Comparison of bacterial profiles in human milk from mothers of term and preterm infants

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Research Article

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Abstract

Background

Bacteria in human milk (HM) can be endogenous or exogenous, and the latter can carry the risk of various infections in very low-birth weight infants because of the possibility of contamination with pathogenic bacteria. The mother's lifestyle and environment have a major influence on such bacterial contamination, and it is thought that there are differences in the number and types of bacteria cultured from HM between term mothers whose infants are at home and mothers of preterm infants in neonatal intensive care units (NICUs). This research aimed to compare the bacterial profiles of HM among mothers of term and preterm infants.

Methods

The data comprised 214 milk samples (term: 75, preterm: 139) donated by 47 registered donors (term: 31, preterm: 16) from January to November 2021. Bacterial culture results were compared between term and preterm HM samples. Differences in the mean total bacterial count and bacterial species count per batch were analyzed using Welch's t-test and Student's t-test, respectively. The bacterial contamination rate was analyzed using Chi-square test or Fisher's exact test.

Results

Coagulase-negative *Staphylococci*, *Staphylococcus aureus*, and *Pseudomonas fluorescens* were frequently found in both term and preterm HM. *Serratia liquefaciens* (p < 0.001) and two other bacteria contaminated term HM, while five types of bacteria, including *Enterococcus faecalis* and *Enterobacter aerogenes* (p < 0.001) contaminated preterm HM. The mean (SD) total bacterial count was 351,141 (1,060,949) CFU/100 µL for term HM and 872,272 (2,324,477) CFU/100 µL for preterm HM (p = 0.026). Similarly, the number of bacterial species in HM was more diverse in preterm donors (p < 0.001).

Conclusions

This study revealed that HM from preterm donors has a higher total bacterial count and greater diversity and characterization of bacterial types compared with HM from term donors. These results also suggested there was a trend toward greater contamination with nosocomial-infection-causing bacteria in the NICU. Enhanced hygiene instructions for preterm donors may reduce the need to dispose of valuable donated HM as well as the risk of BM pathogen transmission to infants in the NICU.

Background
Human milk (HM) is recognized as the most ideal nutrient source for infants, and in particular, for preterm infants. It not only reduces the risk of developing necrotizing enterocolitis [1–3], sepsis, and other diseases but also contributes to better long-term outcomes and the neurodevelopment of the infant [4, 5]. HM contains various kinds of bacteria, such as *Bifidobacteria* spp., *Lactobacillus* spp., coagulase-negative *Staphylococci* (CoNS), diphtheroid, *Acinetobacter* spp., oral *Streptococcus* (viridans group streptococci), *Staphylococcus aureus*, Group B *Streptococcus*, *Escherichia coli*, *Pseudomonas* spp., *Klebsiella* spp., *Enterococcus* spp., *Enterobacter* spp., *Bacillus* spp., and *Moraxella* spp. [6–9], in addition to nutrients and micronutrients.

Bacteria in HM are known to play important roles in infant health that are both positive and negative. The bacteria can be classified into two main categories: endogenous and exogenous (contamination) [10]. The former consists of part of the maternal gastrointestinal microbiota that transitions through the mammary glands via an entero-mammary pathway and is highly beneficial to an infant's intestinal tract development and immune function [11]. The latter, however, comprises contaminants from the external environment, such as normal bacteria on the mother's and infant’s skin, flora in the infant's oral cavity, and bacteria in the breast pump and milk bottle. These bacteria are sometimes pathogenic and may pose a risk of infections in preterm infants.

Contamination with HM is not a major problem for term infants, but this is not the case for preterm infants. There are several pathways resulting in HM contamination, such as breast milk expression, freezing HM at home, shipping, processing in human milk banks (HMBs), and handling in the NICU. Among these, the likelihood of contamination during expression and storage at home are considered to be affected by the donor's living environment conditions and hygiene.

Based on the hypothesis that the type of bacteria in HM is related to the donor's living environment, this study focused on the differences in bacteria in HM between term donors whose infants were at home and preterm donors whose infants were hospitalized in an NICU. The research aim was to compare the bacterial profile of HM from term and preterm mothers.

**Methods**

**Design**

This was a retrospective analysis of data from Japan Human Milk Bank Association (JHMBA) from January to November 2021. The study compared bacterial culture results for HM between term maternal donors (37 to 41 weeks) and preterm maternal donors (<37 weeks). This study was approved by Showa University Research Ethics Review Board (Permit Number: 2714).

**Setting and Participants**

Forty-seven donors who registered at JHMBA from January to November 2021 were included in the study. The donors' health conditions were confirmed based on a health checklist. The donors were screened
based on their detailed medical history, physical examinations, and laboratory data according to the Guidelines for the Establishment and Operation of a Donor Human Milk Bank 2018. Donors who did not fulfill the eligibility criteria were excluded from donating milk.

In Japan, term donors register themselves via the HMB websites, while preterm donors are generally registered through a referral from the medical staff in NICU.

The expression method was either by hand or with an electric or manual pump. From the time of milk expression at the donor’s home, the milk was kept frozen until sent to JHMBA. We pasteurized the donated milk using the Holder pasteurization (HP) method (62.5°C for 30 min), and pre- and post-pasteurization samples were tested for bacterial culture.

The acceptance criteria for donated milk were total bacterial count of \( \leq 10 \) CFU/ml, Enterobacteriaceae count of \( \leq 10^4 \) CFU/ml, \textit{Staphylococcus aureus} count of \( \leq 10^4 \) CFU/ml, and the absence of spore-forming bacteria. The donated HM was accepted as pasteurized donor HM if no bacteria were detected after HP. In addition to the strict safety standards, hygienic instructions for adequate expression, freezing, and shipping were provided to all donors by JHMBA staff at registration.

**Measurement**

For the culture test, we send the samples to the clinical laboratory test company (BML Co.Ltd, Tokyo, Japan). 100 µL sample from each batch (before pasteurization) were cultured on blood agar to allow the growth of aerobic organisms. Bacterial counts are expressed as colony-forming units (CFU) in 100 µL.

**Statistical analyses**

The normality and variability of all parameters were evaluated with the Kolmogorov–Smirnov test and F-test, respectively. The total bacterial count and the bacterial species count per batch are presented as the mean (SD) and compared between term and preterm HM using Welch’s t-test and Student’s t-test, respectively. As for bacterial contamination rates, the number of batches in which a particular bacterial species was detected among all batches of HM is presented as a percentage, and two groups were compared using the Chi-square test or Fisher’s exact test. The rate of milk sample rejection was also analyzed using the Chi-square test.

Donor age, gestational age, birth weight, start date of HM donation (calculated using the expression and delivery dates of the donated milk), and the number of times milk samples were donated per mother are presented as the mean (SD) and median (range) values and compared between two groups using the Mann–Whitney test. All statistical analyses were performed using StatMate V (ATMS Co., Ltd., Tokyo, Japan).

**Results**

**Characteristics of study participants**
The characteristics of all donors are shown in Table 1. Among the 47 donors that registered, 31 were term donors, and the other 16 were preterm donors. The total amount of donated milk batches was 214, including 75 batches from term donors and 139 batches from preterm donors. In some cases, a single donor provided milk multiple times over the study period. There was no significant difference in the mean maternal ages between the groups (term: 32.3, preterm: 32.6; p = 0.75), but there were significant differences in the gestational ages and birth weights of the infants (38.8 weeks vs. 27.6 weeks; 3,080 g vs. 1,040 g; p < 0.001). In addition, mothers who gave birth to preterm infants tended to begin donating breast milk earlier (18.9 weeks vs. 9.8 weeks; p<0.001).

**Total bacterial count and bacterial species count**

The mean total bacterial count was 351,141 CFU/100 μL in the term group and 872,272 CFU/100 μL in the preterm group (Table 2). There was a significant difference in the mean total bacterial count between the two groups (p = 0.026). A total of 29 bacterial species were detected in the batches from both groups. The mean bacterial species count was more diverse in the preterm group than the term group (p < 0.001).

**Bacterial contamination rate**

The bacterial contamination rate of HM is shown in Table 3. *Staphylococcus epidermidis* was the most prevalent bacteria (term: 65.6% of batches, preterm: 85.3% of batches), followed by *Staphylococcus lugdunensis* (14.4%, 32.9%), *Staphylococcus aureus* (20.0%, 21.7%), and *Pseudomonas fluorescens* (12.2%, 27.3%), which were detected at high frequencies in both groups (Figure 1).

Four bacterial species were more prevalent in term HM, *Pseudomonas putida* (p = 0.008), *Serratia liquefaciens* (p < 0.001), *Pantoea agglomerans* (p = 0.014), and *Bacillus cereus* (p = 0.042); while five species were more prevalent in preterm HM, *Pseudomonas fluorescens* (p = 0.027), *Enterococcus faecalis* and *Enterobacter aerogenes* (p < 0.001), *Staphylococcus lugdunensis* (p = 0.01), and *Stenotrophomonas maltophilia* (p = 0.003) (Table 3).

**Discussion**

After bacterial culture studies were conducted on term and preterm HM, the results revealed three major findings. First, high-ranked bacteria were common to both groups. *Staphylococcus epidermidis*, a commensal skin bacterium, was the most frequent bacterial pathogen in both groups, being detected in approximately 80% of the batches. CoNS, *Staphylococcus aureus*, and *Pseudomonas fluorescens* were contaminants found at high rates. These results are in line with previous studies [6–9]. These bacteria were reaffirmed to be common commensal bacteria with a high risk of causing contamination, regardless of the donor's living environment.

Second, the total bacterial count was significantly higher in preterm than term HM, and the bacterial species count was greater in preterm HM.
Third, there were differences in bacterial profiles between term and preterm HM. The characteristics of four bacteria species in the term HM are as follows. *Serratia liquefaciens* and *Pseudomonas putida* are often isolated from water and soil environments [12, 13]; *Pseudomonas putida*, especially, is rarely isolated from clinical specimens [13]. *Pantoea agglomerans* is a Gram-negative bacterium commonly present in fecal material and soil, but it is an uncommon cause of infection in children[14]. The spore-forming bacterium *Bacillus cereus* was detected in three batches. Preterm HM had more *Enterococcus faecalis* and *Enterobacter aerogenes* contamination. These bacteria are classified as enterococci that reside in the human gastrointestinal tract, and they are frequently reported to cause nosocomial infections [15, 16]. There have been cases of outbreaks in the NICU due to contamination of tap water [17]. *Pseudomonas fluorescens* is widely found in water supplies and can also be isolated from medical devices [18]. *Staphylococcus lugdunensis*, the CoNS species, is sometimes clinically treated the same as *Staphylococcus aureus* [19]. It is a commensal skin bacterium [20] and is reportedly a common cause of community-acquired and nosocomial infections [19, 21]. *Stenotrophomonas maltophilia* is commonly isolated from water, soil, and fecal material and detected in hospitals, especially in the water supply [17, 22].

According to a report by Urrea et al. [23, 24], *Enterococcus* species, *Staphylococcus aureus*, and CoNS such as *Staphylococcus epidermidis* are the leading Gram-positive bacteria, while *Escherichia coli*, *Enterobacter* species, *Pseudomonas* species, and *Klebsiella* species have been reported to be the organisms most frequently responsible for nosocomial infections in NICU. Of the five species present at significantly different rates in the preterm HM, four species were relevant. The result suggests that preterm HM tends to be contaminated with bacteria that can cause nosocomial infections in NICUs.

We made three main conclusions based on these results. The first is that preterm donors visit the NICU to meet their infants, and bacteria prevalent in the NICU environment may adhere to their clothing, resulting in them bringing the bacteria home. Therefore, there is a contamination risk by such bacteria, not only when HM is expressed in the NICU but also when expressed at home. Although data related to the expression environment (location, methods) were not collected in this study, it is likely most donors expressed at home because the study period overlapped with the coronavirus outbreak, and many NICUs had restricted visiting times.

Second, several preterm samples included HM that had been expressed before donor registration. First of all, as a basic premise, HMBs always provide hygiene instructions, such as pre-breast expression wiping and disinfection of the breast pump, at donor registration, regardless of whether the donor gave birth prematurely or not. Term mothers may voluntarily register as a donor if they delivered a term baby, are currently breastfeeding, and have excessive breast milk supply. After registration, they donate milk to the HMB. However, preterm mothers may provide HMB with milk, which is kept in stock for infants admitted to the NICU. Therefore, there is a higher risk that preterm HM is contaminated with bacteria.

Our third point is that the unique circumstances of preterm donors should also be considered. Several factors contribute to a stressful expression environment for preterm donors, including physical separation
from their infants, the provision of a structured feeding schedule, the lack of privacy (when expressing in a hospital), the exhaustion and anxiety associated with an infant’s hospitalization, and long expression periods. These factors can also affect HM production [25–28]. Considering the situation, it is understandably more difficult to pay attention to hygiene precautions compared with when expressing at home, and HM may be contaminated by more bacteria.

These results tell us that hygiene education is more important for preterm donors. However, their physical and psychological circumstances need to be taken into consideration. HMBs need to provide less burdensome and more hygienic expression instructions for these mothers. It may also be necessary to communicate more frequently with preterm donors and to follow up the instructions by observing how they are expressing and storing milk at home. In addition, it may be necessary to survey the NICU situation at each institution and discuss hygiene instructions for preterm donors with the NICU staff.

Although the results of this study did not show a significant difference in the pass/fail score according to the bacterial culture test criteria established by the HMB (Table 1), in the future, better hygiene instructions will not only reduce the breast milk transmission risk but also contribute to reducing the wastage of valuable donated HM, a pertinent issue for HMBs.

**Limitations**

We did not investigate the location or method of expression, so the influence of these factors on the culture results is unknown. Additional environmental factors, such as living arrangements and sibling status, may also affect the bacterial profile of HM and will be investigated in the future.

**Conclusions**

This study revealed that preterm HM has a higher total bacterial count and greater diversity of bacterial species. In addition, it tends to be more highly contaminated by nosocomial-infection-causing bacteria in the NICU. Taken together, these findings suggest the need for more focused hygiene education for preterm donors.

**Abbreviations**

HM  
human milk  
NICU  
Neonatal Intensive Care Unit  
CoNS  
coagulase-negative *Staphylococci*  
HMB  
human milk bank
Declarations

Ethics approval and consent to participate

Ethical Approval was obtained from Showa University Research Ethics Review Board (Permit Number: 2714). Additionally, written informed consent was obtained from all donors for using their milk for clinical and research purposes.

Consent for publication

Not applicable.

Availability of data and materials

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

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Competing interests

The authors declare that they have no competing interests.

Author contribution

All authors have read and approved the final manuscript to be published and agree to be accountable for all aspects of the work. K.M., K.M conceived and designed the study; K.M, M.T., M.D., M.I, and N.M contributed to the recruitment and data collection; K.M was responsible for data analysis and writing the manuscript; K.M, M.T. and K.M contributed to reviewing the manuscript critically.

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References


Tables

Tables 1 to 3 are available in the Supplementary Files section.

Figures
Figure 1

**Bacterial species with the highest contamination rate**

The graphs show the bacteria with the highest contamination rates in each of the term and preterm HM.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.xlsx
- Table2TotalbacterialcountandBacterialspeciescountperbatch.docx
- Table3.xlsx