventually supplement as needed the serum levels of Ca, Mg, Na, Cu, Zn, and P.

The content of macronutrients and micronutrients as well as the osmolality of our concentrated HM has similarities to some currently marketed breast milk fortifiers. The human milk-based additive Prolact +4 H2MF (Prolacta Bioscience) when added to 80 mL of preterm mother's milk reaches an energy content of 82 kcal per 100 mL, according to the information provided by the manufacturer, which is similar to our HMCI as shown in Table 2. However, while our product is able to provide higher amounts of carbohydrates and total lipids per 100 mL (Table 2), Prolacta +4 H2MF provides greater amounts of protein (2.3 g), Na (57 mg), Ca (123 mg), Zn (0.97 mg), and P (64 mg). Despite this, a recent study warned that HM fortified

with human milk-based additive does not meet the nutritional recommendations for VLBW infants established by the American Academy of Pediatrics Committee of Nutrition because the supply of protein and vitamins is insufficient when it reaches the recommended caloric intake of 130 kcal/kg [62].

In Brazilian public hospitals, one of the most used HM fortifiers is FM-85 (Nestlé). According to the manufacturer's information, such a bovine milk protein-based additives when added to the HM (100 mL) of the premature mother provides: 85 kcal, 2.5 g of protein, 10.3 g of carbohydrates, 4.02 g of total lipids, 100 mg of Ca, and 59 mg of P. Comparing this nutritional information with the composition of our HMCI (Table 2), we observed similar levels of energy content, protein, and carbohydrates. However, the content of Ca and P present in 100 mL of HMCI is lower than that offered by HM fortified with FM-85 (Nestlé) [22].

Some authors suggest that the use of the FM-85 additive (Nestlé) improves the bone mineralization of VLBW infants, although others report the negative effects of its use [35,36,63]. Sullivan et al. found that the nutritional support of VLBW infants using human milk-based fortifier was associated with lower occurrence of NEC or death when compared to the use of bovine milk protein-based additives [35]. This fact is justified by LH allowing the maternal transfer of adaptive immune defenses, especially secretory IgA, which presents in higher concentration in the maternal milk of mothers of preterm infants [64].

The preservation of nutritional and immunological components as well as the safety of HM processed in HMB is a concern addressed in current literature reviews, as studies and proposals to improve milk manipulation protocols are constantly being generated [32,65]. A study conducted by Ahrabi et al. showed that the process of refrigeration at +4°C for 72 hours followed by frozen storage at -20°C for up to 9 months applied to freshly expressed HM was associated with decreased pH and bacterial counts without affecting the content of total protein, fat, lactoferrin, secretory IgA, and osmolality in the samples [66]. However, Sousa et al. proved that pasteurization of HM colostrum caused a reduction of 20, 51, and 23% in IgA, IgM, and IgG concentrations, respectively [67]. A literature review concluded a similar reduction of IgA, IgM, and IgG concentrations in HM after holder pasteurization; however, the authors warn that clinical practice demonstrates that many beneficial properties remain after pasteurization, which strongly justifies the use of HM processed in HMB for feeding preterm infants [34]. Therefore, our proposal for the nutritional support of VLBW infants with concentrated HM is also strengthened.

The present study generated a new hypothesis which will be elucidated in future studies: the possibility of adding lyophilized HM directly into the mother's own raw milk to produce an improved concentrated milk. A systematic review presents the differences between nutrient contents according to gestational stage (preterm versus term) and lactation time, emphasizing that the colostrum of mothers of preterm infants presented a higher protein mean when compared with mature HM [68]. Thus, it is likely that our lyophilized milk (50 mL matured donated milk) when added to a VLBW infant's mother's own raw milk will produce a concentrated HM with a higher protein content and adequate osmolality that can meet the nutritional needs of preterm infants [37,44,68]. Other authors reinforce the initiative to use the mother's own raw milk during the hospitalization period of preterm infants. Dritsakou et al. suggested that feeding VLBW infants predominantly with the mother's own raw milk results in better neonatal outcomes such as higher body length and head circumference at discharge [69]. In addition, a prospective cohort study conducted with VLBW infants fed exclusively with HM demonstrated an association between the early use of the mother's own raw milk and continued breastfeeding at discharge (OR 2.92; 95% CI [1.94–4.40]), and after 6 months (OR 2.70; 95% CI [1.21–6.03]) [70].

In view of all the arguments presented, the concentrated HM produced in this study is an innovative, viable, and less onerous proposal after initial investment, which can provide many benefits to VLBW infants as well as national and international health systems. Finally, the last aim of this study was to evaluate the stability of the concentrated HM samples stored for 3 and 6 months. After 3 months of storage, comparing samples of HMCI and HM3m, the Ca and P contents increased and the levels of energy, total lipids, and Cu were reduced. However, comparing samples of HMCI and HM6m, a significant increase in the levels of Ca, Mg, K, Zn, and P was observed, despite a slight reduction in levels of energy and total lipids, while the osmolality and other nutrients remained stable, except for Cu. The Cu content presented a different pattern in relation to the rest, since it was the only one that suffered a high reduction after the storage period. Despite significant changes in the nutritional content of concentrated HM stored for 3 and 6 months, we considered clinically relevant only the increase of K and the reduction of Cu in the samples. In view of these results, it is possible to preserve the concentrated HM for 6 months; however, it is necessary to consider the changes in the nutritional content of this product, mainly in the K and Cu elements, which suggest the non-storage of this concentrated product. Currently, target fortification has been tested as a method for adjusting macronutrient and micronutrient content in fortified HM with marketed products that even after fortification may not fully meet the nutritional needs of VLBW infants, unlike the concentrated HM produced in this study [71–72]. Several authors suggest that target fortification is safe and capable of individually optimizing the intake of macronutrients (protein, carbohydrate, and fat) by VLBW infants according to the ESPGHAN 2010 guidelines [73–74]. One limitation of this study was the lack of evaluation of diet composition of the donors during the pre-gestational, gestational, and post-gestational periods.

5. Conclusions

The present study confirms the possibility of formulating concentrated HM, generated from the freeze-drying of the milk donated from the HMB, with osmolality and levels of certain macronutrients and micronutrients compatible with the nutritional needs of VLBW infants. It should be noted that the simplified direct lyophilization method for the formulation of our concentrated HM minimizes the risk of contamination due to minimum product handling as well as present low cost after an initial investment and does not characterize ethical bias since the milk was donated. In addition, the HM samples stored for 3 and 6 months were evaluated and their osmolality stability and nutrient content stability were verified, with both increases and reductions of certain nutrients, such as K and Cu. Another issue to be considered is the promising possibility of HM conservation through the freeze-drying process of donated milk. The present authors are committed to future research looking forward to a randomized controlled double blind clinical trial, phase 1 and 2, whose financial support was recently approved by the Brazilian CNPq (National Council for Scientific and Technological Development), in order to evaluate the safety and tolerability, as well as the initial performance of our HM concentrate to be used as a resource for the nutritional support of VLBW infants.

Supporting information

S1 Fig. Boxplot of the descriptive results of osmolality, macronutrients, and micronutrients in samples of types HM baseline, HMCI, HMC3m, and HMC6m.
(TIF)

S1 Table. Mean and confidence interval 90% for osmolality, macronutrients, and micronutrients expressed per 100 kcal in the samples of types HM baseline, HMCI, HMC3m, and

HMC6m.

(PDF)

S1 Dataset. Database collected available.

(XLSX)

S2 Dataset. Descriptive statistic of macronutrients.

(PDF)

S3 Dataset. Descriptive statistic of micronutrients.

(PDF)

Author Contributions

Conceptualization: Anália R. Heck, Francisco E. Martinez, José S. Camelo, Jr.**Data curation:** Mariana M. Oliveira, Davi C. Aragon, Vanessa S. Bomfim, Tânia M. B. Trevilato, Larissa G. Alves, Anália R. Heck, José S. Camelo, Jr.**Formal analysis:** Mariana M. Oliveira, Davi C. Aragon, Tânia M. B. Trevilato, Larissa G. Alves, Francisco E. Martinez, José S. Camelo, Jr.**Funding acquisition:** José S. Camelo, Jr.**Investigation:** Mariana M. Oliveira, Tânia M. B. Trevilato, Anália R. Heck, Francisco E. Martinez, José S. Camelo, Jr.**Methodology:** Mariana M. Oliveira, Davi C. Aragon, Vanessa S. Bomfim, Tânia M. B. Trevilato, Larissa G. Alves, Francisco E. Martinez, José S. Camelo, Jr.**Project administration:** José S. Camelo, Jr.**Resources:** Tânia M. B. Trevilato, Larissa G. Alves.**Software:** Davi C. Aragon.**Supervision:** Larissa G. Alves, José S. Camelo, Jr.**Validation:** Tânia M. B. Trevilato, José S. Camelo, Jr.**Visualization:** Davi C. Aragon.**Writing – original draft:** Mariana M. Oliveira, José S. Camelo, Jr.

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