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Microbial safety of donor human milk before pasteurization: seasonal, maternal, and procedural determinants

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Abstract

Background Donor human milk (DHM) is a vital alternative for preterm infants when maternal breastfeeding is unavailable, yet its microbial safety remains a critical concern. This study provides the first longitudinal evaluation of DHM contamination patterns in Iran, aiming to identify maternal, procedural, and seasonal risk factors to inform safer milk bank practices.

Methods This repeated cross-sectional study evaluated microbial contamination in DHM over six years (2019–2025) at Valiasr Hospital's Human Milk Bank (HMB) in Tehran. Samples were stratified into two phases following a protocol revision in October 2023, which introduced agar-based culturing and enhanced hygiene practices. Maternal, environmental, and seasonal variables were recorded for each donation. Pathogen-specific contamination rates were analyzed, and multivariate logistic regression identified independent predictors. Firth's penalized likelihood method was applied to ensure statistical stability in sparse data models.

Results Of 509 DHM samples screened, 40.7% were excluded due to microbial contamination, while all 302 pasteurized samples tested pathogen-free. The transition to agar-based culturing and upgraded hygiene protocols in Phase II reduced discard rates and seasonal variation, with *Staphylococcus epidermidis* dropping from 21.3% to 3.2%. *Klebsiella pneumoniae* emerged as the dominant pathogen in Phase II (17.6%), with seasonal peaks in cooler months. Multivariate regression analysis identified manual expression as a significant predictor of *Bacillus cereus* contamination. For *Staphylococcus epidermidis*, longer intervals between childbirth and donation, as well as home refrigeration (vs. freezing), were independently associated with increased contamination risk. No significant associations were found for *Klebsiella pneumoniae*, *Staphylococcus aureus*, or *Escherichia coli*, suggesting pathogen-specific risk dynamics.

Conclusion This study establishes a foundational microbiological profile of DHM in Iran, demonstrating that rigorous pasteurization protocols ensure pathogen-free milk for NICU use. The integration of maternal, procedural, and seasonal variables revealed key contamination drivers and highlighted the impact of methodological upgrades on microbial detection. Findings support the refinement of exclusion thresholds and culture techniques to balance safety with milk availability. These insights provide a critical framework for optimizing DHM governance in emerging milk bank systems.

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Keywords Milk banks, Milk, Human, Pasteurization, Food microbiology

Background

Human milk is universally acknowledged as the optimal source of nutrition for neonates, particularly preterm infants. Its complex composition of bioactive compounds promotes immune system maturation, gut microbiota development, and neurocognitive growth [1, 2]. Exclusive breastfeeding reduces the risk of infections and necrotizing enterocolitis (NEC). Systematic reviews confirm its role in lowering infectious disease incidence during infancy and improving long-term health outcomes [3, 4]. These findings underscore the critical importance of early and sustained breastfeeding practices in preventing neonatal complications and enhancing infant survival.

In the absence of maternal breastfeeding, Donor human milk (DHM) provides a safe and evidence-based alternative to support neonatal nutrition and immune development. However, DHM presents potential risks of bacterial contamination, necessitating rigorous safety protocols to ensure microbiological integrity [5]. The microbiota of raw human milk originates from maternal skin, breast tissue, and intestinal flora. It includes beneficial commensals such as *Bifidobacterium*, *Lactobacillus*, and *Corynebacterium*, as well as opportunistic pathogens including *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* [1, 6]. Although the presence of bacteria in human milk is not inherently pathogenic, excessive microbial load prior to processing can compromise both safety and quality; especially in banked milk intended for premature infants with heightened vulnerability. Bacterial overgrowth in donor human milk may result from improper storage, delayed transport, poor hygiene during expression, or inadequate pump cleaning [7]. Borges et al. (2018) emphasized the importance of hygiene compliance in both home and milk bank settings [8]. Becker et al. (2016) found no consistent contamination differences between hand and pump expression [9]. Laforce-Lavoie et al. (2023) showed that only boiling after soapy cleaning fully eliminates pump-related bioburden [10]. To mitigate these risks, pasteurization is routinely employed, with Holder pasteurization being the most widely used method. This technique effectively eliminates pathogenic organisms while preserving essential bioactive components [11, 12]. A retrospective study by Almutawif et al. (2017) reported a significant reduction in bacterial contamination following pasteurization, reinforcing the importance of controlled processing [13]. Nevertheless, residual contamination has been observed, with strains such as *Staphylococcus aureus*, *Bacillus cereus*, and *Enterococcus faecalis* occasionally detected in post-pasteurized DHM [14–16]. These observations highlight limitations in current sterility protocols and

support the implementation of robust microbial surveillance strategies in human milk banks. Emerging evidence suggests that both maternal and logistical factors—including maternal age, body mass index (BMI), mode of delivery, and environmental exposure—can influence the microbial composition of human milk [17, 18]. However, while these studies highlight maternal and contextual influences, they do not provide direct statistical analysis of storage duration or the time elapsed between milk expressions and processing as independent predictors of contamination.

This is the first study in Iran to systematically evaluate microbial contamination patterns in DHM before and after pasteurization, using multi-year surveillance data from an operational milk bank setting. While previous Iranian research has explored maternal attitudes, donation behaviors, and neonatal outcomes [19–21], none have investigated microbiological safety or contamination risk factors within the milk banking process. Conducted at the HMB of Valiasr Hospital in Tehran, this study offers the first longitudinal analysis of contamination trends in raw DHM, providing actionable insights for quality assurance and risk mitigation in similar healthcare environments. Given the vulnerability of the neonatal intensive care unit (NICU) population, especially preterm infants, understanding the upstream factors that influence microbial safety—such as maternal demographics, seasonal variation, storage duration, and handling practices—is essential. By identifying these determinants, the study supports the refinement of context-specific safety protocols and contributes to broader efforts to enhance DHM integrity in resource-limited settings.

Methods

Study design and setting

This study used a repeated cross-sectional design to examine patterns of microbial contamination in DHM over a six-year period (2019–2025). It was conducted at Valiasr Hospital in Tehran, where the Human Milk Bank (HMB) currently operates under standardized microbiological safety protocols, including routine screening and pasteurization. To reflect major procedural upgrades introduced in October 2023—which included changes in culture methods and hygienic practices—the study period was stratified into two distinct phases: Phase I (November 2019 to September 2023) and Phase II (from October 2023 to February 2025).

Study population and sample eligibility

DHM samples were obtained from lactating mothers who met the eligibility criteria defined by the HMB's clinical guidelines. Donors were required to test negative for HIV, HTLV-1, HTLV-2, hepatitis B surface antigen, hepatitis C antibodies, and syphilis (via VDRL testing). Additional criteria included delivery within the past 12 months, no history of substance abuse, and no known communicable diseases in the donor's spouse. Written informed consent was obtained from all participants prior to sample collection. Only donor milk samples with full microbial culture data before pasteurization and negative infection screening were eligible for inclusion.

Milk collection and storage by donors

Donors received training on hygienic practices for milk expression and storage. In Phase II, this training was expanded and standardized to include detailed instructions on hand hygiene, breast cleansing, and proper pump disinfection. Milk was typically collected in sterile containers provided by the HMB and stored at home in either a refrigerator (2–8 °C) or a freezer (–18 °C or lower), depending on donor preference and equipment availability. However, milk delivered in non-sterile containers or stored beyond the recommended time limits (48 h for refrigerated and 7 days for frozen milk) was still accepted under specific conditions, based on expert assessment and contextual feasibility. Storage type and duration were recorded for each sample and analyzed as potential predictors of microbial contamination.

Transport and processing at the milk bank

Milk was transported under cold chain conditions to preserve sample integrity. Frozen donor human milk was thawed either gradually in the refrigerator (2–8 °C) or in warm water not exceeding 37 °C, following best practice guidelines. Microwaving was strictly avoided to prevent nutrient loss and contamination risk. Upon arrival, each sample underwent microbial screening both before and after pasteurization. The time interval between container distribution and sample submission was recorded for each donation event. Pasteurization was performed using the mp3000 pasteurizer (Matin Promoters of Breast Milk Company, Iran), calibrated monthly with certified thermometric probes (± 0.1 °C accuracy). Temperature logs were automatically recorded and deviations from the 62.5 °C ± 0.5 °C range triggered batch rejection and reprocessing, in alignment with HMBANA standards [22, 23].

October 2023 protocol revision: operational and microbiological upgrades

In October 2023, as part of a comprehensive quality improvement initiative, the Human Milk Bank (HMB) implemented a series of enhanced hygienic protocols

aimed at minimizing microbial risk during milk handling and processing. These institutional upgrades included surgical-grade hand hygiene for all personnel, installation of laminar flow hoods in the sample preparation area, and reinforced environmental controls to reduce airborne contamination. In parallel, donor education protocols were expanded to ensure consistent adherence to hygienic standards during home expression. Donors received structured guidance on proper hand washing techniques, breast hygiene, and the disinfection of breast pump components using validated methods. Instructions also emphasized the use of sterilized containers, immediate freezing of expressed milk, and safe storage and transport practices [10]. These combined measures were designed to strengthen microbial safety across both institutional and donor-controlled stages of milk collection [22, 24].

Microbial culture methods and pathogen detection

Phase I (Nov 2019–Sep 2023): Microbiological screening was conducted using a manual enriched broth method. Samples were inoculated into Tryptic Soy Broth (TSB) supplemented with 0.05% Sodium Polyanethanol Sulfonate (SPS), a medium commonly used for detecting microbial presence in sterile fluids. Samples showing turbidity or signs of growth were plated on selective blood agar for colony enumeration and species identification.

Phase II (Oct 2023 – Feb 2025): Following the protocol revision, DHM samples were screened using direct aerobic agar-based culturing. Samples were plated on Columbia blood agar with 5% horse blood using sterile 1 μ L loops and incubated at 37 °C for 24 h in a 5% CO₂ atmosphere. Colonies were identified using MALDI-ToF mass spectrometry. This transition was documented and incorporated into statistical analyses to ensure methodological consistency.

Thresholds and safety criteria

Although European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) emphasizes microbial safety for preterm infants, it does not specify quantitative thresholds [23]. Our protocol was informed by clinical prudence and national precedents. We adopted a conservative threshold of 800 CFU/mL for any single potentially pathogenic organism. Commensal skin flora (e.g., *Staphylococcus epidermidis*, *CoNS*, *Enterococcus faecalis*) was accepted up to 10⁵ CFU/mL, provided no other pathogens were present. This dual-threshold strategy was tailored to the high-risk NICU population and the early operational phase of our milk bank.

The transition from enriched broth screening (TSB + SPS) to direct agar-based culturing was carefully documented and accounted for in statistical analyses to ensure methodological consistency. While broth-based methods

may offer enhanced sensitivity for detecting low-abundance organisms [25] direct agar-based culturing remains the preferred standard for HMB operations due to its accessibility, reproducibility, and regulatory support [22].

Variables and outcomes

The primary outcome of this study was the presence of microbial contamination in DHM, assessed both before and after pasteurization. To identify potential predictors of contamination, a range of independent variables were examined maternal characteristics included age, BMI, and the time interval between childbirth and milk donation. Logistical factors encompassed the time between milk expression and delivery to the HMB, milk handling variables included the season and year of donation, the volume of milk donated, the method of collection (either manual expression or breast pump), the type of storage container used at home (refrigerator or freezer), and the duration of milk storage prior to submission. Pathogen-specific contamination rates were analyzed with particular focus on clinically relevant organisms, including *Staphylococcus epidermidis*, *Bacillus cereus*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. Less prevalent pathogens were not analyzed due to their low detection frequency.

Statistical analysis

Data were analyzed using SPSS (v.26) and R (v.4.2.2). Descriptive statistics summarized donor demographics and sample characteristics. Continuous variables were reported as means $\pm$ standard deviations (SD); categorical variables as frequencies and percentages.

Temporal trends in contamination were analyzed by month, season, and year at the donor level. To identify predictors of microbial contamination, univariate and multivariate logistic regression models were constructed for each pathogen. All models were stratified by study phase to account for methodological changes.

Variables with p -value (p) ≤ 0.20 in univariate analyses were included in multivariate models. Firth's penalized likelihood estimation was applied when standard maximum likelihood estimation (MLE) failed due to sparse data. Effect sizes were reported as odds ratios (ORs) with 95% confidence intervals (CIs) for categorical variables, and as regression coefficients ($B \pm$ standard error (SE)) for continuous variables. Non-converging models were excluded and documented. All tests were two-tailed, with statistical significance set at $p < 0.05$.

Results

Overview of sample characteristics and pasteurization outcomes

Between November 2019 and February 2025, the Valiasr Hospital HMB received a total of 509 DHM samples. Of these, 302 samples (59.3%) were successfully pasteurized, while 207 samples (40.7%) were excluded due to microbial contamination. All pasteurized samples tested negative for detectable pathogens. The mean age of participants in the contaminated group was 32.08 years ($SD = 5.64$), compared to 31.51 years ($SD = 5.35$) in the non-contaminated group ($p = 0.259$). Additionally, maternal weight and BMI showed no significant differences between the groups, with p -values of 0.399 and 0.119, respectively. These findings indicate that the baseline characteristics of the two groups were comparable.

To facilitate interpretation, findings were stratified based on the methodological transition introduced in October 2023, which included a shift from enriched broth screening (TSB + SPS) to direct agar-based culturing, alongside concurrent upgrades in hygiene protocols. Among the 351 samples screened using the broth method (Phase I), 201 (57.3%) were pasteurized and 150 (42.7%) were excluded due to microbial contamination. In contrast, among the 158 samples processed via agar-based culturing, 101 (63.9%) were pasteurized and 57 (36.1%) were excluded.

Microbial profile of contaminated samples

A total of 509 DHM samples were screened prior to pasteurization using two distinct culture methods. In the Phase I, *Staphylococcus epidermidis* (21.3%) and *Klebsiella pneumoniae* (11.4%) were the most frequently detected organisms, while *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were not identified. In contrast, the Phase II showed a marked increase in *Klebsiella pneumoniae* (17.6%), the emergence of *Pseudomonas aeruginosa* (5.1%) and *Acinetobacter baumannii* (1.3%), alongside a sharp decline in *Staphylococcus epidermidis* (3.2%). Other pathogens—including *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus agalactiae*, and *Citrobacter freundii* were detected in both culture phases, though their prevalence varied. For instance, *Escherichia coli* were identified in 4.4% of samples screened during Phase I and 3.8% of those processed in Phase II. *Staphylococcus aureus* appeared in 3.2% and 3.8% of samples, respectively. *Enterococcus faecalis* showed a modest decline from 3.2% in Phase I to 1.9% in Phase II, while *Streptococcus agalactiae* decreased from 1.7% to 0.6%. *Citrobacter freundii* was detected at low frequencies in both phases (0.6% during Phase I and undetected in Phase II). A detailed comparison of microbial prevalence by culture method is presented in Fig. 1.

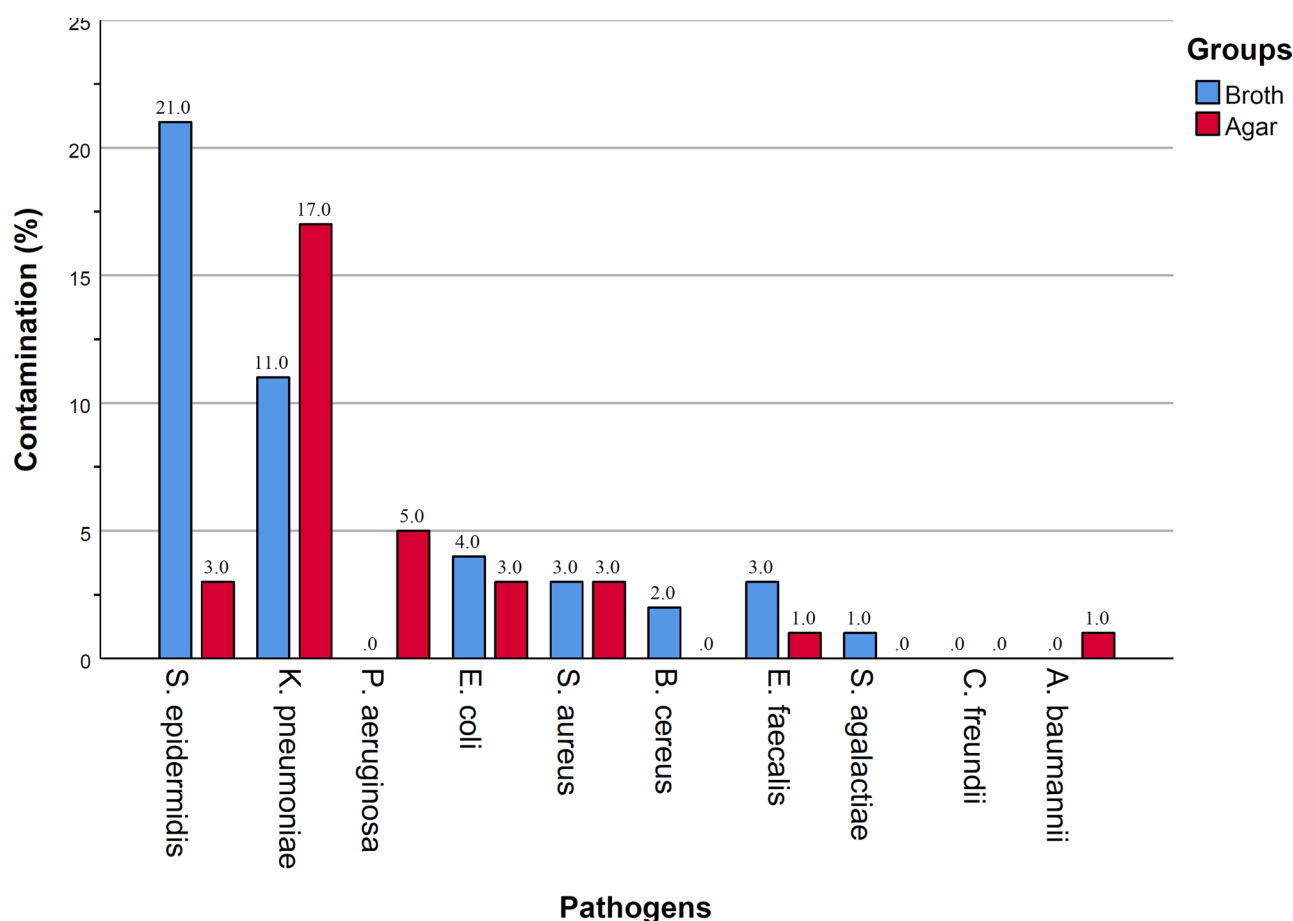


Fig. 1 Prevalence of bacterial contaminants in donor human milk prior to pasteurization in phase I and phase II

Seasonal and temporal trends in contamination

Seasonal and temporal patterns in microbial contamination were analyzed to explore fluctuations across pathogens and study phases. These findings are summarized in Fig. 2A and B, and highlight notable differences in contamination rates between seasons and across Phase I and Phase II.

In Phase I, *Staphylococcus epidermidis* contamination peaked in summer (29.20%) and spring (23.29%), while winter (7.55%) showed the lowest rate. In Phase II, contamination dropped sharply, with summer at 0.00% and winter at 0.99%. *Staphylococcus aureus* displayed a phase-dependent seasonal pattern. In Phase I, contamination remained low across all seasons, with spring (1.47%) and winter (5.77%) being the highest. In Phase II, a marked increase was observed in spring (20.68%), while other seasons remained at 0.00%, suggesting a spring-specific spike. *Klebsiella pneumoniae* showed elevated contamination in cooler seasons. In Phase I, autumn (13.21%) and winter (13.21%) had the highest rates. In Phase II, contamination was highest in spring (24.00%), winter (17.82%), and autumn (12.50%), while summer remained at 0.00%. *Escherichia coli* contamination remained

relatively low and stable. In Phase I, rates ranged from 2.13% (winter) to 5.05% (autumn). In Phase II, contamination was absent in summer and autumn (0.00%), with modest levels in spring (6.89%) and winter (3.96%). *Bacillus cereus* contamination in Phase I was highest in winter (8.47%), with lower levels in autumn (2.83%) and spring (2.55%), and summer at 0.00%. In Phase II, contamination was not detected in any season, indicating a complete drop.

Overall, Phase I demonstrated sharper seasonal contrasts, particularly for *Staphylococcus epidermidis* and *Bacillus cereus*, while Phase II showed reduced seasonal variation. These patterns suggest that seasonal distribution may influence contamination dynamics for select pathogens.

Annual trends in contamination

Figure 3 illustrates the year-by-year contamination rates for six microbial species identified in donor human milk samples collected between 2019 and 2025.

Klebsiella pneumoniae showed the greatest variability, with a peak rate of 50.00% in 2019–2020, followed by elevated levels in 2023–2024 (17.20%) and

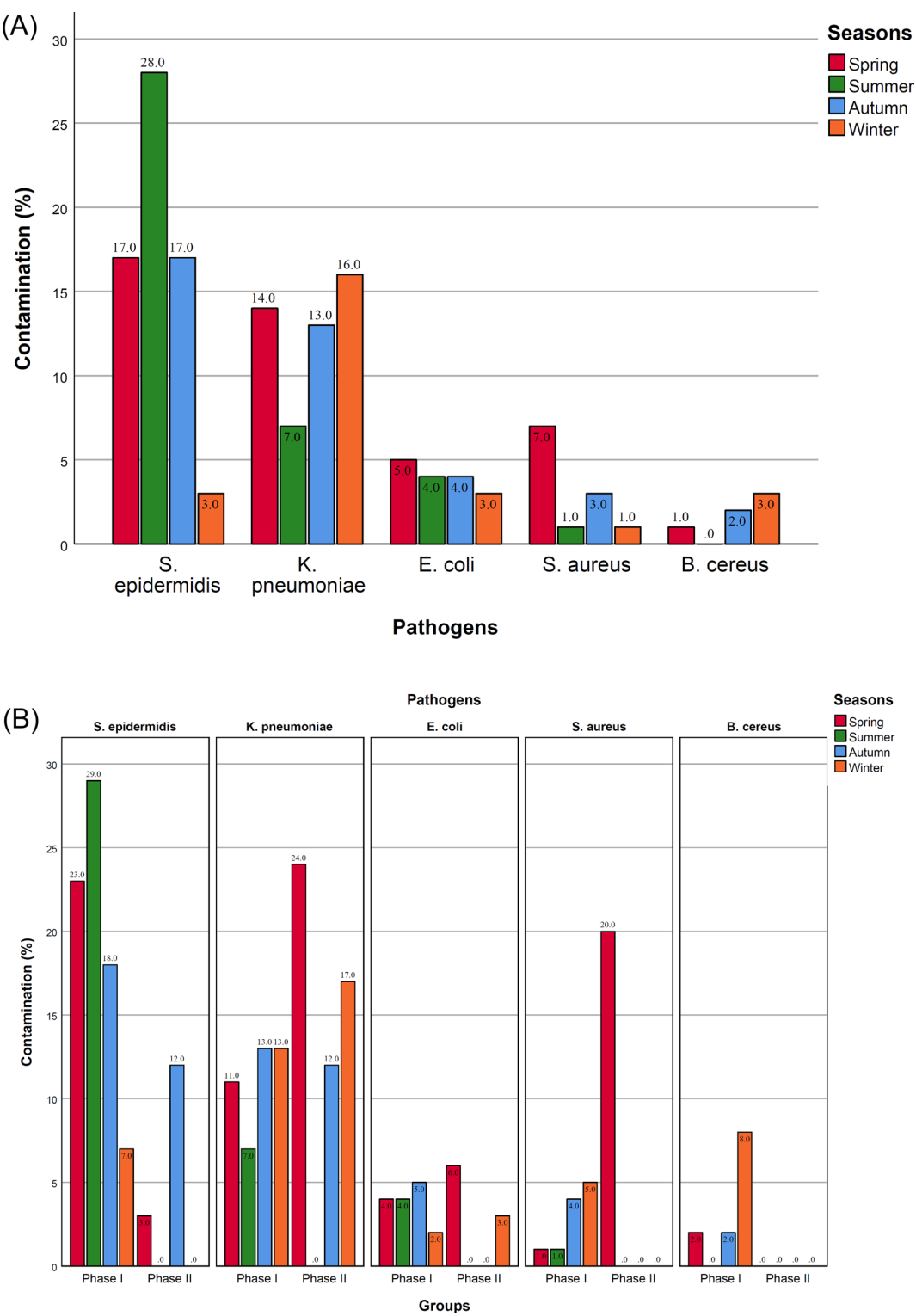


Fig. 2 **A** Seasonal variation in pathogens contamination rates in donor human milk in and all samples. **B** Seasonal variation in pathogens contamination rates in donor human milk in Phase I and Phase II samples

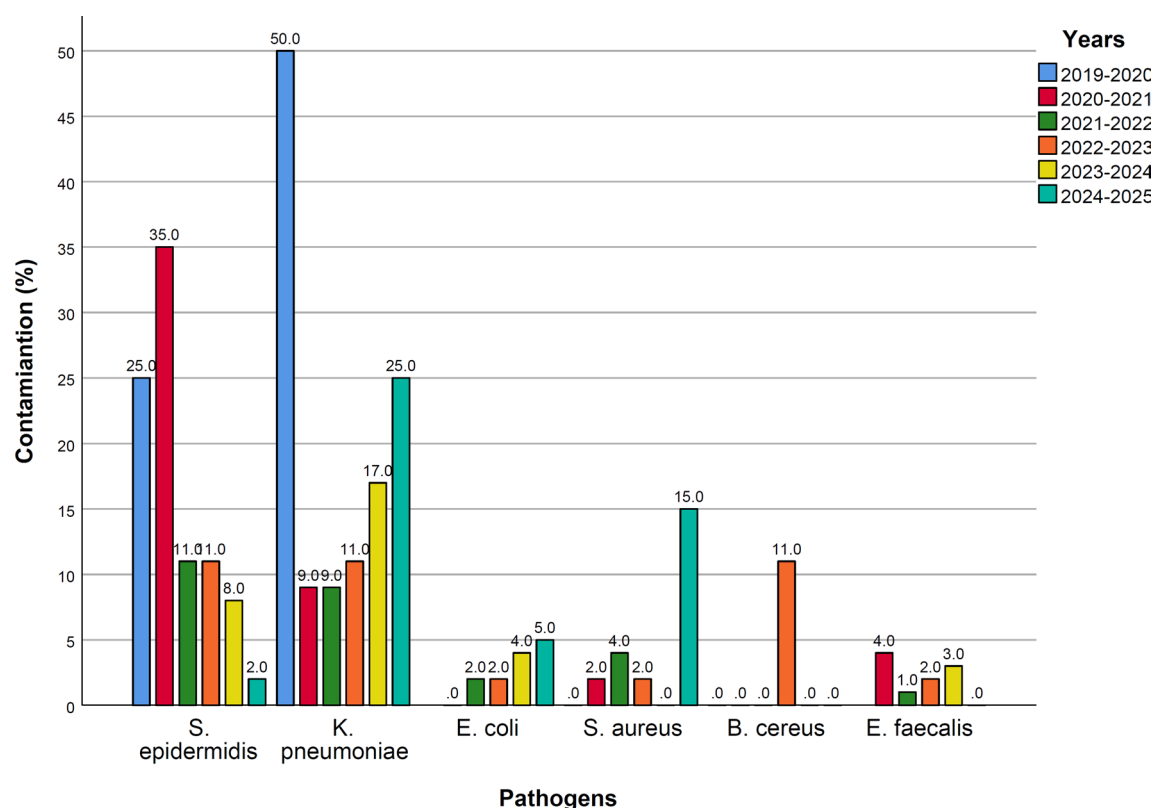


Fig. 3 Annual contamination rates of selected microbial species in donor human milk (2019–2025)

2024–2025 (25.00%). *Staphylococcus epidermidis* reached its highest prevalence in 2020–2021 (35.65%), and then declined steadily over subsequent years, reaching 2.50% by 2024–2025. *Staphylococcus aureus* remained generally low throughout the study period, except for a notable increase to 15.00% in 2024–2025. No detection was recorded in 2019–2020 and 2023–2024. *Escherichia coli* contamination remained relatively stable, ranging from 0.00% in 2020–2021 to 5.00% in 2024–2025, with a modest peak in 2022–2023 (4.46%). *Bacillus cereus* appeared sporadically, with its highest rate of 11.76% in 2022–2023, and no detection in 2021–2022 and 2024–2025. *Enterococcus faecalis* remained consistently below 5% across all years, with no detection in 2019–2020 and 2024–2025.

Regression analysis of contamination risk factors

In this study, maternal and milk-handling variables, including maternal age, BMI, time interval from childbirth to donation, donated milk volume, duration and method of home storage (refrigeration vs. freezing), milk expression technique (manual vs. pump-assisted), and type of collection container (personal vs. sterilized center-provided) were evaluated for their association with microbial contamination. Full regression outputs are presented in Tables 1, 2, 3, 4, 5 and 6.

Bacillus cereus contamination in Phase I showed a significant association with the method of milk expression.

Both univariate and multivariate models indicated that manual expression was associated with a substantially higher risk of contamination compared to pump-assisted expression (adjusted odds ratio [aOR] = 3.48) (Table 1).

For *Staphylococcus epidermidis* in Phase I, five variables met inclusion criteria for multivariate modeling: time from childbirth to donation, milk volume, duration of home storage, storage method, and storage location. Of these, two remained statistically significant in the final model. Shorter intervals between childbirth and donation were associated with reduced contamination risk, while refrigeration (as opposed to freezing) was associated with a markedly increased risk (aOR = 5.78). These findings suggest that both timing and storage conditions play a critical role in *Staphylococcus epidermidis* contamination dynamics. In Phase II, two variables of time from childbirth to donation and total days of home storage, were included in the multivariate model for *Staphylococcus epidermidis*, but neither retained statistical significance, indicating a possible shift in contamination patterns or reduced model power in this phase (Table 2).

No statistically significant associations were observed for *Staphylococcus aureus* (Table 3), *Klebsiella pneumonia* (Table 4), *Escherichia coli* (Table 5) or *Pseudomonas aeruginosa* (Table 6) in either phase, suggesting that the tested donor and handling factors did not meaningfully influence contamination risk for these pathogens.

Table 1 Univariate and multiple logistic regression analysis of factors associated with *Bacillus cereus* contamination in donated human milk samples. Phase I: 10 contaminated samples; phase II: no contamination detected

<i>Bacillus cereus</i>	Phase I (n = 10)		Multivariate analysis *	
	Univariate analysis*			
Variable	B ± SE or OR [95% CI]	P-value	B ± SE or OR [95% CI]	P-value
Mother's Age (years)	0.059 ± 0.058	0.475	-	-
Mother's body mass index	-0.071 ± 0.071	0.314	-	-
Home keeping Days	0.034 ± 0.023	0.146	-0.021 ± 0.019	0.288
Type of Dish (Banking vs. Home)	OR = 2.44 [0.398, 15.02]	0.408	-	-
Milk Volume (ml)	0.000 ± 0.000	0.483	-	-
Storage at Home (Refrigerator vs. Freezer)	Not valid	-	-	-
Delivery to Donor (Days)	0.004 (0.003)	0.408	-	-
Milk Collection Method (Hand vs. Pump)	OR = 0.208 [0.54, 0.802]	0.023	OR = 0.287 [0.495]	0.041

* Firth correction was applied to reduce bias in logistic regression estimates. Values marked with an asterisk (*) reflect Firth-adjusted coefficients. In the absence of an asterisk, results were derived using Standard Maximum Likelihood Estimation (SMLE). Cells marked as "Not valid" indicate that logistic regression estimates were unstable due to quasi-complete separation. In these cases, the variable perfectly predicted the outcome or the number of positive cases was too low for reliable estimation. Therefore, results were not reported. Effect sizes are reported as B ± SE for continuous variables and OR [95% CI] for categorical variables. No contamination was detected in Phase II; therefore, regression analysis was not performed for this phase

Discussion

This study represents the first longitudinal microbiological assessment of DHM in Iran, conducted over a six-year period and incorporating maternal, procedural, and seasonal variables. Among the 509 pre-pasteurization samples screened, 40.7% exceeded established contamination thresholds—substantially higher than rates reported in comparable international studies. For instance, Li et al. (2025) documented a 16.8% rejection rate in the United Kingdom, with only 0.6% contamination following pasteurization [26], Novoselac et al. (2021) reported a 12.6% discard rate in Croatia [27] and Tran et al. (2023) observed 15.1% in Vietnam [15]. Collectively, these findings suggest that DHM banks adhering to standard microbiological criteria typically report discard rates ranging from 10% to 17% before pasteurization, and up to 7% post-pasteurization [15, 26, 27]. In contrast, all 302 pasteurized samples in our study tested negative for

detectable pathogens, underscoring the efficacy of the pasteurization protocol employed.

The elevated discard rate observed in our study reflects a deliberately conservative exclusion threshold of 800 CFU/mL for any single pathogen. In contrast, commensal flora—such as *Staphylococcus epidermidis*, coagulase-negative staphylococci (CoNS), and *Enterococcus faecalis*—were accepted up to 10⁵ CFU/mL, unless co-detected with pathogenic organisms, which triggered exclusion. This threshold is notably stricter than the 10⁴–10⁵ CFU/mL limits recommended by the NICE Clinical Guideline CG93 [26, 28]. Our approach was shaped by three key factors: (i) the high vulnerability of our Level III NICU population, predominantly composed of preterm infants; (ii) the early operational phase of our milk bank; and (iii) the imperative to minimize microbiological risk during implementation.

While NICE CG93 advises excluding DHM samples with total viable counts (TVC) above 10⁵ CFU/mL or with *Staphylococcus aureus* and Enterobacteriaceae exceeding 10⁴ CFU/mL, it does not specify limits for commensal flora [28]. Similarly, ESPGHAN underscores the importance of microbial safety in DHM for preterm infants but offers no quantitative thresholds [23]. Our exclusion strategy aligns with large-scale operational data from the Hearts Milk Bank (UK), where over 6,800 DHM samples were screened using comparable criteria over seven years [26].

Methodological differences between screening phases further influenced discard rates. Phase I (Nov 2019–Sep 2023) employed enriched broth-based screening (TSB + SPS), which enhances sensitivity for low-abundance organisms—including non-pathogenic commensals—and may have inflated rejection rates. Phase II (Oct 2023–Feb 2025) transitioned to direct agar-based culturing, yielding lower discard rates and more clinically relevant detection. Although WHO emphasizes microbiological safety in DHM processing, it does not specify preferred culture methods. In contrast, HMBANA recommends agar-based techniques (e.g., blood agar and MacConkey agar) as standard practice [22].

Diversity and prevalence in raw donor milk

The combined microbial profile of raw DHM across both screening phases revealed a predominance of *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, and *Escherichia coli*, consistent with reports from milk banks in Vietnam and Europe [15, 26, 27]. These organisms likely originate from distinct maternal and environmental sources: *Staphylococcus epidermidis* from maternal skin and breast tissue, *Klebsiella pneumoniae* from environmental exposure during milk expression and handling, and *Escherichia coli* from the gastrointestinal tract [1, 29]. While some of these bacteria are considered commensal

Table 2 Univariate and multiple logistic regression analysis of *Staphylococcus epidermidis* contamination Phase I: 74 contaminated samples; phase II: 5 contaminations detected

<i>Staphylococcus epidermidis</i> Variable	Phase I (n = 74)				Phase II (n = 5)			
	Univariate analysis		Multivariate analysis		Univariate analysis*		Multivariate analysis *	
	B ± SE or OR [95% CI]	P-value	B ± SE or OR [95% CI]	P-value	B ± SE or OR [95% CI]	P-value	B ± SE or OR [95% CI]	P-value
Mother's Age (years)	-0.017 ± 0.023	0.452	-	-	0.048 ± 0.112	0.61	-	-
Mother's Body Mass Index	0.13 ± 0.027	0.635	-	-	-0.038 ± 0.115	0.71	-	-
Home keeping Days	0.008 ± 0.005	0.147	-0.04 ± 0.07	0.591	0.005 ± 0.035	0.087	0.015 ± 0.031	0.623
Type of Dish (Banking vs. Home)	OR = 1.01 [0.478, 2.15]	0.971	-	-	Not valid	-	-	-
Milk Volume (ml)	-0.001 ± 0.000	0.002	0.000 ± 0.000	0.125	0.000 ± 0.001	0.79	-	-
Storage at Home (Refrigerator vs. Freezer.)	OR = 0.164 [0.45, 0.596]	0.006	OR = 0.198 [0.04, 0.851]*	0.029	Not valid	-	-	-
Delivery to Donor (Days)	0.1 ± 0.004	0.004	0.01 ± 0.004	0.012	0.012 ± 0.009	0.065	0.014 ± 0.007	0.045
Milk Collection Method (Hand vs. Pump)	OR = 0.456 [0.233, 0.893]	0.002	OR = 0.7 [0.283, 1.73]	0.441	OR = 1.042 [0.385, 2.84]	0.76	-	-

* Firth correction was applied to reduce bias in logistic regression estimates. Values marked with an asterisk (*) reflect Firth-adjusted coefficients. In the absence of an asterisk, results were derived using Standard Maximum Likelihood Estimation (SMLE). Cells marked as "Not valid" indicate that logistic regression estimates were unstable due to quasi-complete separation. In these cases, the variable perfectly predicted the outcome or the number of positive cases was too low for reliable estimation. Therefore, results were not reported. Effect sizes are reported as B ± SE for continuous variables and OR [95% CI] for categorical variables

under specific conditions, excessive microbial load (particularly in immunocompromised neonates) can pose significant clinical risks [30, 31].

Phase I (broth-based screening) demonstrated a higher detection rate of *Staphylococcus epidermidis* (21.3%), reflecting the method's sensitivity for fast-replicating commensals. In contrast, Phase II (direct agar-based culturing) revealed increased prevalence of *Klebsiella pneumoniae* (17.6%) and enabled detection of *Pseudomonas aeruginosa* (5.1%) and *Acinetobacter baumannii* (1.3%), both absent in Phase I. These findings suggest that agar-based methods enhance detection of Gram-negative opportunistic pathogens relevant to NICU settings [32].

The reduced recovery of *Staphylococcus epidermidis* in Phase II (3.2%) may reflect its limited growth on solid media and improved hygiene protocols. Variations in *Escherichia coli*, *Staphylococcus aureus*, and *Enterococcus faecalis* between phases further underscore the need for methodological standardization in donor milk screening. Notably, *Bacillus cereus* (which was detected sporadically in Phase I) was absent in Phase II, potentially due to both improved hygiene and reduced sensitivity of agar-based methods for spore-forming organisms.

Klebsiella pneumoniae emerged as the dominant organism in Phase II, marking a significant shift in the microbial landscape. As a non-commensal Gram-negative pathogen frequently implicated in NICU-acquired infections—including sepsis, pneumonia, and meningitis [33]—its detection in DHM warrants heightened surveillance. The methodological shift from broth to agar-based culturing likely contributed to this increase, highlighting the importance of technique selection in interpreting microbial data and refining safety thresholds. Given its pathogenic potential and resistance profile, *Klebsiella*

pneumoniae should be prioritized in donor milk screening protocols.

Seasonal and temporal trends in microbial contamination of donor human milk

Our analysis revealed distinct seasonal and temporal patterns in microbial contamination of raw DHM, shaped by both environmental conditions and the methodological transition implemented in October 2023. These findings underscore the dynamic interplay between climate, procedural protocols, and microbial ecology in milk bank operations.

The most pronounced seasonal trend was observed for *Staphylococcus epidermidis*, which peaked during warmer months in Phase I—summer (29.2%) and spring (23.3%)—and declined sharply in winter (7.6%). This pattern aligns with studies showing increased airborne *Staphylococcus* species in warmer seasons, with temperature as a key positive predictor [34]. Following the protocol revision in Phase II, *S. epidermidis* contamination dropped markedly across all seasons, effectively eliminating seasonal variation. This reduction likely reflects the combined impact of enhanced hygiene measures (e.g., surgical-grade hand hygiene, laminar flow hoods) and the shift from broth-based to agar-based culturing, which reduced detection of skin-associated commensals.

In contrast, *Staphylococcus aureus* exhibited a distinct temporal spike during spring 2024–2025, confined to Phase II. This isolated outbreak suggests a year-specific event rather than a recurring seasonal trend. Possible causes include a temporary breach in hygiene protocols, introduction of a novel strain among donors, or localized environmental contamination. While warmer conditions may favor *S. aureus* proliferation [34, 35], the

Table 3 Univariate and multiple logistic regression analysis of factors associated with *Staphylococcus aureus* contamination in donated human milk Samples. Phase I: 11 contaminated samples; phase II: 6 contaminations detected

<i>Staphylococcus aureus</i>	Phase I (n = 11)		Phase II (n = 6)	
	Univariate analysis *		Univariate analysis *	
Variable	B ± SE or OR [95% CI]	P-value	B ± SE or OR [95% CI]*	P-value
Mother's Age (years)	0.03 ± 0.054	0.503	0.038 ± 0.102	0.71
Mother's Body Mass Index	-0.05 ± 0.066	0.453	-0.095 ± 0.115	0.41
Home keeping Days	0.005 ± 0.001	0.628	-0.039 ± 0.045	0.39
Type of Dish (Banking vs. Home)	OR = 0.583 [0.069, 4.95]	0.621	OR = 0.248 [0.42, 14.69]	0.32
Milk Volume (ml)	0.001 (0.001)	0.061	0.018 ± 0.001	0.12
Storage at Home (Refrigerator vs. Freezer.)	Not Valid	-	Not valid	-
Delivery to Donor (Days)	0.0 (0.004)	0.998	0.007 (0.007)	0.29
Milk Collection Method (Hand vs. Pump)	Not Valid	-	OR = 1.36 [0.24, 78.2]	0.74

* Firth correction was applied to reduce bias in logistic regression estimates. Values marked with an asterisk (*) reflect Firth-adjusted coefficients. In the absence of an asterisk, results were derived using Standard Maximum Likelihood Estimation (SMLE). Cells marked as "Not valid" indicate that logistic regression estimates were unstable due to quasi-complete separation. In these cases, the variable perfectly predicted the outcome or the number of positive cases was too low for reliable estimation. Therefore, results were not reported. Effect sizes are reported as B ± SE for continuous variables and OR [95% CI] for categorical variables. In both Phase I and Phase II, only one variable met the inclusion criterion (p-value < 0.20 in univariate analysis); therefore, multiple logistic regression analysis was not performed for either phase

non-recurring nature of this event highlights the importance of integrated surveillance systems that combine seasonal monitoring with longitudinal trend analysis to distinguish endemic patterns from acute public health incidents.

Klebsiella pneumoniae contamination followed a different seasonal trajectory, with higher rates in colder months—autumn and winter in Phase I, and spring and winter in Phase II—and the lowest rates in summer. This pattern is consistent with studies showing enhanced survival of *Klebsiella pneumoniae* at lower temperatures and higher humidity [36]. A progressive increase in its detection over the study period further emphasizes the need

for continuous epidemiological monitoring and targeted interventions.

Bacillus cereus contamination also peaked during winter (8.5% in Phase I), consistent with the stability of bacterial spores in cool, dry environments [14]. The highest annual rate (11.8%) occurred in 2022–2023, prior to the implementation of enhanced quality control measures. Notably, *Bacillus cereus* was undetected in Phase II, likely due to both improved hygiene and the reduced sensitivity of agar-based methods for spore-forming organisms.

Escherichia coli contamination remained relatively stable between phases—4.4% in Phase I and 3.8% in Phase II—but showed a gradual annual increase, reaching 5% by the end of the study. Seasonally, the highest rates were observed in autumn (5.1%) and the lowest in winter (2.1%), though these fluctuations were less pronounced than for other organisms.

Influence of maternal and handling factors on contamination risk

Multivariate analysis identified a significant association between storage method and *Staphylococcus epidermidis* contamination in Phase I. Samples stored in freezers exhibited markedly lower contamination rates than those stored in refrigerators, suggesting that freezing more effectively inhibits the growth of skin-associated bacteria. While evidence supports the microbial stability of refrigerated DHM under controlled clinical conditions for several days [37], the elevated contamination observed in our refrigerated samples likely reflects deviations from standard storage protocols in non-clinical environments. Factors such as prolonged storage duration, temperature fluctuations, or suboptimal hygiene may have contributed. No significant association was found between storage method and contamination by other pathogens.

Longer intervals between childbirth and milk donation were also associated with increased contamination, particularly by *Staphylococcus epidermidis* across both phases. This trend may be explained by the gradual decline of antimicrobial components such as lactoferrin and secretory IgA over time. Luna-Castro et al. (2017) confirmed the antimicrobial role of lactoferrin in human milk, highlighting its capacity to inhibit opportunistic bacterial growth [38]. These findings underscore the importance of timely milk collection, especially for high-risk neonatal populations.

In Phase I, *Bacillus cereus* contamination was significantly associated with manual milk expression, while pump-assisted methods appeared protective. The complete absence of *Bacillus cereus* in Phase II likely reflects both improved hygienic infrastructure and stricter environmental controls. Given the organism's spore-forming capacity, transmission via contaminated expression equipment or storage containers is plausible. Adjidé et al.

Table 4 Univariate and multiple logistic regression analysis of factors associated with *Klebsiella pneumonia* contamination in donated human milk Samples. Phase I: 39 contaminated samples; phase II: 27 contaminations detected

<i>Klebsiella pneumonia</i>	Phase I (n = 39)				Phase II (n = 27)			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis *	
	B ± SE or OR [95% CI]	P-value	B ± SE or OR [95% CI]	P-value	B ± SE or OR [95% CI]	P-value	B ± SE or OR [95% CI]	P-value
Mother's Age (years)	-0.023 ± 0.029	0.441	-	-	0.019 ± 0.044	0.667	-	-
Mother's Body Mass Index	-0.037 ± 0.037	0.325	-	-	0.014 ± 0.05	0.779	-	-
Home keeping Days	0.006 ± 0.006	0.34	-	-	0.006 ± 0.014	0.672	-	-
Type of Dish (Banking vs. Home)	OR = 2.25 [0.976, 5.23]	0.06	OR = 1.91 [0.805, 4.53]	0.142	OR = 1.84 [0.728, 4.65]	0.198	OR = 2.22 [0.785, 6.23]	0.133
Milk Volume (ml)	0.000 ± 0.001	0.424	-	-	0.000 ± 0.001	0.898	-	-
Storage at Home (Refrigerator vs. Freezer.)	Not valid	-	-	-	Not valid	-	-	-
Delivery to Donor (Days)	0.001 ± 0.002	0.801	-	-	0.007 ± 0.005	0.117	-0.01 ± 0.005	0.057
Milk Collection Method (Hand vs. Pomp)	OR = 0.499 [0.219, 1.14]	0.098	OR = 0.467 [0.184, 1.18]	0.108	OR = 1.091 [0.469, 2.54]	0.84	-	-

• Firth correction was applied to reduce bias in logistic regression estimates. Values marked with an asterisk (*) reflect Firth-adjusted coefficients. In the absence of an asterisk, results were derived using Standard Maximum Likelihood Estimation (SMLE). Cells marked as "Not valid" indicate that logistic regression estimates were unstable due to quasi-complete separation. In these cases, the variable perfectly predicted the outcome or the number of positive cases was too low for reliable estimation. Therefore, results were not reported. Effect sizes are reported as B ± SE for continuous variables and OR [95% CI] for categorical variables

Table 5 Univariate and multiple logistic regression analysis of factors associated with *Escherichia coli* contamination in donated human milk Samples. Phase I: 14 contaminated samples; phase II: 6 contaminations detected

<i>Escherichia coli</i>	Phase I (n = 14)				Phase II (n = 6)		
	Univariate analysis		Multivariate analysis		Univariate analysis*		
Variable	B ± SE or OR [95% CI]	P-value	B ± SE or OR [95% CI]	P-value	B ± SE or OR [95% CI]	P-value	P-value
Mother's Age (years)	-0.023 ± 0.047	0.628	-	-	0.084 ± 0.094	-	0.371
Mother's Body Mass Index (BMI)	-0.105 ± 0.067	0.115	-0.112 ± 0.068	0.098	0.040 ± 0.99	-	0.689
Home keeping Days	-0.027 ± 0.016	0.094	-0.015 ± 0.018	0.397	-0.019 ± 0.029	-	0.510
Type of Dish (Banking vs. Home)	OR = 2.16 [0.605, 7.7]	0.236	-	-	OR = 1.31 [0.239, 7.46]	-	0.760
Milk Volume (ml)	0.001 ± 0.001	0.075	-0.001 ± 0.001	0.243	-0.001 ± 0.001	-	0.333
Storage at Home (Refrigerator vs. Freezer.)	Not valid	-	-	-	Not valid	-	-
Delivery to Donor (Days)	0.005 ± 0.003	0.073	0.005 ± 0.03	0.083	0.003 ± 0.008	-	0.743
Milk Collection Method (Hand vs. Pomp)	OR = 0.547 [0.146, 2.05]	0.371	-	-	OR = 0.697 [0.136, 3.57]	-	0.664

• Firth correction was applied to reduce bias in logistic regression estimates. Values marked with an asterisk (*) reflect Firth-adjusted coefficients. In the absence of an asterisk, results were derived using Standard Maximum Likelihood Estimation (SMLE). Cells marked as "Not valid" indicate that logistic regression estimates were unstable due to quasi-complete separation. In these cases, the variable perfectly predicted the outcome or the number of positive cases was too low for reliable estimation. Therefore, results were not reported. Effect sizes are reported as B ± SE for continuous variables and OR [95% CI] for categorical variables. Multiple logistic regression analysis was not performed for Phase II because none of the variables met the inclusion criterion (p-value < 0.20 in univariate analysis)

(2022) similarly reported that *Bacillus cereus* contamination in DHM can originate from environmental sources and is influenced by seasonality, equipment, and collection practices [14].

This study provides the first longitudinal microbiological evaluation of DHM in Iran, offering a comprehensive six-year analysis that spans both pre- and post-pasteurization phases. Conducted during a critical period of operational transition, it uniquely integrates maternal, procedural, and seasonal variables to contextualize contamination patterns. The phased shift from broth-based to agar-based culturing—aligned with HMBANA standards—enhanced methodological precision while enabling real-time quality improvements. By incorporating annual and seasonal trends, the study reveals

dynamic microbial shifts often overlooked in cross-sectional designs. Furthermore, the use of multivariate modeling to identify contamination risk factors adds analytical depth and practical relevance. Collectively, these strengths position the study as a foundational reference for optimizing DHM safety protocols in emerging milk bank systems.

Limitations

This study was conducted within a single milk bank affiliated with a tertiary neonatal care center, which may limit the generalizability of its findings to other institutional settings or geographic regions. Local variations in donor demographics, hygiene infrastructure, and milk-handling practices could influence microbial profiles and discard

Table 6 Univariate and multiple logistic regression analysis of factors associated with *Pseudomonas aeruginosa* contamination in donated human milk Samples. Phase I: 0 contaminated samples; phase II: 8 contaminations detected

<i>Pseudomonas aeruginosa</i>	Phase II (n = 8)	
	Univariate analysis*	
Variable	B ± SE or OR [95% CI]	P-Value
Mother's Age (years)	0.029 ± 0.073	0.693
Mother's Body Mass Index	0.006 ± 0.089	0.947
Home keeping Days	-0.015 ± 0.024	0.520
Type of Dish (Banking vs. Home)	OR = 2.38 [0.56, 10.17]	0.242
Milk Volume (ml)	Not valid	-
Storage at Home (Refrigerator vs. Freezer.)	Not valid	-
Delivery to Donor (Days)	0.009 ± 0.009	0.326
Milk Collection Method (Hand vs. Pump)	OR = 0.19 [0.04, 0.99]	0.049

Firth correction was applied to reduce bias in logistic regression estimates. Values marked with an asterisk (*) reflect Firth-adjusted coefficients. In the absence of an asterisk, results were derived using Standard Maximum Likelihood Estimation (SMLE). Cells marked as "Not valid" indicate that logistic regression estimates were unstable due to quasi-complete separation. In these cases, the variable perfectly predicted the outcome or the number of positive cases was too low for reliable estimation. Therefore, results were not reported. Effect sizes are reported as B ± SE for continuous variables and OR [95% CI] for categorical variables. No contamination was detected in Phase I; therefore, regression analysis was not applicable. In Phase II, only univariate logistic regression was performed. Multiple logistic regression analysis was not conducted because only one variable met the inclusion criterion (p-value < 0.20 in univariate analysis)

rates. Additionally, although both broth-based and agar-based culturing methods were implemented according to standardized protocols, subtle differences in technician technique, incubation conditions, and media sensitivity may have affected detection rates—particularly for low-abundance or spore-forming organisms such as *Bacillus cereus*. These limitations underscore the need for future multi-center studies to validate pathogen-specific thresholds, assess the comparative sensitivity of culture techniques, and support the development of harmonized screening standards for DHM safety.

Conclusion

This study offers the first longitudinal microbiological assessment of DHM in Iran, spanning six years of surveillance and capturing both pre- and post-pasteurization phases. By integrating maternal, procedural, and seasonal variables, it provides a multidimensional view of contamination dynamics rarely addressed in previous literature. The elevated discard rate prior to pasteurization reflects a deliberately conservative exclusion threshold, calibrated to protect a highly vulnerable NICU population. In contrast, the complete absence of detectable pathogens in pasteurized samples confirms the robustness of the applied thermal protocol. These findings also suggest that, with validated safety data, a reassessment of exclusion thresholds—particularly for low-virulence

or commensal organisms—may be warranted to reduce unnecessary discard without compromising microbiological safety. The phased transition from broth-based to agar-based culturing not only improved clinical relevance but also revealed shifts in microbial prevalence—most notably the emergence of *Klebsiella pneumoniae* as a dominant pathogen in later phases. Seasonal trends, such as the summer peak of *Staphylococcus epidermidis* and winter prevalence of *Bacillus cereus*, underscore the influence of environmental conditions on microbial ecology. Temporal spikes, like the isolated *Staphylococcus aureus* outbreak, highlight the need for integrated surveillance systems capable of distinguishing endemic patterns from acute events.

Importantly, the study identifies key maternal and handling factors—such as storage method, expression technique, and donation timing—that significantly influence contamination risk. These findings offer actionable insights for milk bank optimization and neonatal safety. Taken together, the study's methodological rigor, contextual depth, and analytical scope position it as a foundational reference for DHM microbiological governance in emerging systems.

We recommend the development of pathogen-specific validation thresholds and standardized culture protocols adapted to NICU-targeted DHM screening. Future multi-center studies are essential to refine these standards, enhance generalizability, and support global harmonization of donor milk safety practices.

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Author contributions

LS and HD conceptualized the study, HA collected data, LS analyzed the data, and drafted the manuscript. HD, MS, and HA contribute to interpretation, and manuscript revision. All authors read and approved the final manuscript.

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Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Tehran University of Medical Sciences (Approval No. IR.TUMS.IKHC.REC.1403.259). Written informed consent was obtained from all participating donors.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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