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FACULDADE DE MEDICINA DE RIBEIRÃO PRETO
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MARIANA MORAES DE OLIVEIRA

**Desenvolvimento e análise do leite humano concentrado com leite humano liofilizado:
Estudo experimental pré-clínico**

Ribeirão Preto

2021

MARIANA MORAES DE OLIVEIRA

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Estudo experimental pré-clínico**

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Ribeirão Preto da Universidade de São Paulo
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Banca Examinadora

Profa. Dra.: Ana Paula de Carvalho Panzeri Carlotti.

Instituição: Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo.

Julgamento: Favorável.

Profa. Dra.: Daniela Saes Sartorelli.

Instituição: Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo.

Julgamento: Favorável.

Profa. Dra.: Daniela Marques de Lima Mota Ferreira.

Instituição: Universidade Federal de Uberlândia.

Julgamento: Favorável.

*Dedico este trabalho à minha mãe e ao meu pai, pelo
amor, apoio e incentivo incondicional.*

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*“O vento do meu espírito soprou sobre a vida.
E tudo que era efêmero se desfez.
E ficaste só tu, que és eterno ...” (Cecília Meireles)*

RESUMO

OLIVEIRA MM. Desenvolvimento e análise do leite humano concentrado com leite humano liofilizado: Estudo experimental pré-clínico [tese]. Ribeirão Preto: Universidade de São Paulo, Faculdade de Medicina de Ribeirão Preto; 2021.

Introdução: O suporte nutricional do recém-nascido prematuro tem sido alvo constante de estudos nos últimos anos devido, principalmente, à sua contribuição para maior sobrevivência do recém-nascido pré-termo e de muito baixo peso (RNMBP) ao nascer (<1500g). O leite materno foi consagrado como recurso padrão ouro para alimentação do RNMBP. As recomendações atuais enfatizam que o RNMBP deve receber o leite da própria mãe como primeira escolha e, quando não disponível, a segunda opção é o leite humano (LH) doado fortificado com aditivos comerciais específicos. A maioria de tais aditivos têm como matéria prima o leite de vaca, ou seja, uma fonte de proteína heteróloga para o organismo humano a qual está associada ao aumento da morbimortalidade do RNMBP. Diante disso, a hipótese desta tese é que o LH doado possa ser liofilizado e utilizado como aditivo natural do próprio LH e, com isso, aumentar a concentração de macro e micronutrientes essenciais a fim de atender as necessidades nutricionais do RNMBP. **Objetivos:** Desenvolver um LH concentrado a partir da adição do LH liofilizado e analisar a segurança microbiológica, a osmolalidade, o valor energético e a composição de macro e micronutrientes essenciais além de elementos potencialmente tóxicos. **Métodos:** O LH concentrado foi desenvolvido por meio dos processos de liofilização, reconstituição e pasteurização. A avaliação da segurança microbiológica foi obtida por meio do teste simplificado de detecção de coliformes totais. A osmolalidade foi obtida pelo método “Osmometria de Ponto de Congelamento”. O valor energético e o teor de macronutrientes foram determinados pela técnica de espectroscopia no infravermelho em equipamento denominado *Human Milk Analyzer* MIRIS. O teor de micronutrientes foi determinado pela técnica de espectrofotometria de absorção atômica com chama e método colorimétrico automatizado. O teor dos elementos potencialmente tóxicos foi determinado pela técnica de espectrometria de massa com plasma indutivamente acoplado. **Resultados:** Artigos publicados em revistas científicas indexadas. **Conclusão:** Esta tese comprova a formulação do LH concentrado a partir de método direto, simples, pouco oneroso e isento de viés ético de liofilização do LH doado e sua utilização como aditivo natural do próprio LH.

Palavras-chave: Leite humano. Liofilização. Nutrientes.

ABSTRACT

OLIVEIRA MM. Development and analysis of human milk concentrated with human milk lyophilized: Pre-clinical experimental study [thesis]. Ribeirão Preto: Universidade de São Paulo, Faculdade de Medicina de Ribeirão Preto; 2021.

Introduction: The nutritional support of premature newborns has been a constant target of studies in recent years, mainly due to its contribution to greater survival of preterm and very low birth weight newborns (VLBW) at birth (<1500g). Breast milk has been established as the gold standard resource for feeding the VLBW. Current recommendations emphasize that the VLBW should receive the mother's own milk as the first choice, however, when not available, the second option is human milk (HM) donated fortified with specific commercial additives. Most of these additives are based on cow's milk that contains a heterologous protein source to the human organism, which is associated with increased morbidity and mortality of VLBW. Therefore, the hypothesis of this thesis is that the HM donated can be lyophilized and used as a natural additive of HM and increase the concentration of essential macro and micronutrients in order to meet the nutritional needs of VLBW. **Aim:** Develop a HM concentrated from the addition of HM lyophilized and analyze the microbiological safety, osmolality, energy value and composition of essential macro and micronutrients, in addition to potentially toxic elements. **Method:** HM concentrated was developed through lyophilization, reconstitution and pasteurization. The evaluation of microbiological safety was obtained through the simplified test for detection of total coliforms. Osmolality was obtained by the "Freezing Point Osmometry" method. The energy value and the macronutrient content were determined by the infrared spectroscopy technique in equipment called Human Milk Analyzer MIRIS. The micronutrient content was determined by flame atomic absorption spectrophotometry technique and automated colorimetric method. The content of potentially toxic elements was determined by the inductively coupled plasma mass spectrometry technique. **Results:** Articles published in indexed scientific journals. **Conclusion:** This thesis proves the formulation of HM concentrated using a direct, simple, inexpensive and ethically free method of lyophilization of HM donated and its use as a natural additive to HM itself.

Keywords: Human milk. Lyophilization. Nutrients.

LISTAS DE ABREVIATURAS E SIGLAS

AAP	Academia Americana de Pediatria
BLH	Banco de Leite Humano
EN	Enterocolite Necrosante
FMRP	Faculdade de Medicina de Ribeirão
HC	Hospital das Clínicas
LH	Leite Humano
OMS	Organização Mundial da Saúde
rBLH	Rede de Bancos de Leite Humano
rBLH-BR	Rede Brasileira de Bancos de Leite Humano
RNMBP	Recém-Nascido pré-termo e de Muito Baixo Peso
USP	Universidade de São Paulo
UTIN	Unidade de Terapia Intensiva Neonatal

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1. INTRODUÇÃO

1.1 Recém-Nascido pré-termo e de Muito Baixo Peso (RNMBP)

A taxa de nascimento prematuro está aumentando em todo o mundo (Chang; Tseng; Chen, 2020; WHO, 2012). O nascimento prematuro, de acordo com a Organização Mundial da Saúde (OMS), ocorre quando os nascidos vivos não completaram 37 semanas de idade gestacional (WHO, 2012, 2019). Tal desfecho de nascimento pode ser causado por vários fatores categorizados em socioeconômicos, demográficos, comportamentais, complicações obstétricas, saúde reprodutiva da mulher além de condições médicas e nutricionais do binômio mãe-bebê (Laelago; Yohannes; Tsige, 2020). Sendo assim, a incidência mundial de nascimento prematuro é bastante variável, porém significativamente mais elevada nos países com recursos financeiros escassos e alta taxa de fertilidade como na África Subsaariana e no sul da Ásia (Purisch; Gyamfi-Bannerman, 2017). Entretanto, países como Índia, China, Brasil e Estados Unidos também têm apresentado um número alarmante de nascimentos prematuros, o que demonstra ser um problema significativo em todos os contextos de renda (WHO, 2019). Estima-se que no ano de 2014 a taxa mundial de nascimento prematuro foi de 9 a 12% (12,65 a 16,73 milhões) dentre os nascidos vivos, ou seja, mais de 1 em cada 10 bebês nasceram prematuros no mundo (Chawanpaiboon et al., 2019; Laelago; Yohannes; Tsige, 2020; WHO, 2012).

Os recém-nascidos pré-termo apresentaram maior risco de morbidades e, consequentemente, mortalidade (Chang; Tseng; Chen, 2020; WHO, 2019). A cada ano, no mundo, cerca de 2,5 milhões de recém-nascidos morrem durante os primeiros 28 dias de vida, sendo que, aproximadamente 80% desses nasceram com baixo peso e dois terços nasceram prematuramente. Felizmente, de acordo com a OMS, a taxa global de mortalidade neonatal diminuiu nos últimos anos (WHO, 2019). Estudo de coorte de nascimentos realizado no Brasil ratifica os dados da OMS e destaca que a mortalidade dos recém-nascidos pré-termo e de muito baixo peso ao nascer (<1500g) diminuiu nos últimos 33 anos, entretanto, enfatiza que tais prematuros ainda contribuem com mais da metade dos óbitos neonatais (Victora et al., 2018).

A redução da mortalidade do recém-nascido pré-termo deve-se aos notáveis avanços técnico-científicos aplicados aos cuidados obstétricos e neonatais durante o pós-natal imediato (Hay, 2008; Victora et al., 2018). A maior sobrevivência do recém-nascido pré-termo está relacionada ao melhor uso de antibióticos, à reanimação neonatal, ao controle da temperatura e redução do estresse por hipotermia, à nutrição adequada que inclui o aleitamento materno, ao controle de líquidos e eletrólitos e ao uso de oxigênio, surfactante e respirador neonatal

(Harrison; Goldenberg, 2016). Tais informações sustentam o fato da taxa de sobrevivência do recém-nascido pré-termo ser mais alta em países desenvolvidos, ou seja, com alta renda e que dispõem de melhores recursos aplicados no cuidado neonatal (Purisch; Gyamfi-Bannerman, 2017; Victora et al., 2018; WHO, 2019). Entretanto, muitos recém-nascidos pré-termo ainda morrem devido à falta de atendimento adequado em diversos países de baixa e média renda (Chawanpaiboon et al., 2019). Portanto, é necessário o contínuo aprimoramento e fortalecimento do cuidado neonatal para melhorar cada vez mais a sobrevivência e o bem-estar do recém-nascido pré-termo, especialmente em países subdesenvolvidos de baixa e média renda (Chawanpaiboon et al., 2019; deRegnier, 2018).

1.2 Nutrição do RNMBP

O suporte nutricional do recém-nascido pré-termo tem sido alvo constante de estudos nos últimos anos devido, principalmente, à sua contribuição para maior sobrevivência do prematuro extremo (<28 semanas de idade gestacional) e com peso muito baixo (<1500g) ao nascer (Harding et al., 2017; Hay, 2008; McNelis; Fu; Poindexter, 2017). Além disso, estratégias para otimizar o cuidado nutricional desempenham um papel importante no crescimento, na redução das morbidades e no neurodesenvolvimento a longo prazo desses recém-nascidos pré-termo (Belfort; Ehrenkranz, 2017; Horbar et al., 2015).

A otimização da nutrição do recém-nascido pré-termo tem como objetivo, além de promover a sobrevivência, permitir que esse recém-nascido extremamente imaturo cresça e se desenvolva como se ainda estivessem no ambiente intra-uterino (Asbury et al., 2019; Hay, 2008; Villar et al., 2015). Sendo assim, os padrões de crescimento pós-natal do recém-nascido pré-termo devem ser os mesmos que o de um feto normal de mesma idade gestacional que cresce no ambiente intra-uterino (AAP, 1985; Villar et al., 2015). Entretanto, apesar dos esforços nos últimos anos para otimizar o suporte nutricional praticado na Unidade de Terapia Intensiva Neonatal (UTIN), muitos recém-nascidos com 501-1500g apresentam déficit de crescimento pós-natal (Horbar et al., 2015).

O déficit de crescimento pós-natal do RNMBP durante o período de hospitalização inicial está relacionado às exigências nutricionais elevadas, ao aporte insuficiente de nutrientes e à fisiopatologia das morbidades associadas à prematuridade (Asbury et al., 2019; Hay, 2008; Martinez; Camelo Jr, 2001).

O nascimento prematuro impede que o recém-nascido vivencie parte ou todo o último trimestre gestacional e, com isso, o crescimento e desenvolvimento que deveriam ocorrer neste

período são prejudicadas. Assim, quanto mais acentuada a prematuridade e menor o peso de nascimento, maiores são as dificuldades enfrentadas pelo recém-nascido devido suas limitações morfofuncionais, morbidades e ao estresse associados à prematuridade (Martinez; Camelo Jr, 2001). Com isso, o crescimento de RNMBP é dificultado por problemas fisiopatológicos relativamente frequentes como hipotensão, hipóxia, acidose, infecções, intervenções cirúrgicas, além de tratamentos farmacológicos com corticosteroides, e também pela imaturidade fisiológica como a limitada motilidade gastrointestinal (Hay et al, 1999).

A nutrição adequada é fundamental para prevenir o retardo do crescimento pós-natal precoce e otimizar o crescimento e o neurodesenvolvimento a curto e longo prazo dos recém-nascidos pré-termo (Belfort; Ehrenkranz, 2017; Embleton; Pang; Cooke, 2001). Portanto, o crescimento e a maturação estrutural satisfatórios, particularmente das estruturas cerebrais, exigem o aporte quantitativo e qualitativo adequado de nutrientes visando evitar deficiências nutricionais assim como indesejáveis sobrecargas metabólico-digestivas (AAP, 1985; CMAJ, 1995; Coviello et al., 2018).

Suprir as necessidades nutricionais do RNMBP é um desafio visto que a prematuridade exige uma demanda aumentada de energia, proteína e micronutrientes essenciais tais como Ferro, Zinco e Cálcio devido ao baixo estoque corporal e rápido potencial de crescimento pós-natal do recém-nascido pré-termo (Agostoni et al., 2010; Kumar et al., 2017; Raiten; Steiber; Hand, 2016). Sabe-se que o aporte aumentado de energia e proteínas durante a primeira semana pós-natal está associado ao melhor neurodesenvolvimento e crescimento aos 18 meses de recém-nascidos com baixo peso (Stephens et al., 2009). Além disso, a ingestão de energia durante a primeira semana de vida também está associada ao crescimento até a idade corrigida de 2 anos de recém-nascidos extremamente prematuros (Hiltunen et al., 2018). Sendo assim, existe uma correlação positiva entre a nutrição enteral precoce adequada e o crescimento, a maturação cerebral e o neurodesenvolvimento de recém-nascidos pré-termo (Cormack et al., 2020; Coviello et al., 2018; Martin et al., 2009; Miller et al., 2014).

1.3 Nutrição enteral precoce exclusiva com Leite Humano (LH)

A nutrição enteral precoce exclusiva com LH é considerada uma estratégia segura, vantajosa e econômica para o suporte nutricional do RNMBP visto que está associada à redução da incidência de Enterocolite Necrosante (EN), da mortalidade neonatal e também dos custos e do tempo de hospitalização desses recém-nascidos pré-termo (Assad; Elliott; Abraham, 2016; Colaizy et al., 2016; Cortez et al., 2018; Sullivan et al., 2010).

O leite materno fornece nutrientes ideais e fatores bioativos de proteção que auxiliam o desenvolvimento dos sistemas imunológico e gastrointestinal, além de apoiar o neurodesenvolvimento a longo prazo do RNMBP (Andreas; Kampmann; Le-Doare, 2015; Brown; Walsh; McGuire, 2019; Patra et al., 2017; Sammallahiti et al., 2017; Walker, 2010). Apesar de alguns estudos afirmarem que a alimentação com fórmula alimentar artificial resulta em maior ganho de peso e melhor crescimento linear e da circunferência da cabeça do recém-nascido pré-termo, tal fórmula também está associada ao aumento do risco de desenvolvimento de EN (Quigley; Embleton; McGuire, 2019). Importante destacar que a terapia com LH durante o período de hospitalização está associada ao melhor neurodesenvolvimento do RNMBP aos 18-20 meses de idade corrigida (Patra et al., 2017; Vohr et al., 2006). Assim, diante dos benefícios dos componentes nutricionais e bioativos do LH, o aleitamento materno foi consagrado como recurso padrão ouro para alimentação do recém-nascido pré-termo (AAP, 2012; Andreas; Kampmann; Le-Doare, 2015; Mosca; Gianni, 2017; Walker, 2010).

As recomendações atuais enfatizam que o recém-nascido pré-termo deve receber o leite da própria mãe como primeira escolha (Dutta et al., 2015; Kumar et al., 2017). Entretanto, as mães de recém-nascidos pré-termo internados na UTIN frequentemente apresentam produção tardia e limitada de leite materno durante as primeiras semanas após o parto (Ikonen et al., 2018; Sethi et al., 2017). Sendo assim, quando o leite da própria mãe não estiver disponível, a segunda escolha é o LH doado ao Banco de Leite Humano (BLH), pasteurizado e, quando clinicamente permitido e/ou necessário, enriquecido com aditivos comerciais específicos para LH (Bertino et al., 2013; Colaizy et al., 2012; Quigley; Embleton; McGuire, 2019; Valentine et al., 2017).

1.4 Aditivos específicos para LH

A imaturidade estrutural e funcional do sistema gastrointestinal do RNMBP não permite que o prematuro tolere o volume de LH capaz de suprir as quantidades necessárias de nutrientes, principalmente energia e proteínas, para garantir o seu adequado crescimento e desenvolvimento durante o período de internação na UTIN (Harding et al., 2017; Seii-Biarag; Mirghafourvand, 2020). Assim, a alimentação predominante com LH cru ou pasteurizado pode ser nutricionalmente insuficiente para o RNMBP culminando em déficits de crescimento e ganho de peso, além de outras complicações relacionadas à subnutrição aguda (Colaizy et al., 2012; Quigley; Embleton; McGuire, 2019). Embora os benefícios nutricionais e imunológicos do LH para o recém-nascido pré-termo já estejam bem estabelecidos, diversos autores defendem a necessidade de fortificação do LH com aditivos específicos com intuito de

fornecer nutrientes na quantidade e qualidade ideais para promover o adequado crescimento e desenvolvimento do RNMBP (Colaizy et al., 2012; Harding et al., 2017; Kumar; Yadav, 2019; Schanler et al., 2018).

Tradicionalmente, os produtos comerciais utilizados como aditivos de LH têm como matéria prima o leite de vaca enriquecido com determinados nutrientes (Arslanoglu et al., 2019; Koo; Tice, 2018). Entretanto, a exposição precoce à proteína do leite de vaca, considerada nutricionalmente heteróloga para o organismo humano, está associada ao déficit de crescimento pós-natal, à maior intolerância alimentar e ao aumento de morbidades como EN cirúrgica e não cirúrgica (Assad; Elliott; Abraham, 2016; Ganapathy; Hay; Kim, 2012; Sullivan et al, 2010).

Recentemente, por meio de técnicas avançadas de lacto-engenharia, os aditivos específicos para o LH derivados exclusivamente do próprio LH foram desenvolvidos com a finalidade de evitar a utilização dos produtos derivados da proteína do leite de vaca e, com isso, reduzir a morbidade e mortalidade do recém-nascidos pré-termo associada ao consumo de proteína heteróloga (Premkumar; Pammi; Suresh, 2019; Sullivan et al, 2010). Apesar de vários estudos apontarem as vantagens da nutrição exclusiva com LH, incluindo a utilização de aditivos à base de LH, pouco se discute a respeito das questões éticas e do custo elevado envolvidos na comercialização de tais produtos provenientes de substância humana, ou seja, matéria prima biológica humana (Arslanoglu et al., 2019; Assad; Elliott; Abraham, 2016; Sullivan et al., 2010). Atualmente, a empresa privada norte americana *Prolacta Bioscience*[®] é a única que produz e comercializa aditivos à base de LH, segundo dados disponíveis em <https://www.prolacta.com>. Entretanto, considerando a realidade econômica das UTINs públicas e as legislações vigentes brasileiras, a utilização dessa única opção disponível de aditivos exclusivamente à base de LH é considerada economicamente inviável além de eticamente questionável (Brasil, 2006, 2016).

1.5 Justificativa e hipótese do estudo

Estudos realizados previamente demonstram que há a possibilidade de desenvolver aditivos líquido, creme e em pó derivados exclusivamente do LH por meio de vários processos de manipulação desse leite tais como a remoção da lactose e a liofilização (Grance et al., 2015; Lucas et al., 1980; Thomaz et al., 2012).

A hipótese da presente tese é que o LH doado ao BLH possa ser liofilizado e utilizado como aditivo natural do próprio LH e, com isso, aumentar a concentração de macronutrientes e micronutrientes essenciais visando atender com qualidade e segurança as necessidades

nutricionais do RNMBP. A expectativa é que este estudo comprove o desenvolvimento e viabilidade do LH concentrado a partir da liofilização direta do LH doado por meio de um método simplificado e pouco oneroso com etapa final de pasteurização, segundo os critérios da Rede Brasileira de Bancos de Leite Humano (rBLH-BR). Além disso, o propósito desse estudo é demonstrar a possibilidade de implantação do método de liofilização e concentração do LH na rotina da rBLH-BR, gerando assim uma alternativa viável ao uso dos aditivos comerciais à base de proteína do leite de vaca atualmente utilizados nas UTINs brasileiras. Secundariamente, os resultados deste estudo permitirão atualizar as informações a respeito da composição do leite materno maduro proveniente de doadoras brasileiras.

1.6 Objetivos geral e específicos

O objetivo geral deste estudo foi desenvolver um LH concentrado pasteurizado a partir da adição do LH liofilizado com adequada segurança microbiológica, osmolalidade e composição nutricional a fim de ser utilizado como recurso para o suporte nutricional do RNMBP durante o período de internação na UTIN. Assim, os objetivos específicos foram:

- Avaliar a segurança microbiológica do LH linha de base e do LH concentrado logo após a pasteurização. Reavaliar a segurança microbiológica do LH concentrado após 6 meses de congelamento (-20°C).
- Determinar a osmolalidade do LH linha de base e do LH concentrado.
- Determinar o valor energético (Kcal/100mL) e a concentração dos seguintes macronutrientes no LH linha de base e no LH concentrado: proteína, carboidrato, lipídios totais, sólidos totais e proteína verdadeira;
- Determinar a concentração dos seguintes micronutrientes essenciais no LH linha de base e no LH concentrado: Cálcio (Ca), Cobre (Cu), Cromo (Cr), Ferro (Fe), Fósforo (P), Magnésio (Mg), Manganês (Mn), Potássio (K), Selênio (Se), Sódio (Na) e Zinco (Zn);
- Determinar a concentração dos elementos potencialmente tóxicos no LH linha de base e no LH concentrado: Alumínio (Al), Arsênio (As), Cádmio (Cd), Chumbo (Pb), Estanho (Sn), Mercúrio (Hg), Níquel (Ni) e Tálcio (Tl);
- Avaliar a estabilidade da osmolalidade, do valor energético e da concentração de determinados macronutrientes (proteína, carboidrato, lipídios totais, sólidos totais, proteína verdadeira) e micronutrientes (Ca, Mg, Na, K, Cu, P e Zn) essenciais no LH concentrado após 3 e 6 meses de congelamento (-20°C).

- Avaliar a adequação das concentrações de macro e micronutrientes essenciais no LH concentrado confrontando a literatura científica atual referente às recomendações nutricionais para recém-nascidos pré-termo.

2. MATERIAIS E MÉTODOS

A presente tese foi realizada no BLH e Laboratório de Pediatria (Seção de Metais e Doenças Raras), ambos pertencentes ao Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo (HC-FMRP-USP). Além disso, parte deste estudo teve a parceria das seguintes instituições: Laboratório de Ecotoxicologia e Parasitologia Ambiental da Escola de Enfermagem de Ribeirão Preto, Universidade de São Paulo; Laboratório de Toxicologia e Saúde Ambiental, Universitat Rovira i Virgili (Espanha); Laboratório de Ciência do Solo, Universitat de Barcelona (Espanha). Esta tese foi realizada com material biológico humano e, devido a isso, foi necessária a submissão e aprovação pelo Comitê de Ética em Pesquisa em Seres Humanos do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto - USP (Parecer HREC nº 738.080).

O LH utilizado neste estudo foi proveniente de doações da produção excedente das doadoras as quais foram informadas sobre a natureza do estudo e, aquelas que se dispuseram a participar, assinaram o “Termo de Consentimento Livre e Esclarecido”. Após a triagem clínica e sorológica preconizada pela rBLH-BR, as doadoras consideradas aptas para participarem do estudo receberam orientações sobre como massagear e ordenhar os seios além de como rotular e pré-estocar o LH cru ordenhado em frasco de vidro estéril e inerte previamente fornecido pelo BLH do HC-FMRP-USP (BLH-IFF/NT-09.18; BLH-IFF/NT-16.11; BLH-IFF/NT-17.11; BLH-IFF/NT-18.11). A equipe do BLH em questão foi responsável pelo transporte em caixas isotérmicas, recepção e degelo das amostras de LH cru ordenhado (BLH-IFF/NT-19.11; BLH-IFF/NT-20.11; BLH-IFF/NT-21.11; BLH-IFF/NT-24.11).

No BLH do HC-FMRP-USP, as amostras doadas de LH cru ordenhado descongeladas passaram pelos processos de seleção e classificação como determinado pela rBLH-BR (BLH-IFF/NT-23.11). Os critérios de seleção incluíram a verificação das condições da embalagem, de sujidades, da cor, do *flavor* e da acidez Dornic (BLH-IFF/NT-25.11; BLH-IFF/NT-26.11; BLH-IFF/NT-27.11; BLH-IFF/NT-28.11; BLH-IFF/NT-29.11). As amostras que não preencherem os parâmetros de normalidade referentes aos critérios de seleção foram consideradas impróprias para consumo e descartadas como lixo hospitalar (BLH-IFF/NT-08.11).

Os critérios de classificação incluíram a verificação do período de lactação, a acidez Dornic e o conteúdo energético determinado pelo Crematócrito (BLH-IFF/NT-23.11).

A acidez Dornic do LH cru ordenhado foi obtida por meio da acidez titulável determinada com o auxílio de uma solução padrão de hidróxido de sódio (NaOH), um titulante alcalino. A técnica da acidez titulável baseia-se em uma reação estequiométrica entre o titulante alcalino padrão NaOH e os constituintes com caráter ácido presentes no LH cru ordenhado, até a completa neutralização. O ponto final da reação é revelado através de uma solução indicadora que gera uma mudança de cor de acordo com a mudança de pH. Neste estudo, a solução titulante foi o NaOH N/9, também conhecido como Solução Dornic, sendo que cada 0,01mL gasto para neutralizar 1mL de leite humano ordenhado corresponde a 1 grau Dornic (1°D). Sendo assim, foram pipetadas 3 alíquotas de 1mL da amostra de LH cru e acondicionadas, respectivamente, em 3 tubos de crioscopia com capacidade para 5 mL. Após a adição de uma gota da solução indicadora de fenolftaleína à cada alíquota, procedeu-se à titulação com o gotejamento de NaOH N/9. Durante o procedimento, o tubo de ensaio contendo LH foi constantemente e levemente agitado para evitar a incorporação de ar no produto. O procedimento foi interrompido quando ocorreu a viragem do indicador, ou seja, quando o produto assumiu coloração róseo-clara. Com isso, a leitura do volume da solução titulante utilizados foi utilizada para determinar a acidez da amostra lembrando que cada 0,01mL de NaOH gasto para neutralizar 1mL de leite humano ordenhado correspondeu a 1°D (BLH-IFF/NT-29.11).

O Crematócrito foi a técnica analítica empregada para determinação do teor de creme o qual permitiu o cálculo estimado do teor de gordura e do conteúdo energético do leite humano cru ordenhado (BLH-IFF/NT-30.11). A técnica consistiu em homogeneizar manualmente as amostras e pipetar 1mL do LH cru em um tubo de ensaio (5mL). Após aquecimento em banho-maria (40°C por 15 minutos), as amostras foram homogeneizadas por agitação em vórtex e, em seguida, foram coletadas de forma independente 3 alíquotas com auxílio de tubos capilares com uma das extremidades vedada com massa. Os tubos capilares foram centrifugados por 15 minutos a 3.500 rpm e procedeu-se a leitura em milímetros das colunas de creme e soro formadas. Após a leitura, aplicou-se os resultados nas fórmulas descritas abaixo para obter os valores de % de Creme, % de Gordura e Kcal/L. O valor final correspondeu à média aritmética das 3 alíquotas avaliadas.

1. Teor de Creme: $\text{Coluna de Creme (mm)} \times 100 / \text{Coluna total (mm)} = \% \text{ de Creme.}$
2. Teor de Gordura: $(\% \text{ Creme} - 0,59) \div 1,46 = \% \text{ de Gordura.}$
3. Conteúdo Energético Total: $(\% \text{ de Creme} \times 66,8 + 290) = \text{Kcal/L.}$

Os critérios de inclusão das amostras foram o LH cru ordenhado doado com período de lactação maior que 15 dias, denominado LH cru maduro, e com um valor de acidez Dornic até 8°D. Considerando um valor de 0,36 para o desvio padrão em relação à média esperada de concentração proteica (2,20 g/dL) e um valor de erro absoluto de 0,1, com nível de confiança de 95%, foi necessário obter 50 amostras. Após o descongelamento do LH cru ordenhado e realização dos procedimentos de seleção e classificação no BLH do HC-FMRP-USP, sob campo de chama, cada amostra de mesma doadora foi subdividida em frascos contendo alíquotas de 80mL, 75mL e 50mL. As alíquotas de 80mL foram denominadas “LH linha de base”. As alíquotas de 50mL foram congeladas em freezer -30°C por 24 horas, liofilizadas e reconstituídas com as alíquotas de 75mL da mesma doadora. Detalhadamente, o processo de liofilização das alíquotas de 50mL congeladas foi realizado utilizando o liofilizador de bancada Lyophilizer L108-LioTop® o qual promoveu a desidratação do LH por sublimação após as amostras terem sido submetidas ao vácuo seguido de aumento gradativo da temperatura. Após 72 horas, as amostras foram retiradas do liofilizador e mantidas em cadeia de frio para serem reconstituídas com 75 mL do LH da mesma doadora em cabine de segurança biológica e sob campo de chama. Tais amostras foram então denominadas “LH concentrado”.

As amostras de LH linha de base e de LH concentrado foram pasteurizadas a fim de assegurar a qualidade sanitária final do LH ordenhado. Assim, a pasteurização foi o tratamento térmico aplicado ao LH ordenhado com o objetivo de inativar 100% dos microrganismos patogênicos e 99,99% da microbiota saprófita, que é equivalente a um tratamento para inativação térmica da *Coxiella burnetti*. Tal tratamento térmico compreendeu um período de pré-aquecimento entre o momento da colocação dos frascos a serem pasteurizados no banho-maria até a estabilização da temperatura a 62,5°C. Após o pré-aquecimento, os frascos com LH ordenhado permaneceram em banho-maria a 62,5°C por 30 minutos. O banho-maria utilizado neste estudo não disponha de agitador automático, portanto, o pesquisador responsável pela pasteurização agitou manualmente cada frasco, sem retirá-lo do banho-maria, de 5 em 5 minutos. Transcorridos os 30 minutos relativos à letalidade térmica, foi conduzido o resfriamento dos frascos pela imersão dos mesmos em um banho de água e gelo até que o LH pasteurizado atingisse temperatura $\leq 5^{\circ}\text{C}$ (BLH-IFF/NT-34.11). Imediatamente após o resfriamento das amostras, em cabine de segurança biológica e sob campo de chama, foram coletas pequenas quantidades de cada amostra de LH linha de base e de LH concentrado para a avaliação da segurança microbiológica e determinação da osmolalidade, do valor energético (Kcal/100mL), da concentração de macro e micronutrientes essenciais e também de elementos potencialmente tóxicos. Parte das amostras coletadas foram imediatamente congeladas a -20°C

para as análises após 3 e 6 meses de armazenamento e também para as análises as serem realizadas no Laboratório de Pediatria (Seção de Metais e Doenças Raras) do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo (Ribeirão Preto, São Paulo, Brasil) e também no Laboratório de Toxicologia e Saúde Ambiental da Faculdade de Medicina da Universitat Rovira i Virgili (Reus, Catalunha, Espanha).

O controle da qualidade microbiológico das amostras de LH linha de base e LH concentrado pasteurizadas foi realizado no BLH do HC-FMRP-USP por meio do teste simplificado de detecção de coliformes totais seguindo a lógica preconizada para alimentos que institui a utilização de micro-organismos indicadores de qualidade sanitária, como determinado pela rBLH-BR. O método do teste, conduzido em cabine de segurança biológica e sob campo de chama, consistiu no inóculo de quatro alíquotas de 1mL cada, pipetadas de forma independente, em tubos com 10mL de Caldo Verde Brilhante (BGBL) a 5% p/v, com tubos de Durham em seu interior. Após a inoculação e incubação a $36 \pm 1^\circ\text{C}$ por 24-48 horas, a presença de gás no interior do tubo de Durham caracteriza resultado presuntivo positivo. Neste caso, o tubo presuntivo positivo deve ser repicado, com auxílio de alça bacteriológica, para tubos contendo BGBL na concentração de 40g/L. Após a incubação destes tubos a $36 \pm 1^\circ\text{C}$ por 24-48 horas, a presença de gás confirma a existência de microrganismos do grupo coliforme e caracteriza o LH pasteurizado como impróprio para consumo (BLH-IFF/NT-40.11). Após 6 meses, parte das amostras de LH concentrado armazenadas a -20°C foram descongeladas em banho-maria a 37°C por 15 minutos e reavaliadas quanto a qualidade microbiológica.

A osmolalidade foi determinada nas amostras de LH linha de base e de LH concentrado por meio do método “Osmometria de Ponto de Congelamento” utilizando o equipamento Osmômetro Microprocessado PZL-1000 (PZL) alocado no BLH do HC-FMRP-USP e que gera resultados expressos em mOsm/Kg H_2O . Após 3 e 6 meses, parte das amostras de LH concentrado armazenadas a -20°C foram descongeladas em banho-maria a 37°C por 15 minutos e reavaliadas quanto a osmolalidade.

A determinação do valor energético (Expresso em Kcal/100mL) e da concentração dos macronutrientes proteína, carboidrato, lipídios totais, sólidos totais e proteína verdadeira (Expressos em g/100mL) no LH linha de base e no LH concentrado foi realizado por meio do equipamento *Human Milk Analyzer* MIRIS (Miris, Uppsala, Suécia) alocado no BLH do HC-FMRP-USP. As amostras foram aquecidas em banho-maria a 37°C por 15 minutos e homogeneizadas com o equipamento *Sonicator* MIRIS (Miris, Uppsala, Suécia) imediatamente antes de serem inseridas no *Human Milk Analyzer* MIRIS, o qual realiza análises com base na técnica de espectroscopia no infravermelho. Após 3 e 6 meses, parte das amostras de LH

concentrado armazenadas a -20°C foram descongeladas em banho-maria a 37°C por 15 minutos, sonicadas e reavaliadas quanto a concentração dos macronutrientes acima citados.

A determinação dos micronutrientes essenciais Ca, Mg, Na, K, Cu, Zn e P foi realizada no Laboratório de Pediatria (Seção de Metais e Doenças Raras) do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo (Ribeirão Preto, São Paulo, Brasil). Inicialmente congeladas no BLH do HC-FMRP-USP, as amostras foram encaminhadas para o Laboratório de Pediatria e descongeladas em banho-maria à 37°C. Após sonicação das amostras por 10 minutos, as diluições adequadas foram realizadas de acordo com as curvas de calibração específica para cada micronutriente a ser dosado pela técnica de Espectrofotometria de Absorção Atômica com Chama em equipamento EAA 55B VARIAN no qual se utiliza lâmpadas de catodo oco específicas, exceto para o micronutriente fósforo. O método colorimétrico automatizado foi utilizado para dosar o P nas amostras.

As concentrações dos micronutrientes essenciais (Fe, Cr, Mn e Se) e dos elementos potencialmente tóxicos (Al, As, Cd, Pb, Sn, Hg, Ni e Tl) nas amostras de LH linha de base e LH concentrado foram determinadas pelo Laboratório de Toxicologia e Saúde Ambiental da Faculdade de Medicina da Universitat Rovira i Virgili (Reus, Catalunha, Espanha) após um preparo prévio realizado no Laboratório de Pediatria (Seção de Metais e Doenças Raras) do Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo (Ribeirão Preto, São Paulo, Brasil). Inicialmente congeladas no BLH do HC-FMRP-USP, as amostras foram encaminhadas para o Laboratório de Pediatria e descongeladas em banho-maria à 37°C. Após sonicação das amostras por 10 minutos, alíquotas de 1 mL foram digeridas quimicamente com 1 mL de ácido nítrico Suprapur 65% (Merck KGaA®, Darmstadt, Alemanha) em recipientes de Teflon hermético os quais foram submetidos à estufa (FANEM®, Modelo 315 SE, São Paulo, Brasil) com temperatura controlada de 80°C por 8 horas e posterior resfriamento em temperatura ambiente por 12 horas. As amostras digeridas foram diluídas 1:5 com água ultrapura, acondicionadas em tubos Falcon e enviadas para o Laboratório de Toxicologia e Saúde Ambiental da Faculdade de Medicina da Universitat Rovira i Virgili. Após o recebimento das amostras, os técnicos do Laboratório de Toxicologia realizaram uma diluição 1:5 com ácido nítrico 1% (Merck KGaA, Darmstadt, Alemanha) e determinaram a concentração de Fe, Cr, Mn, Se, Al, As, Cd, Pb, Sn, Hg, Ni e Tl nas amostras de LH linha de base e LH concentrado por meio da técnica da espectrometria de massa com plasma indutivamente acoplado em equipamento ICP-MS da PerkinElmer® NexION 350D e que gera resultados expressos em µg/L.

A análise estatística descritiva se propôs a apresentar o valor médio mais o desvio padrão referente a cada análise das dosagens da osmolaridade, do valor energético e dos macronutrientes e micronutrientes essenciais Ca, Mg, Na, K, Cu, Zn e P. Quanto às concentrações dos micronutrientes essenciais e elementos potencialmente tóxicos Fe, Cr, Mn, Se, Al, As, Cd, Pb, Sn, Hg, Ni e Tl, a estatística descritiva se propôs a apresentar o valor médio, desvio padrão e valor mínimo e máximo de cada análise. Além disso, modelos lineares bayesianos de efeito misto foram ajustados usando OpenBUGS para estimar a diferença média e intervalos confiáveis de 95% ao comparar as amostras de LH linha de base e LH concentrado.

3. RESULTADOS

A presente tese será apresentada no formato de coletânea de artigos de estudos publicados nas revistas científicas indexadas, multidisciplinares e de acesso livre *PLOS ONE* e *Environmental Research* contemplados, respectivamente, nos seguintes capítulos 1 e 2. As publicações abordam detalhadamente o desenvolvimento do LH concentrado com LH liofilizado assim como as análises da osmolalidade, da segurança microbiológica, do valor energético e do teor de determinados macronutrientes e micronutrientes essenciais e também elementos potencialmente tóxicos.

3.1 Capítulo 1

O capítulo 1 apresenta a publicação na revista *PLOS ONE* referente ao desenvolvimento do LH concentrado a partir do LH liofilizado e análise da osmolalidade, da segurança microbiológica, do valor energético e do teor de determinados macronutrientes e micronutrientes essenciais (proteína, carboidrato, lipídios totais, sólidos totais, proteína verdadeira; Ca, Mg, Na, K, Cu, P e Zn) no LH linha de base e no LH concentrado, seguido de avaliação da estabilidade da segurança microbiológica e da composição nutricional.

3.2 Capítulo 2

O capítulo 2 apresenta a publicação na revista *Environmental Research* referente à determinação da concentração dos micronutrientes essenciais Cr, Fe, Mn e Se além dos elementos traço potencialmente tóxicos Al, As, Cd, Pb, Sn, Hg, Ni e Tl no LH linha de base e no LH concentrado.

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.

study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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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 (60.42μg; 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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Essential and toxic elements in human milk concentrate with human milk lyophilizate: A preclinical study

Mariana M. Oliveira^a, Tânia M.B. Trevilato^b, Susana I. Segura-Muñoz^c, Davi C. Aragon^a, Larissa G. Alves^d, Martí Nadal^e, Montse Marquès^e, José L. Domingo^e, Jordi Sierra^{e,f}, José Simon Camelo Jr.^{a,*}

^a Department of Pediatrics, Children's Hospital, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

^b Section of Metals and Rare Diseases, Laboratory of Pediatrics, Clinics Hospital of Ribeirão Preto, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

^c Laboratory of Ecotoxicology and Environmental Parasitology, Ribeirão Preto College of Nursing, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

^d Human Milk Bank, Clinics Hospital of Ribeirão Preto, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

^e Laboratory of Toxicology and Environmental Health, School of Medicine, IISPV, Universitat Rovira i Virgili, Reus, Catalonia, Spain

^f Laboratory of Soil Science, Faculty of Pharmacy, Universitat de Barcelona, Catalonia, Spain

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ABSTRACT

Concentrated human milk (HM-concentrate) can be obtained from the simple and inexpensive method of donated breast milk direct lyophilization. A previous study reported that HM-concentrate contains the adequate amount of main macro- and micronutrients for use as a nutritional resource for preterm infants with very low birth weight admitted to neonatal intensive care units. However, further details need to be elucidated about HM-concentrate composition, particularly its content of essential and potentially toxic trace elements. Therefore, this study aimed to determine the concentration of essential and toxic elements in human milk considered baseline (HM-baseline) and HM-concentrate, as well as to quantify changes in concentration of these elements after the HM concentration process. The concentration of Aluminum, Arsenic, Cadmium, Chromium, Iron, Mercury, Manganese, Nickel, Lead, Selenium, Tin, and Thallium was analyzed by inductively coupled plasma-mass spectrometry (ICP-MS). Moreover, Bayesian linear mixed effect models were applied to estimate the mean difference between HM-baseline and HM-concentrate samples. After comparison (HM-concentrate versus HM-baseline), a significant increase in concentration was observed only for Manganese (0.80 µg/L; 95% CrI [0.16; 1.43]) and Selenium (6.74 µg/L; 95% CrI [4.66; 8.86]), while Lead concentration (−6.13 µg/L; 95% CrI [−8.63; −3.61]) decreased. This study provides latest and reliable information about HM composition. After milk concentration by lyophilization, there was a significant increase only in the essential elements Manganese and Selenium. The essential micronutrient content in HM-concentrate was similar or higher than that in preterm mothers' milk, which suggests it is viable for nutritional support of preterm infants. In addition, the low concentrations of potentially toxic elements in HM-concentrate indicates that it is safe for consumption by premature newborns.

1. Introduction

Despite rising preterm survival rates, studies show that worldwide, an estimated 9–12% (12.65–16.73 million) of live births are premature (Chawanpaiboon et al., 2019). This significant figure suggests a need for improvement in neonatal nutritional support for preterm infants, especially in low and middle-income countries. Exclusive early enteral nutrition with human milk (HM) is an effective nutritional support strategy for feeding very low birth weight (VLBW) preterm infants.

Such practice is associated with lower incidence of necrotizing enterocolitis and neonatal mortality, with reduction in time and cost of hospitalization (Assad et al., 2016; Colaizy et al., 2016; Cortez et al., 2018).

Breast milk provides optimal nutrients and protective factors that enhance immune and gastrointestinal systems, as well as supports long-term neurodevelopment of VLBW preterm infants (Andreas et al., 2015; Brown et al., 2019; Patra et al., 2017; Sammallahti et al., 2017; Walker, 2010). Current recommendations emphasize that preterm newborn

* Corresponding author. Ribeirão Preto Medical School - University of São Paulo Bandeirantes, Av., 3900 - 5th floor - Off D506 - HC Criança, SP, Brazil.
E-mail addresses: jscamelo@fmrp.usp.br, jscamelo85@gmail.com (J.S. Camelo).

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Fig. 1. Flowchart of processes applied to HM-baseline and HM-concentrate samples.

infants should receive the mother's milk or HM donated to the Human Milk Bank (HMB), plus commercial fortifiers to improve the milk composition, since milk is the singular nutritional source for infants (Bertino et al., 2013; Colaizy et al., 2012; Dutta et al., 2015; Valentine et al., 2017).

An innovative recent study produced HM-concentrate from a simple and inexpensive method of direct lyophilization of donated breast milk (Oliveira et al., 2019). This concentrated HM demonstrated microbiological safety, acceptable osmolality, and suitable nutritional composition of the main macro- and micronutrients needed by a VLBW preterm infant.

These findings can be translated to clinical trials to test this nutritional resource in preterm infants admitted to neonatal intensive care units. However, there are still not so well-defined aspects of HM-concentrate composition, such as essential and potentially toxic elements content, which could represent a risk for the health of premature

infants. Therefore, this study aimed to determine the essential and potentially toxic elements concentration in HM-baseline and HM-concentrate samples, as well as to quantify changes in concentration of these elements after the HM concentration process. The expectation is that our findings confirm and reinforce the viability of HM-concentrate for nutritional support of very low birth weight preterm infants. Furthermore, these findings may have clinical relevance, and update information about mature breast milk composition, which will also be useful for HMB networks worldwide.

2. Materials and methods

This cross-sectional study was part of a recently published report approved by the Human Research Ethics Committee of the Clinics Hospital, Ribeirão Preto Medical School - USP (HREC Report No. 738.080), regarding development of a HM-concentrate with HM

lyophilizate (Oliveira et al., 2019). Voluntary donors of surplus HM production were informed about the nature of the study, signed a free and informed consent form, and underwent clinical and serological screening. Criteria included being healthy, not smoking more than 10 cigarettes per day, not consuming alcohol or illegal drugs, and providing medical and laboratory exams.

2.1. HM sample collection

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 samples passed through the selection and classification processes recommended by the Brazilian HMB Network (available at: <http://www.redeblh.fiocruz.br>). A total of 50 samples (≥ 220 mL) were collected, falling within a standard deviation of 0.36 of the expected average of protein concentration (2.20 g/dL), absolute error value of 0.1, and confidence level of 95%. Surplus mature breast milk with a Dornic acidity value of up to 8°D was included in this study.

2.2. HM-baseline and HM-concentrate sample generation

Procedures applied to formulate HM-Baseline and HM-Concentrate samples in the HMB at the Clinics Hospital of Ribeirão Preto were recently reported (Oliveira et al., 2019). Briefly, HM-baseline samples were initially separated into two aliquots of 80 and 125 mL each: the 80 mL aliquot was considered the HM-baseline sample. To formulate the HM-concentrate sample, the 125 mL aliquot was further divided into two aliquots of 50 and 75 mL. The 50 mL aliquot was transferred to an inert, sterile glass container and was frozen (-20 °C for 24 h). Subsequently, the frozen sample was lyophilized under vacuum (Lyophilizer L108, LioTop®, São Carlos - SP - Brazil). After 72 h, the lyophilized sample was reconstituted into the other (75 mL) aliquot reserved for the formulation of HM-concentrate. All samples were subjected to pasteurization and microbiological quality control as recommended by the Brazilian HMB Network. In collection tubes, aliquots were separated and immediately frozen (-20 °C) for transport and subsequent analysis. Procedures applied to HM-baseline and HM-concentrate samples are shown in Fig. 1.

2.3. Essential and toxic element analysis

Concentrations of essential and potentially toxic elements were analyzed in HM-baseline and HM-concentrate samples at two different timepoints. Preliminary sample preparation was performed in the Laboratory of Pediatrics, Section of Metals and Rare Diseases, Clinics Hospital of Ribeirão Preto, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil. Samples were thawed at room temperature followed by heating in a water bath (37 °C) and homogenized by sonication for 10 min. Aliquots (1 mL) of the samples were chemically digested with 1 mL of 65% Suprapur nitric acid (Merck KGaA®, Darmstadt, Germany) in hermetic Teflon vessels at room temperature overnight (12 h), followed by heating (80 °C) for 8 h in a hot plate (FANEM®, Model 315 SE, São Paulo, Brazil). Digested samples were diluted by 1:5 using ultrapure water and were sent for analysis by the Laboratory of Toxicology and Environmental Health, School of Medicine, Universitat Rovira i Virgili (Reus, Catalonia, Spain). Following 1:5 dilution with 1% nitric acid (Merck KGaA, Darmstadt, Germany), the concentration of Aluminum (Al), Arsenic (As), Cadmium (Cd), Chromium (Cr), Iron (Fe), Mercury (Hg), Manganese (Mn), Nickel (Ni), Lead (Pb), Selenium (Se), Tin (Sn), and Thallium (Tl) was analyzed by inductively coupled plasma-mass spectrometry (ICP-MS; PerkinElmer® NexION 350D). Reference certificates, duplicates and laboratory blanks were used as quality control to ensure method accuracy and precision. The limit of quantification (LOQ) and limit of detection (LOD) were, respectively: 12.5 and 6.25 µg/L for Al; 0.1 and

0.05 µg/L for As, Cd, and Tl; 2.0 and 1.0 µg/L for Cr; 0.2 and 0.1 µg/L for Hg; 20.0 and 10.0 µg/L for Fe; 1.25 and 0.625 µg/L for Mn, Ni, Pb and Sn; and 2.5 and 1.25 µg/L for Se.

2.4. Statistical analysis

Mean values, standard deviation (SD), and minimum and maximum values were used to statistically analyze the essential and toxic element concentrations. Bayesian linear mixed effect models were adjusted using OpenBUGS to estimate mean difference and 95% credible intervals when comparing essential and toxic elements concentrations between HM-baseline and HM-concentrate samples.

3. Results and discussion

3.1. Characteristics of study population

After selection and classification processes based upon donor inclusion criteria ($n = 50$ donors), the mean Dornic acid value was 4.34°D and the standard deviation (SD) was 1.59. The mean age, weight, height, and gestational age of the donors was, respectively, 30 years (SD = 6), 67.5 kg (SD = 11.9), 1.63 m (SD = 0.59), and 38.4 weeks (SD = 2.3). All donors reported that they did not smoke or consume alcohol and/or drugs at the time of clinical screening.

3.2. Descriptive results

Descriptive statistics (Mean, SD, maximum and minimum values) for essential and potentially toxic elements concentration (µg/L) in HM-baseline and HM-concentrate are summarized in Table 1.

One sample was discarded from the statistical analysis due to possible external contamination. Comparative results of essential and toxic elements concentration in HM-baseline and HM-concentrate samples are presented in Table 2.

After comparison between HM-concentrate (T2) and HM-baseline (T1), a significant increase was observed in the concentration of only the essential elements Mn (0.80 µg/L; 95% CrI [0.16; 1.43]) and Se (6.74 µg/L; 95% CrI [4.66; 8.86]), while the concentration of the toxic element Pb (-6.13 µg/L; 95% CrI [-8.63 ; -3.61]) decreased. There was no significant difference in the concentration of other elements (Al, As, Cd, Cr, Fe, Hg, Ni, Sn, and Tl) between HM-concentrate (T2) and HM-baseline (T1).

Table 1

Descriptive statistics (mean, standard deviation (SD), and maximum and minimum values) of essential and potentially toxic elements concentration (µg/L) in HM-baseline and HM-concentrate ($n = 49$).

Element	HM-baseline			HM-concentrate		
	Mean (SD)	Min	Max	Mean (SD)	Min	Max
Al	211.06 (90.06)	112.30	574.60	202.21 (69.30)	121.00	413.90
As	0.29 (0.13)	< LOQ	0.63	0.29 (0.14)	< LOQ	0.80
Cd	0.37 (0.19)	< LOQ	0.96	0.35 (0.25)	0.10	1.18
Cr	4.39 (1.57)	2.32	8.93	5.65 (5.34)	2.63	29.81
Fe	673.55 (264.05)	321.40	1608.90	756.95 (279.39)	313.40	1762.50
Hg	0.39 (0.25)	< LOQ	1.68	0.42 (0.20)	< LOQ	1.25
Mn	5.07 (1.28)	2.75	9.14	5.88 (1.92)	2.94	15.08
Ni	6.34 (3.46)	2.96	25.64	6.06 (3.72)	2.65	20.42
Pb	12.79 (8.19)	2.17	41.94	6.69 (3.14)	2.72	19.63
Se	7.89 (4.63)	< LOQ	27.60	14.60 (7.16)	< LOQ	27.50
Sn	2.78 (1.48)	< LOQ	9.46	3.26 (4.19)	< LOQ	25.01
Tl	< LOQ (0.06)	< LOQ	0.30	0.10 (0.12)	< LOQ	0.63

LOQ: Limit of quantification.

Table 2

Mean difference between essential and potentially toxic elements concentration ($\mu\text{g/L}$) in HM-baseline (T1) and HM-concentrate (T2) ($n = 49$).

Element	Comparison	Mean difference	95% CrI Lower limit	95% CrI Upper limit
Al	T2 - T1	-7.99	-39.65	23.67
As	T2 - T1	-0.004	-0.05	0.04
Cd	T2 - T1	-0.02	-0.11	0.07
Cr	T2 - T1	1.25	-0.31	2.70
Fe	T2 - T1	72.76	-29.93	171.65
Hg	T2 - T1	0.03	-0.05	0.12
Mn	T2 - T1	0.80	0.16	1.43
Ni	T2 - T1	-0.28	-1.62	1.17
Pb	T2 - T1	-6.13	-8.63	-3.61
Se	T2 - T1	6.74	4.66	8.86
Sn	T2 - T1	0.48	-0.70	1.68
Tl	T2 - T1	0.03	-0.01	0.06

T1 – HM-baseline; T2 – HM-concentrate; 95% CrI: Credible Interval 95%.

3.3. General findings

This study evaluated the concentration of essential and potentially toxic trace elements in HM-baseline and HM-concentrate samples and quantified changes in the levels of these elements after the concentration process. A significant increase (HM-concentrate versus HM-baseline) in the concentration of the essential elements Mn and Se was found, while concentration of potentially toxic element Pb decreased. Interestingly, the present study provides updated and reliable information about the composition of mature breast milk (HM-baseline) processed by the Brazilian HMB Network, which should be also useful for HMB networks worldwide.

3.4. Essential elements in HM

Premature and low birth weight infants have slightly higher requirements of essential micronutrients such as Cr, Fe, Mn, and Se due to the rapid postnatal growth and development, iatrogenic losses, and limited body storage of these elements. Premature birth interrupts the mother's transfer of these nutrients during the third trimester of gestation. Thus, such infants are at increased risk of developing nutritional deficiencies, such as iron deficiency anemia (Finch, 2015; Harding et al., 2017).

Iron plays a crucial role in the metabolic pathway of energy production, in oxygen transport, and in erythropoiesis. It also aids in the growth and neurodevelopment of preterm infants (Moreno-Fernandez et al., 2019). A recent systematic review indicates that long-term Fe supplementation results in improved Fe levels and a reduction in iron deficiency anemia in VLBW preterm infants (McCarthy et al., 2019). The present study highlights that Fe is a predominant essential micronutrient in HM-baseline and HM-concentrate samples (Table 1). Previous studies conducted in Greece (Leotsinidis et al., 2005), Sweden (Björklund et al., 2012), Chile (Castro et al., 2014), Australia (Mohd-Taufek et al., 2016), Switzerland (Sabatier et al., 2019), and Brazil (Alves Peixoto et al., 2019) detected lower Fe content in breast milk compared to the values found in the present study (Table A1). Furthermore, Fe content in HM samples collected 1–2 months postpartum was higher than in samples collected after 6–7 months and 12 months of lactation (Taravati Javad et al., 2018). Therefore, despite the higher baseline Fe content of the HM-baseline used in the present study, the concentration process maintained the high Fe content (Table 2).

Furthermore, a cross-sectional study demonstrated a positive correlation between Fe and Mn concentration at all stages of lactation (Li et al., 2016). Therefore, like Fe, Mn content also decreases over the lactation stages of colostrum, transitional, and mature milk (Björklund et al., 2012). In addition, Mn concentrations in human milk differs among specific populations (Poland, USA, Argentina, and Namibia;

Table A1) (Klein et al., 2017). Interestingly, a study conducted on Brazilian breast milk showed a very low Mn content (Cardoso et al., 2014). However, our study found a higher Mn content in HM-baseline, as well as a significant increase of $0.80 \mu\text{g/L}$ in HM-concentrate (Table 2). Manganese is an enzymatic cofactor in carbohydrate and lipid metabolism, and also essential micronutrient for growth and bone development (ATSDR, 2012c; Kumar et al., 2017). The higher Mn content in HM-concentrate could positively impact the health of VLBW preterm infants. Likewise, it has been suggested that Fe and Mn content in mothers' milk does not meet the nutritional requirements of preterm infants (Table A1). Consequently, due to its high Fe and Mn content, we believe that our HM-concentrate may be a viable alternative for the nutritional support of premature infants.

Selenium is another essential micronutrient with a high concentration in HM-baseline and a significant increase in concentration in HM-concentrate (Tables 1 and 2). Studies conducted in Sweden (Björklund et al., 2012), Chile (Castro et al., 2014), Australia (Mohd-Taufek et al., 2016), Switzerland (Sabatier et al., 2019), and Slovenia (Jagodic et al., 2020; Snoj Tratnik et al., 2019) indicate similar Se concentrations to those reported in the present study (Table A1). Significantly, Se has been highlighted as an important element for optimal function of antioxidant defense systems in preterm infants (Tindell and Tipple (2018)). Selenoenzymes, including glutathione peroxidase, protect the body against free radical damage that contributes to risk for prematurity diseases such as bronchopulmonary dysplasia, retinopathy of prematurity, and necrotizing enterocolitis (Finch, 2015). The Se content in HM-concentrate is similar to that in mother's milk of preterm infants. Consequently, it indicates suitability of HM-concentrate for use in premature newborns (Sabatier et al., 2019). This speculation is supported by the fact that the milk from mothers of preterm infants has a mean Se content of $16.1 \mu\text{g/L}$, which meets the nutritional requirements of premature newborns (Alves Peixoto et al., 2019). In contrast, micronutrients such as Se and Cr are absent in most commercial human milk fortifiers, suggesting that they do not meet the nutritional needs of premature newborns (Koo and Tice (2018)).

Small doses of the essential nutrient Cr are required for proper energy metabolism (ATSDR, 2012b). The mean Cr level in HM-concentrate was similar to that found in a recent study (Samiee et al., 2019) of Iranian breast milk (Table A1). However, previous surveys conducted in Sweden (Björklund et al., 2012) and Brazil (Cardoso et al., 2014) reported very low Cr content in HM (Table A1). Importantly, Holder pasteurization by thermal treatment applied to HMB, as performed in the present study, does not affect Fe, Mn, and Se content in breast milk (Alves Peixoto et al., 2019; Mohd-Taufek et al., 2016).

3.5. Potentially toxic elements in HM

Breast milk is non-invasive biomonitoring matrix of exposures to harmful elements such as Al, As, Cd, Hg and Pb. Toxic organic substances, as well as toxic trace elements, which have been accumulate in the tissues of mothers can cross the mammary glands, exposing newborns to postnatal contamination through HM intake. Potential damage to neonatal growth and development caused by these toxic elements may be severe and permanent (Bansa et al., 2017; Bassil et al., 2018; Bastos et al., 2018; Gil and Hernández (2015); Letinić et al., 2016; Rebelo and Caldas, 2016). Early exposure to excessive amounts of these potentially toxic elements can contribute to long-term adverse health effects, such as neurodevelopmental disorders and damage to immune and respiratory functions (Cao et al., 2016; Heyer and Meredith (2017)). In general, breast milk has a low concentration of potentially toxic elements. Importantly, the concentration of the toxic microelements Al, As, Cd, and Pb decrease rapidly and significantly over the stages of lactation (Chao et al., 2014; Martínez et al., 2019). Thus, it is expected that mature milk used in our HM-concentrate may be safe for the nutritional support of premature newborns.

Aluminum is the most abundant metal in the earth's crust (soil,

water, and air), as well as the most frequent and highly concentrated element in breast milk (ATSDR, 2008; Bastos et al., 2018). However, Al concentration in our HM-baseline and HM-concentrate was lower than that reported by studies conducted in Iran (Taravati Javad et al., 2018) and Spain (Martínez et al., 2019) (Table A2). It is important to note that only a small amount of Al enters the infant's body through breastfeeding (ATSDR, 2008). However, the sources of Al exposure may be also contaminated by Pb, As, and Cd (Weidenhamer et al., 2017), where As is the most toxic of these elements.

Exposure of lactating mothers to As may occur through drinking water, as well as via consumption of contaminated rice/cereals, fish/seafood, mushrooms, and poultry (ATSDR, 2007; Bassil et al., 2018). The "Priority List of Substances" released in 2017 by the Agency for Toxic Substance and Disease Registry ranked Arsenic as the potentially most hazardous element to human health. The low As concentration in our HM samples was comparable to other studies performed in Sweden (Björklund et al., 2012), Chile (Castro et al., 2014), USA (Carignan et al., 2015), Turkey (Kılıç Altun et al., 2018), and Slovenia (Jagodic et al., 2020; Snoj Tratnik et al., 2019) (Table A2). However, two studies (Bassil et al., 2018; Klein et al., 2017) showed higher mean As concentrations than reported in the present study ($> 2 \mu\text{g/L}$). Nevertheless, only a negligible amount of As is excreted in breast milk, even in highly exposed mothers (Fängström et al., 2008). Furthermore, it has been emphasized that mixed breastfed babies ingested higher As amounts than exclusive breastfeeding group (Castro et al., 2014). Thus, our innovative proposal based on exclusive breast milk diet could be protective to VLBW against As exposure.

Lead, Hg, and Cd are harmful toxic elements that must be taken into account and analyzed in certain regions, particularly in developing countries, to avoid the risk of exposure to infants (ATSDR, 2019; Klein et al., 2017; Pajewska-Szmyt et al., 2019; Rebelo and Caldas, 2016; Samiee et al., 2019).

Mobilization of Pb stored in mother's bones is the main route of HM contamination, and consequent infant exposure may cause damage to neurological development (Chao et al., 2014). Fortunately, the Pb content in breast milk is low despite high Pb concentration in maternal blood (Baranowska-Bosiacka et al., 2016). The mean Pb concentration in breast milk ranges from 2.0 to 16.8 $\mu\text{g/L}$, as reported by a major multicenter study conducted by the World Health Organization (WHO) (WHO/IAEA, 1989). Recently, two cross-sectional studies conducted in Iran detected high Pb levels in HM. Gasoline, food, water, dust, and cosmetics (lipstick) were identified as possible contamination sources (Khanjani et al., 2018; Vahidinina et al., 2019). However, the present study found a mean Pb concentration in HM-baseline similar to another Brazilian study (Marques et al., 2013), and the values were within acceptable WHO limits (WHO/IAEA, 1989). Interestingly, the concentration process to produce HM-concentrate resulted in a significant reduction of Pb content compared to HM-baseline (Table 2). A study of lactating Lebanese women found a slightly higher mean Pb concentration compared to the concentration observed in the current survey (Table A2) (Bassil et al., 2018). This study also demonstrated a positive association between pre-pregnancy smoking and Pb and Cd levels in breast milk (Bassil et al., 2018).

Cadmium is found in the earth's crust (air, water, and soil) and is associated with Zn, Pb, and Cu ores (ATSDR, 2012a). Tobacco leaves accumulate high Cd levels, and consequently maternal consumption of tobacco or exposure to tobacco smoke enhanced the risk of infant exposure (ATSDR, 2012a; Bassil et al., 2018). Although smoking during pregnancy increased Cd levels in HM by 37%, there was a non-significant increase associated with the number of smoked cigarettes per day (García-Esquinas et al., 2011). Moreover, an Iranian study detected a higher Cd concentration in breast milk of non-smoking mothers (Khanjani et al., 2018). Cadmium concentrations in breast milk from nursing Brazilian mothers ranged from < 0.05 to $7.0 \mu\text{g/L}$ (Cardoso et al., 2014), while the present study detected even lower Cd concentrations in HM-concentrate samples. In addition, these values do not

exceed the WHO reported limit ($1 \mu\text{g/L}$) (WHO/IAEA, 1989), which is not surprising since Brazil strongly recommends against smoking during prenatal care.

Further, a low level of Ni was found in our HM samples, being the Ni concentration lower than that reported in previous studies conducted in Spain (Martínez et al., 2019) and Iran (Salmani et al., 2016) (Table A2). However, investigations conducted in Sweden (Björklund et al., 2012) and Brazil (Cardoso et al., 2014) found Ni levels in HM even lower than observed in the present study (Table A2). Nickel concentrations in breast milk ranged from 4.9 to $16.1 \mu\text{g/L}$ according to WHO (WHO/IAEA, 1989). The Agency for Toxic Substance and Disease Registry indicates that the Ni concentration in breast milk is either similar to or less than that in cow's milk-based or soy-based infant formula (ATSDR, 2005). Contaminated water and food, as well as inhalation of tobacco smoke may increase the Ni concentration in breast milk (Salmani et al., 2016). The low Pb, Cd, and Ni concentrations found in this study are possibly due to the non-smoking habits reported by our breast milk donors.

The present study also identified low Hg content in HM samples. Similar to Pb, Hg is excreted in breast milk from exposed mothers, and may affect infant neurodevelopment, causing irreversible damage (ATSDR, 1999; WHO, 2008). A multicenter WHO study identified that the normal range for Hg concentration in HM is $1.4\text{--}3.3 \mu\text{g/L}$ (WHO/IAEA, 1989). Moreover, recent studies have shown that breast milk Hg levels range from 0.5 to $7.0 \mu\text{g/L}$ worldwide (Cunha et al., 2013; García-Esquinas et al., 2011; Rebelo and Caldas, 2016; Vahidinina et al., 2019). Although some studies have reported a positive correlation between fish consumption and Hg accumulation in breast milk, a recent cross-sectional study showed no association between these factors (Behrooz et al., 2012; Letinić et al., 2016; Vahidinina et al., 2019).

Overall, our study confirms the optimal quality of donated breast milk, since only low concentrations of the potentially toxic elements Al, As, Pb, Cd, and Hg were detected in HM samples. Lastly, studies related to the concentration of Sn and Tl in breast milk remain scarce. A multicenter WHO study reported an Sn concentration ranging from 1.4 to $3.3 \mu\text{g/L}$ in HM, which is comparable with our findings (WHO/IAEA, 1989). Although prenatal Tl exposure could increase the risk of prematurity and low birth weight (Qi et al., 2019; Wu et al., 2019), Tl content in breast milk has not yet been investigated (WHO/IAEA, 1989).

3.6. Study limitations

Limitations of this study include the fact that the dietary patterns of the milk donors were not studied, which could have been integrated with the analysis of breast milk elemental composition. Furthermore, the number of donors was limited number due to the difficulties of collecting samples as human milk donations are scarce. Furthermore, the number of donors was rather limited due to the difficulties of collecting samples, taking into account that human milk donations are scarce. Nevertheless, the results of this investigation provide updated and reliable information about breast milk composition. Furthermore, we demonstrate that the HM lyophilization and concentration processes are safe, since only low concentrations of the potentially toxic elements Al, As, Pb, Cd, and Hg were detected in HM-concentrate samples. However, further studies of HM-concentrate are still necessary in order to assess the safety and tolerability for the effective nutritional support of preterm infants.

4. Conclusions

Assessment of essential and toxic elements concentration in HM-concentrate confirms and reinforces the viability of this product specially developed to support the effective nutritional requirements of premature infants. Essential micronutrient content in HM-concentrate was similar to or better than that in preterm mothers' milk, which

clearly suggests it can be used for preterm infants. In addition, the low content of toxic elements indicates a low risk for premature newborns. Furthermore, the present study provides updated and reliable information about the composition of mature breast milk (HM-baseline), which should be also useful for HMB networks worldwide.

Phase 1, randomized, controlled, double-blinded clinical trial is underway to assess the safety, tolerability, and initial performance of our HM-concentrate as a nutritional support resource for VLBW infants.

Author contribution

Mariana M. Oliveira: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft. Tânia M. B. Trevilato: Conceptualization, Data curation, Investigation, Methodology, Resources, Validation Writing - Writing - review & editing. Susana I. Segura-Muñoz: Data curation, Investigation, Methodology, Resources, Validation and Writing - review & editing. Davi C. Aragon: Formal analysis Writing - Writing - review & editing. Larissa G Alves: Investigation Resources. Martí Nadal: Investigation, Methodology, Resources, Validation Writing - Writing - review & editing. Montse Marquès: Validation and Writing - review & editing. José L. Domingo: Investigation, Methodology, Resources, Validation and Writing - review & editing. Jordi Sierra: Investigation, Methodology, Resources, Validation and Writing - review & editing. José Simon Camelo Jr: Conceptualization, Funding acquisition, Project administration, Resources and Writing - review & editing.

Declaration of competing interest

No conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2020.109733>.

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4. DISCUSSÃO

A presente tese comprova a possibilidade de formulação do LH concentrado pasteurizado a partir de um método direto, simples e pouco oneroso de liofilização do LH maduro doado e sua utilização como aditivo natural do próprio LH, seguido de etapa final de pasteurização. Além disso, o LH concentrado pasteurizado apresentou segurança microbiológica, osmolalidade aceitável e o aumento significativo da concentração de macro e micronutrientes essenciais, o que torna esse produto uma alternativa viável e segura para o adequado suporte nutricional do RNMBP durante o período de internação na UTIN. Sendo assim, consideramos como mérito desse estudo a possibilidade de formulação de um LH concentrado pasteurizado com alta qualidade nutricional e comprovada segurança microbiológica a partir de um método pouco oneroso e isento de viés ético, visto que o LH utilizado foi proveniente de doações da produção excedente de leite materno por doadoras recrutadas pelo BLH.

A primeira publicação científica, apresentada no capítulo 1 desta tese, relata os métodos empregados na formulação do LH concentrado assim como a determinação do valor energético, da concentração de macro e micronutrientes essenciais, da osmolalidade, além das análises da segurança microbiológica e da estabilidade nutricional e microbiológica após 3 e 6 meses de congelamento. A comprovação e quantificação da concentração final do LH após acréscimo do LH liofilizado foi obtida mediante comparação entre o LH linha de base e o LH concentrado ambos pasteurizados. Assim, foi possível detectar o aumento significativo do valor energético e da concentração de macro e micronutrientes essenciais tais como proteína, carboidratos, lipídios totais, Ca, Mg, Na, K, Cu, Zn e P no LH concentrado. A segurança microbiológica foi comprovada e se manteve por até 6 meses de armazenamento congelado. A estabilidade nutricional do LH concentrado foi estatisticamente parcial após 3 e 6 meses de armazenamento congelado (-20°C), porém tais alterações são consideradas clinicamente pouco significantes.

O capítulo 2 desta tese apresenta a publicação científica referente a análise de elementos essenciais (Cr, Fe, Mn e Se) e potencialmente tóxicos (Al, As, Cd, Pb, Sn, Hg, Ni e Tl) tanto no LH concentrado quanto no LH linha de base com o propósito de complementar o estudo anterior descrito no capítulo 1. Assim, foi possível comprovar o aumento significativo apenas dos micronutrientes essenciais Mn e Se no LH concentrado, o que reforça a viabilidade e segurança do LH concentrado a ser utilizado no suporte nutricional do RNMBP durante o período de internação na UTIN.

A presente tese também demonstrou a possibilidade de implantação do método de liofilização e desenvolvimento do LH concentrado na rotina da rBLH-BR, reforçando assim a utilização do LH maduro doado liofilizado como alternativa promissora para o desuso dos aditivos comerciais utilizados nas UTINs brasileiras.

Atualmente, os aditivos comerciais específicos para LH mais utilizados na prática clínica neonatal dos hospitais públicos brasileiros são o FM-85® (Nestlé®) e o Enfamil HMF® (Mead Johnson®), os quais têm como matéria prima o leite de vaca enriquecido com macro e micronutrientes essenciais (Arslanoglu et al., 2019; Brown et al., 2016; Thomaz et al., 2012).

Diversos autores defendem a necessidade de fortificação do LH com aditivos específicos com o objetivo de aumentar o conteúdo de nutrientes e, com isso, tentar atender as necessidades nutricionais especiais do RNMBP e garantir o adequado crescimento e desenvolvimento desse recém-nascido durante o período de internação na UTIN (Colaizy et al., 2012; Harding et al., 2017; Kumar, Yadav, 2019; Raiten; Steiber; Hand, 2016; Schanler et al., 2018). Entretanto, estudos relatam que a exposição precoce à proteína do leite de vaca, considerada heteróloga para o organismo humano, está associada ao déficit de crescimento pós-natal, à maior intolerância alimentar, ao esvaziamento gástrico lento, ao tempo prolongado de nutrição parenteral, ao aumento de morbidades tais como EN e sepse e, conseqüentemente, maior mortalidade neonatal (Assad; Elliott; Abraham, 2016; Ganapathy; Hay; Kim, 2012; Hair et al., 2018; Perrella et al., 2015; Sullivan et al, 2010). Além disso, recente e extenso estudo destaca que tais aditivos comerciais de LH não atendem satisfatoriamente as necessidades nutricionais do RNMBP visto que muitos micronutrientes essenciais estão ausentes ou presentes em quantidades bastante inadequadas (Koo; Tice, 2018).

Diante do dilema da utilização de aditivos à base de proteína do leite de vaca, técnicas avançadas de lacto-engenharia permitiram o desenvolvimento de fortificantes líquido e cremoso derivados do próprio LH, os quais foram associados à desfechos satisfatórios tais como a maior velocidade de ganho de peso e de crescimento, redução do tempo de internação hospitalar, redução de morbidade por sepse e EN, além de menor mortalidade do RNMBP (Abrams et al., 2014; Cristofalo et al., 2013; Hair et al., 2014; Hair et al., 2016a; Hair et al., 2016b; Sullivan et al, 2010).

O estudo inicial referente ao desenvolvimento do método para obtenção do LH concentrado foi publicado na década de 80 e, desde então, vem sendo continuamente explorado e aprimorado. O método original de concentração do LH baseia-se na centrifugação desse leite

para obtenção de um creme, seguido de extração da lactose por diálise simples e, finalmente, liofilização (Lucas et al., 1980).

Inspirados no estudo anterior, pesquisadores brasileiros comprovaram a possibilidade de formulação de dois aditivos, líquido e em pó, derivados exclusivamente do LH por meio dos processos de retirada da gordura, evaporação para concentração dos nutrientes e remoção da lactose e, por fim, liofilização (Thomaz et al., 2012). Anos depois, o mesmo grupo de pesquisa publicou o aprimoramento da técnica de desenvolvimento do suplemento homólogo de LH a partir da remoção da lactose e liofilização, o que minimizou etapas de manipulação, mudanças de temperatura e do estado físico do leite com o propósito de melhorar a conservação dos nutrientes (Grance et al., 2015). Ainda assim, o processamento e a preparação dos aditivos derivados do LH demandam uma avançada tecnologia e a extensa manipulação do leite para a obtenção da concentração dos componentes nutricionais e fatores bioativos.

Atualmente, a empresa privada norte americana denominada *Prolacta Bioscience*® é a única que produz e comercializa o total de seis modalidades de aditivos à base de LH para diferentes volumes e valores calóricos finais do LH fortificado, cujas informações completas estão disponíveis em <https://www.prolacta.com>. Tais produtos foram clinicamente testados em diversos estudos: randomizado controlado, observacional, comparativo e retrospectivo. Tais autores comprovaram os desfechos satisfatórios de redução do tempo de internação hospitalar, redução de morbidades tais como EN cirúrgica e não cirúrgica, sepse tardia, displasia bronco pulmonar e retinopatia da prematuridade, além da promoção de maior ganho de peso, melhor crescimento longitudinal e maior ganho de circunferência da cabeça dos RNMBP (Cristofalo et al., 2013; Hair et al., 2013; Hair et al., 2014; Hair et al., 2016a; Hair et al., 2016b; Huston et al., 2018; Sullivan et al., 2010).

Os benefícios associados à utilização dos aditivos produzidos pela empresa *Prolacta Bioscience*® são promissores, entretanto, consideramos inviável a utilização de tais produtos pelas UTINs brasileiras devido ao custo elevado e, principalmente, pelas questões éticas envolvidas na produção e comercialização desses aditivos provenientes de substância humana (Ganapathy; Hay; Kim, 2012). No Brasil, de acordo com o parágrafo 4º do artigo 199 da Constituição da República Federativa de 1988, é vedado todo tipo de comercialização de substâncias humanas como o leite materno, fato que talvez inviabilizasse legalmente a utilização dos aditivos da *Prolacta Bioscience*® (Brasil, 2016). Sendo assim, consideramos como mérito dos estudos apresentados nesta tese a possibilidade de formulação de um LH concentrado pasteurizado a partir de um método pouco oneroso, isento de viés ético e que, após

incorporado na rotina da rBLH-BR, disponibilizará um produto não monetizado (sem fins lucrativos) com alta qualidade nutricional e segurança microbiológica para ser utilizado como recurso nutricional para o RNMBP durante o período de internação na UTIN. Importante destacar que a Resolução RDC/Anvisa nº. 171 de 2006 proíbe a comercialização dos produtos coletados, processados e distribuídos pelo BLH e Posto de Coleta de LH (Brasil, 2006).

A alta qualidade nutricional e a segurança microbiológica do LH concentrado desenvolvido no presente estudo foi comprovada a partir de vários métodos de análise. Inicialmente, foi importante a avaliação da osmolalidade do leite após o processo da concentração visto que alguns autores sugerem que a alta osmolalidade da nutrição enteral pode estar associada à eventos adversos tais como disfunções gastrointestinais e EN em prematuros (Book et al., 1975; Pearson; Johnson; Leaf, 2013).

As recentes diretrizes referentes à alimentação enteral do recém-nascido pré-termo não incluem um nível superior recomendado para a osmolalidade (Agostoni et al., 2010; Koletzko; Poindexter; Uauy, 2014; Kumar et al., 2017; Raiten; Steiber; Hand, 2016). Além disso, atual revisão sistemática aponta que não há evidências consistentes de que a osmolalidade da alimentação enteral na faixa de 300 a 500 mOsm/kg H₂O esteja associada à sintomas gastrointestinais adversos em neonatos (Ellis et al., 2019). Entretanto, apesar de ultrapassada, a recomendação vigente da Academia Americana de Pediatria (AAP) orienta que a osmolalidade não exceda 450 mOsm/kg H₂O (ou, aproximadamente, a osmolaridade de 400 mOsm/L) para o leite materno ou fórmulas infantis (Barness et al., 1976).

A osmolalidade natural do leite materno é de aproximadamente 300 mOsm/kg H₂O, entretanto, o acréscimo de aditivos específicos de LH à base de proteína do leite de vaca proporciona um aumento para níveis superiores a 400 mOsm/kg H₂O (Chandran et al., 2017; Kreissl et al., 2013; Rochow et al., 2013; Rosas et al., 2016).

O presente estudo detectou valores médios de osmolalidade no LH linha de base (289.48 mOsm/kg H₂O) compatível com a osmolalidade natural do leite materno, assim como o LH concentrado (452.12 mOsm/kg H₂O) apresentou osmolalidade aceitável e semelhante ao leite com adição de aditivos comerciais à base de proteína do leite de vaca, como citado acima e observado na Tabela 2 do capítulo 1 desta tese.

Estudos anteriores referentes aos métodos de concentração do LH apostaram na remoção da lactose e posterior liofilização do LH com objetivo de ajustar a osmolalidade do produto final aos valores considerados aceitáveis pela AAP (Grance et al., 2015; Lucas et al., 1980; Thomaz et al., 2012). Entretanto, tais autores detectaram valores de osmolalidade (335-

438 mOsm/kg H₂O) semelhantes aos nossos (Grance et al., 2015). Diante disso, é importante destacar que o método simplificado proposto pelo presente estudo, ou seja, a mínima manipulação do leite por meio da liofilização direta sem redução da lactose, possibilitou a formulação do LH concentrado com valor aceitável de osmolalidade e possível de ser tolerada pelo RNMBP. Além disso, tais valores de osmolalidade não apresentaram um aumento significativo durante os períodos de 3 e 6 meses de armazenamento (-20°C), fato sustentado por outro estudo que não detectou o aumento da osmolalidade do leite materno cru armazenado congelado (-20°C) por até 9 meses (Ahrabi et al., 2016).

A segurança microbiológica é o principal critério a ser cumprido no que diz respeito a alimentação enteral fornecida aos recém-nascidos pré-termo. Sendo assim, para garantir a segurança microbiológica do LH concentrado desenvolvido neste estudo foi aplicado o tratamento térmico de pasteurização com objetivo de inativar termicamente agentes virais e bacterianos a partir de uma fase inicial de aquecimento rápido, seguido da manutenção da temperatura de 62,5°C por 30 minutos e, finalmente, uma fase final de resfriamento rápido ($\leq 5^{\circ}\text{C}$) (Capriati et al., 2019; Moro et al., 2019; Peila et al., 2016; Wesolowska et al., 2019). Sabe-se que tal tratamento térmico reduz parcialmente as concentrações de determinadas proteínas específicas de ação imunológica como imunoglobulinas (IgA, IgM e IgG), lactoferrina, lisozima e lactoperoxidase (Buffin et al., 2018; Peila et al., 2016; Picaud; Buffin, 2017; Sousa; Delgadillo; Saraiva, 2014). Entretanto, o método de pasteurização não afeta negativamente importantes componentes nutricionais e bioativos do LH tais como o conteúdo de determinados ácidos graxos essenciais e vitaminas lipossolúveis extremamente relevantes para o desenvolvimento do sistema nervoso central de recém-nascidos (Peila et al., 2016; Picaud; Buffin, 2017). Portanto, a pasteurização é o procedimento recomendado por diretrizes nacionais e internacionais de BLH para o garantir a qualidade nutricional e a segurança microbiológica do LH doado oferecido ao recém-nascido, fato que corrobora o método aplicado no presente estudo (Brasil, 2006, 2008; Moro et al., 2019; Peila et al., 2016). Após os testes, as análises de segurança microbiológica realizadas nas amostras no período imediato e após o armazenamento (-20°C) confirmaram a adequada qualidade sanitária do produto por até 6 meses.

Os efeitos do processo de concentração do LH podem ser observados nas publicações referentes aos capítulos 1 e 2 desta tese. É importante destacar que o valor energético e a concentração de macro (proteína, carboidrato, lipídios totais, sólidos totais, proteína verdadeira) e micronutrientes (Ca, Mg, Na, K, Cu, Zn, P, Mn e Se) essenciais avaliados apresentaram um aumento estatisticamente significativo no LH concentrado quando comparado com o LH linha

de base, exceto o conteúdo de Cr e Fe. Além disso, é importante destacar que não houve aumento estatisticamente significativo na concentração de elementos potencialmente tóxicos (Al, As, Cd, Pb, Sn, Hg, Ni e Tl) no LH concentrado, fato possivelmente justificado pela favorável baixa concentração de tais elementos no LH linha de base. A literatura científica referente ao procedimento de liofilização sustenta nossos resultados visto que tal método preserva importantes propriedades nutricionais e bioativas do LH tais como ácidos graxos essenciais e imunoglobulinas (Albarrán et al., 2017; Cortez; Soria, 2016; Hahn et al., 2019; Lozano et al., 2014). Sendo assim, comprovamos a possibilidade de concentração do LH por meio do processo de liofilização direta seguida de reconstituição em mesmo leite (LH linha de base) e, portanto, a formulação de um produto à base de LH exclusivo seguro do ponto de vista microbiológico e com alta qualidade nutricional.

O armazenamento do LH concentrado congelado (-20°C) por 3 e 6 meses proporcionou efeitos diversos na concentração de macro e micronutrientes essenciais, como descrito no capítulo 1 desta tese. Primeiramente, foi detectada uma redução do valor energético e nas concentrações de lipídios totais e Cu no LH concentrado após o armazenamento congelado. Além disso, as amostras de LH concentrado apresentaram um ligeiro aumento nas concentrações de Ca, Mg, K, P e Zn após congelamento. Entretanto, é importante destacar que as concentrações de carboidrato e proteínas se mantiveram estáveis por 3 e 6 meses de armazenamento a -20°C.

Estudo recente valida nossos resultados pois comprova que o armazenamento do LH congelado (-20°C) por até 9 meses não afetou seu conteúdo de proteínas totais e imunoativas (lactoferrina e IgA secretora), o que sugere a preservação de importantes componentes nutricionais e imunológicos do leite materno (Ahrabi et al., 2016). Entretanto, embora alguns autores reportem o aumento significativo de 13.4% na concentração de proteínas no LH pasteurizado após 8 meses congelado (-20°C), estudo recente não encontrou alterações nos teores de proteínas no leite materno após 2 meses em mesma condição de armazenamento (Kim et al., 2019; Waard et al., 2018). Além disso, estudo destaca o não comprometimento do conteúdo energético e dos teores de gordura e carboidrato no LH pasteurizado durante o período de 8 meses de armazenamento congelado (-20°C) (Waard et al., 2018). Porém, semelhantes aos nossos resultados, estudos relatam que o armazenamento sob congelamento (-20°C) por 3 e 6 meses reduziu discretamente o conteúdo energético e o teor de gordura do LH pasteurizado enquanto o conteúdo de proteínas e carboidratos permaneceu estável (García-Lara et al., 2012; García-Lara et al., 2013; Tanriverdi et al., 2019). Sendo assim, tal variabilidade das

concentrações de macro e micronutrientes sugere uma estabilidade estatisticamente parcial do LH concentrado armazenado congelado (-20°C) porém clinicamente pouco significativa.

Finalmente, atendendo ao último objetivo específico desta tese, foi possível avaliar se as concentrações de macro e micronutrientes essenciais presentes no LH concentrado pasteurizado atendem as necessidades nutricionais específicas do recém-nascido pré-termo, de acordo com as recomendações nutricionais vigentes. Sendo assim, as quantidades de carboidratos (11.5g), lipídios totais (5.0g), K (126.8mg), Cr (0.7µg) e Se (1.8µg) presentes no LH concentrado (média expressa por 100 kcal) atendem plenamente às recomendações nutricionais estabelecidas pela *European Society of Pediatric Gastroenterology, Hepatology, and Nutrition* e pela *Life Sciences Research Office*, referentes à alimentação enteral adequada do recém-nascido pré-termo (Agostoni et al., 2010; Tudehope et al., 2013).

Diversos estudos relatam que a ingestão adequada de energia e lipídios promove melhor ganho de peso, crescimento, maturação cerebral e neurodesenvolvimento do recém-nascido pré-termo (Cormack et al., 2020; Coviello et al., 2018; Hiltunen et al., 2018; Schneider et al., 2018; Westin et al., 2018). Além disso, recente estudo revela que o LH produzido pela mãe do recém-nascido pré-termo apresenta maior conteúdo de energia e de teor de lipídios durante as primeiras semanas após o parto, comparado ao leite materno produzido pela mãe do recém-nascido a termo (Fischer Fumeaux et al., 2019; Thakkar et al., 2019). Sendo assim, tal informação sugere uma possível adequação natural do LH concentrado desenvolvido frente às necessidades nutricionais do prematuro visto ser um produto adequado quanto ao conteúdo de carboidratos, lipídios e, consequentemente, energia. Diante de tais fatos, os resultados desta tese sustentam a viabilidade nutricional do LH concentrado desenvolvido como proposta para o adequado suporte nutricional do RNMBP. Entretanto, é importante relatar que os demais nutrientes essenciais avaliados atendem parcialmente as recomendações de ingestão enteral para o recém-nascido pré-termo (Agostoni et al., 2010; Tudehope et al., 2013).

As concentrações de proteínas e de determinados micronutrientes essenciais (Ca, Mg, Na, P, Cu, Zn, Fe e Mn) no LH concentrado, as quais atendem parcialmente as necessidades nutricionais do recém-nascido pré-termo, podem gerar preocupações e, por isso, justificam o monitoramento sérico e a necessidade de suplementação isolada.

O teor de proteínas no LH concentrado (1.68-2.02g/100kcal) está aquém das recomendações nutricionais vigentes para o recém-nascido pré-termo (Agostoni et al., 2010; Tudehope et al., 2013). Entretanto, o LH pasteurizado e fortificado com FM-85® (Nestlé®) na diluição de 4%, o aditivo comercial comumente utilizado nas UTIs brasileiras, fornece apenas

3.7g de proteína (média expressa por 100 kcal) e pode não atender plenamente as necessidades nutricionais de proteínas (3.2-4.1g/100kcal) do RNMBP (Agostoni et al., 2010; Thomaz et al., 2012). Corroborando com este alerta, autores afirmam que o LH fortificado com FM-85® (Nestlé®) não atende a alta necessidade nutricional tanto de proteínas quanto de Ca, P e Mg do recém-nascido extremamente prematuro (Corvaglia et al., 2010; Loui et al., 2002). Além disso, parte deste montante de proteínas fornecido pelo LH fortificado com FM-85® (Nestlé®) é composto por proteína heteróloga proveniente do leite de vaca comprovadamente maléfica para o recém-nascido pré-termo (Arslanoglu et al., 2019; Brown et al., 2016).

Estudos atuais sugerem que a ingestão de proteínas de alto valor nutricional e bioativo, naturalmente presentes no LH, quando associadas à ingestão adequada de energia resultam no crescimento de melhor qualidade do RNMBP constatado pela maior taxa de massa corporal livre de gordura, que pode gerar melhores desfechos metabólicos no futuro para o prematuro (Cerasani et al., 2020; Gianni; Roggero; Mosca, 2019; Morlacchi et al., 2018). Além disso, proteínas bioativas (ex.: proteínas antimicrobianas, citocinas, lactoferrina, lisozimas e imunoglobulinas) presentes no LH desempenham um papel crucial na maturação do sistema imunológico e na proteção do recém-nascido pré-termo proporcionando a redução da incidência de morbidades relacionadas ao sistema imune (ex.: EN, alergias, displasia bronco pulmonar e retinopatia da prematuridade) desse prematuro (Lewis et al., 2017; Lönnnerdal, 2017). É interessante observar que o LH fortificado com os produtos da *Prolacta Bioscience*® (Prolact+ 4, 6, 8 e 10), aditivo comercial à base exclusivamente de LH, apresentam concentrações (expressa por 100 kcal) de proteínas (2.3-3.3g), carboidratos (8.0-9.9g), Na (53.0-61.4mg), K (86.7-105.3mg), P (63.2-76.3mg), Mg (8.1-10.2mg), Mn (5.0-6.4µg), Cu (88.8-104.1µg) e Se (4.4-5.4µg) as quais atendem apenas parcialmente as recomendações nutricionais vigentes para o recém-nascido pré-termo, quando referente à adição do fortificante em LH maduro de mães de recém-nascidos a termo segundo os dados disponíveis em <https://www.prolacta.com> (Agostoni et al., 2010; Tudehope et al., 2013). Além disso, é importante destacar que tais produtos da *Prolacta Bioscience*® não atendem as recomendações para Zn e Fe, assim como o LH concentrado apresentado nesta tese (Agostoni et al., 2010; Tudehope et al., 2013). Apesar de tais incompatibilidades frente às recomendações nutricionais citadas, diversos estudos clínicos randomizados e controlados comprovam relevantes desfechos favoráveis relacionados à utilização desse aditivo à base de LH, o que corrobora para a viabilidade e segurança do LH concentrado proposto nesta tese como alternativa para o adequado suporte nutricional do

RNMBP durante o período de internação na UTIN (Cristofalo et al., 2013; Hair et al., 2014; Hair et al., 2016b; Sullivan et al, 2010).

5. CONCLUSÃO

A presente tese comprova a possibilidade de formulação do LH concentrado pasteurizado a partir de um método direto, simples, pouco oneroso e isento de viés ético de liofilização do LH maduro doado e sua utilização como aditivo natural do próprio LH, seguido de etapa final de pasteurização. Tal LH concentrado pasteurizado apresentou segurança microbiológica, osmolalidade aceitável e o aumento significativo da concentração de macro e micronutrientes essenciais, o que torna esse produto uma alternativa viável e segura para o adequado suporte nutricional do RNMBP durante o período de internação na UTIN. Além disso, os resultados desta tese permitirão atualizar as informações a respeito da composição do leite materno maduro proveniente de doadoras brasileiras e processado pelo BLH, informação que pode ser útil para demais rBLH do mundo.

Recentemente foi concluído o estudo clínico fase 1 (duplo-cego, randomizado e controlado) o qual avaliou a segurança, tolerabilidade e desempenho inicial do LH concentrado utilizado no suporte nutricional do RNMBP com a intenção de ser uma alternativa viável para o desuso dos aditivos comerciais à base de proteína do leite de vaca atualmente utilizados nas UTINs brasileiras.

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ANEXOS

O anexo a seguir é referente ao estudo clínico fase 1 (duplo-cego, randomizado e controlado) no qual atuei como pesquisadora na avaliação da segurança e tolerabilidade do LH concentrado utilizado no suporte nutricional do RNMBP. O resumo do estudo foi publicado em dois renomados eventos internacionais, a *International Conference on Clinical Neonatology 2020* e o *Grand Challenges Global Meeting 2020*. Recentemente, o resumo do trabalho também foi submetido ao *United Nations Science, Technology and Innovation Forum 2021*. O estudo completo já está em fase de submissão e será publicado em breve.

Seguem as referências bibliográficas das publicações acima citadas:

J.S. Camelo Jr.¹, M.C. Achcar¹, A. Carnevale-Silva¹, M.M. Mussi¹, F. Carmona¹, D.C. Aragón, F. Ued¹, M. Oliveira¹, L.M. Monti², V. Bonfim³, L.G. Alves³, A. Heck³, F.E Martinez¹, V. Nogueira-Pileggi¹. **The Project Lioneo: Nutrition of Very Low Birthweight Newborns Using a Concentrate with Human Milk Lyophilisate: Phase 1 Study for Safety and Tolerability.** International Conference on Clinical Neonatology 2020, Torino, Italy, virtual, published as a Selected Abstract in the American Journal of Perinatology: Am J Perinatol 2020; 37(S 02): S89-S100 (doi:10.1055/s-0040-1716981).

J.S. Camelo Jr.¹, M.C. Achcar¹, A. Carnevale-Silva¹, M.M. Mussi¹, F. Carmona¹, D.C. Aragón, F. Ued¹, M. Oliveira¹, L.M. Monti², V. Bonfim³, L.G. Alves³, A. Heck³, F.E Martinez¹, V. Nogueira-Pileggi¹. **The Project Lioneo: Nutrition of Very Low Birthweight Newborns Using a Concentrate with Human Milk Lyophilisate: Phase 1 Study for Safety and Tolerability.** Grand Challenges Global Meeting 2020, virtual, Abstract published in the Gates Open Research: Gates Open Res 2020, 4:133 (poster) (doi:10.21955/gatesopenres.1116671.1).

PROJECT LIONE: NUTRITION OF VERY LOW BIRTH WEIGHT NEWBORNS USING A CONCENTRATE WITH HUMAN MILK LYOPHILISATE – PHASE 1 STUDY FOR SAFETY AND TOLERABILITY

Authors: J.S. Camelo Jr.¹, M.C. Achcar¹, A. Carnevale-Silva¹, M.M. Mussi¹, F. Carmona¹, D.C. Aragón, F. Ued¹, M. Oliveira¹, L.M. Monti², V. Bonfim³, L.G. Alves³, A. Heck³, F.E. Martinez¹, V. Nogueira-Pileggi¹.

Affiliations: 1. Depart. Pediatrics, Ribeirão Preto Medical School, University of São Paulo; Ribeirão Preto Nursing School, University of São Paulo; 3. Human Milk Bank, Clinics Hospital, Ribeirão Preto Medical School, University of São Paulo.

Introduction: A human milk concentrate using lyophilizate from banked human milk has been recently developed. Preclinical tests showed that the human milk with human lyophilizate has an adequate nutrient composition, physical-chemical and bacteriological stability, immediately and after 3 and 6 months stored in freezer -20 degrees Celsius. This study aims to assess safety and tolerability of the human milk with human milk additive compared to the standard additive (cow's protein milk). **Materials and Methods:** The study is a double-blind, randomized, controlled clinical trial. 40 very low birth weight infants (VLBWI) were divided in 2 groups, human milk plus human lyophilizate (LioNeo) and human milk plus commercial additive (LHFM85). Inclusion criteria: preterm babies (<37 weeks GA), birth weight 750-1500g, small or adequate for gestational age, receiving exclusively human milk, volume equals to 100mL/kg/day or higher, hemodynamically stable, and whose parents or responsables signed the informed consent. Exclusion criteria were large for gestational age, malformations, grade III and IV for peri and intraventricular hemorrhages and children from underage mothers without a responsible companion. Participants were computer randomly assigned to receive either LioNeo or LHFM85 during 21 consecutive days. **Main outcomes:** Necrotizing enterocolitis (NEC), late-onset neonatal sepsis and death, gastrointestinal bleeding and perforation, diarrhea, regurgitation, vomiting, abdominal distension (>10%). **Results:** The main characteristics were similar between the groups: 1220±200g and 30.4±2,7w GA for LioNeo group and 1219±204g and 29.7±1.8w GA for LCFM85 group at baseline. The regression model

based on a double Poisson distribution showed no differences between groups comparing the means of adverse outcomes. Diarrhea, gastrointestinal perforation, NEC and neonatal sepsis were not observed in the group LioNeo and therefore were not statistically compared (1 late onset sepsis and 1 NEC in group LCFM85). We also compared groups using a regression model with total volume of milk as an adjusted variable and there were no differences between the groups. We had no deaths during the study. Babies from both groups grew up similarly.

Conclusion: The lyophilizate of human milk was considered safe and tolerable to use in hemodynamically stable VLBWI.

Key words: Human milk; human milk bank; preterm newborn; lyophilisate; very low birth weight infants.

Conflicts of interest: None declared.