**ORIGINAL ARTICLE**

# THE EFFECT OF STORAGE TIME ON THE GROWTH OF MICROORGANISMS IN PASTEURIZED AND UNPASTEURIZED DONOR HUMAN MILK IN A TERTIARY HOSPITAL IN DAVAO CITY: A QUASI-EXPERIMENTAL STUDY

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The authors declare that the data presented are original material and has not been previously published, accepted or considered for publication elsewhere; that the manuscript has been approved by all authors, and all authors have met the requirements for authorship.

## ABSTRACT

**Background:** Donor Human Milk (DHM) is the recommended food of infants whenever mom's own milk (MOM) is not available. However, due to the pathogenic microbiological component of DHM, concerns on the safety of the milk are inevitable.

**Objective:** To determine the effect of storage time on the microbial growth of pasteurized and unpasteurized Donor Human Milk maintained at a constant temperature of -20°C.

**Methodology:** This is a Quasi-experimental Research done in the Newborn Care Unit (NCU) and Bacteriology Section of a private tertiary hospital in Davao City. The effect of storage time to the microbial growth of pasteurized and unpasteurized DHM was determined using Friedman Test 2-way Analysis of Variance by Ranks. Pairwise comparison of microbial growth between pasteurized and unpasteurized DHM at different storage times was determined using the Mann-Whitney U test.

**Results:** Baseline DHM samples had moderately heavy bacterial growth of *Staphylococcus epidermidis*. There was a decrease from moderately heavy to light growth of the same species in the 24-hour storage time for both pasteurized and unpasteurized DHM. Pasteurized DHM did not have any microbial isolates at 48h, 72h, 4w, 8w and 12w while unpasteurized DHM had *Acinetobacter baumanii, Staphylococcus warneri, Kocuria kristinae,* and *Staphylococcus saprophyticus* growths. The analysis revealed that there is a statistically significant difference in the microbial growth in both pasteurized and unpasteurized DHM samples when stored at different times, $\chi^2(6) = 28.457$, $p = 0.00$.

**Conclusions:** Storage time significantly interacts with the microbial growth on both pasteurized and unpasteurized DHM samples. Therefore, microbial growth in DHM samples may be affected by the length of time stored at a constant temperature of -20°C. Pasteurized DHM samples when stored at -20°C for more than 48 hours resulted to a statistically reduced microbial growth.

**KEYWORDS:** *Human milk, Pasteurization, Storage time*

## INTRODUCTION

Human breastmilk, with its known time-tested benefits, is the most natural perfect food for babies and is highly recommended in the medical field. As proven by clinical and research studies, human breastmilk is the best source of nutrition for the infant because of its compelling advantages in terms of nutrition, immunoprotection, neurodevelopment, psychologic, socio-economic, and environment.[1]

In the Philippines, Expanded Breastfeeding Promotion Act of 2009 states that *"the state shall promote and encourage breastfeeding and provide the specific measures that would present opportunities for mothers to continue expressing their milk and or breastfeeding their infant or young child"* (Republic Act No. 10028). However, if breastfeeding is not possible, international authorities like the World Health Organization (WHO), American Academy of Pediatrics (AAP), European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHN) recommend the use of donated human breastmilk as the first alternative, and pasteurized Donor Human Milk as the most adequate alternative especially for preterm infants.[1,2,6] Nevertheless, the main concern regarding Donor Human Milk is its microbiological safety especially during storage.[3-7] Current official protocols include pasteurization and freezer storage at –20°C to eliminate hazards for newborns and preserve bioactive compounds.[1-5]

Milk pasteurization is a heating process that kills any harmful bacteria or viruses that may be present in the milk. A pasteurization process at 62.5°C for 30 minutes, also known as Holder Pasteurization, is currently recommended in all international guidelines.[3] This process preserves most of the milk's nutrients, immune properties, and other health components.[3-8] Milk is pasteurized to ensure safety from infectious agents potentially contaminating human milk, especially Donor Human Milk.

There are a few guidelines and research studies available regarding the optimum storage time and temperature of pasteurized Donor Human Milk. Thus, evidence-based standard protocol for milk handling and storage pre- and post-pasteurization in hospitals where milk pasteurizers are available is needed. Given the importance of milk storage and handling of expressed human milk to both mother and infant, it is of equal importance to determine and know the potential impact of storage time on Donor Human Milk after pasteurization, hence this study was conducted.

This study intended to determine the effect of storage time on the microbial growth on unpasteurized and pasteurized Donor Human Milk kept at a constant temperature of -20 °C. Specifically, to describe microbiota through bacterial culture of unpasteurized and pasteurized Donor Human Milk (bacteria profiling) and to determine and compare the presence or absence of microorganisms in unpasteurized, pasteurized Donor Human Milk samples when stored at -20°C for 24, 48, and 72 hours and at 4, 8 and 12 weeks.

## MATERIALS AND METHODS

### *Study Design and Setting*

This study utilized a quasi-experimental research design and was conducted at the neonatal care unit (NCU) and Bacteriology Section (Laboratory) of a tertiary hospital in Davao City, Philippines. The NCU of the institution does not do milk banking, only milk pasteurization.

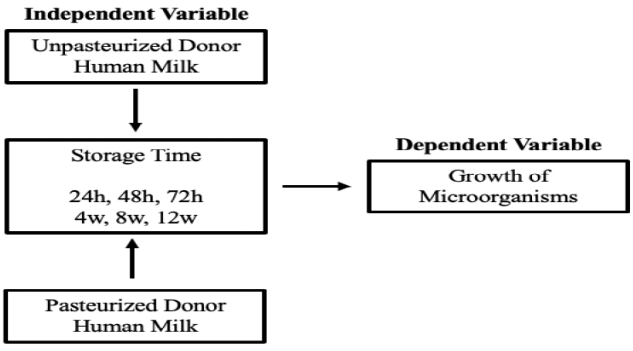


Figure 1. Conceptual Framework on the Effect of Storage Time on the Microbiota of Unpasteurized and Pasteurized Donor Human Breastmilk.

### *Procedures of the Study*

**Procurement of permission to conduct study.** The primary investigator secured permission from the Medical Director of a tertiary hospital to conduct this study and obtain access to the NCU. This study

followed the Donor Human Milk pasteurization protocol of the hospital, which is adapted from the Philippine Human Milk Banking Manual of Operation (MOO) of the Department of Health (DOH).[1] In addition, this study was submitted and approved by the Institutional Ethics Review Committee of the Davao Doctors Hospital prior to data collection. This study was conducted in accordance with the principles that have their origin in the Declaration of Helsinki and is consistent with the International Conference on Harmonization Tripartite Guidelines and the Good Clinical Practice Guidelines (ICH-GCP). All patient information were anonymized and kept confidential. The primary investigator declares that there was no conflict of interest in the conduct of this study. There was no funding received from any individual nor institution.

**Donor recruitment.** Milk samples were obtained from mothers who delivered in the said institution. Four mothers who were in their immediate postpartum period consented to participate in this study as milk donors. These donors were interviewed and screened using a standard interview and screening forms which were taken from the Philippine Human Milk Banking MOO of DOH.[1] Physical examination of the breast was done by the primary investigator, and the antenatal test results (e.g., HBsAg, Anti-HBs, VDRL, RPR) at the time of their hospital admission were utilized in this study as an integral part of the screening process. All mothers had negative test results for the above mentioned tests.

**Milk collection**. Hand washing with soap and water is required upon entry to the NCU. Milk expression was done in the breastfeeding section of the NCU wherein only the primary investigator, NCU nurse, and milk donor were present. The primary investigator instructed the donor to clean her nipple and areola with the use of cotton and water. The recommended and standard method of manual milk expression (Marmet technique) for milk donation prior to pasteurization in the NCU was employed. The milk donor was instructed to use the thumb and forefinger of one hand and to position them one to two centimeters outside the areola as well as to perform the cycle of "Push-Compress-Release" repeatedly for several minutes to stimulate milk ejection. The breast milk was allowed to flow freely to a sterilized wide-mouth container and was made sure not to touch the nipple to avoid contamination. Visual presentations, such as videos and pictures, were used for assistance. After the manual expression, the container was covered with a sterile cap, and both breasts were cleaned with water. Expressed breast milk was stored in a sterile plastic container properly labelled and was frozen within five to ten minutes from milk expression.

**Assignment to different treatment groups**. Each milk donor was able to express an average of 60-90 ml of breastmilk. A total of 360 ml donor human milk was collected and pooled. This was considered as one milk batch. One milliliter (1 ml) aliquot from the pooled milk was sent immediately to the laboratory for baseline culture, which was done by a registered medical technologist. This milk batch was then distributed equally into two groups, the pasteurized group (Group A) and the unpasteurized group (Group B). Group A underwent the pasteurization process at 62.5$^{o}$C for 30 minutes (Holder Pasteurization) while Group B remained unpasteurized. Three replicates were made per group at different storage times. Triplicates were done for each batch for validation of observed results. Each replicate was stored in a freezer at -20$^{o}$C using a Traceable® Jumbo-Display Fridge/Freezer Digital Thermometer for 24h, 48h, 72h, 4w, 8w, and 12w. Bacteriologic testing of each replicate after a designated storage time was done thereafter.

**Bacteriologic Testing.** The streak plate method using selective and differential culture media were employed to isolate bacteria from the baseline, unpasteurized and pasteurized milk samples at different storage times. Species identity was determined via microscopy and susceptibility testing. To determine whether there is a significant difference in the growth of microorganisms between the unpasteurized and pasteurized milk samples at different storage times, relative to the baseline cultures, a scoring system based on the percent coverage was used and followed. This was based on

standard laboratory interpretations of the plating method as adapted from Bailey and Scott's Diagnostic Microbiology and has been the practice in the laboratory of the institution.[9] If no visible colonies of bacteria were noted, it was labelled as No Growth (NG) and assigned with a score of 0. Visual presentation of bacterial growth based on the percent occupied by the microorganisms on the zone of inoculation of the culture media for Light Growth (LG) was scored as 25, Moderate Growth (MG) as 50, Moderately Heavy Growth (MHG) as 75, and Heavy Growth (HG) as 100 (Figure 2).

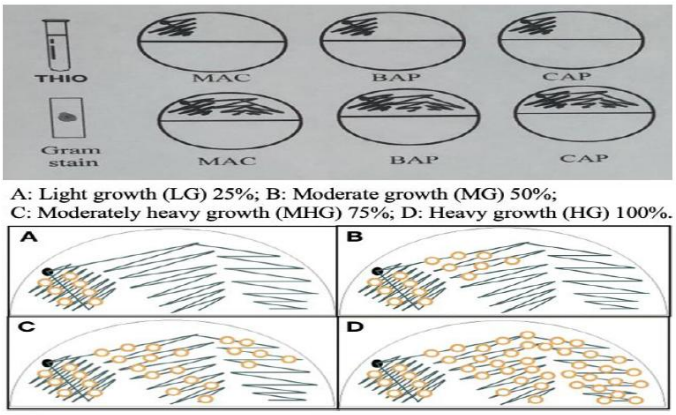


Figure 2: Streak plate method and visual presentation of bacterial growth based on the percent occupied by the microorganisms on the zone of inoculation of the culture media.

The specimen was inoculated on 1/2 blood agar plate, 1/2 chocolate agar plate, and 1/2 MacConkey agar plate; and a smear for gram staining was made. The slide was stained and examined under oil immersion field. After 16 to 24 hours of incubation, the plates were examined for growth.

Microbial growth on MacConkey agar indicates the presence of lactose/non-lactose fermenting type of organisms; and biochemical and susceptibility testing was done thereafter. Those that have microbial growth on the blood agar plate, gram staining and catalase test was done. For those gram-positive cocci, coagulase and susceptibility testing were done. Those with negative catalase test, possible organisms were alpha-hemolytic, hence Optochin and susceptibility testing were done. Otherwise, possible organisms were beta-hemolytic, hence Streptex/Pastorex and susceptibility testing were done. For growth on chocolate agar, gram staining was done to bacterial colony that were translucent. Smears were examined under oil immersion and short rods/pleomorphic microorganisms were identified. Once microorganisms were identified, workup for *Haemophilus influenza*, factors X and V, and susceptibility testing were done. The BIOMERIEUX Vitek® 2 Compact System was used for all identification and susceptibility testing.

If no bacterial growth was observed after 16 to 24 hours of incubation and the thioglycolate medium was clear, the plates was re-incubated up to 48 to 72 hours with daily inspection before releasing as no growth (Figure 3). These processes were done by a licensed senior medical technologist assigned in the bacteriology section of the hospital laboratory who followed standard procedure of accepting specimen for culture.

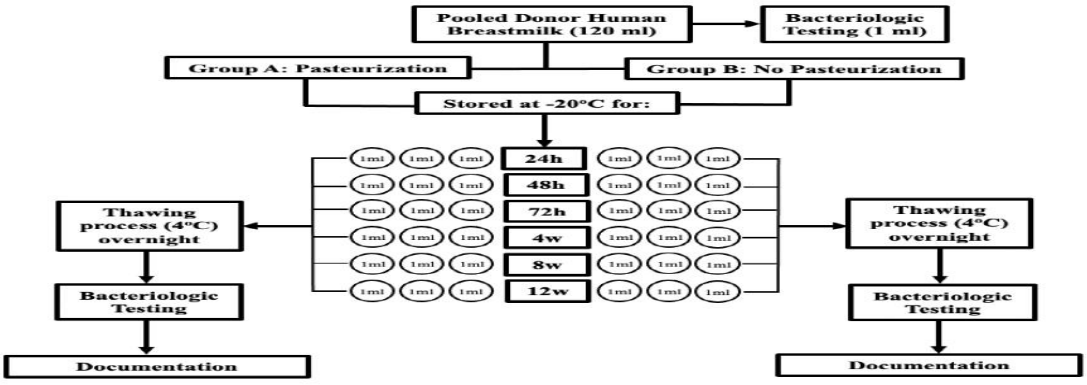


Figure 3: Flowchart of the research procedure.

*Data Entry and Statistical analysis*

Data collected was entered into a Microsoft Excel for the purpose of coming out with a .csv file type in preparation for the statistical analysis. This study was limited to non-parametric tests due to the qualitative nature of the variable of interest – the ordinal level of measurement of the percentage growth of bacteria. The effect of storage time on the microbial growth of pasteurized and unpasteurized DHM was determined using Friedman Test 2-way Analysis of Variance by Ranks. In addition, pairwise comparison of microbial growth between pasteurized and unpasteurized DHM at different storage times was determined using the Mann-Whitney U test.

**RESULTS**

*Bacterial Isolates from Donor Human Milk (DHM)*

Selective and differential culture media were employed to isolate bacteria from all milk samples and species identity was determined via microscopy and susceptibility testing. Table 1 shows the dominant bacterial species isolated from both pasteurized and unpasteurized donor human milk samples at different storage times. The microbial isolates which occupied majority of the section on the zone of inoculation was considered dominant. This has been the practice of releasing culture results in the laboratory.[9]

The baseline DHM samples showed growth of *Staphylococcus epidermidis.* Similarly, among the pasteurized milk samples, only those stored at 24 hours showed growth of the same species as the baseline. For the pasteurized DHM samples, no growth of microorganisms was observed at 48 hours, 72 hours, 4 weeks, 8 weeks, and 12 weeks. On the other hand, unpasteurized DHM samples exhibited a more diverse growth of microorganisms.

*Acinetobacter baumanii*, a gram-negative, strictly aerobic, non-fermenting and non-fastidious bacterium was isolated at 24 hours of storage time.[10] Two Staphylococcus species were isolated namely *Staphylococcus warneri*, at the 48-hour storage time, and *Staphylococcus saprophyticus*, at the 4-week and 12-week storage times. *Kocuria kristinae* was isolated at 72 hours storage time for unpasteurized DHM samples. At 8 weeks storage time, both unpasteurized and pasteurized DHM samples showed no growth of microorganisms.

Table 1: Dominant bacterial species isolated from pasteurized and unpasteurized donor human milk samples across different storage time.

| | Species Identity | |
|---|---|---|
| 0 hour (baseline) | *Staphylococcus epidermidis* | |
| Storage Time | Pasteurized | Unpasteurized |
| 24 hours | *Staphylococcus epidermidis* | *Acinetobacter baumanii* |
| 48 hours | none | *Staphylococcus warneri* |
| 72 hours | none | *Kocuria kristinae* |
| 4 weeks | none | *Staphylococcus saprophyticus* |
| 8 weeks | none | none |
| 12 weeks | none | *Staphylococcus saprophyticus* |

*Effect of Milk Pasteurization and Storage Time on Bacterial Growth*

The baseline samples, in all replicates, exhibited a moderately heavy bacterial growth (Figure 4A) of *Staphylococcus epidermidis*, and light growth of the same species was also present in pasteurized samples at 24-hour storage time (Figure 4B). At 48-hours post-pasteurization (Figure 4C), pasteurized DHM samples were found to have no bacterial growth up to 12 weeks of storage time.

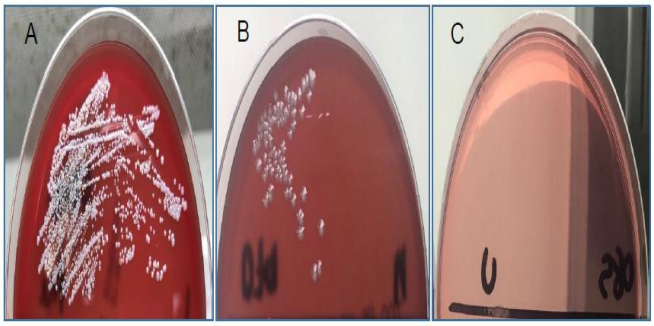


Figure 4: Bacterial cultures at **(A)** baseline samples showing moderately heavy bacterial growth, **(B)** 24-hour storage time of the pasteurized samples showing light bacterial growth, and **(C)** 48-hour storage time of the pasteurized samples showing no bacterial growth.

Unpasteurized and pasteurized DHM samples were compared for bacterial growth at different storage times as shown in Table 2. Baseline DHM samples had moderately heavy bacterial growth of *Staphylococcus epidermidis* and although similar species was isolated at 24 hours storage time for the pasteurized samples, it was noted that there was a decrease from moderately heavy to light growth of the said species. No microbial growth was observed at 48 hours until 12 weeks storage time for the pasteurized DHM samples. This was also noted at 8 weeks storage time for unpasteurized DHM samples. Light growth of *Acinetobacter baumanii*, *Staphylococcus warneri, Kocuria kristinae, and Staphylococcus saprophyticus* were isolated at 24 hours, 48 hours, 72 hours, 4 weeks, and 12weeks of storage time, respectively, for unpasteurized DHM samples.

*Microbial Growth Comparison among Treatment*

The effect of storage time on the microbial growth of pasteurized and unpasteurized donor human milk was determined using Friedman Test two-way Analysis of Variance by Ranks. The analysis revealed that there is a statistically significant difference in the microbial growth in both pasteurized and unpasteurized DHM samples when stored at different times, $\chi^2(6) = 28.457$, $p = 0.00$ (Figure 5).

This result signifies the interaction of storage time with the microbial growth on both pasteurized and unpasteurized DHM; thus, microbial growth in DHM samples may be affected by the length of time stored in a freezer at a constant temperature of -20°C.

Table 2: Comparison of bacterial growth between pasteurized and unpasteurized donor human milk (DHM) samples at different storage times.

| Storage Time | Pasteurized Milk | | | Unpasteurized Milk | | |
|---|---|---|---|---|---|---|
| | Replicate 1 | Replicate 2 | Replicate 3 | Replicate 1 | Replicate 2 | Replicate 3 |
| 0 hour (Baseline) | MHG | MHG | MHG | MHG | MHG | MHG |
| 24 hours | NG | LG | LG | LG | LG | LG |
| 48 hours | NG | NG | NG | NG | LG | LG |
| 72 hours | NG | NG | NG | LG | LG | LG |
| 4 weeks | NG | NG | NG | LG | LG | LG |
| 8 weeks | NG | NG | NG | NG | NG | NG |
| 12 weeks | NG | NG | NG | LG | LG | LG |

Legend: NG - No growth, LG - Light growth, MG - Moderate growth, MHG - Moderately Heavy growth, HG - Heavy growth.

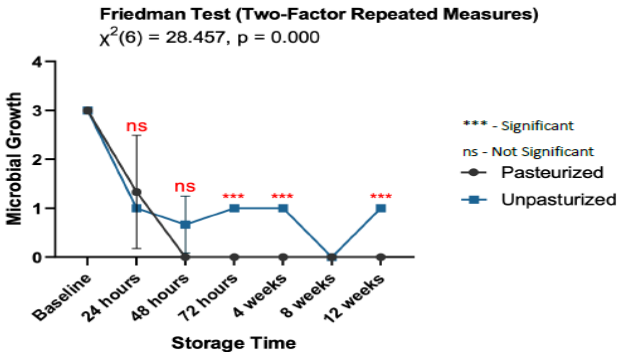


Figure 5: Interaction of Storage Time with Microbial Growth on Pasteurized and Unpasteurized Donor Human Milk

Despite the significant interaction between the storage time and microbial growth, the multiple comparison analysis (Table 3) of related samples between the baseline microbial growth and the succeeding measurements in varying storage times revealed that there is not enough evidence to support statistically significant differences ($p>0.05$) in the microbial growth from the baseline culture samples with other samples stored at different times. In other words, for example, although there was a reduction in the [light] bacterial growth of the unpasteurized DHM samples stored at -20°C for 24 hours compared to the [moderately heavy bacterial growth of] baseline DHM samples, this was not statistically different from each other. This may warrant further investigation using additional test DHM samples.

Table 3: Multiple Comparison of Microbial Growth in Pasteurized and Unpasteurized Donor Human Milk Stored in Various Storage Times.

| Storage Time | Pasteurized | | Unpasteurized | |
|---|---|---|---|---|
| Baseline vs. | Z | Asymptotic Sig. (2-tailed) | Z | Asymptotic Sig. (2-tailed) |
| 24 hours | -1.633 | 0.102 | -1.732 | 0.083 |
| 48 hours | -1.732 | 0.083 | -1.633 | 0.102 |
| 72 hours | -1.732 | 0.083 | -1.732 | 0.083 |
| 4 weeks | -1.732 | 0.083 | -1.732 | 0.083 |
| 8 weeks | -1.732 | 0.083 | -1.732 | 0.083 |
| 12 weeks | -1.732 | 0.083 | -1.732 | 0.083 |

In addition, the Mann-Whitney U pairwise comparison test (Table 4) revealed that microbial growth in pasteurized and unpasteurized DHM samples stored at 24 hours and 48 hours do not significantly differ ($p>0.05$) from each other while those milk samples stored in 72 hours, 4 weeks, and 12 weeks, has statistically significant difference ($p<0.05$). The analysis implies that pasteurized DHM stored in longer duration may result to reduced microbial growth. Therefore, pasteurized DHM poses greater advantage over unpasteurized DHM in terms of microbial growth after storage. Furthermore, this analysis also supports that storage time may have an interaction to pasteurization that may have resulted to reduced microbial growth.

Table 4: Mann-Whitney U Pairwise Comparison of Microbial Growth between Pasteurized and Unpasteurized Donor Human Milk at Different Storage Times.

| Storage Time | Mann-Whitney U | Z statistic | Asymptotic Sig. (2-tailed) |
|---|---|---|---|
| Baseline | 4.50 | 0.00 | 1.00 |
| 24 hours | 3.00 | -1.00 | 0.317$^{ns}$ |
| 48 hours | 1.50 | -1.58 | 0.114$^{ns}$ |
| 72 hours | 0.00 | -2.24 | 0.025* |
| 4 weeks | 0.00 | -2.24 | 0.025* |
| 8 weeks | 4.50 | 0.00 | 1.00 |
| 12 weeks | 0.00 | -2.24 | 0.025* |

* Significant ($p<0.05$), ns-not significant ($p>0.05$)

## DISCUSSION

Human milk has high nutritional content which includes proteins, fats, carbohydrates, vitamins, minerals, and essential amino acids that can support a rich microbiota.[11, 12] It may, however, also be a vehicle for microorganisms derived from the mother or the environment during its collection, storage, and handling. Freshly collected breast milk is rarely sterile and normally contains bacteria originating from the maternal skin and nipple duct microflora, but it also sometimes contains potential pathogens. Although it is questioned whether it is possible to aseptically collect human milk, culture-dependent methods have confirmed the presence of bacteria in assumed aseptically collected milk. Bacterial contamination in milk can originate through a variety of sources which includes teat apex, milking equipment, air, and other environment by which it is exposed.[13-15] These microorganisms are known to play several roles such as facilitating dairy fermentations (e.g., *Lactococcus* and *Lactobacillus*), causing spoilage (e.g., *Bacillus a*nd *Clostridium*), promoting health (e.g., *Lactobacilli* and *Bifidobacteria*) or causing diseases (e.g., *Listeria, Salmonella, Escherichia coli* and *Campylobacter*).[12] One of the most commonly isolated bacterial species from human milk include *Staphylococcus epidermidis*, an emerging leading cause of subacute mastitis in both women and veterinary medicine. The baseline DHM samples in this study, which came from a single pooled milk

batch from four donors, demonstrated a moderately heavy growth of *Staphylococcus epidermidis*, suggesting that one or more of the milk donors could have the said condition. Furthermore, the same species was isolated in the pasteurized milk samples at 24 hours storage time. This species is an opportunistic human pathogen which is one of the leading causes of nosocomial infections and are also commonly associated with hospital-acquired medical device infections.[16,17] Its capacity to form biofilms, exopolymers, and various defense mechanisms give it protection from antibiotics and host defenses, making it difficult to eradicate.[18] The biofilms of such species are temperature-sensitive but can still maintain some level of cell viability even after exposure at 60$^{o}$C for 1 hour.[17] This could partly explain why Holder pasteurization was not able to fully eradicate *S. epidermidis* from the samples used in this study. Its growth seemed to be inhibited beginning at 48 hours post-pasteurization, possibly due to the preserved bactericidal properties in the breastmilk. Another possibility is that pasteurization may not have fully eradicated this organism within the 24-hour mark, but the heating process was enough to make the organisms incapable to proliferate further. Coagulase-negative Staphylococci (CoNS) have traditionally been part of the normal skin microbiota. It represents a regular part of the microbiota of the skin and mucous membranes of humans and animals.[19] The differences in skin thickness and folds as well as hair follicles and glands densities define distinct differing microbiota including CoNS. In humans, *S. epidermidis* is the most frequently recovered CoNS species.[19] While the virulence of these organisms is relatively low, their opportunistic behavior can cause clinically significant infections of the bloodstream and other tissue sites.

Unpasteurized DHM samples were observed to have a consistent light growth of microorganisms from 24 hours until 12 weeks of storage time, except for the cultures at two months storage time which showed no growth at all. Microorganisms grown in closed culture follow a reproducible growth pattern referred to as the growth curve that consists of four phases namely the lag, exponential, stationary, and death phases. It could be possible that microorganisms found in unpasteurized DHM when stored at -20$^{o}$C for two months may be in transition between the stationary and the death phases of the growth curve, meaning that at this point, microbes may begin to decrease in number of living bacterial cells. Four microbial isolates were identified in the unpasteurized DHM samples, namely, *Acinetobacter baumanii, Staphylococcus warneri, Kocuria kristinae,* and *Staphylococcus saprophyticus*. These were either typical skin commensals, part of the normal flora of the human oropharynx, or species that are ubiquitous in nature.

*Acinetobacter baumanii*, isolated from the unpasteurized milk samples at 24 hours of storage time, is a gram-negative, strictly aerobic, non-fermenting and non-fastidious bacteria that is usually pathogenic. The possibility that this isolated microorganism could be a contaminant is highly favored since the processes of milk collection until milk culture inoculation were done in a hospital setting, specifically the NICU and Bacteriology Section of the Laboratory. Another possibility, though least probable, is that one of the milk donors belong to the few percentages of the population that harbor the rare species as part of their natural skin microbial flora. Two Staphylococcus species were also isolated from the unpasteurized DHM samples namely *Staphylococcus warneri*, at the 48-hour storage time, and *Staphylococcus saprophyticus*, both at 4 weeks and 12 weeks of storage time. Staphylococcus species are gram-positive cocci that form clumps and are traditionally divided based on their coagulase reaction. *Staphylococcus aureus* and *Staphylococcus intermedius* are the only known coagulase positive Staphylococci while the rest are known to be coagulase negative. Many Coagulase Negative Staphylococci (CoNS) such as *S. warneri* and *S. saprophyticus* are common commensals on the skin and membrane linings, although several species are known to cause infections in both

humans and animals.[20] *S. warneri* is a gram-positive skin commensal which rarely causes disease, but also occasionally cause infections particularly in immunocompromised patients.[21] It has already been reported as an emerging pathogen although there is still a lack of scientific data on the pathogenesis and epidemiology of the species.[22] Previous studies reported the clinical significance of *S. warneri* in orthopedic infections, pediatric and adult bacteremia, septicemia, endocarditis, urinary tract infections, as well as its pathogenicity in neonates being a predominant CNS isolated from the hands of nurses.[22-26] On the other hand, *S. saprophyticus* has been widely documented as one of the leading causes of urinary tract infections (UTI), second to *Escherichia coli*, and is commonly found in the gastrointestinal tract particularly exhibiting rectal, vaginal and urethral colonization.[27,28] Furthermore, the bacteria have been found to contaminate various food samples in a study conducted in Sweden with high prevalence in raw beef and pork and has been isolated from rectal swabs from cattle and pigs.[29,30] Among the more severe complications that it can cause includes acute pyelonephritis, septicemia, nephrolithiasis and endocarditis.[31-34] Another flora found is the *Kocuria kristinae,* a gram-positive coccus, which is also a natural skin and mucous membrane commensal and usually non-pathogenic was isolated from the unpasteurized samples at 72 hours storage time.[35] Members of the genus *Kocuria* are responsible for different types of infections, mostly in immunocompromised host with serious underlying conditions.[36,37] Opportunistic infections caused by this species in patients with malignancy has also been reported.[38] Furthermore, it has also been reported to cause infections in premature babies and immunocompromised pediatric patients which highlights its expanding infection spectrum.[39] There is still limited information on the epidemiology and virulence of Kocuria species, but the formation of biofilms has been suggested to mediate adhesion, colonization and subsequent infection.[40]

Current official protocols for donor human milk include pasteurization and freezer storage at -20$^{o}$C to eliminate hazards for newborns and preserve bioactive compounds. In order to strike a balance between microbiological and immunological safety, Low Temperature Long Time (LTLT) milk pasteurization is usually employed to reduce microbial load and viable pathogenic bacteria, to limit the number of spoilage microorganisms that can cause foodborne diseases, and to ensure safety for human consumption.[8] Holder pasteurization has been proven to effectively remove any detectable bacteria from samples in a previous study using routine bacterial cultures for Staphylococcus, Streptococcus and Enterococcus species.[4] This method is also often simulated in small aliquots rather than being performed in compliance with Human Milk Bank (HMB)-implemented protocols, which are causing huge variability of results in many published studies using the same technique.[3] Although pasteurization assures the microbiological safety of human milk, the mechanism of thermal inactivation of bacteria is detrimental to the bioactivity of the milk since most of the proteins will denature when exposed to heat.[41] Several studies have shown that Holder pasteurization reduces, to some extent, the activity of important immunomodulating components. Hence, thermal treatment may not only impair the beneficial antibacterial properties of human milk but may also increase its susceptibility to subsequent bacterial contamination.[42] However, in this study, the pasteurized DHM samples showed no microbial growth from 48 hours to three months storage time. Despite such result, there is still not enough basis to attribute this effect to pasteurization alone as light bacterial growth was observed at 24-hour storage time post-pasteurization, like that of the unpasteurized DHM samples. Holder pasteurization significantly reduces (50–70%) the bactericidal effect, but if the pasteurized donor milk is kept refrigerated, the residual bactericidal capacity remains stable for up to 72 hours.[3,43] This suggests that other processes are contributing to the decline of the microbial flora between the 24-hour and 48-

hour mark, and that this was sustained up to three months of storage time.

The evidence for the benefits of pasteurized donor milk is limited, and the exact effect of frozen storage time after pasteurization on human milk composition is not clear. There is yet no complete agreement about storage times, however it is always preferable to store milk for as short a time as possible to ensure minimal growth of bacteria and minimal loss of antibodies and nutrients. Milk is typically stored at colder temperatures that reduce the growth of most bacteria, except for cryo-tolerant microorganisms that can proliferate under these conditions and become a major cause of milk spoilage.[13] Freezing breast milk at --20$^{o}$C for up to three months has been recommended as optimal. This 12-week time frame is within the recommended storage guidelines for milk by the National Institute for Health and Care Excellence, which is 6 months after expression, and to pasteurize within 3 months after expression.[44] Human milk has been known to have a natural bactericidal capacity which provides defensive factors against many disease-causing microorganisms, although this property can be altered during the storage of milk and post-processing events such as pasteurization.[42,45]

Literatures on the effect of storage duration on the bactericidal capacity of milk are limited. Refrigeration for less than 48 hours does not modify the bactericidal capacity of human milk, thus the protective properties for the nursing infant remain intact. However, if storage is extended beyond this time, then bactericidal capacity decreases, and the loss of this protection is very significant statistically after 72 hours. This property of maternal milk is lost over a period in which other components remain stable and within the limits advised by usual protocols. When frozen storage is employed, just like in this study, milk stability is prolonged. Frozen storage is an option when longer storage periods are needed.

Results of this study showed that storage time significantly interacts with the microbial growth on both pasteurized and unpasteurized DHM samples as determined by using the Friedman Test two-way Analysis of Variance by Ranks. Therefore, microbial growth in DHM samples may be affected by the length of time stored in a freezer at a constant temperature of -20$^{o}$C. Despite this, the multiple comparison analysis of related samples between the baseline microbial growth and the succeeding measurements in varying storage times revealed that there is not enough evidence to support statistically significant differences ($p>0.05$) in the microbial growth from the baseline culture samples with other samples when stored at different times. This means that among the unpasteurized DHM samples, for example, the microbial growth is relatively the same at different storage times. The absence of considerable differences of bacterial growth among these samples can be attributed to the pooling of milk samples from different donors, and the qualitative nature of the data obtained from bacterial culture techniques, all of which could possibly limit the explanatory power of the independent variables being tested. The Mann-Whitney U pairwise comparison test revealed that microbial growth in pasteurized and unpasteurized DHM samples stored at 24 hours and 48 hours do not significantly differ ($p>0.05$) from each other while those milk samples stored in 72 hours, 4 weeks, and 12 weeks, in contrast, has statistically significant difference ($p<0.05$). The analysis implies that pasteurized DHM stored in longer duration may result to reduced microbial growth. It is hypothesized therefore that this was because of a possible positive feedback to the bactericidal capacity after most bacterial contaminants were eradicated post-pasteurization. The innate bactericidal properties that are still preserved after pasteurization were able to enhance their function in regulating the remaining microbiota during the prolonged storage period at low temperature condition (-20$^{o}$C). Previous studies have reported that bactericidal activity of human milk is better preserved by means of holder pasteurization compared to other pasteurization methods, and that its microbiological quality can be maintained when properly handled and refrigerated at 4-

6°C.[44,46] It is therefore worth investigating in future studies whether the changes in biochemical and microbial composition after pasteurization enhances the bactericidal capacity of and if this enhanced effect is only applicable for certain types of bacteria. Therefore, pasteurized DHM poses greater advantage over unpasteurized DHM in terms of microbial growth after storage. Furthermore, this analysis also supports that storage time may have an interaction to pasteurization that may have resulted to reduced microbial growth.

Nevertheless, this study highlighted the importance of pasteurization in preventing the growth of several bacterial species such as *Acinetobacter baumanii, Staphylococcus warneri, Staphylococcus saprophyticus* and *Kocuria kristinae*. The absence of these bacteria in the pasteurized DHM samples highlights the benefit that pasteurization can provide in terms of reducing microbial populations which have the potential to cause infections and put consumers such as newborns at risk. Furthermore, this benefit can be enhanced with the proper storage at low temperatures to extend its shelf life while maintaining the essential nutrients present and keeping it safe for consumption.

## CONCLUSION AND RECOMMENDATION

In conclusion, storage time significantly interacts with the microbial growth on both pasteurized and unpasteurized DHM samples as determined by the Friedman Test Two-way Analysis of Variance By Ranks. Therefore, microbial growth in DHM samples may be affected by the length of time stored at a constant temperature of -20°C. In addition, the Mann-Whitney U pairwise comparison test revealed that pasteurized DHM samples when stored at -20°C for more than 48 hours resulted to a statistically significant reduced microbial growth. Furthermore, this analysis supports the result that storage time may have an interaction to pasteurization which have resulted to a reduction in the growth of microorganisms.

It is recommended to continue the practice of pasteurizing donor human milk in the NICU and to store them in a freezer at a constant temperature of -20°C as this will pave the way for the institution to develop and establish a local milk bank which will be very useful for the community. Also, it is worth investigating whether the changes in biochemical and microbial composition of Donor Human Milk (DHM) after pasteurization enhances the bactericidal capacity components of breastmilk and if this enhanced effect is only applicable for certain types of bacteria, as well as the viability to reproduce the recalcitrant microbes that grew. Furthermore, an equally spaced time intervals is suggested in data gathering to better capture the events of microbial growth within the duration of a study. It is also worth exploring what would happen to the quality of pasteurized DHM, in terms of its nutritional aspect, beyond 3 months of storage.

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