Bacterial content of fortified and unfortified Holder pasteurized donor human milk during prolonged refrigerated storage

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Abstract:

Objective: To assess the microbial growth in unfortified and fortified Holder pasteurized donor human milk (HPDHM) during 96 hours of refrigerated storage in a clinical setting.
Methods: Thirty-six unfortified samples and 77 fortified samples of HPDHM were prepared in a neonatal intensive care milk preparation room and stored in the NICU refrigerator at 4°C to simulate a real-life feeding environment. One milliliter aliquots were removed at 24-hour intervals and cultured in duplicate for bacterial growth on solid blood agar medium. Viable bacterial colonies were characterized using standard microbiological methods. **Results:** 96.5% of milk samples manipulated in a vertical laminar flow hood were negative for bacterial growth. In the remainder 3.5% of the samples, the maximum growth was 1 colony forming unit/0.1 ml plated. Higher colony counts were observed when the laminar hood was not used. In all cases, the colonies represented common skin bacteria and demonstrated an inconsistent and unsustained growth. Fortifier status and storage time were not significantly associated with increased bacterial growth ($P > 0.05$). **Conclusions:** Unfortified and fortified HPDHM remain largely free of bacterial growth for up to 96 hours of refrigerated storage in NICU settings. Sample handling techniques are important for preventing microbial contamination.

Keywords: donor human milk | donor milk | storage | fortified

Article:

What Is Known

- Unfortified Holder pasteurized donor human milk remains free of microbial growth for up to 96 hours of refrigerated storage in a laboratory setting.
- Neonatal intensive care units in the United States routinely fortify human milk for very low birth weight infants.

What Is New

- Unfortified and fortified Holder pasteurized donor human milk remains largely free of microbial growth for up to 96 hours of refrigerated storage in a neonatal intensive care unit setting, with appropriate handling protocols.
- Growth is sporadic in nature, represents common skin bacteria, and does not increase with time.

Mother's milk is the ideal source of nutrition for premature infants, providing benefits in host defense, gastrointestinal maturation, infection rate, neurodevelopmental outcomes, and long-term cardiovascular and metabolic disease [(1)]. The American Academy of Pediatrics (AAP) recommends that mother's own milk, fresh or frozen, appropriately fortified, should be the primary diet of enteral feeds for very low birth weight (VLBW) infants [(2)]. If mother's own milk is unavailable, despite significant lactation support, pasteurized donor milk is considered the most physiologic alternative [(2–4)].

The use of donor milk in United States neonatal intensive care units (NICUs) is increasing, with over 65% of level 3 and level 4 NICUs reporting some use in a 2015 Center for Disease Control survey [(5)]. The Human Milk Banking Association of North America (HMBANA) recently extended their refrigerated storage recommendations for Holder pasteurized donor human milk (HPDHM) from 24 to 48 hours, based primarily on evidence of the storage of unfortified HPDHM [(6–9)].

Fortification of human milk for the preterm infant is recommended by the AAP [(2)] and is common practice in the United States, with over 90% of NICUs reporting that they use human milk fortifiers [(5)]. A 2018 literature review on the refrigerated and frozen storage of HPDHM found only a single study that had assessed storage of fortified HPDHM [(10)]. Given that HPDHM is routinely fortified for preterm infants, the objective of this study was to assess microbial growth in both unfortified and fortified HPDHM over 96 hours of refrigerated storage in "real life" NICU settings. We hypothesize that unfortified and fortified HPDHM, because of inherent antimicrobial properties, will maintain microbiological purity for 4 days of refrigerated storage in the NICU.

METHODS

This was an observational, descriptive study designed to determine the bacterial content in HPDHM after extended storage for 96 hours at 4ºC in a human milk refrigerator for all patients located in a level IV NICU. The study was approved by the IRB at New York Medical College and Westchester Medical Center.

All neonates with birth weight ≤1500 g and gestational age (GA) ≤32 weeks, patients with congenital gastrointestinal (GI) or cardiac anomalies, and patients recovering from NEC receive

exclusive human milk diets (EHM). An EHM consists of mothers expressed breast milk (EBM), donor milk (when EBM is unavailable), and fortification with a human milk-derived fortifier, which is added when the patient is tolerating 80 mL · kg^{-1} day^{-1} of enteral feeds and adjusted based on the patient's growth. This diet is maintained until 34 weeks corrected GA after which we switch to a bovine milk-derived fortifier, and donor milk is replaced with preterm formula. All other infants receive preterm or term formula as a supplement or full diet and EBM is fortified with a bovine milk fortifier.

Samples were collected at Maria Fareri Children's Hospital (Valhalla, NY), in a dedicated human milk preparation room by a trained milk technician. Bottles of HPDHM were obtained from the New York Milk Bank (Valhalla, NY), licensed by the New York State Department of Health and accredited by HMBANA. HPDHM is dispensed from HMBANA banks only if postpasteurization cultures demonstrate no bacterial growth. The milk arrives at hospitals frozen and is stored in the freezer at −20ºC until needed. In this study, 100 mL bottles of HPDHM were thawed per our NICU protocol and aliquoted for individual patients daily. If indicated, fortification was done using human milk-derived human milk (HUM) fortifier (Prolacta Bioscience, City of Industry, CA) or bovine-derived (BOV), sterile human milk fortifier (Enfamil Human Milk Fortifier Acidified Liquid, Mead Johnson, Chicago, IL). HPDHM was fortified either in the fortifier manufacturer's container (Prolacta), sterile specimen cups (Sterile Specimen Container, Medegen Medical Products), or aseptic airtight, leak-resistant containers (Snappies, Mead Johnson, Chicago, IL), depending on the volume needed. Clean techniques were used when preparing infant feeds including: practicing hand hygiene; wearing clean gowns, caps, masks, and gloves; disinfecting counters; and using measuring containers, which are supplied in sealed sterile packages.

Prepared HPDHM was stored at 4ºC in the NICU refrigerator. In the first 24 hours, every 3-4 hours nurses removed milk for feedings using a sterile syringe and fresh gloves. After the feeding removal, unused HPDHM was placed back in the refrigerator until the next feeding. HPDHM containers were accessed 6 to 8 times on average by 2 or 3 different nurses in the first 24 hours of storage. After 24 hours in the refrigerator, HPDHM was considered “expired” for clinical use. For the study, the first 1 mL sample, designated as time 0, was removed immediately after preparation of the HPDHM using a sterile syringe and clean gloves and placed into a sterile study container for transfer to the microbiology laboratory. Beyond the 24-hour refrigeration, the bottles of “expired” HPDHM continued to be stored in the NICU refrigerator for 4 days in a locked, fenestrated box to ensure exposure to the same storage environment. Bottles were accessed only for the purpose of drawing additional study samples. One milliliter samples were removed at 24, 48, 72, and 96 hours. Each sample was removed using a new sterile syringe, sterile gloves, and placed in a sterile container. During the study time (96 hours), it was estimated that the milk room refrigerator was opened dozens of times daily by different nurses. The milk room countertop was disinfected at each step of milk handling.

All samples were transported to a Biosafety Level 2 (BSL-2) microbiology laboratory in sterile containers in a cooler for plating on standard (5%) Sheep's Blood Agar medium. The study is divided into 2 phases based on the movement of the microbiology lab, which resulted in the use of 2 different microbiological approaches. In the first, samples were plated on a freshly disinfected laboratory benchtop (BENCH) employing standard aseptic microbiological

techniques. In the second, samples were plated inside a biosafety cabinet equipped with vertical laminar airflow (LAMINAR) to eliminate background contamination.

Samples were cultured in duplicate per HMBANA guidelines. Briefly, 0.1 ml of HPDHM was placed on plates that were then incubated for 48 hours at 34°C. The number of colonies grown on each plate was enumerated by a colony counter. Cultures were classified based on the number of CFUs per the HMBANA standard operating procedure of: 0 (accept); 1 to 5 CFUs (retest); 6+ (reject). Colonies were characterized by morphology, hemolytic pattern, Gram-staining, cellular morphology, and pertinent biochemical tests. To ensure that the microbiological laboratory environment, or the sheep blood agar plates themselves were not contributing to the number of growing bacterial colonies in these experiments, 2 control plates were "mock" plated without milk using a sterile disposable spreader and incubated along with the milk samples.

Statistical analysis was performed using SAS 9.4 Enterprise Edition (SAS Software, Cary, NC). *P* value 0.05 or less was considered statistically significant. Samples were categorized by the number of timepoints that showed any growth (timepoint 0–4; growth = yes/no). Differences in categorical data were evaluated using a Fisher exact test. A mixed model was used to analyze repeated measures of the same sample for the main effects of time (0, 24, 48, 72, and 96 hours) and fortifier type.

RESULTS

The 113 bottles of HPDHM included in this study reflect the clinical need based on our census and established feeding protocol. When sporadic bacterial growth was observed in BENCH samples, timepoint 0 was added to the study protocol to determine if bacteria were introduced into the HPDHM by the milk technician. Therefore, each bottle had between 4 (when timepoint 0 was not done) and 5 timepoints, for a total of 521 timepoints assessed. Baseline data (at time 0) was not available for 44 of the samples collected during BENCH culturing. As stated previously, each timepoint was cultured in duplicate (except for 2 time points), for a total of 1040 test cultures, along with 80 background control cultures. BENCH culturing, which occurred between June and August 2016, included 73 bottles of HPDHM, and LAMINAR culturing, which occurred in January 2017, included 40 bottles. Table 1 contains a summary of the study samples.

Table 1. Summary of types of Holder pasteurized donor human milk included in the study

Type of HPDHM	June to August 2016	January 2017
No. HPDHM samples	73	40
No. timepoints	321	200
No. culture	640	400
Biosafety level of culture Lab and plating environment	BSL-2 disinfected benchtop	BSL-2 laminar hood
Unfortified samples	16 (22%)	20 (50%)
HUM fortified samples	55 (75%)	0 (0%)
BOV fortified samples	2 (3%)	20 (50%)

BOV = bovine-derived fortifier; BSL = biosafety level; HPDHM = Holder pasteurized donor human milk; HUM = human-derived fortifier.

Results by Lab Environment

Forty-two percentage (47/113) of the samples had no cultures exhibiting growth at any timepoint, 29% (33/113) had growth in 1 timepoint, 12% (13/113) had growth in 2 timepoints, 12% (13/113) had growth in 3 timepoints, and 6% (7/113) had growth in 4 timepoints. There was no growth observed in control samples. Of the 519 timepoints cultured in duplicate, 76% (395/519) had no growth in either culture, 7% (36/519) had growth in 1 of the 2 plates, and 17% (88/519) had growth in both plates. Results were compared between BENCH and LAMINAR because of the different plating environments. There was a significant difference ($P < 0.0001$) in the prevalence of samples that had no growth at any time point between BENCH (20/73, 27.4%) and LAMINAR (27/40, 67.5%). BENCH had a higher rate of samples with 2 or more timepoints exhibiting growth than LAMINAR (32/73, 43.8% vs 1/40, 2.5%; $P < 0.0001$).

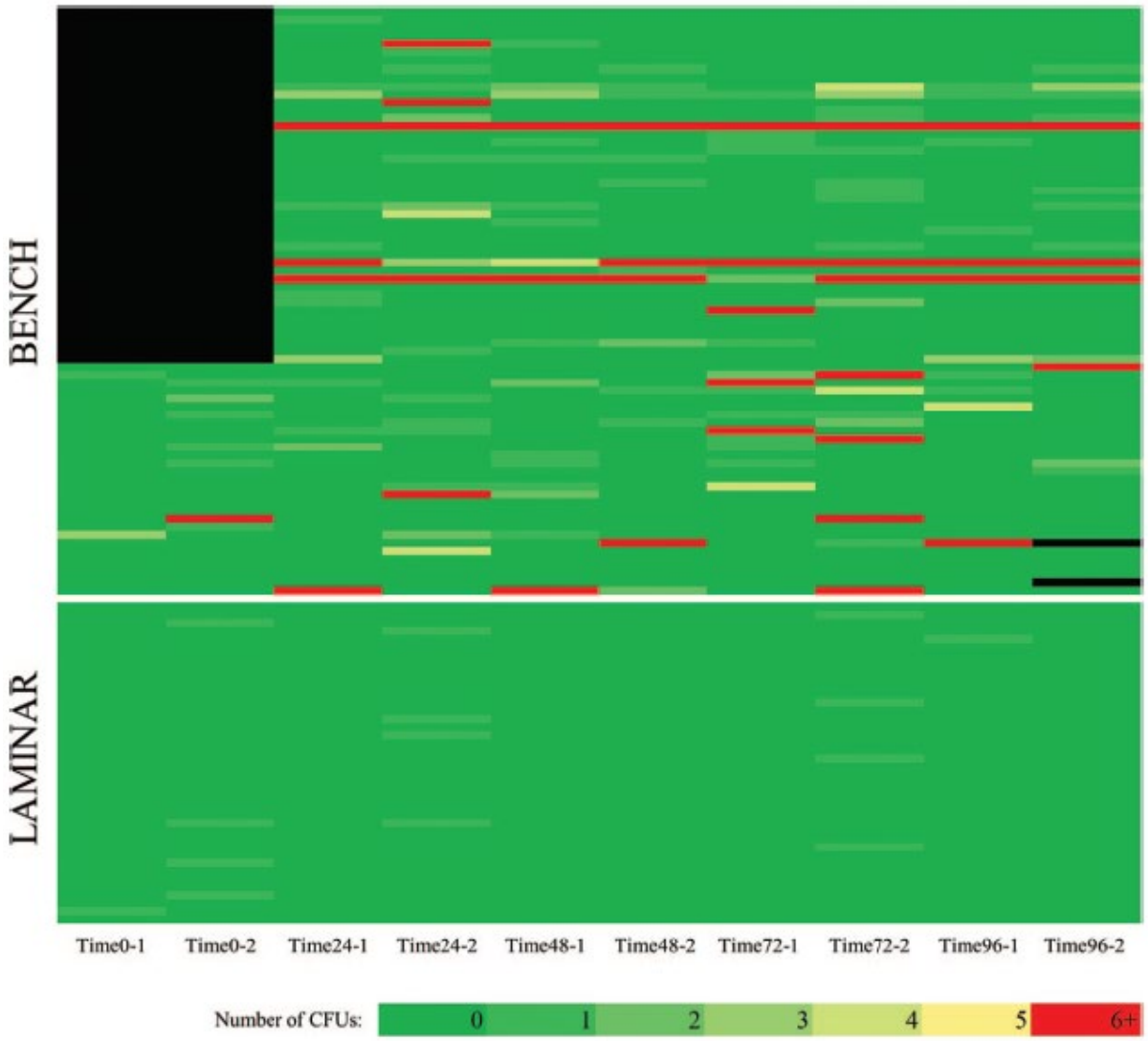

Figure 1. Heat map of duplicate cultures at 0, 24, 48, 72, and 96 hours for 113 samples of Holder pasteurized donor human milk. BENCH—data represent 73 samples collected between June and August 2016 and cultured on a disinfected benchtop in a BSL-2 lab. LAMINAR—data represent 40 samples collected during January 2017 and cultured in a laminar flow cabinet in a BSL-2 lab. CFU—colony forming unit per 0.1 mL of sample; black shading—no data available. Each line represents a sample in duplicate over time.

There were 0 CFUs in 85% (885/1040) of the cultures tested. Eleven percentage (118/1040) of cultures grew 1 to 5 CFUs, and 4% (37/1040) of cultures grew 6 or more CFUs. Only 1/113 (0.09%) samples grew 6 or more CFUs on all cultures at all timepoints. One sample (0.09%) grew 6 or more CFUs on duplicate cultures at 3 time points. And 1/113 (0.09%) sample grew 6 or more CFUs on duplicate cultures at 2 time points. There was a significant difference ($P <$ 0.001) in the percentage of cultures exhibiting any growth between BENCH (141/640, 22.0%) and LAMINAR (14/400, 3.5%). A heat map representing all study cultures by phase is presented in Figure 1.

To determine if the laboratory environments were associated with different outcomes, we performed a sub-analysis on only the unfortified samples. Overall, there were 344 cultures from unfortified HPDHM and 11% (37/344) exhibited growth. The prevalence of unfortified cultures exhibiting growth in BENCH was 20% (29/144) compared with 4% (8/200) in LAMINAR, which was statistically significant ($P < 0.0001$).

Microbiological identification was done on 40 representative colonies, based on Gram stain, morphology, hemolysis, catalase test, mannitol test, TSI test: 30/40 (75%) colonies grew *Staphylococcus epidermidis*. Six of 40 (15%) colonies grew *Staphylococcus aureus*. Four of 40 (10%) colonies grew Gram-positive/variable Bacilli. No Gram-negative organisms were identified.

Effects of Fortifiers

The 2 different culture environments (BENCH vs LAMINAR) were also associated with the use of different fortifier types (neutral HUM vs acidic BOV), therefore, the primary comparison we made was between unfortified and fortified samples within each unique culture environment (Table 2). Within BENCH, there was no difference in the distribution of samples by the number of timepoints with growth between fortified and unfortified samples (P =0.7035). The percentage of samples with 2 or more timepoints exhibiting any growth was 43.8% (25/57) for fortified samples and 43.8% (7/16) for unfortified samples. There was a statistically significant difference between the unfortified and fortified BENCH samples in the distribution of cultures by CFU classification ($P = 0.0029$), with unfortified samples having a higher prevalence of cultures with 6+ CFUs (discard) (10.4% vs 4.4%, respectively) and a lower prevalence of cultures containing 1 to 5 CFUs (retest) (9.7% vs 18.2%, respectively). Within LAMINAR samples, there was no difference in the distribution of samples by the number of timepoints containing any growth ($P =$ 0.3008) or the distribution of cultures by CFU classification ($P = 0.7868$) between fortified and unfortified samples. There was no growth in 96.5% of the cultures in LAMINAR, and the maximum growth on any 1 culture was 1 CFU. Additionally, all cultures that showed growth in the LAMINAR environment had no growth on their duplicate culture plates. These results are summarized in Table 2.

Effects of Time Within Phases

The proportion of samples exhibiting any growth at each 24-hour interval is summarized in Table 3 by culture technique. There was no observed difference in samples with any growth

by time for either fortified or unfortified samples in BENCH or LAMINAR ($P > 0.24$). When assessing the number of CFUs per culture using a repeated measures analysis to account for multiple cultures over 96 hours, neither time nor fortifier status were significant for BENCH or LAMINAR ($P > 0.05$).

Table 2. Proportion of samples by number of timepoints with any growth, and number of cultures by range of colony forming units within each study phase

	Unfortified	Fortified	*P* value
BENCH			
No. HPDHM samples (73 total)	16	57	
Samples by number of timepoints with growth			0.7035
0 timepoints w/ growth	6 (37.5)	14 (24.6)	
1 timepoint w/ growth	3 (18.8)	18 (31.6)	
2 timepoints w/ growth	2 (12.5)	10 (17.5)	
3 timepoints w/ growth	4 (25.0)	9 (15.8)	
4 timepoints w/ growth	1 (6.3)	6 (10.5)	
Number of cultures (640 total)	144	496	
Number of cultures by number of CFUs			0.0029
0 CFUs (accept)	115 (79.9)	384 (77.4)	
1 to 5 CFUs (retest)	14 (9.7)	90 (18.2)	
6+ CFUs (reject)	15 (10.4)	22 (4.4)	
LAMINAR			
No. HPDHM samples (40 total)	20	20	
Samples by number of timepoints with growth			0.3008
0 timepoints w/ growth	12 (60.0)	15 (75.0)	
1 timepoint w/ growth	8 (40.0)	4 (20.0)	
2 timepoints w/ growth	0 (0.0)	1 (5.0)	
3 timepoints w/ growth	0 (0.0)	0 (0.0)	
4 timepoints w/ growth	0 (0.0)	0 (0.0)	
No. cultures (400 total)	200	200	
No. cultures by number of CFU			0.7868
0 CFUs (accept)	192 (96.0)	194 (97.0)	
1 to 5 CFUs (retest)	8 (4.0)	6 (3.0)	
6+ CFUs (reject)	0 (0.0)	0 (0.0)	

Date represent quantities (%) of samples and cultures with growth. Differences in categorical classifications were evaluated using a Fisher exact test. BENCH represents samples cultured on a disinfected benchtop of a BSL-2 lab (June to August 2016); 55 samples were fortified with a human-derived fortifier and 2 samples were fortified with a bovine-derived fortifier. LAMINAR represents samples cultured in a laminar airflow cabinet of a BSL-2 lab (January 2017); the 20 fortified samples were all fortified with a bovine-derived fortifier. CFU = colony forming unit per 0.1mL of sample; HPDHM Holder pasteurized donor human milk.

Table 3. Prevalence of growth at each 24-hour interval by fortifier type and study phase

	Unfortified	Fortified	*P* value
BENCH			
Total number of HPDHM samples	16	57	
Total number of timepoints	73	248	
Timepoints with growth, n (%)	23 (31.5)	89 (35.9)	0.577
Prevalence of any growth by time			>0.24
Time 0	0 (1.4)	8 (3.2)	
Time 24	8 (11.0)	21 (8.5)	
Time 48	5 (6.9)	19 (7.7)	
Time 72	6 (8.2)	24 (9.7)	
Time 96	3 (4.1)	17 (6.9)	
LAMINAR			

Total number of HPDHM samples	20	20	
Total number of timepoints	100	100	
Timepoints with growth, n (%)	8 (8.0)	6 (6.0)	0.783
Prevalence of any growth by time			>0.24
Time 0	4 (4.0)	1 (1.0)	
Time 24	1 (1.0)	3 (3.0)	
Time 48	0 (0.0)	0 (0.0)	
Time 72	2 (2.0)	2 (2.0)	
Time 96	1 (1.0)	0 (0.0)	

Data represent quantities (%) of timepoints with any bacterial growth. Differences in categorical classifications were evaluated using a Fisher exact test. In BENCH (June to August 2016), samples were cultured on a disinfected benchtop and 55 samples were fortified with a human-derived fortifier and 2 samples were fortified with a bovine-derived fortifier. In LAMINAR (January 2017), samples were cultured under a laminar airflow hood and the 20 fortified samples were all fortified with a bovine-derived fortifier.

DISCUSSION

Our study is the first to address the combined effects of extended refrigeration storage and fortification with both bovine and human milk-derived fortifiers in "real life NICU" not laboratory settings. In our study, we observed sporadic growth of common skin bacteria in unfortified and fortified HPDHM. Importantly, we observed higher bacterial growth when culturing on a benchtop than when culturing under a laminar hood. Using a disinfected laboratory benchtop, 5.7% (37/640) of the cultures contained 6 or more CFUs, which would be indicative of milk discarding as per HMBANA guidelines, whereas none (0/400) of the cultures under a laminar hood exhibited this magnitude of bacterial growth. Cohen et al reported culturing HPDHM under sterile conditions and finding no growth in unfortified HPDHM at 24 to 122 hours postthawing, but 33% (2/6) of HPDHM with a powdered bovine fortifier were bacterial culture-positive [(7)]. Vickers et al [(8)] and Meng et al (9) who studied bacterial growth during storage in a laboratory setting, support Cohen's finding of no bacterial growth in unfortified HPDHM for 4 to 7 days of refrigerated storage.

It has been reported that powdered infant formulas may expose infants to pathogenic bacteria [(11)]. The fortifiers used in this study were liquid fortifiers, which may explain the lower rate of positive cultures we observed in BENCH (112/496, 23%) and LAMINAR environments (6/200, 3%) as compared with those reported by Cohen et al (2/6, 33%), where a powdered fortifier was used. In addition, this study was a time line investigation where the majority of bacterial growth was not systemic, but sporadic and apparently unrelated to the longer storage times. Meng et al reported that bacteria introduced into HPDHM through exposure to the microbiota in the infant mouth through cup or bottle feeding decreased during refrigerated storage [(9)], which is likely because of antimicrobial proteins that are partially preserved during Holder pasteurization [(12)]. This may explain why the bacterial growth that we observed in our study was sporadic in nature and did not increase with time.

In a benchtop environment, 56% of the samples (41/73) had growth at 1 or fewer time points, and the maximum growth density was too many CFUs to be effectively counted. In contrast, in a laminar flow hood, 97.5% of the samples (39/40) had growth in 1 or fewer time points, and the maximum growth density was 1 CFU. The distinct results observed between the 2 culture environments warrants some discussion. The lower growth rates seen in LAMINAR could be the result of the different laboratory culture environments where a laminar flow hood would reduce

external sample contamination. In addition, in LAMINAR an acid fortifier was added, which could also aid in reduction of bacterial growth. When limiting our analysis to just the unfortified samples, which were used in both the BENCH and LAMINAR study arms, we observed a significant difference in the percentage of cultures with any growth between BENCH (20%, 29/144) and LAMINAR (4%, 8/200), which was statistically significant ($P < 0.0001$), suggesting that the laboratory plating environments may have contributed to the observed differences between phases. It is also possible that different milk technicians and nurses during the 2 study periods were handling the milk differently, thus contributing to differences that were observed. It is reassuring that even if bacteria might be introduced during handling, they did not demonstrate a continuous consistent or increasing growth patterns.

Information on the impact of fortification on other characteristics of HPDHM during prolonged storage is limited and is an important area of future research. Donovan et al [13] studied the impact of an acidic and a neutral bovine-based fortifier on mother's milk and HPDHM over 24 hours of refrigerated storage and reported changes in protein, lipase activity, pH, and osmolality. More research is warranted into how different fortifier types impact the nutrients, bioactive factors, and physical properties of HPDHM. Although our study suggests HPDHM inhibits bacterial growth in both unfortified and fortified samples during 96 hours of refrigerated storage, evidence is emerging that retort-processed shelf-stable human milk has significantly lower retention of antimicrobial proteins than HPDHM; therefore, more research is warranted into appropriate storage of human milk products that have been pasteurized using techniques other than Holder pasteurization [14,15].

Limitations

The use of 2 different laboratory plating environments is a limitation of this study. We could not compare results between BOV and HUM fortifiers because of the use of different plating environments; therefore, more research is warranted to see if HUM fortifiers produce low rates of bacterial growth when cultured under a laminar hood. After 24 hours of storage, the samples were only accessed once a day, thus greatly reducing the multiple nurse-exposure points for additional study days. Most samples cultured on a benchtop did not have a baseline measurement, at time 0, thus, 1 could not establish growth levels at the time the HPDHM was considered appropriate for clinical use. Where such baseline data was available, all samples were cultured under a laminar hood and there was very limited and sporadic growth of common skin bacteria and neither fortifier status nor storage time were significantly associated with bacterial growth. As molecular typing was not used, we cannot speculate on the source of the observed bacterial growth.

CONCLUSIONS

This is the first large-scale study of the storage of fortified HPDHM in a clinical setting and contributes to the growing body of evidence regarding the refrigerated storage of HPDHM. Our research suggests that unfortified and fortified Holder pasteurized donor human milk can remain free of pathogenic bacterial growth, with limited presence of common skin microbiota, for up to 96 hours of refrigerated storage when appropriate handling techniques are used in the feeding preparation room and laboratory. Our study supports the recently updated HMBANA guidelines

for extending refrigeration storage time of defrosted HPHDM to 48 hours [6]. More research is needed into how fortification type and storage time impact other factors in Holder pasteurized donor human milk including bioactive factors, micronutrients and macronutrients, and physical properties of the milk. More research is also warranted regarding the impact of fortification and prolonged refrigerated storage on shelf-stable human milk products.

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