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Bacterial growth in breast milk expressed under hygienic control: a pilot study

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Abstract

Objective In this pilot study, we verified safe practices for breast milk expression, storage, and duration, based on bacteriological results.

Results We collected breast milk samples from three healthy lactating volunteers and analyzed the bacterial flora and changes in the viable bacterial counts (including those of *Staphylococcus*) of the samples. Although no consistent change could be observed in the abundance of a particular bacterial group in samples expressed under hygienic control conditions, viable bacterial counts were higher in self-expressed milk than in milk expressed under hygienic control conditions. In conclusion, increased hygiene awareness is vital during breast milk expression and storage.

Keywords Breast milk, Nipple skin wiping, Bacteria, Storage, Hygiene

Introduction

In Japan, hand expression of breast milk involves prior hand washing with soap and wiping the nipple and areola with clean cotton. However, preparation techniques are midwife-dependent. Certain midwives advise that wiping the nipple and areola before breastfeeding is unnecessary as the mucus secreted by the sebaceous glands of the areola protects the nipple, although this practice has not been validated. Furthermore, there are different recommendations concerning expressed breast milk storage duration. The Academy of Breastfeeding Medicine recommends the following optimum milk storage conditions: 4 h at room temperature (16–29 ℃) and 4 days in the refrigerator (~4 ℃) [1].

Unfortunately, studies verifying the bacterial contamination of breast milk expressed with and without any hygienic control (wiping the nipple and areola before milk expression) are scarce. Therefore, bacterial contamination-related bacteriological safety assessment of expressed breast milk is of utmost importance.

We examined the bacteriological safety of breast milk expressed using different methods, assessing bacterial growth under different storage conditions, including duration and location.

Methods

Study design

We performed a pilot study on bacterial growth in breast milk. The Research Ethics Committee of the Shiga University of Medical Science approved this study (No. R2021-096). The research was conducted between September 24 and December 31, 2021.

Participants

Three lactating women with no abnormalities during their pregnancy, delivery, and postpartum period were included. The selection criteria were as follows: age≥20

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years, ability to express 10 mL of breast milk from each breast in one batch, and provision of written consent. The exclusion criteria comprised formula feeding owing to the lack of sufficient daily breast milk supply and puerperal breast and nipple problems.

To recruit participants, we distributed leaflets at the nursery school of our university. We explained the study verbally to the participants, and then arranged the date and site (a seminar room at our university or the participant's home) for milk collection.

We collected the following information through a verbal interview: number of days postpartum; feeding interval on the day of the survey; formula use; last time of milk discharge; presence of breast milk leakage; breast pad use; last time of breast pad change; breast or nipple problems; and last intake of oral medication since the day of the survey. The use of medications (especially antimicrobials) was investigated since they might affect bacteriological results.

Breast milk collection

We collected approximately 10 mL of breast milk from both breasts of the participants and classified the samples into two groups based on whether they were obtained under hygienic conditions by the researcher or through self-expression performed by the participant. Only one expressing session was performed per participant.

The expression process under hygienic conditions by the researcher was as follows: the researcher disinfected their hands with alcohol gel sanitizer and put on sterile gloves.

Four pieces of absorbent cotton (soaked with pure water and sterilized in an autoclave) were used to wipe off the nipple and areola in a circular motion from the center to the outside of the nipple. Subsequently, the first few drops of milk were discharged onto a tissue, the sterile gloves were changed, and a sterile tube was used to collect the milk sample.

The milk extraction process by the participants was as follows: the participants washed their hands and fingers with soap and running water. The first few drops of milk were discharged onto a tissue, and a sterile tube was used to collect the milk sample.

We immediately transferred the collected milk samples into a cooler with ice and a temperature maintained near 0 °C, took them to the laboratory, and labeled them as samples I–III according to participants 1–3, respectively.

16 S rRNA bacterial flora analysis

Immediately after collection, we submitted the six samples to 16 S rRNA bacterial flora analysis by Repertoire Genesis Inc [2].

Viable bacterial count in breast milk samples

Viable bacterial counts were measured in milk samples collected immediately, in those stored at room temperature (27.4±0.12 °C) for 1, 4, and 8 h, and in those refrigerated (4 °C) for 24 h, 4 days, and 8 days after collection. Isolates were obtained based on the Food Hygiene Inspection guidelines [3] for viable bacterial counts.

Bacterial identification

We performed bacterial identification according to the guidelines of the Japanese Society of Public Health for bacteria and fungi identification [4], including Gram staining, catalase testing, culturing in Gifu anaerobic semisolid medium (Nissui Pharmaceutical Co., Ltd.), tube coagulase testing with Rabbit Plasma (Eiken Chemical Co., Ltd.), and culturing on Sheep Blood Agar (Eiken Chemical Co., Ltd.).

Antibacterial breast milk whey test against Staphylococcus aureus (broth microdilution method)

We tested breast milk whey susceptibility to *S. aureus* (ATCC25923) using microdilution in 96-well plates as previously described [4].

Results

The number of postpartum days of the three participants was 292, 278, and 656. The last breastfeeding time ranged between 1 and 5 h before sample collection.

We detected *Haemophilus*, *Neisseria*, *Leptotrichia*, *Veillonella*, *Streptococcus*, *Lactobacillus*, *Granulicatella*, *Staphylococcus*, *Prevotella*, and *Actinomyces* as predominant bacterial genera in all samples (Additional File 1). The bacterial flora of the participants differed. However, no expression technique was correlated with a consistent decline in any bacterial groups.

We measured the viable bacterial count used as a safety standard (5.0×10^4 colony forming units [CFU]/mL) for general foods in Japan, and registered higher values in the self-expressed milk than in the control (Fig. 1). Self-expressed sample III yielded the highest viable bacterial count of $2.5\pm0.31\times10^4$ CFU/mL, remaining below the 5.0×10^4 CFU/mL standard.

All the bacteria were gram-positive cocci, with approximately 50% *Staphylococcus* and 50% *Streptococcus.* The number of staphylococci was higher in the self-expressed samples than in the control (Fig. 1), with most being coagulase-negative staphylococci (CNS). We only detected a few *S. aureus* in samples I and II. All streptococci were alpha-hemolytic or non-hemolytic.

In all samples, the viable bacterial count increased over time after storage at room temperature. The standard was exceeded after room temperature storage for 4 h in self-expressed samples I and III, and after storage for 8 h in self-expressed sample II, but not in the control, even

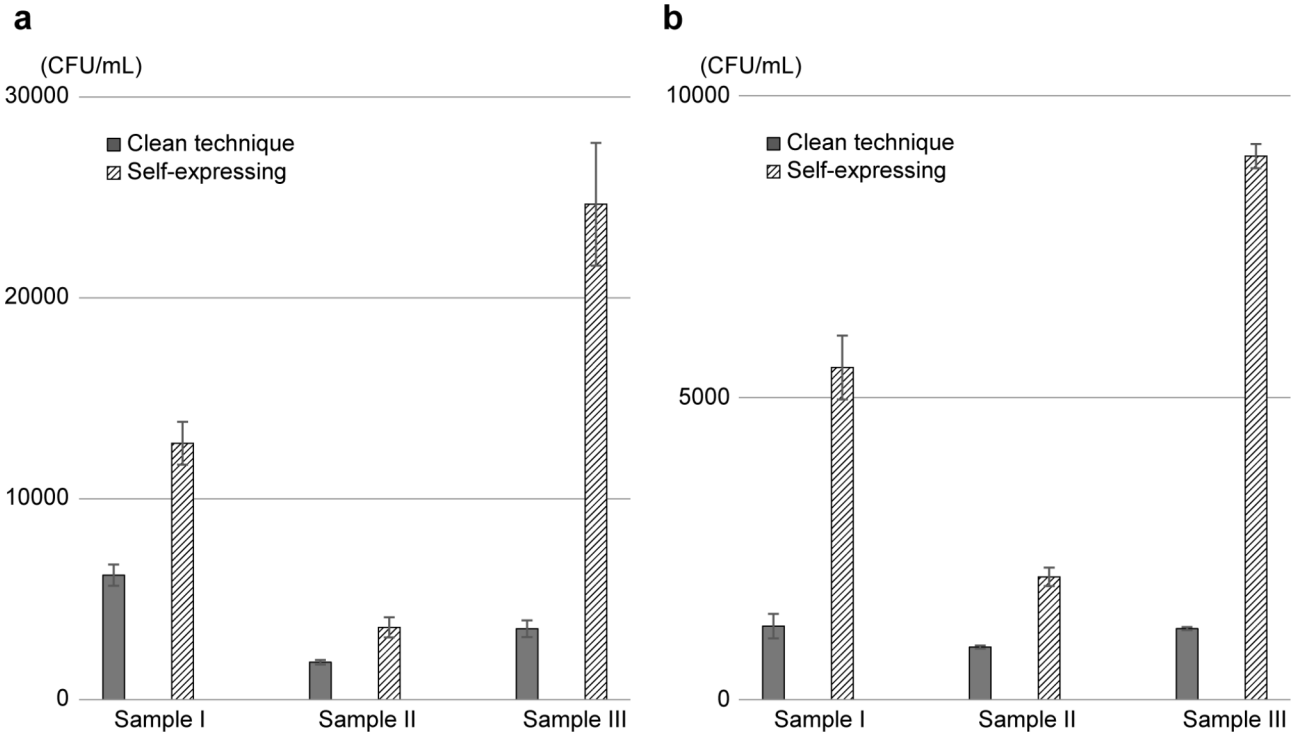

Fig. 1 Comparison of the viable bacterial count (CFU/mL) (**a**) and staphylococci number (CFU/mL) (**b**) in milk using different expressing techniques immediately after collection when expressed by the researcher using a hygienic technique or by self-expression by the participant. Each data point is presented as the mean and standard deviation of experiments performed in triplicate

upon storage at room temperature for 8 h (Fig. 2). During room temperature storage, the number of staphylococci in the milk increased with time for CNS but not *S. aureus* (Additional File 2).

The viable bacterial count of all samples decreased over time after storage in the refrigerator (Fig. 3). The number of staphylococci decreased over time (Additional File 3).

Staphylococcus aureus growth was not significantly inhibited by whey but increased whey concentration-dependently (Additional File 4), suggesting that whey does not exert a direct antimicrobial effect on *S. aureus* and that whey components serve as bacterial nutrients.

Discussion

In this study, we performed a 16 S rRNA bacterial flora analysis and detected relatively large anaerobic bacterial numbers, likely of infant oral origin, as well as the genera *Streptococcus* and *Staphylococcus*, which are reportedly predominant in milk [5–8]. Kim et al. [8] demonstrated that sterile water (rather than antiseptics) was previously used for pre-expression nipple care, such as in this study.

The percentage of bacterial groups of presumably infant oral origin was higher in this study than in a previous one [9], which was potentially related to milk expression during weaning.

We primarily identified non-pathogenic and resident streptococci. We mostly observed bacteria with low pathogenicity and rarely detected *S. aureus*, the most virulent *Staphylococcus*, and pathogenic *Escherichia coli*. This study provides important evidence of the low pathogenicity of bacterial populations in breast milk fed to neonates and infants with immature immune systems.

Since no obvious bacterial groups were present that could cause food poisoning, we deemed the milk bacteriologically safe based on the viable bacterial count (5.0×10^4 CFU/mL) [10]. This count was in the range of 10^3–10^4 CFU/mL immediately after collection, similar to previously reported counts [11], and did not exceed the standard. This result suggests that safety criteria can be met using common expressing methods if the milk is immediately ingested after collection.

Nipple wiping practices before breastfeeding vary. In a previous study in Japan, researchers only examined the effect of removing bacteria by nipple cleaning [12]. A novelty of our study is that it allowed examination of differences in the number of bacteria in breast milk expressed under hygienic control conditions versus self-expression.

The viable bacterial count in self-expressed samples I and III exceeded the standard when stored for 4 h at room temperature. Although the guideline recommends a room temperature storage time of 4 h or less, we also evaluated milk storage at room temperature for 8 h owing to the possibility of longer storage times due to extended

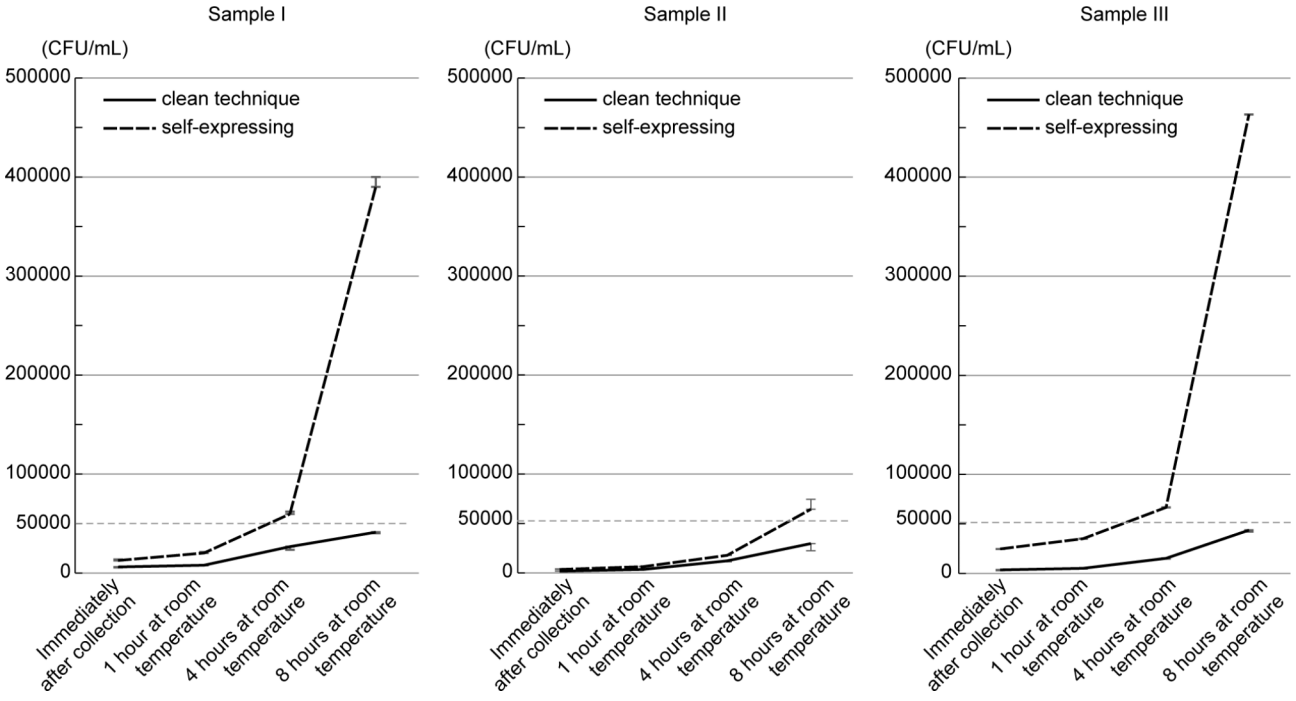

Fig. 2 Temporal changes in the viable bacterial count of milk after storage at room temperature. Changes in viable bacterial counts (CFU/mL) in milk expressed by the researcher using a hygienic technique and milk self-expressed by the participant immediately after collection up to 8 h of storage at room temperature are shown for participants I, II, and III. Dotted lines (5.0×10^4 CFU/ml) indicate the safety standard. Each data point is presented as the mean and standard deviation of experiments performed in triplicate

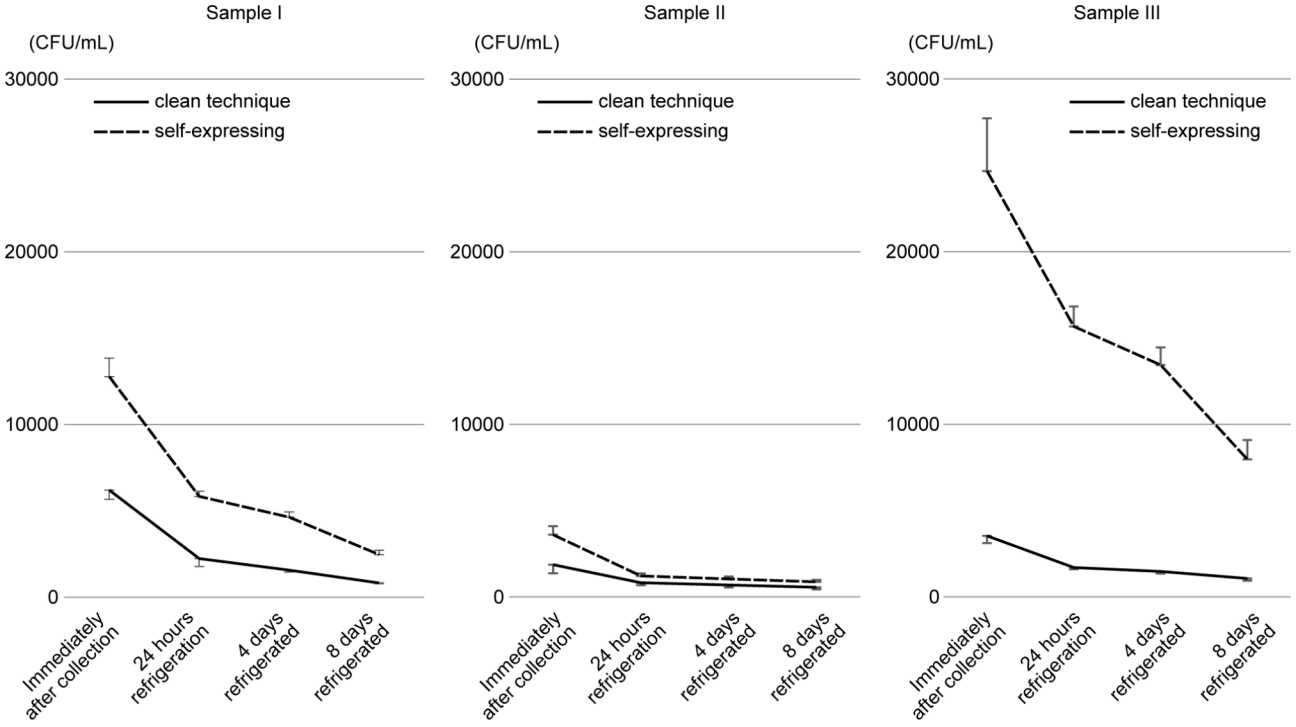

Fig. 3 Temporal changes in the viable bacterial count of milk after storage in the refrigerator. Changes in the viable bacterial count (CFU/mL) in milk expressed by the researcher using a hygienic technique and in milk self-expressed by the participant immediately after collection up to 8 days of storage in the refrigerator are shown for participants I, II, and III. Each data point is presented as the mean and standard deviation of experiments performed in triplicate

lactation intervals with infant growth and longer expression times related to low maternal milk production. The bacterial counts of all three self-expressed samples exceeded the standard when stored for 8 h at room temperature. In contrast, the viable bacterial counts of all control samples increased over time during room temperature storage but did not exceed the standard. Therefore, the viable bacterial count is relatively suppressed, even during room temperature storage, if few contaminating bacteria are present, but it exceeds the standard during room temperature storage, even for a short time, when the sample contains multiple contaminating bacteria. During storage in the refrigerator, the viable bacterial count decreases over time [11]. If the viable bacterial count in milk does not exceed the standard immediately after collection, the sample is considered bacteriologically safe.

In this study, CNS grew during room temperature storage, but *S. aureus* did not. In contrast, another study reported that *S. aureus* growth in milk was attributed to storage at room temperature [13]. Antimicrobial testing of whey in this study did not validate its antimicrobial effect on *S. aureus*. Therefore, the lack of *S. aureus* growth might not have been owing to the immune component in milk but due to the fact that the number of *S. aureus* species in the milk was very low, such that other bacterial groups might have suppressed its growth. *Staphylococcus aureus* is present on the nipple skin of adult women [14], contaminates breast milk during expression [6], and might grow when stored at room temperature. In contrast, the abundance of staphylococci decreased over time during storage in the refrigerator, consistent with previous reports [13].

Since nipple wiping reportedly reduces the number of contaminating bacteria in milk, nipple wiping and hand washing before expression should be promoted as a standard practice.

Based on our findings, the recommended storage duration of 4 h at room temperature may not be safe. Guidance is required based on bacteriological evidence that milk should be refrigerated or frozen as soon as possible after expression if it is not immediately fed to the infant. Additionally, a cold storage environment should be maintained when transporting the milk.

Limitations

First, freezer storage of milk was not assessed owing to the short study period. Second, the sample size was small, potentially influencing the findings related to *S. aureus* or CNS and their subsequent growth. Third, the collection period and the timing after the last breastfeeding was not the same for all participants. Fourth, factors that could affect the generalizability of the findings were not assessed, including the infection history of the mothers.

In future studies, we would like to examine differences in the distribution of the bacterial flora among different participants as well as longitudinal data from the same participants at different collection periods.

Conclusions

Hand washing before expression as well as nipple cleanliness and wiping should be promoted. Expressed breast milk should be refrigerated as soon as possible and kept in a cold environment during transportation instead of storage at room temperature.

Abbreviations

CFU Colony forming unit
CNS Coagulase-negative staphylococci

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13104-024-06933-2.

Supplementary Material 1 : **Table S1**. Top bacterial groups determined using 16S rRNA bacterial flora analysis present in all samples expressed by the researcher using the hygienic technique and self-expression by the participants.

Supplementary Material 2 : **Figure S1**. Temporal changes in the number of staphylococci in milk after storage at room temperature. Changes in the number of staphylococci (CFU/mL) in milk are shown for participants I, II, and III. This includes milk expressed by the researcher using a hygienic technique and self-expressed milk by the participant. The changes are from immediately after collection up to 8 h of storage at room temperature. Each data point is presented as the mean and standard deviation of experiments performed in triplicate.

Supplementary Material 3 : **Figure S2**. Temporal changes in staphylococci numbers in milk after storage in the refrigerator. Changes in the number of staphylococci (CFU/mL) in milk expressed by the researcher using a hygienic technique and in milk self-expressed by the participant immediately after collection up to 8 days of storage in the refrigerator are shown for participants I, II, and III. Each data point is presented as the mean and standard deviation of experiments performed in triplicate.

Supplementary Material 4 : **Figure S3**. Antibacterial test for whey against Staphylococcus aureus in breast milk. The value of each sample is shown with the absorbance of only phosphate-buffered saline and bacterial suspension set as 100%. Each data point is shown as the mean and standard deviation of the value obtained for the sample expressed by the researcher using the hygienic technique and the sample self-expressed by the participant for participants I, II, and III.

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Author contributions

Mika Miyatake: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing-original draft; Yukihiro Tambe: Formal analysis, Investigation, Methodology, Resources, Writing-original draft; Yumiko Tateoka: Conceptualization, Methodology, Project administration, Supervision, Writing-original draft. All authors read and approved the final manuscript.

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

Declarations

Ethics approval and consent to participate
All methods were carried out in accordance with the Declaration of Helsinki and relevant guidelines and regulations. The Research Ethics Committee of the Shiga University of Medical Science approved this study (No. R2021-096). Written informed consent was obtained from participants.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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