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Antimicrobial advantage: Augmenting pasteurized pooled donor human breast milk from human milk bank with human milk fortifier and probiotics against pathogens linked to necrotizing enterocolitis

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Abstract

Breast Milk is essential for neonates, providing vital nutrients and immunological protection. When a mother's Own Milk (MOM) is unavailable, Pasteurized pooled donor Human Breast Milk (PHBM) from Human Milk Bank (HMB) is a suitable alternative. This study investigates the antimicrobial properties of freshly Expressed Breast Milk (EBM), Unpasteurized Pooled Donor Breast Milk (UPHBM), and PHBM, including various fortifications with Human Milk Fortifiers (HMF) and probiotics *Bifidobacterium breve*-M16-V (*B. breve*) and *Limosilactobacillus reuteri*-DSM17938 *(L. reuteri)*. Findings indicate that long storage and pasteurization significantly diminish the bactericidal activity of breast milk. However, fortifying PHBM with HMF and specific probiotics enhances its antimicrobial effects against bacteria associated with Necrotizing Enterocolitis (NEC). Despite these enhancements, Methicillin-Resistant *Staphylococcus aureus* (MRSA) remained unaffected, highlighting pathogen-specific variability. Overall, fortifying PHBM at HMB may help restore nutritional and protective qualities lost during pasteurization, offering improved antimicrobial protection for preterm infants at risk of NEC.

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Introduction

Breast milk, often called "nature's first food," is a complex and dynamic biological fluid that provides essential nutrients and bioactive compounds necessary for the optimal growth and development of infants (Baker *et al.*, 2023). It is the primary food source for neonates, offering not only nutrition but also immune protection and microbiota that shape the infant's gut, leading to short term and long-term health benefits (Viazis *et al.*, 2007; Wagner *et al.*, 1996). Human breast milk contains a range of bioactive agents, such as Human Milk Oligosaccharides (HMOs), lactoferrin, immunoglobulins, antimicrobial proteins, and commensal bacteria, which contribute to immune modulation, gut maturation, and defence against infection (Chen and Allen, 2001; Sharma *et al.*, 2018; Lubiech and Twarużek, 2020). Despite the recognized importance of breast milk, some infants, particularly preterm neonates, are unable to receive sufficient breast milk from their mothers due to various reasons. For these infants, pasteurized donor milk from HMB serves as the second-best option (Baker *et al.*, 2023; Chen and Allen, 2001). However, the pasteurization process (Holder pasteurization at 62.5°C for 30 minutes) at HMB, while effective in inactivating viral and bacterial contaminants, can also reduce the antimicrobial and immunological properties of the milk (Silvestre *et al.*, 2008). This loss of activity is particularly concerning for preterm infants, who have higher nutritional demands and are more vulnerable to infections such as neonatal sepsis and NEC (Chen and Allen, 2001; Lubiech and Twarużek, 2020). Preterm neonates, due to their immature immune systems and underdeveloped digestive tract, are at a higher risk of developing severe infections like sepsis and NEC. Although breast milk alone is beneficial, it may not fully meet the heightened nutritional and immunological needs of these infants (Olivares *et al.*, 2006; Kalidoss *et al.*, 2022). Fortification of breast milk with HMF is a standard practice in neonatal care to enhance its nutritional value (Baker *et al.*, 2023; Lubiech and Twarużek, 2020).

The potential of HMF to improve the antimicrobial properties of PHBM is not well understood. Additionally, probiotics, particularly strains such as *B. breve* and *L. reuteri* have been documented to confer beneficial effects on gut health and immunity (Lubiech and Twarużek, 2020), which we also used to demonstrate in our study. These probiotics could offer an innovative approach to restoring the lost protective properties of PHBM by enhancing its antimicrobial activity against pathogens that commonly linked to neonatal sepsis and NEC. This study aims to investigate whether fortifying PHBM from Human Breast Milk Bank with HMF and probiotics *B. breve* and *L. reuteri* can enhance its antimicrobial properties. Specifically, the study focuses on whether fortification can restore the protective effects lost during pasteurization process and improves the milk's ability to defend against neonatal pathogens linked to NEC. Key objectives of the study include assessing the impact of pasteurization on the antimicrobial properties of donor milk by comparing EBM against PHBM additionally we also assessed UPHBM from HMB to gain insight on the impact of storage on antimicrobial activity. Evaluating the antimicrobial properties of PHBM + HMF, Exploring the potential of probiotics by adding probiotics along with HMF as alone and in combination (PHBM + HMF + *B. breve*), (PHBM + HMF + *L. reuteri*), (PHBM + HMF + *B. breve* + *L. reuteri*).

Objective of this study understands the interactions between breast milk components, fortification strategies, and microbial communities; this study seeks to contribute to the development of innovative interventions that can reduce the incidence of neonatal infections like sepsis and NEC. This research may have significant implications for improving the health outcomes of preterm infants by offering enhanced nutritional and antimicrobial support through fortified donor milk.

Materials and methods

Source of study

Nectar of Life - Mother's Milk Bank, Sri Ramakrishna Hospital, Coimbatore, India.

Study design

Observational Study, Period of Study: From 1st April 2024 to 31st May 2024.

Ethical committee approval

This study was conducted in accordance with the Declaration of Helsinki and approved by Institutional Human Ethical Committee (IHEC) of Sri Ramakrishna Hospital, Coimbatore India. IHEC no: EC/2024/1602/CR-23 dated 16-Feb-2024 under ICH – GCP and New drugs and Clinical Trail Rule 2019. Consent to participate in the study is not applicable.

Study population and sample size

2000 ml of Human Breast Milk. Collection and Transport: The samples were collected aseptically from HMB, stored in freezer box and transported to laboratory within 30 minutes.

Study groups

Group A: Fresh EBM

Group B: Stored UPHBM

Group C: PHBM

Group D: PHBM + HMF (25ml of PHBM + 1gm of HMF)

Group E: PHBM + HMF + *B. breve* (2 billion CFU)

Group F: PHBM + HMF + *L. reuteri* (0.1 billion CFU)

Group G: PHBM + HMF + *B. breve* (2 billion CFU) + *L. reuteri* (0.1 billion CFU)

Antimicrobial susceptibility test

The antimicrobial activity was evaluated via agar well diffusion method on Mueller-Hinton Agar (MHA) Plates. 7 common pathogens linked to NEC were *Salmonella typhi, Escherichia coli, Staphylococcus epidermidis, Klebsiella pneumoniae, Enterococcus faecalis, Pseudomonas aeruginosa*, Methicillin-Resistant *Staphylococcus aureus* was tested. Test organisms were prepared in 0.1 peptone broth incubated for 24 hrs. Each organism was swabbed on 7 MHA plates (Total 49 plates) 5 wells of 8mm were cut using sterile tips where the following volumes of milk added to each well: 20 µL, 40 µL, 60 µL, 80 µL, and 100 µL and incubated at 37°C for 24 hours. After incubation, the zone of inhibition (ZOI) around each well was measured in millimetres to determine the antimicrobial effect (Sharma *et al.*, 2018). The results were interpreted based on the Clinical Laboratory Standards Institute (CLSI) guidelines. This methodology enlightens the antimicrobial properties of human breast milk before and after pasteurization, as well as the potential enhancement of these properties through fortification with HMF and probiotics.

Results

Fresh EBM demonstrated significant inhibitory activity against *S. typhi*, *S. epidermidis* and *E. faecalis*. UPHBM, PHBM both exhibited antimicrobial activity solely against *S. typhi* and showed no inhibition for the other six pathogens. PHBM + HMF, antimicrobial activity was observed against both *S. typhi* and *E. coli*. Further enhancement was seen when PHBM + HMF + *B. breve*, broadening the antimicrobial spectrum to include *S. epidermidis* and *K. pneumoniae*, in addition to *S. typhi* and *E. coli*. The addition of PHBM + HMF + *L. reuteri* inhibited for five of the seven pathogens tested: *S. typhi*, *E. coli*, *S. epidermidis*, *K. pneumoniae*, and *P. aeruginosa*. Notably, PHBM + HMF + *B. breve* + *L. reuteri* significantly enhanced the antimicrobial spectrum of PHBM by inhibiting six out of seven test organism *S. typhi, E. coli, S. epidermidis, K. pneumoniae, E. faecalis, P. aeruginosa* (Table 1).

However, *S. aureus* (MRSA) remained unaffected, indicating pathogen-specific variability in response to fortification. These findings suggest that probiotics not only help in gut colonization and immune regulation but also enhance the milk's ability to directly inhibit bacterial growth. Across all groups (Table 2 and 3), *Salmonella typhi* was the most sensitive pathogen, with the highest zone of inhibition observed in fresh milk (26 mm at 100 µL). Pasteurization was associated with a reduction in antimicrobial activity, as evidenced by smaller zones of inhibition in PHBM compared to fresh or unpasteurized milk. However, fortification with HMF and probiotics effectively restored and even enhanced antimicrobial activity, demonstrating the potential of these interventions to compensate for the loss of activity caused by pasteurization.

Table 1. Sensitivity pattern of the organisms tested against the various combinations

Sl	Bacterial culture	Gram	No intervention group				Intervention group		
			Fresh milk	UPHBM	PHBM	PHBM + HMF	PHBM + HMF + *B. breve*	PHBM + HMF + *L. reuteri*	PHBM + HMF + *L. reuteri* + *B. breve*
1	*Salmonella typhi*	-	S	S	S	S	S	S	S
2	*Escherichia coli*	-	R	R	R	S	S	S	S
3	*Staphylococcus epidermidis*	+	S	R	R	R	S	S	S
4	*Klebsiella pneumoniae*	-	R	R	R	R	S	S	S
5	*Enterococcus faecalis*	+	S	R	R	R	R	R	S
6	*Pseudomonas aeruginosa*	-	R	R	R	R	R	S	S
7	*Staphylococcus aureus* (MRSA)	+	R	R	R	R	R	R	R

*R- Resistant, S- Sensitive

Table 2. Zone of inhibition of fresh breast milk, stored unpasteurized EBM and pasteurized human breast milk

Sl	Culture	Fresh Milk (ZOI mm)					Stored UPHBM (ZOI mm)					PHBM (ZOI mm)				
	Volume of milk sample (µL)	20	40	60	80	100	20	40	60	80	100	20	40	60	80	100
1	*Salmonella typhi*	22	22	24	25	26	20	21	23	24	24	-	-	14	17	17
2	*Escherichia coli*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	*Staphylococcus epidermidis*	-	10	15	19	20	-	-	-	-	-	-	-	-	-	-
4	*Klebsiella pneumoniae*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	*Enterococcus faecalis*	-	-	-	13	15	-	-	-	-	-	-	-	-	-	-
6	*Pseudomonas aeruginosa*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	*Staphylococcus aureus* (MRSA)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3. Zone of inhibition exhibited by the intervention combinations added to pasteurized milk in study groups

Sl	Culture	PHBM + HMF (ZOI mm)					PHBM + HMF + *B. breve* (ZOI mm)					PHBM + HMF + *L. reuteri* (ZOI mm)					PHBM + HMF + *B. breve* + *L. reuteri* (ZOI mm)				
	Volume of milk sample (µL)	20	40	60	80	100	20	40	60	80	100	20	40	60	80	100	20	40	60	80	100
1	*Salmonella typhi*	18	18	20	22	25	19	20	22	23	24	20	20	21	22	25	15	20	20	23	25
2	*Escherichia coli*	15	17	17	19	20	15	15	20	20	23	10	12	17	18	20	10	13	14	20	25
3	*Staphylococcus epidermidis*	-	-	-	-	-	-	12	12	13	15	-	10	12	12	16	8	8	10	12	15
4	*Klebsiella pneumoniae*	-	-	-	-	-	14	14	20	20	20	-	-	18	20	27	9	13	15	18	20
5	*Enterococcus faecalis*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9	12	15	17	20
6	*Pseudomonas aeruginosa*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10	-	-	-	10	12
7	Staphylococcus *aureus* (MRSA)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Discussion

In our study, we confirmed that EBM possesses inherent antimicrobial activity against *S. typhi, S. epidermidis* and *E. faecalis*. However, the absence of antimicrobial activity against *E. coli, K. pneumoniae, P. aeruginosa* and *S. aureus* (MRSA) in fresh breast milk aligns with previous research indicating that not all pathogens are equally affected by breast milk's natural defences. This variability likely reflects differences in the resistance mechanisms of specific bacteria and the unique bioactive compounds present in human milk. Our findings did not show bactericidal activity against *E. coli*, which contrasts with some other studies (Tully *et al.*, 2001; Martínez-Costa *et al.*, 2007). Our study also revealed that both UPHBM and PHBM lost their antimicrobial activity against *S. epidermidis* and *E. faecalis* when compared to EBM. We did not specifically investigate how storage time affects antimicrobial activity before pasteurization, but it is well-documented that subsequent storage and pasteurization diminish the levels of bioactive components like lysozyme,

lactoferrin, and immunoglobulins specific to *E. coli* which may explain its resistance in our tests (Tully *et al.*, 2001).

Pasteurization, while effective in inactivating harmful and beneficial microorganisms, inevitably degrades some essential bioactive components that contribute to the antimicrobial properties of human milk (Viazis *et al.*, 2007; Chen and Allen, 2001). Immunoglobulin A (IgA) and lysozyme, both of which play significant roles in providing antimicrobial protection, are particularly susceptible to heat degradation (Martínez-Costa *et al.*, 2007; McPherson and Wagner, 2001). Studies by Wills *et al.* and Bjorksten *et al.* have reported reductions of 20–33% in IgA levels and 23–33% in lysozyme levels after pasteurization, a loss that likely impacts the overall protective properties of PHBM (McPherson and Wagner, 2001; Wills *et al.*, 1982). IgA plays a key role in secreting antibodies against specific pathogens in the infant's gut, helping to prevent their penetration of the gut's epithelial barriers and thereby reducing the risk of enteric diseases (Sharma *et al.*, 2018; Tully *et al.*, 2001; Koenig *et al.*, 2005). Lysozyme, another critical component, works by disrupting bacterial cell walls through the degradation of N-acetyl-D-glucosamine and N-acetyl-D-muramic acid, essential components for bacterial cell wall synthesis (Baker *et al.*, 2023; Lubiech and Twarużek, 2020). Instead of isolating specific agents responsible for the antimicrobial effects such as we sought to restore and enhance the lost bactericidal activity in PHBM through fortification. The introduction of HMF to PHBM (Group D) partially restored its antimicrobial activity, extending its inhibitory effect to *E. coli* in addition to *S. typhi*. This indicates that fortification, commonly used to meet the nutritional needs of preterm infants, may also contribute to improving the functional bioactivity of donor milk due to its influence on osmolarity of milk.

However, the antimicrobial effect remained limited to just two pathogens, suggesting that additional interventions are necessary to further enhance the protective properties of fortified PHBM. The inability of HMF alone to restore broad-spectrum activity suggests that while it offers some benefit, it may not be sufficient to compensate for the loss of multiple protective components post-pasteurization. To the best of our knowledge, no prior studies have specifically examined the effects of fortifying PHBM from HMB with Probiotics and assessed its dynamics on antimicrobial properties making this research a novel contribution to the field. *B. breve* is a Gram positive, rod shaped, anaerobic, non-motile, non-spore forming, GRAS certified probiotic first isolated from an infant gut in 1963, clinically trailed over 4000 infants in over 36 clinical trials including 120 NICU units and was added to commercial infant formulas in 1982 due to its beneficial effects (Björkstén *et al.*, 1980; Patole *et al.*, 2014).

In our study the fortification of *B. breve* M-16V to PHBM + HMF increases the spectrum of antimicrobial activity to *S. epidermidis* and *K. pneumoniae* i.e out of 7 test organism 4 was suspectable in the presence of *B. breve* M-16V and it has been documented that *B. breve* M-16V exhibits antimicrobial effects by lowering pH through the production of lactic and acetic acids, which inhibits the harmful pathogens (Patole *et al.*, 2014; Bozzi Cionci *et al.*, 2018). It also plays a key role in boosting the immune system by increasing Toll-like receptor 4 (TLR4) expression in mesenteric lymph nodes and intraepithelial compartments, and by raising intestinal IgA levels (McPherson and Wagner, 2001). This strengthens the intestinal immune response, especially in preterm neonates by reducing the short chain fatty acid butyrate. Various studies support its role in reducing infections and NEC in infants (Patole *et al.*, 2014). *L. reuteri* is gram positive, rod shaped, facultative anaerobic, non-sporing forming bacteria found in gastrointestinal tract, urinary tract, skin, and human breast milk capable of producing antimicrobial metabolites like reuterin, reutericyclin, lactic acid, acetic acid, which acts as a broad-spectrum antimicrobial by inhibiting pathogens during glycerol metabolism. In addition to reuterin, organic acids, bacteriocins, and ethanol further enhance antimicrobial activity by creating a hostile

environment for pathogens (Bozzi Cionci *et al.*, 2018; Woodman *et al.*, 2018).

In the context of our study the fortification has extended the inhibitory effect to 3 additional pathogens such as *S. epidermidis, K. pneumoniae* and *P. aeruginosa*. The extended coverage in Group G aligns with prior research showing that probiotics, particularly *B. breve* and *L. reuteri* in combination exhibits highest antimicrobial activity by inhibiting 6 out of 7 pathogens including *E. faecalis* which was originally inhibited only by EBM. This suggests that multi-strain probiotic approach is more effective in restoring the antimicrobial activity lost during pasteurization and offers enhanced protection against a wider range of pathogens. Breast milk contains various antimicrobial compounds, but Methicillin-resistant *S. aureus* is not easily inhibited by it due to several factors.

First, MRSA has evolved resistance to many antibiotics, including methicillin, via the mecA gene, which alters its penicillin-binding proteins and renders it less susceptible to breast milk's natural antimicrobial agents like lactoferrin and lysozyme. Additionally, MRSA forms protective biofilms that shield the bacteria from both the immune system and breast milk's antimicrobial compounds, preventing these agents from effectively targeting the bacteria. Moreover, MRSA employs immune evasion mechanisms, such as producing proteins that block immune responses, making it difficult for breast milk's immune factors to neutralize the pathogen (Mu *et al.*, 2018). Breast milk has variable effects on different bacterial strains, with some demonstrating substantial inhibition and others showing little to no response.

Conclusion

In conclusion, our study highlights the importance of fortifying PHBM with multiple probiotics, specifically *B. breve* M16 and *L. reuteri* DSM 17938 to enhance its antimicrobial activity against pathogens such as *S. typhi, E. coli, S. epidermidis, K. pneumoniae, E. fecalis,* and *P. aeruginosa*. While Storage and Pasteurization reduce the natural protective properties of breast milk, this fortification strategy can restore and even surpass the antimicrobial efficacy of fresh milk. The ability to restore and even improve the protective qualities of PHBM through fortification and probiotic supplementation could have important implications for reducing the incidence of neonatal sepsis and NEC, two major causes of morbidity and mortality in preterm population. This approach is particularly relevant for infants relying on donor milk, offering a promising solution. Further studies are needed to explore the clinical outcomes associated with these interventions and to determine optimal probiotic combinations for enhancing the safety and efficacy PHBM.

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