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A Study On Metabolomic Changes In Pasteurized Pooled Human Breast Milk From Human Milk Bank: Impact Of Pasteurization, Fortification, And Probiotic Supplementation

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Abstract
Background: *Breastfeeding is the natural and universally recommended method of infant feeding, supporting optimal growth, development, and both short- and long-term health outcomes. Human milk is thought to have evolved over time to meet the specific needs of lactating women and their infants* [1,2]*. This adaptation is evident in the significant and the composition of breast milk varies significantly based on a range of maternal factors, including diet, age, body mass, and health conditions such as diabetes mellitus and hypercholesterolemia, which reflects differences in placental structures, lactation patterns, offspring growth rates, and environmental conditions. These variations highlight the intricate relationship between milk composition and the unique biological and ecological demands of each species* [3]*. While when Mother's Own Milk (MOM) is unavailable or not sufficient for the preterm infant Pasteurized Pooled Human Breast Milk (PHBM) from Human Milk Bank (HMB) offers significant benefits, regardless there is limited data on the nutritional status and nutritional losses due to the process of pasteurization at HMB, especially in India where more than 90 HMB are functional and expanding year on year.*
Objective: *To evaluate the impact of holder pasteurization on the macronutrient composition of PHBM and the role of fortification with human milk fortifier (HMF) and probiotics (Bifidobacterium breve M16V and Limosilactobacillus reuteri DSM 17938).*
Methods: *A pre/post observational study was conducted at Nectar of Life Human Milk Bank, Sri Ramakrishna Hospital, Coimbatore, India. A pooled 1000 mL PHBM sample was analyzed in various combinations: unpasteurized, pasteurized, and fortified with HMF and probiotics. Macronutrient analysis included Protein, Fat, Carbohydrate along with energy, Major Minerals Calcium, and Phosphorus.*
Results: *The results revealed significant losses in the macronutrient composition of PHBM due to pasteurization. Pasteurization reduced energy content by 19.2%, carbohydrate by 22%, protein by 24.3%, and fat by 23.1%. However, fortification with HMF resulted in significant increases in energy (27.8%), carbohydrate (26.3%), protein (138%), and fat (25.3%). The addition of probiotics further boosted energy (37.5% higher than PHBM)*

and carbohydrates (42.5% higher than PHBM). In terms of major minerals, pasteurization resulted in a reduction in calcium (14.3%) and phosphorus (4.8%), but fortification with HMF enhanced calcium (256%) and phosphorus (299.4%). Addition of Probiotics had no impact on the Protein and Mineral content.

Conclusion: *Pasteurization negatively impacts the macronutrient content of PHBM. Fortification with HMF and probiotics effectively compensates for these losses, offering a nutrient-rich alternative in absence or insufficient of MOM, crucial for preterm and low-birth-weight infants. Further optimization of these formulations is needed to meet recommended nutritional standards of fortified breastmilk.*

Keywords: *Pasteurization, Human Milk Fortifier, Human Milk Bank, Metabolome.*

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I. Introduction

Breast milk provides optimal nutrition for infants with all bioactive components. WHO recommends exclusive breastfeeding for the first 6 months of life followed by continued breastfeeding till 2 years of life [(1)]. When MOM is unavailable the PHBM from HMB become the next best alternative. It is often presumed that breastfeeding delivers uniform nutritional benefits to all infants. Breastfeeding practices also influence milk composition, with variations depending on milk volume, lactation duration, the time elapsed since the last feeding, and even within a single feeding session [(2,3)]. Furthermore, maternal genetic differences add another layer of complexity to the nutritional profile of breast milk [(3)]. The primary role of breastmilk bank is to provide PHBM to sick and needy preterm neonates.

Donor milk from human milk banks is collected from eligible donors, pooled and pasteurized at 62.5^{o} C for 30 minutes (Holder Pasteurization), which is then stored at - 20^{o} C (in a deep freezer) after microbiological screening. Pooling the breastmilk from donors and pasteurization is a tedious process involving aseptic techniques and meticulous screening prior to dispensing the needy infants [(3,4)]. The process of collection, refrigeration, pasteurization, storage, and thawing affects the constitutional elements of breast milk. Current pasteurization technique is standard practice used by milk banks to ensure microbiological safety of donor milk [(5,6)]. While effective at eliminating microbiological pathogens, this process of rapid heating, followed by a constant temperature phase and rapid cooling, may negatively impact the macronutrient composition of the milk [(4,7)]. Exact data on the composition of pooled PHBM is scarce. Our study aims to compare the loss of metabolic composition of breastmilk post pasteurization and highlight the importance of fortification in providing optimal nutrition to preterm neonates who require PHBM.

What this study adds?

This observational exploratory study aims to determine the degree of impact of holder pasteurization and subsequent interventions as storage, freezing and thawing on the macronutrient composition of PHBM from human milk banks.

The composition of PHBM is equally essential as fresh breast milk, any metabolomic losses in the PHBM can cause decreased nutrition delivery against the recommended RDA and fortified breastmilk standards which may impact the growth trajectory of the preterm infant.

II. Materials And Methods

Study Methodology:

- **Type of Study:** Pre/post observational exploratory study.

- **Objective**: To evaluate the macronutrient and major mineral composition of PHBM before and after holder pasteurization and the subsequent impact of fortification and probiotics on metabolome.
- **Location**: Nectar of Life - Human Milk Bank, Sri Ramakrishna Hospital, Coimbatore, India.
- **Ethical Approval:** Institutional Human Ethical Committee (IHEC) approval obtained. IHEC no: EC/2024/1602/CR-23 dated 16-Feb-2024 under ICH – GCP and New drugs and Clinical Trail Rule 2019.

Study overview:

The sample was collected between April 2024 to May 2024 at Nectar of Life, Human Milk Bank, Sri Ramakrishna Hospital, Coimbatore, India. Demographics and gestational age data were not collected since this is a pooled sample study. Macronutrient composition of PHBM were evaluated before and after Holder pasteurization following the addition of Human Milk Fortifier (HMF) and probiotics *Bifidobacterium breve* M16V (*B. breve*) and *Limosilactobacillus reuteri* DSM 19738 (*L. reuteri*). The purpose of adding probiotics to PHBM is to evaluate does the addition of probiotics improves the antimicrobial activity or not. This paper discusses only about the metabolome.

From Human Milk Bank Pre- and post-pasteurized samples were separately collected and labelled. A 1000 ml milk sample was obtained from the bank and transported in a thermostable bag to the analyzing laboratory. The samples were divided and analyzed under the following combinations:

1. Unpasteurized Pooled Human Breast Milk (UPHBM)
2. Pasteurized Pooled Human Breast Milk (PHBM)
3. PHBM + HMF (4g of HMF per 100 ml)
4. PHBM + HMF + *B. breve* (2 billion CFU)
5. PHBM + HMF + *L. reuteri* (0.1 billion CFU)
6. PHBM + HMF + *B. breve* (2 billion CFU) + *L. reuteri* (0.1 billion CFU)

The method used for analyzing the components was High Performance Liquid Chromatography - Photodiode Array (HPLC-PDA), Inductively Coupled Plasma- Optical Emission Spectroscopy (ICP-OES). Analyses included Energy (kcal/100 mL), Protein, Fat, and Carbohydrate (g/100 mL), Calcium and Phosphorous (mg/100 mL). This comprehensive study aims to provide valuable insights into the nutritional adequacy and potential improvements in PHBM to better support neonatal growth and development.

III. Results

The macronutrient composition of the analyzed groups is described in Table 1.

Energy:

UPHBM has the energy content of 71.10 kcal per 100 ml while due to the impact of pasteurization PHBM energy content was reduced to 57.40 Kcal which is 19.2% loss, while adding HMF energy increases significantly to 73.40 Kcal which is 27.8% increase. Adding probiotics further boosts energy with PHBM + HMF + *B. breve* + *L. reuteri* having the highest of 78.94 Kcal which is 37.5% higher than the PHBM.

Carbohydrate:

UPHBM has the carbohydrate content of 7.79 gram per 100 ml while due to the impact of pasteurization PHBM carbohydrate content was reduced to 6.07 gram which is 22% loss, while adding HMF carbohydrate increases significantly to 7.67 gram which is 26.3% increase. Adding probiotics further boosts carbohydrate with PHBM + HMF + *B. breve* + *L. reuteri* having the highest of 8.65 gram which is 42.5% higher than the PHBM.

Protein:

UPHBM has the Protein content of 1.15 gram per 100 ml while due to the impact of pasteurization PHBM protein content was reduced to 0.87 gram which is 24.3% loss, while adding HMF protein increases significantly to 2.07 gram which is 138% increase. Adding probiotics protein with PHBM + HMF + *B. breve* + *L. reuteri* having very little impact on protein of 2.08 gram which is 139% higher than the PHBM.

Fat:

UPHBM has the Fat content of 4.11 gram per 100 ml while due to the impact of pasteurization PHBM fat content was reduced to 3.16 gram which is 23.1% loss, while adding HMF fat increases significantly to 3.96 gram which is 25.3% increase. Adding probiotics further boosts fat with PHBM + HMF + *B. breve* + *L. reuteri* having the highest of 4.15 gram which is 31.3% higher than the PHBM.

Calcium:

UPHBM has the Calcium content of 28.60 milligram per 100 ml while due to the impact of pasteurization PHBM Calcium content was reduced to 24.50 milligram which is 14.3% loss, while adding HMF Calcium increases significantly to 87.2 milligram which is 256% increase. Adding probiotics has no effect on Calcium content.

Phosphorous:

UPHBM has the Phosphorous content of 12.30 milligram per 100 ml while due to the impact of pasteurization PHBM Phosphorous content was reduced to 11.70 milligram which is 4.8% loss, while adding HMF Phosphorous increases significantly to 46.74 milligram which is 299.4% increase. Adding probiotics has no effect on Phosphorous content.

Pasteurization have negatively impacted on metabolomic content of Human Breast Milk such as Energy, Carbohydrate, Protein, Fat, Calcium, and Phosphorus showing notable losses. However, addition of probiotics has no impact on calcium and phosphorus levels.

IV. Discussion

Human milk is not a stable body fluid, it keeps changing over the period of lactation. The composition of foremilk is different from hind milk; colostrum varies from mature milk [6,7]. HMB provide PHBM which is the next best alternative to MOM [8]. However, the composition of PHBM need to be analyzed to ensure that those needy infants receive optimal nutrition too. Although PHBM offers significant advantages, only limited data is available on the nutritional losses associated with pasteurization, particularly among Indian donors.

Our study shows that holder pasteurization significantly reduces the macronutrient composition especially carbohydrate, protein, and fat which in turn contribute to lower energy delivery (Table 2). The decrease in energy content is could be due to the fat loss during pasteurization. Vieira et al reported 5.5% and 3.9% loss of fat and protein concentration respectively after pasteurization [9]. Garcia-lana et al analysis of 34 samples of frozen breast milk shows 3.5 % reduction in fat and 2.8% reduction in energy content post pasteurization. In our study 23.1% fat and 24.3% protein loss occurred after pasteurization, led to a notable decline in energy content, likely due to heat-induced degradation. Adherence of fat materials to the bottle wall may also be the reason for loss as stated in few studies [10].

The European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recommends providing 115–160 kcal/kg/day for optimal growth in preterm neonates and highlights the importance of fortifying donor human milk or pasteurized MOM to achieve this caloric target [11]. Our findings showed that fortifying PHBM increased its calorie content to levels comparable to the fortified breastmilk reference standard. However, fortification with HMF effectively restored and, in some cases, enhanced these levels, emphasizing its crucial role in meeting the nutritional needs of preterm or low-birth-weight infants.

The addition of probiotics further influenced macronutrient composition in distinct ways as the storage medium of added *L.reuteri* and *B.breve* were associated with increased protein, fat, and carbohydrate levels. The highest energy and carbohydrate content were observed when both probiotics were added together, suggesting a potential synergistic effect that could enhance nutrient availability or digestion. Pasteurization impact on major minerals Calcium and phosphorous is much lower than macronutrients. Nearly 80% phosphorous and all the calcium is stored in bones and sufficient phosphorous intake is needed for energy generation and lean mass accretion (12). The observed enhancements in calcium and phosphorus content due to HMF addition further reinforce its critical role in ensuring adequate bone mineralization. ESPGHAN recommends 120 200 mg/kg/day of calcium and 70 – 115 mg/kg/day of phosphorous for optimal growth and development (11). Adding HMF post-pasteurization nearly meets this recommendation; however, probiotics have little effect on calcium and phosphorus levels after fortification. Given the growing body of evidence supporting the benefits of probiotics in neonatal nutrition, their inclusion in fortified breast milk formulations may offer additional advantages in gut health and immune function.

These findings highlight the need for tailored breast milk fortification strategies to compensate for pasteurization-related nutrient losses. While pasteurization ensures microbiological safety, combining HMF with carefully selected probiotics may optimize the caloric and macronutrient profile of human milk. Further research is needed to explore the mechanisms behind these nutrient alterations and assess their long-term effects on infant growth and development.

V. Conclusion

PHBM composition is highly varied before and after pasteurization. Because of the varied macronutrient components accompanied with losses during storage and pasteurization, fortification and adding probiotics could impact beneficially on the development of gastrointestinal tract and immune system. Our research adds importance to fortifying the PHBM with bovine based HMF and probiotics for delivering optimal nutrition in par with mother's own milk in case of unavailability. The combined formulation of PHBM + HMF + *B. breve* + *L. reuteri* demonstrated the most balanced and nutrient-dense composition, making it an ideal choice for addressing the elevated nutritional needs of preterm or low-birth-weight infants. However, the observed reduction in the macronutrient content across all fortified formulations warrants further investigation to optimize nutritional delivery in such specialized feeds that meets the recommend RDA and fortified breastmilk reference standard.

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Table 1: Nutritional Profile of Pasteurized Human Breast Milk with Fortification and Probiotic additions.

Parameters	UPHBM	PHBM	PHBM + HMF	PHBM + HMF + *L. reuteri*	PHBM+ HMF+ *B.breve*	PHBM + HMF + *B.breve+ L.reuteri*	Fortified Breast Milk Reference Range
Energy (Kcal Per 100ml)	71.10	57.40	73.40	75.14	77.20	78.94	84
Carbohydrate (gm / 100 mL)	7.79	6.07	7.67	7.67	8.65	8.65	8.8
Protein (gm / 100 mL)	1.15	0.87	2.07	2.08	2.07	2.08	2.8
Fat (gm / 100 mL)	4.11	3.16	3.96	4.15	3.96	4.15	4.4
Calcium (mg / 100 mL)	28.60	24.50	87.2	87.2	87.2	87.2	88.72
Phosphorous (mg / 100 mL)	12.30	11.70	46.74	46.74	46.74	46.74	49.56

Table 2: Energy Profile of HMB Pre & Post Pasteurization, Fortification and Probiotic additions.