

Research Paper



Microbiota composition of human milk based on storage and processing methods in Korea

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ABSTRACT

BACKGROUND/OBJECTIVES: The microbiota present in human milk (HM) plays a crucial role in infant health and development. Maintaining it during storage and thawing is vital for preserving its beneficial properties; however, the effects of storage period, temperature, and thawing conditions on HM are not well understood.

SUBJECTS/METHODS: HM samples from lactating mothers were collected and stored under different temperature conditions (room temperature [RT], refrigeration, and -20°C) for different durations (1 week and 4 weeks), and thawed using different methods (RT, bottle warmer, and microwave), followed by analysis of their microbiota.

RESULTS: Storing HM samples at RT for a week and at -20°C for four weeks resulted in a significant reduction in microbiota diversity, whereas the other treatments did not lead to a significant change in beta diversity. In the microbiota distance analysis, HM samples stored at -20°C for a week showed the highest similarity to the initial microbiota compared to those after one week of storage at RT or in a refrigerator. Microwave thawing after 1 week of freezing resulted in the greatest alteration in the initial microbiota composition, whereas thawing at RT or with a bottle warmer showed comparable microbial profiles.

CONCLUSION: The most suitable method for storing and thawing HM while preserving the microbiota is to store it at -20°C for up to one week and thaw it using a bottle warmer.

Keywords: Human milk; breastfeeding; microbiota; pasteurization; Korea

INTRODUCTION

Human milk (HM) is well known to be essential for the growth and development of neonates and infants [1,2]. The macronutrients such as carbohydrates, proteins, and fats contained in HM not only serve as energy sources but also play a role as prebiotic effects such as intestinal colonization and functionalization and organization of cells [3,4]. In addition, HM contains growth factors and immunologic factors as well as various vitamins and micronutrients, and many studies have been conducted on these components [3-5].

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Conflict of Interest

The authors declare no potential conflicts of interests.

Author Contributions

Conceptualization: Yi DY; Data curation: Oh C; Writing - original draft: Oh C; Writing - review & editing: Kang I, Kang B, Yi DY.

Meanwhile, with the development of new testing techniques such as next-generation sequencing (NGS), it was found that HM also contains various microbiota [6,7]. The microbiome of HM plays an important role in colonization in infant intestine and is known to affect infant's immune regulation and endocrine metabolism [4,8]. The factors affecting the microbiota of HM have not been clearly identified, however, it remains difficult to rule out the possibility that its origin may be due to contamination from the oral cavity or skin [9-11]. In addition, it was confirmed that it may change due to factors such as maternal diet or taking probiotics, or after pasteurization [12,13].

For various reasons, including the increase in working women, mothers cannot directly breastfeed their children, and they often freeze HM and then thaw when necessary to lactate them. However, in such a general home environment, it is practically impossible to store, thaw, and lactate under laboratory pasteurization conditions, but HM studies related to this realistic situation are insufficient [14]. Therefore, through this study, we tried to determine how the microbiota of HM changes according to the situation of storing or thawing HM.

SUBJECTS AND METHODS

Participants and sample collection

The participants were nursing mothers at 2 weeks to 5 mon after delivery, and colostrum was excluded from the analysis. Participants with pregnancy-related diseases, such as eclampsia, gestational diabetes, or other chronic diseases, were excluded. A questionnaire was provided to collect information, such as the lactating mother's age, birth history, medication history, meal diary for the last 3 days, infant's age, and postnatal weight change of the infant. Through the questionnaire, cases with a history of antibiotics, probiotics, or herbal medicine within the last one month were excluded.

Fifty milliliters of HM samples were collected in sterile bags by manual or pump expression from 15 lactating mothers, who agreed to participate in the study. Before expressing milk, the hands and nipples were cleaned. The first ten drops of HM were discarded. Fresh HM was delivered either immediately after collection or within 24 h under refrigeration. The delivered HM (40–50 mL) was immediately aliquoted for further analysis. Fifteen lactating mothers were enrolled in the study. Five mothers had vaginal deliveries, and 10 had caesarean deliveries. The mean maternal age was 34.4 yrs, and while 9 mothers were primiparous, 6 were multiparous. The average postpartum period was 84.6 days at the time of sample collection. The average gestational age of the infants was 35 weeks.

Analysis of HM samples according to storage and treatment methods

Ten milliliters of the collected milk were used for calorie analysis, and the results were provided to the participants. The remaining samples were classified into 8 situations (**Fig. 1**); #1, initial sample: analysis of the HM microbiome immediately after collection; #2, microwave: analysis of the HM microbiome after subjecting it to microwave heating (700 W) for 1 min immediately after collection; #3, room temperature (RT, approximately 20°C) for 1 week: analysis of the HM microbiome after storage at RT for 1 week; #4, 4°C for 1 week: analysis of the HM microbiome after storage in a refrigerator (4°C) for 1 week; #5, -20°C for 1 week & RT: analysis of the HM microbiome after 1-week of storage at -20°C followed by thawing at RT (approximately 20°C) for 10 min; #6, -20°C for 1 week & bottle warmer: analysis of the HM microbiome after 1 week of storage at -20°C followed by

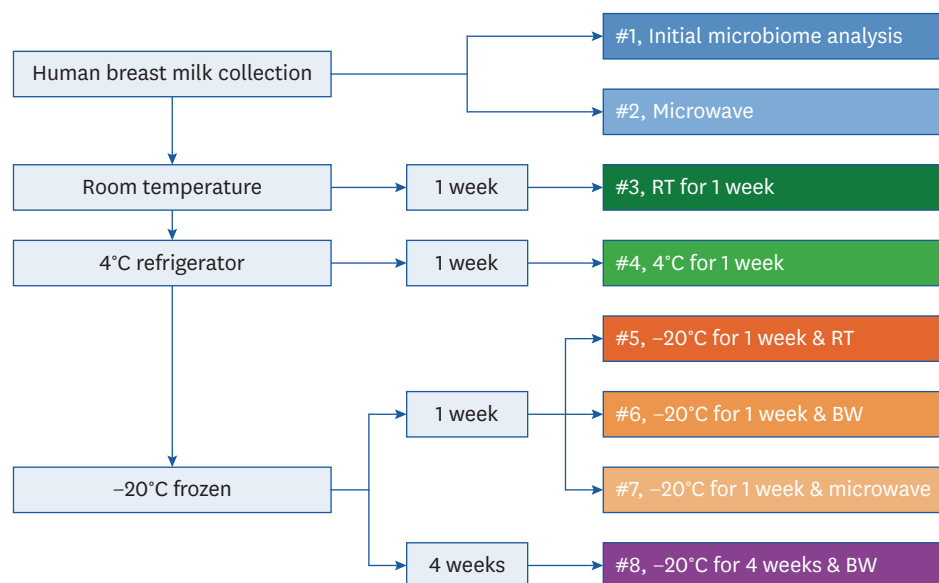


Fig. 1. Diagram of human milk storage and thawing process.
RT, room temperature; BW, bottle warmer.

warming with a bottle warmer (20°C) for 3 min; #7, -20°C for 1 week & microwave: analysis of the HM microbiome after 1 week of storage at -20°C followed by heating with microwave for 1 min; #8, -20°C for 4 weeks & bottle warmer: analysis of HM microbiome after 4-week of storage at -20°C followed by warming with a bottle warmer (20°C) for 3 min. Changes in the microbiome were compared by categorizing the samples based on different condition: microwave heating (#1 vs. #2), storage temperature (#1 vs. #3, #4, and #6), thawing method (#1 vs. #5, #6, and #7), and storage period (#1 vs. #6 and #8). A total of 120 samples were collected from 15 lactating mothers.

DNA extraction and NGS for microbiome analysis

Microbial DNA was isolated using the PowerMax Soil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) following the manufacturer's protocol. For library preparation, the 16S rRNA gene amplicons were generated in accordance with the Illumina 16S Metagenomic Sequencing Library Preparation Guide (Illumina, San Diego, CA, USA). Specifically, the V3–V4 hypervariable regions were targeted using primer sets 519F and 816R. The concentration and purity of extracted DNA were assessed using PicoGreen fluorescence dye and a NanoDrop spectrophotometer. A total of 10 ng of genomic DNA was amplified by polymerase chain reaction (PCR) using fusion primers with barcodes, including 341F (5'-CCTACGGGNGGCWGCAG-3') and 806R (5'-GACTACHVGGGTATCTAATCC-3'). The amplified products were quantified using quantitative PCR following the KAPA Library Quantification protocol (KAPA Biosystems, Wilmington, MA, USA) for Illumina platforms and analyzed with the LabChip GX HT DNA High Sensitivity Kit (PerkinElmer, Waltham, MA, USA). Sequencing was conducted by Macrogen Inc. (Seoul, Korea) using the Illumina MiSeq™ system, generating paired-end reads of 2 × 300 bp.

Of the 15 participants, samples from one were excluded owing to failed bacterial DNA extraction. Finally, HM samples from 14 participants were analyzed. Twelve samples were excluded due to low read counts. After filtering out ambiguous, wrong-prefix, low-quality reads and chimeras, 100 samples remained, with an average sequencing depth

of 42,428.5 reads. The rarefaction curve plateaued with increasing sequencing depth, suggesting that the microbial population was sufficiently represented (**Supplementary Fig. 1**). The values of Q20 values (base quality > 20 and error rate < 0.01) consistently exceeded 97% and Q30 values (base quality > 30; error rate < 0.001) consistently surpassed 92%, suggesting a low probability of sequencing error (**Supplementary Figs. 2 and 3**).

Identification of microbial species

FASTQ files were generated from the raw reads obtained through the Illumina MiSeq platform using real-time analysis software and the bcl2fastq v2.20.0.422 pipeline. To reconstruct full-length reads, paired-end sequences from each sample were merged using FLASH v1.2.11. Quality control was subsequently performed using CD-HIT-OUT, which involved filtering out low-quality reads and eliminating potential chimeric sequences. The cleaned reads were then clustered into operational taxonomic units (OTUs) based on a 97% sequence similarity threshold. For taxonomic identification, representative sequences from each OTU were aligned against the NCBI 16S rRNA gene reference database using BLASTN v2.4.0. Taxonomic assignments were made based on the highest similarity match. Sequences with a best-hit identity or alignment coverage below 85% were considered unclassified due to insufficient similarity to known sequences.

Microbiome data analysis

The Shannon index of a sample was calculated using the phylogenetic distance and the number of observed OTUs and analysis of variance (ANOVA) was performed using R package 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria). Dilution analysis was performed based on a customized Perl script that calculated the mean, minimum, and maximum OTUs for each group (8 groups in total) per 100 reads. Data were analyzed using one-way ANOVA followed by Dunnett's *post-hoc* test to determine statistical differences between each treatment group and the control group.

Alpha diversity and beta diversity

To analyze structural changes in the ecological community of HM under different handling conditions, we first employed various alpha diversity. These included observed OTUs, Chao1 richness, the Shannon Index, and the inverse Simpson's Index. To determine the similarities and differences among microbial communities, we conducted beta diversity analysis on all samples. Phylogenetic methods using Unifrac distance were applied to quantify beta diversity, and Principal Coordinate Analysis (PCoA) was used to visualize changes in the HM microbiota under various handling conditions.

Comparative microbiome analysis

To analyze the microbiome data, QIIME 1.9³⁵ and customized Perl scripts were used to 1) understand the overall characteristics of the microbial community in HM samples, 2) compare HM microbiomes in the four thawing conditions, and 3) compare the microbial community structure in the 4 thawing conditions. For a detailed analysis, customized Perl scripts were developed to combine microbial species into groups and calculate bacterial abundance and proportions at the species, genus, family, and order levels. The results of each script analysis were exported in Excel for additional analyses.

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of the Institutional Review Board (IRB) of Chung-Ang

University Hospital, Seoul, Korea (IRB No.: 1821-004-352). Informed consent was submitted by all subjects when they were enrolled.

RESULTS

HM microbial alpha diversity

The alpha diversity results of HM microbiota are shown in **Fig. 2**. As expected, the highest alpha diversity was observed in the initial sample (#1) and the initial sample after microwave heating (#2), with all other conditions showing reduced diversity. Notably, significantly decreased alpha diversity was observed in sample stored at RT for 1 week (#3), and in those frozen for 4 weeks and thawed using bottle warmer: #8 across all 4 diversity metrics, while no significant differences were observed on other conditions (**Fig. 2**). Among the storage temperature conditions (#3, #4, and #6), sample #3 resulted in a significant decrease in both richness (Chao1 and observed OTUs) and evenness (Shannon and Simpson), indicating that storing HM at RT for 1 week substantially alters its microbiota. Although samples #4 and #6 also showed reduced alpha diversity, these changes were not statistically significant. In comparing different thawing methods after one week of storage at -20°C (#1 vs. #5, #6, and #7), all treatments showed reduced alpha diversity compared to the control, though the reductions were not statistically significant. Finally, we compared the impact of storage

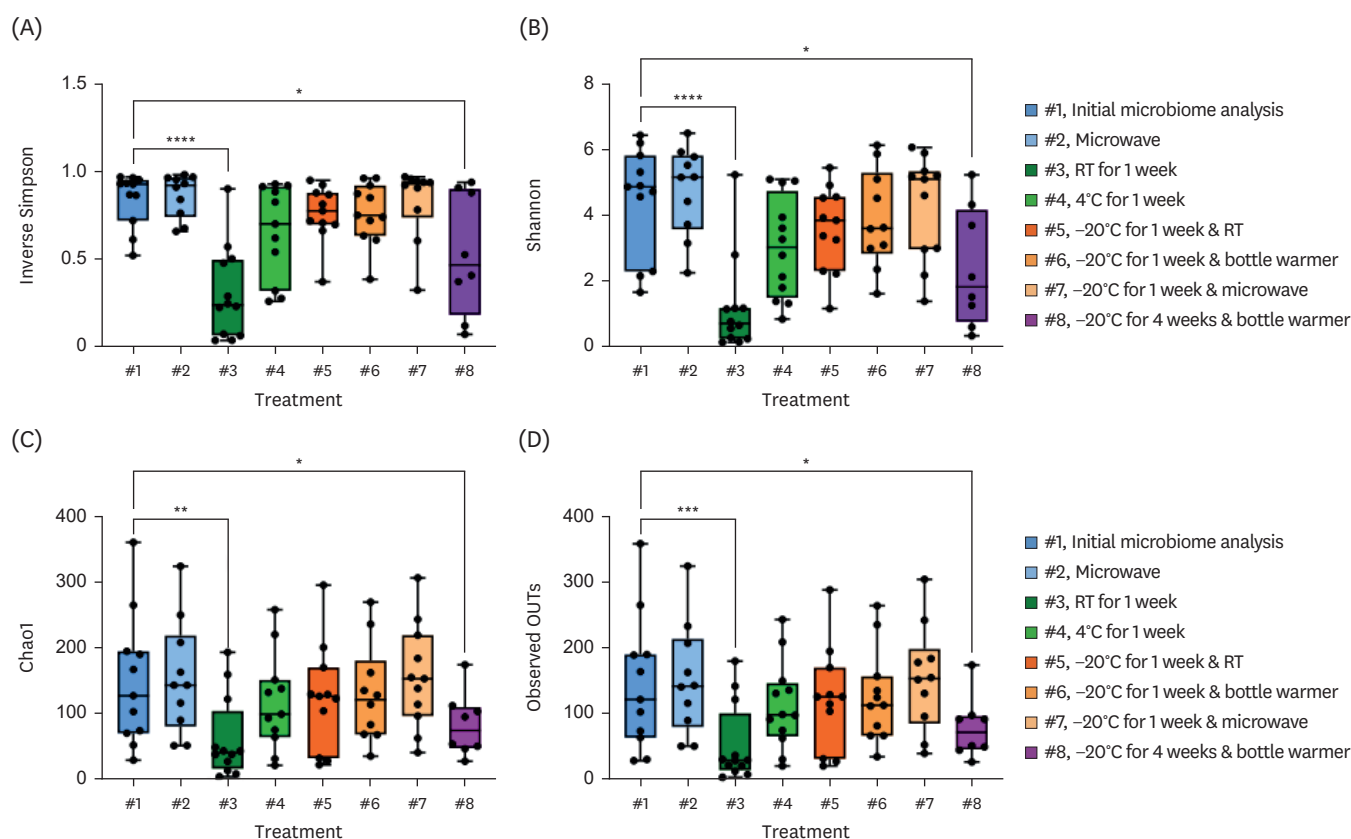


Fig. 2. Comparison of the microbial alpha diversity of human milk under different storage and handling conditions. (A) Inverse Simpson, (B) Shannon, (C) Chao1, and (D) Observed OTUs. Boxes represent the minimum to maximum values, respectively, and the horizontal line inside the box defines the median. Whiskers represent the lowest and highest values, respectively. Statistical significance was determined by one-way ANOVA followed by Dunnett's *post-hoc* test. OTUs, operational taxonomic units; ANOVA, analysis of variance; RT, room temperature. P-values are indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

period at -20°C (1 week vs. 4 weeks). Interestingly, storing HM for 4 weeks (#8) resulted in significant decrease in both richness and evenness compared to the initial sample (#1), while 1 week of freezing (#6) did not significantly affect alpha diversity.

Beta diversity of HM microbiota

The beta diversity results of HM microbiota are shown in **Fig. 3**. This analysis revealed a distinct separation of samples into 3 clusters: #3, #8, and the remaining samples (#1 and others) (**Fig. 3A**), indicating that the microbiota in #3 and #8 differed notably from the others.

To statistically analyze the microbial community differences from the initial sample (#1), we generated a comparison plot of weighted Unifrac distance (**Fig. 3B**). As expected, microwave (#2) is the closest to the initial sample (#1). Among the storage methods comparison, sample #3 (RT for 1 week) was the most distant from #1, while sample #6 (frozen at -20°C for 1 week) was the closest. For thawing method comparisons following 1 week of freezing, sample #5 (thawed at RT) showed the smallest distance from #1, suggesting that the RT thawing preserves the microbiota most similarly to the initial state. In contrast, sample #8 (frozen for 4 weeks and thawed with a bottle warmer) was significantly more distant from sample #1, indicating greater microbiota alteration with longer storage.

HM microbiota composition

As shown in **Figs. 2 and 3**, both alpha and beta diversity in sample #3 and #8 differed significantly from the initial sample (#1). To further investigate these differences, we analyzed the microbiota composition at the phylum and genus levels.

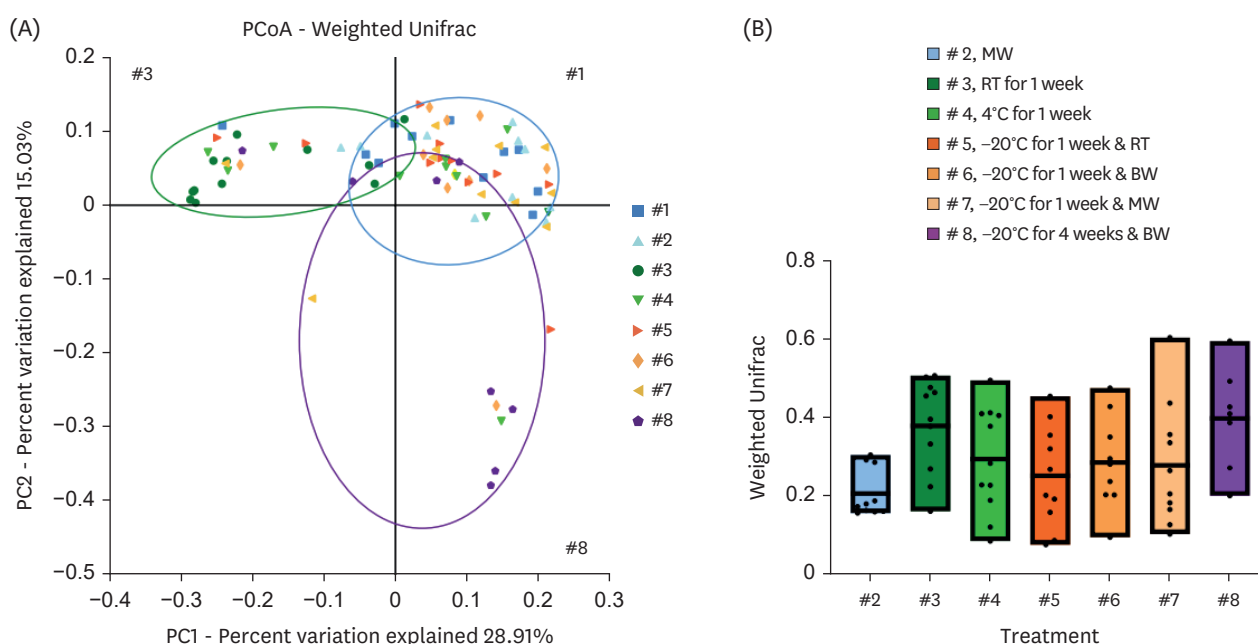


Fig. 3. Comparison of the microbiota in human milk under various storage and handling conditions. (A) PCoA plots showing beta diversity measure using weighted UniFrac distance. The distance from treat #1 in each sample group (derived from the same mother) was plotted in different color; blue (#1), light blue (#2), dark green (#3), green (#4), dark orange (#5), orange (#6), light orange (#7), and purple (#8). The distributed areas of each treatment groups were circled using the same color as the corresponding group. Only significantly different groups' areas are shown in the plot. (B) Average UniFrac distance with respect to #1 was plotted.

PCoA, Principal Coordinate Analysis; RT, room temperature; BW, bottle warmer; MW, microwave.

A total of 22 bacterial phyla were detected across all HM samples. The 4 most dominant phyla in the initial sample (#1) were *Firmicutes* (53%), *Bacteroidetes* (21%), *Actinobacteria* (12%), and *Proteobacteria* (12%), which remained dominant across different treatment groups (**Fig. 4A**). The significant alterations in alpha and beta diversities in sample #3 and #8 were primarily associated with shifts in the relative abundance of *Firmicutes* and *Bacteroidetes*. Specifically, *Firmicutes* significantly increased in samples #3 (86%), #4 (59%) and #6 (71%) compared to #1 (**Fig. 4A, C, and D** top). In contrast, *Bacteroidetes* significantly decreased in samples #3 and #8 (**Fig. 4C and D** bottom).

At genus level, a total of 735 genera were identified, with the 16 most dominant genera shown in **Fig. 4B**. In the initial sample (#1), *Streptococcus* (18%) and *Staphylococcus* (16%) were the most abundant genera. The relative abundance of *Staphylococcus* significantly increased in sample #3 (71%), while *Lactobacillus* was significantly increased in sample #8 (44%) compared to #1 (**Fig. 4B, E and F**).

When comparing different storage temperatures, *Firmicutes* significantly increased after storing BM at RT for 1 week (#3) (**Fig. 4A, C, and D**) and *Staphylococcus epidermidis* was identified as the significantly enriched species in this condition (**Fig. 4B, E, and F, Supplementary Fig. 4**). In sample #4, relative abundance of *Streptococcus lactarius* showed a slight increase (**Supplementary Fig. 4**). Among the storage temperatures, #6 showed a microbiota composition most similar to #1, with a slight increase in *Firmicutes* and a decrease in *Bacteroidetes*.

Regarding thawing methods, no significant alterations were observed in bacterial relative abundances at either phylum and genus level, consistent with the alpha and beta diversity results. In the comparison of storage periods, storing HM at -20°C for 4 weeks (#8) showed a significant increase in *Firmicutes*, and enrichment of *Lactiplantibacillus plantarum* (**Fig. 4A and B, Supplementary Fig. 4**).

DISCUSSION

The importance of the formation and modulation of the gut microbiome in human health is well established [4,15-16]. More recently, the concept of gut-brain axis and disorders of gut-brain interaction have emphasized that the gut microbiome can influence neurological pathways and, consequently, gastrointestinal and other organs in the body [17,18]. Therefore, early establishment of the microbiome and intestinal colonization is critical. Since HM is the most important and often the only nutritional source introduced orally in the gastrointestinal tract after birth, the microbial contribution of HM may be essential for shaping long-term immunity and health [19]. Therefore, maintaining a microbiota as close as possible to that of fresh milk would be important. While direct breastfeeding is the most effective and ideal option, many mothers in South Korea are unable to do so for socioeconomic or health-related reasons and instead express and store milk for feeding later [20,21]. Our present study provided meaningful data on the optimal duration and methods of HM storage for minimizing changes in the milk microbiome.

Generally, HM is known to be stored well at RT for up to 4 h, in a refrigerator (4°C) for 4 days, and in a freezer for up to 6 mon [22]. In our study, microwave heating of the initial sample did not result in substantial alterations of the microbiota. However, as is well established, the use of microwave heating is not recommended because it generates uneven

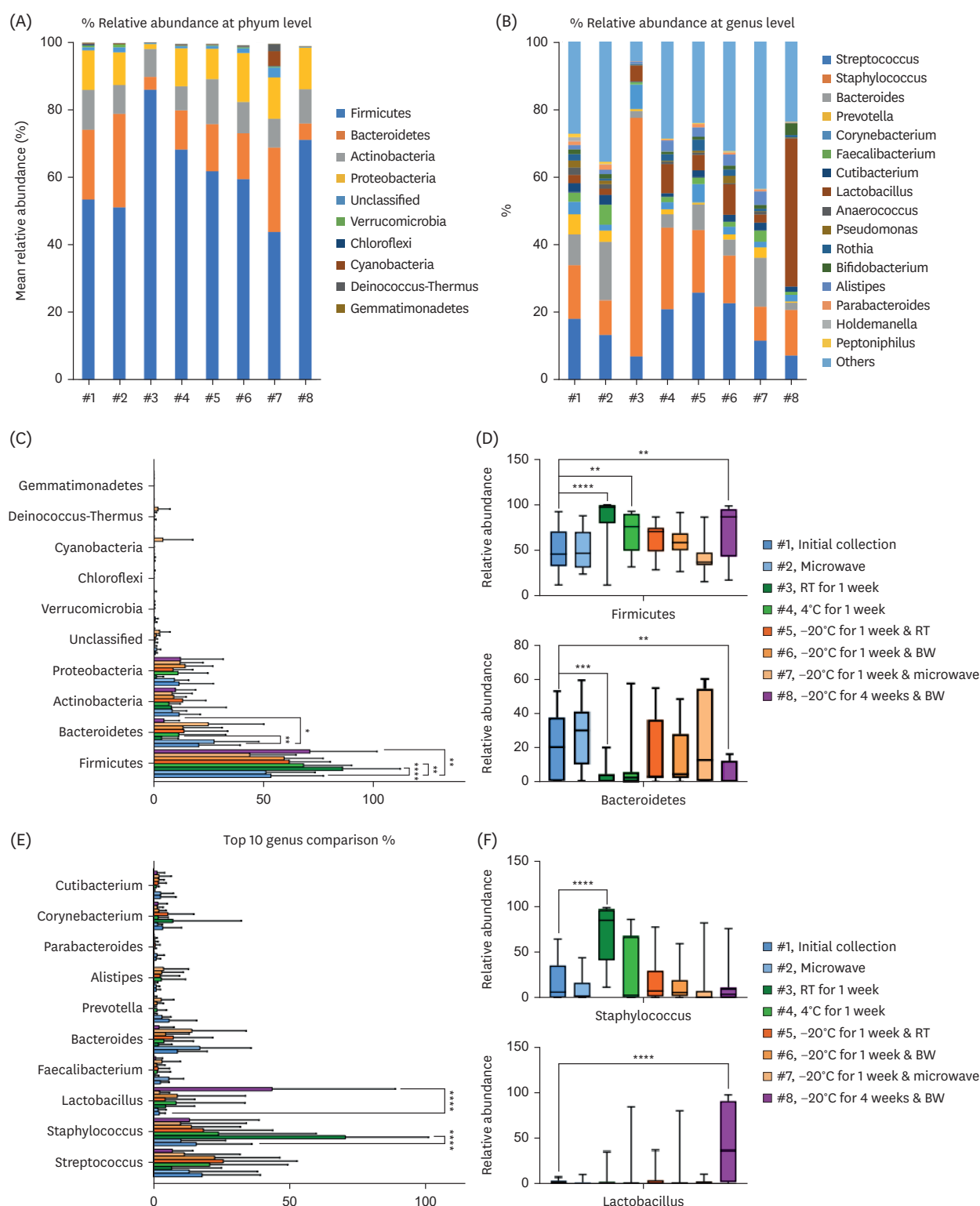


Fig. 4. Mean relative abundances of bacterial phyla and genera in human milk across the different treatment groups. Mean relative abundances of the top 10 most abundant bacterial phyla (A) and genera (B). (C) Comparison of relative abundance of each phylum in the 8 treatment groups; only the top 10 phyla are shown. (D) Comparison of relative abundance of *Firmicutes* (top) and *Bacteroidetes* (bottom) among the 8 treatment groups. (E) Relative abundance of each genus in the treatment groups; only top 10 genera are shown. (F) Relative abundance of *Staphylococcus* (Top) and *Lactobacillus* (bottom) across the 8 treatment groups. Statistical significance was determined by one-way ANOVA followed by Dunnett's *post-hoc* test. ANOVA, analysis of variance; RT, room temperature; BW, bottle warmer. P-values are indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

thermal distribution and can compromise the integrity of other components, including key nutrients such as proteins and lipids [14]. In addition, when thawing is performed using a microwave, the bacterial communities are likely to be destroyed by heat. Therefore, although the microbiota composition measured by DNA analysis may appear similar, these microorganisms would not be viable and thus unlikely to colonize the infant's oral cavity or intestine. However, relevant studies on this topic are limited, and although clinical investigation may not be feasible, this could be further explored through animal studies. Even one week of refrigeration caused microbial changes. Our study demonstrated that storing HM at -20°C for one week and thawing it using a bottle warmer or at RT preserved the microbial diversity and composition best. Although RT thawing is often practiced in domestic settings, it can lead to secondary contamination or introduce timing issues regarding feeding the infant. Therefore, methods with slightly greater microbial variability are not necessarily unsafe or entirely unsuitable. To minimize microbiota disruption, we recommend rapid freezing, short-term storage, and gentle thawing at safe temperatures.

In one sample stored at RT for a week, we observed an increase in *S. epidermidis*. As a known skin commensal, *S. epidermidis* is presumed to have originated from the mother's breast or nipple during sample collection and is one of the most commonly found species in HM [4]. We chose not to exclude such findings from our analysis, since our aim was to reflect the actual microbiome profile of the milk ingested by infants. Even skin-derived bacteria likely contribute to the gut microbial development through digestive processing and immune modulation. The significant increase in *S. epidermidis* during room-temperature storage of HM is likely associated with its origin as a skin commensal and its ability to proliferate efficiently at ambient temperatures [23,24]. Interestingly, we observed an increase in *L. plantarum* in milk stored at -20°C . *Lactobacillus* spp. exert inhibitory activity against pathogens such as *Shigella* spp., *Salmonella* spp., and *Escherichia coli* by preventing intestinal adhesion [25]. *L. plantarum* is frequently exposed to refrigerated and freezing conditions during fermented food production, which might naturally contribute to the development of cold tolerance [26]. In our study, *L. plantarum* was the only taxon that notably increased after storage at -20°C . This could likely be due to a relative increase caused by the decline of other taxa during freeze-thaw stress, rather than true proliferation under frozen conditions. Further research would be recommended to determine whether the strain really exhibits greater resistance to environmental and storage-related stresses; if so, it could have potential commercial applications in microbiome-based or probiotic formulations.

The composition of human milk microbiome has been well-characterized in previous studies, with *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Propionibacterium* commonly identified as the core genera. Togo *et al.* [27] confirmed the diversity of human milk microbiota through a systematic review and identified commonly found species, namely *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus agalactiae*, *Cutibacterium acnes*, *Enterococcus faecalis*, *Bifidobacterium breve*, *E. coli*, *Streptococcus sanguinis*, *Lactobacillus gasseri*, and *Salmonella enterica* [27]. Our analysis of fresh samples yielded similar findings. However, the exact role of each species in infant gut remains unclear. The bacteria likely coexist and synergistically support early immunological development, protect against infections, and aid in nutrient absorption. Therefore, instead of promoting or suppressing specific strains, preserving the overall microbiota in a state as close to its original composition as possible would be essential. Hence, if direct breastfeeding is not possible, immediate freezing, limited storage duration (preferably less than one week), and gentle thawing to minimize microbial shifts would be recommended.

Our study had several limitations. Due to practical constraints in human milk research, the sample size was limited. This may have led to overlooking meaningful differences or influenced the variability observed in the box plots. Further investigations with a larger number of samples will be conducted to validate these findings. There were also differences depending on the comparison criteria. For instance, sample #3 showed a more pronounced difference from the initial sample than sample #8. However, this study was not designed to compare all 10 conditions simultaneously; it focused solely on differences according to storage temperature or thawing method. Therefore, these variations were not emphasized. In addition, collecting samples under real-world conditions involves some variability and difficulty in bacterial extraction. Further, we were unable to evaluate the actual clinical or gut-level effects of microbial changes in infants. However, such limitations are common in human clinical research, and we plan to address them in future well-designed animal studies.

In conclusion, this study is meaningful in that it investigates the microbial changes in HM caused by storage and thawing methods that can realistically occur or be mistakenly applicable in daily domestic practice, rather than under strictly controlled laboratory settings. Our findings provided essential evidence for future workplace milk collection policies and product development. Furthermore, they can serve as a foundation for national policies promoting breastfeeding and supporting childbirth and childcare.

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SUPPLEMENTARY MATERIALS

Supplementary Fig. 1

Summary of sample collection and treatment information.

Supplementary Fig. 2

Average rarefaction curves of samples from each treatment group.

Supplementary Fig. 3

Quality assessment of NGS data.

Supplementary Fig. 4

Relative abundance of bacterial species in HM samples.

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