Study Protocol
ClinicalTrials.gov Study ID: NCT03123874

Title: Milk in Life Conditions (MiLC): Bacterial Composition of Human Milk Pumped and Stored in "Real-Life" Conditions

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Internal Review Board at Cornell University
Unique Protocol ID: 1608006566**.**
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Study Protocol
Milk in Life Conditions (MiLC) Trial

Background and Objective: Women use a wide range of practices for pumping their milk (1-4). The effects of these practices on the bacterial composition of the milk are unclear. The objective of the MiLC trial was to test the effect of women's own pumping practices on the bacterial content of their milk compared to aseptic collection.

Study Design: The MiLC trial will be a randomized, controlled cross-over trial of 2 breast pump set-ups: mother's own and sterile. Mother's own pump set-ups will consist of the mother's own electric pump and own collection kits, cleaned at home using her usual practices. Sterile pump set-ups will consist of a multi-user electric pump (Medela Symphony) and a sterile collection kit. The pump each woman will use first will be randomized using stratified randomization based on infant feeding status (human milk only *v*. human milk and complementary foods).

Recruitment: We will recruit women in Ithaca, NY and the surrounding area. Participants will be recruited on a continuous basis through flyer advertisements at pediatric clinics, day-care centers, graduate-student housing at Cornell University, and Ithaca College, in WIC offices, and in cafes and local children's boutiques. Posters will briefly state the purpose of the study and that participation is voluntary and anonymous. We will also use snowball, or word-of-mouth, sampling, whereby recruited participants may refer other potential participants to the study. We will also advertise by word-of-mouth at La Leche League meetings. Written informed consent will be obtained according to the study protocol approved by the Institutional Review Board at Cornell University (Unique Protocol ID: IRB #: 1608006566).

Inclusion/exclusion criteria: We will include lactating women over the age of 18 years who: i) self-report as healthy, ii) pump their milk with an electric pump, iii) are confident of their ability to donate 1 oz of milk from one breast during each of two consecutive pumping sessions where pumping sessions are 3 hours ± 30 minutes apart and between 0700-1100 hours, iv) are able to store their donated milk at home for 30 days, v) describe their infants as being healthy, and vi) have infants who do not consume formula or only consume formula episodically as long as the most recent formula-feeding occurred > 2 weeks before the day their mothers pump for this study. We will *exclude* women who: i) are not confident of their ability donate 1 oz of milk from one breast during each of two consecutive pumping sessions, 3 hours ± 30 minutes apart and between the hours of 0700-1100 hours, ii) have an infant who has consumed formula in the past 2 weeks, iii) have current indication of breast infection (e.g., breast pain, discomfort, lumps, mastitis with fever, red streaks, or hard red portions of the breast), iv) have breast pain that the woman does not consider "normal" for lactation/breastfeeding, and v) self-reports that she or her infant showed signs/symptoms of acute Illness in the past 7 days including: fever (rectal or temporal temperature ≥99.5 ºF), dark green nasal discharge, diarrhea (abrupt onset of 3 or more excessively "loose" stools in one day), vomiting (where infant vomiting is not associated with feeding), or severe cough.

Sample Collection: Lactating women (n=50) will donate 1 oz of pumped milk during each of two consecutive pumping sessions, for a total volume of 2 oz donated on one day. Women will be asked to fully express one breast during each pumping session. Women will pump once with

their own pumps and bottles and once with the sterile pump and sterile bottles (provided by the research team). Women will be randomized to which pump they will use first. Randomization will be done using stratified randomization by infant feeding status (human milk only vs. human milk and complementary foods). All pumping sessions will occur at participants' homes between 0700 and 1100 hours. The second pumping session must begin 3 hours ± 30 minutes after the beginning of the first pumping session (e.g. the first pumping session at 7:30 am and the second at 10:30 am). Women will elect from which breast they donate their milk and that breast will be used for both pumping sessions. Women will be asked not to nurse from or pump that breast during the 2 hours before to the first pumping session and not until after the second pumping session (a total of ~5.5 hours). From the milk produced during each pumping session (which could be up to ~6 oz), we will collect 1 oz using a sterile, plastic syringe. Women will keep the remaining volume of milk. Each ounce of milk collected will be separated into 5 sterile containers that we will provide. Women will store donated milk at their home until it is picked up by a researcher 2, 4, and 30 days after pumping.

Bacterial Composition of Human Milk: The bacterial content of milk will be assessed via aerobic culturing and high-throughput sequencing of the bacterial 16S rRNA gene from DNA extracted from the milk. Total aerobic bacterial counts will be reported as colony-forming unit (CFU)/mL estimated using standard methods. Sequence data from 16S rRNA analysis will be reported as bacterial community richness and diversity using observed taxa (amplicon sequence variants) and the Shannon diversity index, respectively.

Sample size calculations: Inasmuch as the variability of our outcomes differs widely, we decided to base our sample size calculations on 16S rRNA data from our bacterial community analysis because these data are more variable than those from culture-related assays. However, there is not sufficient evidence on which to base sample size calculations. We are aware of one on-going multi-site study in which each site has 40 participants (5). We have sufficient resources to analyze the milk of 50 women. This sample size is also within the range of most culture-based investigations; thus, this will be our target sample size.

Outcomes:

- **Bacterial Community Richness:** Richness is the total number of different bacterial taxa (amplicon sequence variants) detected in the sample. This metric will be assessed on data derived from high-throughput sequencing of the bacterial 16S rRNA gene present in milk.
- **Bacterial Community Diversity:** Bacterial community diversity will be assessed using the Shannon diversity index. The Shannon diversity index is a type of entropy measure and is a function of the distribution of the total number of organisms across all of the species. If S is the total number of species in the sample and p_i is the number of organisms in the i-th species divided by the total number of organisms, then Diversity = $-\Sigma\ p_i \log(p_i)$. This metric will be assessed on data derived from high-throughput sequencing of the bacterial 16S rRNA gene present in milk.
- **Total Live Aerobic Bacterial Counts:** Number of live total aerobic bacteria in milk assessed by aerobic culturing of milk on plate count agar. Reported as colony-forming units (CFU)/mL.

Statistical Analysis: Statistical analyses will be based on our primary hypothesis that the bacterial content of expressed human milk pumped with mothers' own pump set-up differs from the bacterial content of expressed human milk pumped with a sterile pump set-up. To test this hypothesis, we will use linear mixed effects regression. Our 3 primary outcomes (e.g., bacterial counts, observed taxa, and Shannon diversity index) will serve as response variables in separate models. For all models, the fixed effect of interest will be pump set-up (own *v.* sterile pump set-up). In addition, models will be adjusted for covariates relevant to our study design, namely infant feeding status (human milk feeding only *v.* human milk and complementary foods) and randomization schedule (which pump each woman used first). For all models, the random effect will be participant ID to account for multiple observations per participant.

References

1. Felice JP, Geraghty SR, Quaglieri CW, Yamada R, Wong AJ, Rasmussen KM. "Breastfeeding" without baby: A longitudinal, qualitative investigation of how mothers perceive, feel about, and practice human milk expression. *Matern Child Nutr* 2017;13(3):e12426.
2. Felice JP, Geraghty SR, Quaglieri CW, Yamada R, Wong AJ, Rasmussen KM. "Breastfeeding" but not at the breast: Mothers' descriptions of providing pumped human milk to their infants via other containers and caregivers. *Matern Child Nutr* 2017;13(3):e12425.
3. Labiner-Wolfe J, Fein SB, Shealy KR, Wang C. Prevalence of breast milk expression and associated factors. *Pediatrics* 2008;122(suppl 2):S63-8.
4. Labiner-Wolfe J, Fein SB. How US mothers store and handle their expressed breast milk. *J Hum Lact* 2013;29(1):54-8.
5. McGuire MK, Meehan CL, McGuire MA, Williams JE, Foster J, Sellen DW, Kamau-Mbuthia EW, Kamundia EW, Mbugua S, Moore SE, et al. What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically. *Am J Clin Nutr* 2017;105(5):1086-100.