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Bacterial Analysis of Refrigerated Human Milk Following Infant Feeding

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BACTERIAL ANALYSIS OF REFRIGERATED HUMAN MILK FOLLOWING INFANT FEEDING

A THESIS
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by
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Contents

Dedicated to my Mother,
Dolores Rae Harrison,
without whom I would not be here

and to my Husband,
Fred Brusseau,
who sold his beloved Jeep,
so I could afford to be here.

Acknowledgements

This project would not have been possible without the help of various people. First of all I'd like to thank my daughter Emily for giving me the idea to do the project. She provided great motivation to stay interested in the project, and incentive to always represent the data correctly and completely. Thank you to my family for watching Emily at odd times of the day and night when I had to come into the lab and work on this project. Thank you to the LACTNET members at www.lactnet.com. This forum of 1600 International Board Certified Lactation Consultants (IBCLC) provided me with a great opportunity to gather practical input for the project. Thank you to Leroy Maki my distant cousin, and retired microbiology professor, who helped me interpret and understand my results. Lastly, thank you to Dr. Gary Hanson, who always had an open door when I needed it. Sometimes I tease him about his constant reminders, but it was this reminding that helped me stay on schedule with this immense project.

SUMMARY

The number of infants who are breastfed is on the rise, as is the number of women in the workforce. Many women who choose breastfeeding after returning to work, express milk during the day and store this milk for a future feeding. When infants do not finish a bottle of expressed breastmilk, doctors recommend unfinished portions be thrown away. This study examined bacterial levels in expressed, partially consumed breastmilk that was stored for 48 hours at 4-6° C. A portion of unconsumed milk was examined as a control. Samples were taken every 12 hours for bacterial analysis. Tests were performed to identify total colony counts, pathogenic Staphylococci, coliforms and β-hemolytic Streptococci. This study showed no significant difference between bottles that were partially consumed and those that were not exposed to the baby's mouth for 5 out of 6 participants. All milk samples had colony counts in the acceptable range of $< 10^5$ colony forming units per milliliter (CFU/ml). Although this project provides evidence that it may be safe to refeed a child a bottle of breastmilk, due to the small sample size, further tests should be performed.

INTRODUCTION

The American Academy of Pediatrics (AAP) identifies breastfeeding as the ideal method of feeding and nurturing infants and recognizes breastfeeding as primary in achieving optimal infant and child health, growth, and development. Research provides strong evidence that human milk feeding decreases the incidence and/or severity of several health problems including diarrhea (Dewey 1995), lower respiratory infection (Wright 1995), ear infection (Aniansson 1994), bacterial meningitis (Istre 1985), botulism (Aron 1984), urinary tract infection (Pisacane 1992), and necrotizing enterocolitis (Covert 1995). Breastfeeding has also been related to possible enhancement of cognitive development (Wang 1996).

Breastfeeding also provides significant economic benefits to both parents and the nation. It has been estimated that the 1993 cost of purchasing infant formula for the first year after birth was $855 (Montgomery 1997). Through the WIC program, this expense is passed along to tax payers (Tuttle 1996). Equally important, breastfeeding could contribute to reduced health care costs and reduced employee absenteeism for care attributable to child illness.

Many women choose to stop breastfeeding when they return to work. In the December 1997 issue of *Pediatrics*, the AAP encouraged working mothers to pump and store the breastmilk instead of supplementing with formula. Significant research has been done in regards to the safe pumping and storage of breastmilk. It has been shown that breastmilk contains microorganisms similar to those found on the skin of a nursing mother, such as "coagulase-negative Staphylococci which make up about 87% of the skin's flora" (Skinner, 1978). It has been shown that bacterial counts of human milk stored in the refrigerator decrease significantly when stored for 72 hours (Barger 1987). Unfortunately, no research has been done regarding bacterial levels in breastmilk that has been expressed, partially consumed, and then stored for a later feeding. Health officials recommend throwing out human milk that has been partially consumed (Kaiser 1997). Many working mothers find it challenging to keep up their milk supply when they are using breast pumps. This problem is compounded when previously collected breastmilk is thrown out.

It was this study's intention to find out if there is a difference in the amount and types of bacteria found in milk that is stored in the refrigerator after partial infant feeding.

MATERIALS AND METHODS

This study followed the bacterial growth rate of human milk that was expressed, stored in a 4-6° C refrigerator for 12-36 hours, warmed to 37° C, partially fed to an infant and then stored in the refrigerator for 48 hours. It was important to emulate realistic storage conditions, therefore clean, but not aseptic techniques were followed during expression, initial storage and feeding. However, standard aseptic techniques were followed during plate preparation and bacterial analysis.

Collection: Six women ages 17-26 with breastfed babies age 1-9 months participated in the study. One week prior to the study, women were given a packet which included detailed instructions (Appendix A), an Informed Consent Form (Appendix B), a questionnaire (Appendix C), two 8-ounce clean bottles with lids, a small cooler with ice, and a thermometer.

Women were instructed to express 6-8 ounces of breastmilk 12-36 hours before coming into the laboratory to feed to their infant. Milk was collected by electric breast pumps. Before pumping, women were instructed to wash their hands in hot water and

soap for one minute. The bottles provided to the women in their kits were cleaned by a dishwasher, because this is the method many women use to clean bottles. The breasts were not sanitized prior to collection because previous studies have shown cleaning the breast prior to collection does not decrease the amount of bacteria found in the milk (Thompson 1997), and because it did not fit "real life" criteria. There are conflicting reports as to whether discarding the first 5 ml of expressed milk will decrease the total amount of bacteria present in human milk (Asquith 1979 and West 1979). I chose to use the entire sample of milk because most women use it all for future feedings. The milk was stored 12-36 hours in a 4-6° C refrigerator. The participants used a small cooler with ice pack for transport to the laboratory the next day.

Feeding: After arrival at the laboratory, the expressed breastmilk was poured into two clean, 4-ounce bottles and warmed in a 37° C hot water bath for 10-20 minutes. Each bottle was labeled E for experimental or C for control. Individual participants were assigned a letter, A - F. A clean nipple was placed on all bottles. The experimental bottle was fed to the infant for one minute, or until one ounce was gone. The control bottle was not fed to the infant. Plastic nipple covers were placed on the bottle before transport down the hallway to the lab for storage and culturing.

Plate Preparation, and Bacterial Analysis: One day before the study all agar plates were prepared in accordance with the Difco Manual using 100 mm disposable plastic petri dishes. All plates were labeled with the participant's code letter (A - F), the milk sample used [control (C) or experimental (E)], time of collection (0, 12, 24, 36 or 48), type of media [plate count (Pc), 5% sheep blood (Bl), mannitol salt (Mn) or MacConkey (Mc)], and amount of milk plated (10 μl or

Figure 1. Sample Petri Dish Label

12 A E Bl 100 μl

Key:
12 = Milk sample was plated 12 hours after baby ate.
A = Participant A
E = Experimental Sample
Bl = 5% Sheep Blood Agar Plate
100 μl = 100 μl of milk plated

100 μl). Figure 1 shows a sample label. Plates were stored in a 4° C refrigerator until use.

Storage: All bottles (control and experimental) were stored at 4° C for a total of 48 hours. Cultures from all bottles were analyzed at 0, 12, 24, 36 and 48 hours post-feeding. Bottles were removed one at a time for analysis. Each bottle was inverted 25 times, or until the milk was homogeneous. Care was taken to return each bottle to the refrigerator as quickly as possible (<2 minutes).

Colony Counts: Tryptone glucose extract agar (TGEA) was used to perform colony counts. This media is used by the dairy industry to do standard plate counts (Richardson, 1985). 1:10 and 1:100 serial dilutions of all milk were plated onto correspondingly labeled plates. Plates were incubated under aerobic conditions at 35° C for 48 hours (Difco, 1969).

Mannitol Salt Agar Plates with 7.5% NaCl and Phenol Red indicator: 100 μl of milk was pipetted and distributed onto correspondingly labeled plates. 100 μl was used, because it should identify bacteria that are present in concentrations of 10 CFU/ml or higher. Plates were incubated under aerobic conditions at 37° C for 36 hours.

MacConkey Agar Plates: 100 μl of milk was pipetted and distributed onto correspondingly labeled plates. Plates were incubated under aerobic conditions at 37° C for 16-18 hours.

5% Sheep Blood Agar Plates: 100 μl of milk was pipetted and distributed onto correspondingly labeled plates. Plates were incubated under aerobic conditions at 37° C for 36 hours.

Previous microbiological studies with human milk incubated plates in 10% CO_2 conditions (Jocson 1997), 5% CO_2 conditions (El-Mohnades 1993), and aerobic conditions (West 1979 and Pardou 1994). Because of the expense of equipment needed and scope of the study, I chose to incubate all plates in aerobic conditions.

To aid in the removal of plates from the incubator, a table was created and posted on the incubator (Appendix D).

RESULTS

Results of the Questionnaire are recorded in Table 1.

Table 1 - Questionnaire Results

	Average (n = 6)	Range
Age of Mother (years)	22.8	17-26
Child's Age (months)	5.9	2-10
Amount of Water Mother Drinks Per Day (8 ounce Glasses)	3.5	0-8
Amount of Sleep Mother Gets Per 24-hour Day (hours)	6.0	4-8
Amount of Times Per Day Child is Breastfed	5.5	2-10
Time Milk was Refrigerated Prior to Feeding (Hours)	22.5	12-36
Total Amount of Milk Collected (Ounce)	6.2	6-8
Temperature Milk was Stored at Prior to Feeding (°C)	5.3	4-6
Mothers who Take Prenatal Vitamins	50 %	
Mothers who smoke Cigarettes	16.6%	
Mothers on Medications	16.6% (antibiotics)	
	16.6 % (thyroid medication)	
Mothers with Current Breast Infections	16.6%	
Mothers who Exercise	16.6 %	
Infant's Crawling	33.3%	
Infant's Walking	16.6%	
Infants Supplemented with expressed breastmilk	50 % yes	
Infants Supplemented with formula	50 % yes	
Infants Supplemented with Other foods	66.6 %	
Infants with Recent Illness	50 % (flu, stuffy nose, reflux in lungs)	

Education Completed	33 % High School	33 % Some College	33% College
Income Bracket	16.6 % <25000	50 % $25,001-$50,000	16.6 % $50,000-100,000

Results of the microbiological analysis are as follows.

Plate Count Agar: Colony counts on the TGEA plates were performed using a Quebec colony counter. The 10 μl plates (1:100 dilution) were counted first. Each colony on the 10 μl plates represents 100 live bacterium per one ml of milk, commonly referred to as CFU/ml. If the 10 μl plate had less than 25 colonies, then the corresponding 100 μl plate was counted. Each colony on the 100 μl plates represents 10 individual bacterium in one milliliter of milk. Average CFU/ml in the control and experimental milk were compared for each time period. T-tests were performed to establish if there was a significant difference in the amount of bacteria found before the child drank the milk and after. The average CFU/ml and results of the t-tests can be found on the next page in Table 2. The numbers in Table 2 were calculated by two methods. First, all of the participants were averaged together, and CFU/ml were calculated for *each* time period. Second, individual participants CFU/ml were calculated using *all* time periods. Figure 2 shows the average trend of

bacterial growth in control and experimental milk over time. All participants CFU/ml were averaged together when making this graph. Figure 3, on the next pate, shows each individual's average CFU/ml. More detailed counts and t-test calculations can be found in Appendix E & F.

Table 2 - Average Total Bacteria Counts Before and After Infant Feeding

Time (post-feeding)	Milk Before Feeding (mean CFU/mL)	Milk After Feeding (mean CFU/mL)	t-value	Critical t-value $\alpha = .10$	Significantly Different?
Analysis by Individual Storage Times					
0 hours	11,900	12,137	0.15	2.13	no
12 hours	10,665	13,355	1.43	2.13	no
24 hours	12,032	13,627	1.78	2.13	no
36 hours	12,053	12,472	0.42	2.13	no
48 hours	12,347	12,062	-0.16	2.13	no
Analysis by Individual Participants					
Participant A	810	1,000	1.28	2.02	no
Participant B	1,028	980	-.037	2.02	no
Participant C	618	762	1.96	2.02	no
Participant D	11,920	9,320	-0.12	2.02	no
Participant E	26,180	25,880	-0.12	2.02	no
Participant F	30,240	36,040	3.62	2.02	yes

Figure 2 - Average of All Participants Total Bacteria Counts Before and After Infant Feeding

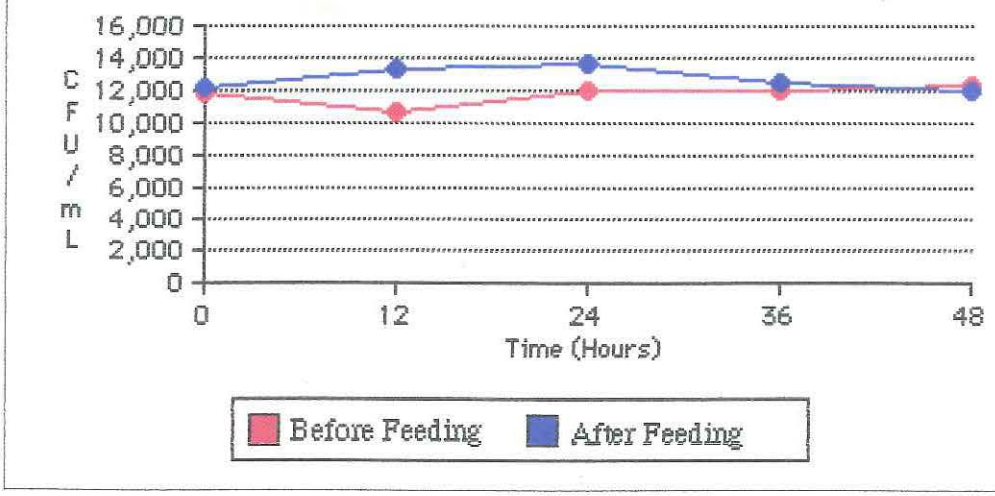
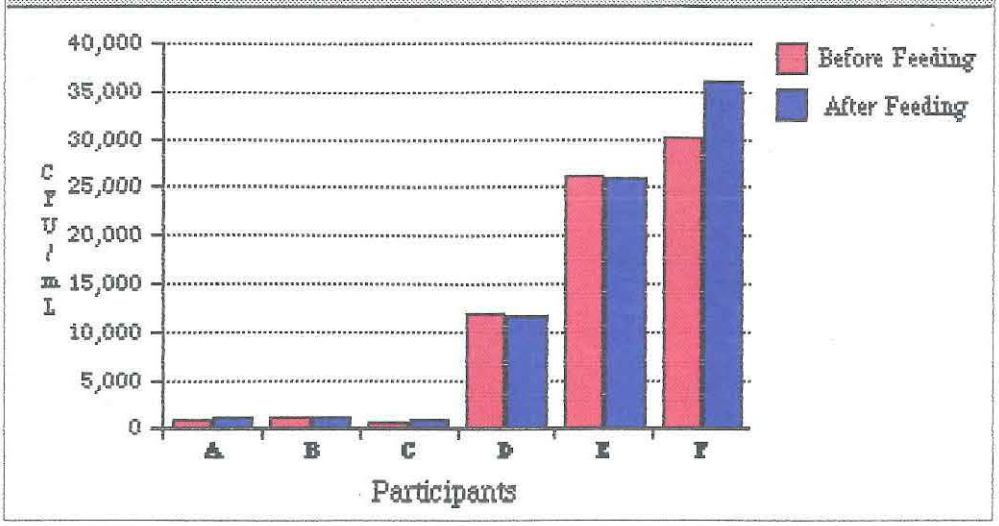


Figure 3 - Average of Total Bacteria Counts Before and After Infant Feeding



Mannitol Salt Agar Plates: Any colony with a yellow ring around it was considered a positive mannitol fermenter (Difco, 1969). 11 out of the 30 mannitol salt plates inoculated with milk the infant had partially eaten were positive for mannitol fermentation. Similarly, 11 out of the 30 mannitol salt plates inoculated with control milk were also positive for mannitol fermentation. See Table 3 for results of the mannitol fermentation test. Colonies within the yellow zones were gram stained and tested for coagulation and catalase activity. All colonies tested were coagulase-negative, catalase-positive, gram-positive cocci.

Table 3 - Number of Mannitol Plates with Positive Mannitol Fermentation

Storage Time (hours)	0 C	0 E	12 C	12 E	24 C	24 E	36 C	36 E	48 C	48 E
Participant A	0	1	0	0	1	1	1	1	1	1
Participant B	0	1	0	0	0	0	0	0	0	0
Participant C	0	0	0	0	0	0	0	0	0	0
Participant D	1	0	0	0	0	0	1	1	0	0
Participant E	1	1	1	1	1	1	1	1	1	1
Participant F	0	0	0	0	0	0	1	0	0	0
SUM	**2**	**3**	**1**	**1**	**2**	**2**	**4**	**3**	**2**	**2**

SUM of all Control (C) 11
SUM of all Experimental (E) 11

MacConkey Agar Plates: Any pink or red colony grown on MacConkey agar was considered a positive lactose fermenter (Figure 4). 5 out of the 30 MacConkey plates

inoculated with milk were positive lactose fermenters. See Table 4 for results of lactose fermentation. Gram staining was performed on all morphologically different colonies. All colonies were gram-negative rods, with exception of the dark purple colonies, which were gram-positive cocci. Oxidase tests were performed on all gram-negative rods. Red, pink and bright purple colonies were catalase negative. Enterotube II tubes were inoculated with these organisms. Organisms were identified as Klebsiella pneumoniae, Acinatobacter sp., and Escherichia coli respectively.

Figure 4 - MacConkey Plate

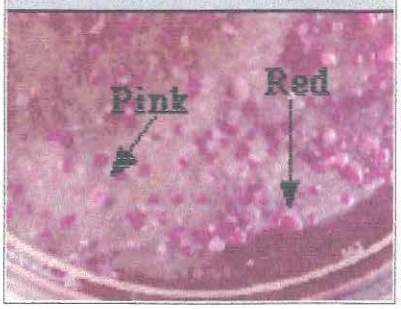


Table 4 - Number of Plates with Positive Lactose Fermentation on MacConkey Agar

Storage Time (hours)	0 C	0 E	12 C	12 E	24 C	24 E	36 C	36 E	48 C	48 E
Participant A-C	0	0	0	0	0	0	0	0	0	0
Participant D	1	1	0	0	0	0	0	0	0	0
Participant E	0	1	1	0	0	0	0	0	0	0
Participant F	0	0	1	0	0	0	0	0	0	0
SUM	**1**	**2**	**2**	**0**	**0**	**0**	**0**	**0**	**0**	**0**

Control Milk = C
Experimental Milk = E

5% Sheep Blood Plates: All of the blood agar plates had various colonies growing on them, however no hemolytic zones were formed around any of the colonies. A β-hemolytic bacteria (*Staphylococcus aureus*) from the laboratory culture stock was plated onto one of the blood agar plates. It showed clear zones of β-hemolysis.

DISCUSSION

Plate Count Agar: To determine if the total bacteria levels I found in the study were safe, I need to first define "safe". Currently, "no agreed-upon guidelines exist regarding the acceptable microbiological quality of collected human milk" (El-Mohandes, 1993). The human milk banking industry does have standards, but they are very strict, because most of

the milk from human milk banks is fed to pre-term infants with compromised immune systems (Hamosh, 1996).

I found varying requirements for "safe" milk. Three examples are: 10^3 CFU/ml with no enteropathogens (Sauve 1984), 10^5 CFU/ml excluding *Staphylococcus aureus*, group ß Streptococci, Pneumonocci or coliforms (Tyson 1982), and 2.5 X 10^4 CFU/ml with no enterobacteria, (except non-lactose-fermenting enterobacteria), with *Staphylococcus aureus* levels below 1.0 X 10^3 CFU/ml (Williamson 1978).

With exception of participant F there was no change in total bacteria found in expressed human milk that has been partially fed to an infant. Where I did see variance was between the individual participants. For example both the control and experimental milk from participants A-C ranged from 600-1,000 CFU/ml, while participants D-F ranged from 12,000 to 36,000 CFU/ml (Figure 3). It is interesting to note that due to personal scheduling problems, participants A and B's experiment was performed two days prior to C, D, E and F. It is also interesting to note that C, D, E and F's code letters were assigned randomly and did not reflect the order in which any tests were performed.

Since participants E-F were showing a significantly higher amount of growth than participants A-C I examined the questionnaire to see if I could find any common factors among each group. Due to small sample size it was not possible to statistically analyze the information, but I could not find anything outstanding that participants A-C did differently from participants E-F. For example both groups had children that were crawling and eating solids. The general health and average sleep amongst mothers was consistent throughout. In future studies I would suggest the use of a more detailed questionnaire to help identify the sources of variance.

According to Margit Hamosh, an accomplished human milk researcher, the "great individual variations among lactating women" found in my study was similar to other studies (Hamosh, 1998). A 1987 study by Jan Barger illustrates this point. The study showed expressed, refrigerated milk to have an average of 2000 CFU/ml with a range of 0-113,000 CFU/ml. This variation can be explained with a variety of reasons.

There may be several sources of contamination, including mothers' hands,

nasopharyngeal secretions, breast skin flora, and distal milk ductules as well as collection and storage equipment (El-Mohanas, 1993). In attempt to reduce contamination from the hands and nose I asked the mothers to wash their hands prior to collection. In regards to the skins natural flora, studies have shown that cleaning the breast with Phisoderm prior to pumping does not decrease the amount of bacteria in the milk when compared to breasts cleaned with water alone (Thompson, 1997). Since I did not have the women rinse their breasts with water maybe milk from a previous feeding remained on the breast, or bra, which contaminated the results. A probable source of contamination could be due to the collection technique. Perhaps the breast pumping equipment was not clean. Due to the difficulty of cleaning the hollow, bent collection devise, it is possible that the apparatus was not thoroughly sanitized between uses.

Mannitol Salt Agar: The mannitol salt agar was used to identify the possible presence of *Staphylococcus aureus*. The high salt content inhibits gram-negative organisms, and many gram-positive organisms other than staff. Many Staphylococci ferment mannitol, therefore the second coagulase test was done to confirm the presence of *S. aureus*, which also forms a β-hemolytic zone on 5% sheep blood agar. Since all samples taken from the yellow areas of the mannitol salt agar plates were coagulase-negative, and because none of the blood agar plates showed β-hemolytic zones, I will conclude that *S. aureus* was not present in any of the samples. Since this test's intent was to rule out the presence of *S. aureus*, and not to identify every organism in the milk, I did no further testing with these plates.

5% Sheep Blood Agar: Since the 5% sheep blood agar did not show any hemolytic zones, I will also conclude that there were no β-hemolytic Streptococci in any of the samples.

MacConkey Agar: MacConkey agar is used for detection and isolation of coliforms and enteric pathogens. It inhibits gram-positive organisms. Participants A-C had zero growth at all time periods for both the control and experimental milk. Participants D-F had a lactose fermenting enterobacteriaceae, commonly known as coliforms.

To sum up my results I found total average bacterial counts of 1.2 X 10^4 CFU/ml, with no *Staphylococcus aureus*, or group ß Streptococci. Two of the participants had the presence of coliforms. Although high bacterial levels, and high coliform levels were found in the milk, it is important that they were found in the control sample as well as the experimental sample. This study showed high variability among participants, but no significant difference between the quality or quantity of bacteria found in breastmilk that has been partially consumed.

It would be interesting to see how bacterial counts would be affected if the nipple were stored off of the bottle, the bottle were stored at room temperature, the milk were previously frozen before fed to child, and the milk were warmed again before plating. It would also be interesting to see how bacterial counts in stored, used human milk compared to stored, used infant formula. The major flaw in this experiment was the small sample size. I cannot be confident that the control and experimental samples were actually statistically similar, or just appeared that way because of the low number of participants.

The most important lesson we can learn from this data is that in spite of high bacterial levels found both control and partially consumed milk, none of the babies became ill. This provides some evidence that different standards need to be made for healthy full-term infants. "The rationale for less stringent recommendations for storage of a mother's own milk that is fed to her (own) healthy, full-term infant ... is that the microorganisms are probably less hazardous than the organisms from an unrelated donor, because a mother secretes antibodies in her milk that reflect her own immunologic experience" (Hamosh 1996). It is believed that protection is provided by secretory IgA that mothers produce in their milk against potential pathogens in their gastrointestinal tracts (Narayanan, 1981). In fact while gastrointestinal intolerance and infection have been associated with expressed human milk that contains greater than 10^3 to 10^6 CFU/ml (Botsford, 1986), other researchers have not found any adverse effects when milk with the same high levels of bacteria was ingested (Law, 1989).

Appendix A - Letter to Participant

Dear Participant,

First of all let me thank you very much for taking part in this study. Your willingness to participate may shed light on the question at hand "Is it safe to store breastmilk in the refrigerator after partial infant feeding?". This paperwork includes an explanation of your role in the study, instructions regarding your participation, a questionnaire and a consent form. You should also have received a "cooler kit". This kit contains a thermometer, two clean 8 ounce bottles with lids, an ice pack and a carrying case. Use of these materials is explained below.

Please read all instructions before participating in the study. Completely fill out the attached questionnaire and consent form. Review your "cooler kit" contents, and place the enclosed ice pack in your home freezer.

On the day of the experiment, please bring with you: Completed paperwork, baby (a little hungry), and 6-8 ounces of expressed milk. I have a large variety of clean nipples. They include orthodontic, standard, latex and silicon. If your baby uses a Platex nurser, please call me, because I am limited on these supplies, but I can arrange for their availability.

Instructions for expression: Please express 6 - 8 ounces of breastmilk 12 - 36 hours before arriving. (That would be after 9 PM on Friday, but before 9 PM on Saturday.) Please wash hands for one minute in hot soapy water before expressing. Express in your normal manner, and note method on questionnaire. Use enclosed bottles for expression. "Double pumpers" can use both bottles provided. Just combine the milk into one bottle before storing in the refrigerator. Single pumpers can just use one bottle.

Please do whatever needed to get 6 - 8 ounces of breastmilk. This would include pumping both breasts, or multiple pumping sessions within the 12-36 hour window. Note time and date of collection, and store the milk in your refrigerator until Sunday morning. I have enclosed a thermometer (° C), because I would like you to measure the temperature of your refrigerator. Just write down the temperature on the enclosed questionnaire. While in transit to Concordia, please place milk into cooler with ice pack.

Please arrive at Concordia University on Sunday, March 8th at 9 AM. Meet in Luther Building, Room 316. (See attached map) You can park in the west parking lot.

What to expect on Sunday: When you and your baby arrive, we will place the eight ounce bottle of expressed breastmilk into a warm water bath for 5-10 minutes, or until 98° F. While waiting, baby will be given two sterile swabs for saliva collection. Once heated, the bottle will be shaken and split between two sterile four ounce bottles. Clean nipples will be placed on both bottles. One bottle will be fed to baby for one minute, or until one ounce is consumed. The other bottle will be used as a control. Both bottles will be capped and refrigerated. Bacterial analysis will be performed over the next 4 days.

Thank you in advance for your participation.

Sincerely,
Rachel R. Brusseau
[redacted]

Appendix B - Informed Consent Form

Informed Consent Form

I, _____, agree to participate in this
(your name here)
research project on safe bottle feeding. Specifically the study will attempt to determine how long it is safe to store breast milk in a bottle that has been partially consumed. I agree to express breastmilk at my home 12 - 36 hours before the study. I agree to bring my child to Concordia University Laboratory at 9:00 am on March 8, 1998. I will feed my child 1 ounce of previously expressed breastmilk. Rachel will keep the remainder of the milk for analysis. I understand that participating in this study in no way infers that I do not feed my infant safely. Participation merely provides the samples and data needed for analysis. I understand there is no risk to my child or myself to participate in this study. Rachel Brusseau has offered to answer any questions I may have regarding the study and my role in it. I understand at no time during the study will my name be used in in connection with the results. All personal data and results will be kept confidential. I understand that my participation is voluntary and that I am free to withdraw from the project at any time. I will be provided with a final copy of the paper in Spring of 1998.

I have read the above information and agree to take part in this study.

Participants's Name: _____ (last) , _____ (first)

Signature of Participant:_____ Date: ___/___/___

Signature of Legal guardian : _____
(if Participant is under 18 years of age)

Address: _____ (Street) _____ (City) ____ (State) _____ (Zip)

Child's Name: _____ (first) Child's birth date: ___/___/___ (mon day yr)

Phone Number: _____ (Daytime) , _____ (Evening)

Best Time to Call:_____

If you have any questions about this study please contact Rachel Brusseau at:

[redacted]

Appendix C - Questionnaire

Questionnaire

Let me remind you that the information you provide here will be kept completely confidential. It will only be used to help analyze and interpret the results within the context of the experiment. At no time will your name be connected with the results, or any information provided on this survey. I realize that some of the questions may seem personal, or irrelevant. I assure you that each question *is* necessary for proper interpretation of this study, so please answer them as truthfully as possible. When completed, please seal questionnaire in envelope provided, and return to me on Sunday, March 8th.

Information about milk collection:

How was the milk expressed? [examples: electric double pump, electric single pump, battery operated pump, manual pump, manually (no pump), or other (if other please specify)] _____

Date of collection:_____ Time of collection: _____(AM or PM)

Amount of milk collected:_____ (ounces) Temperature of Refrigerator:_____ ° C

Information about you:

Your Name: _____Your Age:_____years

Is your education level: Less than High School, High School, Some college, College, Beyond College? (Please circle appropriate answer).

Is your yearly household income bracket: under $25,000; $25,001-$50,000; $50,000-$100,000; above $100,000? (Please circle appropriate answer).

Do you usually eat very nutritious, somewhat nutritious, somewhat non-nutritious or very non-nutritious foods? (please circle appropriate answer)

Do you have a high fat, average fat, low fat or non-fat diet? (please circle appropriate answer)

Do you exercise? _____If yes, how much per week? _____

On average, how many glasses of water do you drink a day? _____

On average, how much sleep do you get at night? _____

Do you take vitamins? _____ If yes, what kind? _____

Do you smoke? _____ If yes, how much per day? _____

Are you taking any medications or drugs? _____ If so, what? _____

Have you recently had a breast infection? _____ If so, when? _____

Appendix C (cont) - Questionnaire

Information about your child:

Is your child crawling? _____

Is your child walking? _____

How many times does you child breastfeed a day? _____

Do you supplement with expressed breastmilk? _____ If so, how much per day? _____

Do you supplement with formula?_____ If so, how much per day? _____

Does your child eat foods other than breastmilk? _____ If so what? (eg. vegetables, cereal, meat, etc.) _____

Has your child recently had thrush? _____ If so, when? _____

Has your child had any other recent illnesses? _____ If so, what? _____

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end of survey

Appendix D - Removal From Incubator Chart

Plates To Be Removed From Incubator

Saturday, March 7, 1998	9:00 AM	A-B 0 Mc & Bl
Saturday, March 7, 1998	9:00 PM	A-B 0 Mn
Saturday, March 7, 1998	9:00 PM	A-B 12 Mc & Bl
Sunday, March 8, 1998	9:00 AM	A-B 0 Pc
Sunday, March 8, 1998	9:00 AM	A-B 12 Mn
Sunday, March 8, 1998	9:00 AM	A-B 24 Mc & Bl
Sunday, March 8, 1998	9:00 PM	A-B 12 Pc
Sunday, March 8, 1998	9:00 PM	A-B 24 Mn
Sunday, March 8, 1998	9:00 PM	A-B 36 Mc & Bl
Monday, March 9, 1998	9:00 AM	A-B 24 Pc
Monday, March 9, 1998	9:00 AM	A-B 36 Mn
Monday, March 9, 1998	9:00 AM	A-B 48 Mc & Bl
Monday, March 9, 1998	9:00 AM	C-F 0 Mc & Bl
Monday, March 9, 1998	9:00 PM	A-B 36 Pc
Monday, March 9, 1998	9:00 PM	A-B 48 Mn
Monday, March 9, 1998	9:00 PM	C-F 0 Mn
Monday, March 9, 1998	9:00 PM	C-F 12 Mc & Bl
Tuesday, March 10, 1998	9:00 AM	A-B 48 Pc
Tuesday, March 10, 1998	9:00 AM	C-F 0 Pc
Tuesday, March 10, 1998	9:00 AM	C-F 12 Mn
Tuesday, March 10, 1998	9:00 AM	C-F 24 Mc & Bl
Tuesday, March 10, 1998	9:00 PM	C-F 12 Pc
Tuesday, March 10, 1998	9:00 PM	C-F 24 Mn
Tuesday, March 10, 1998	9:00 PM	C-F 36 Mc & Bl
Wednesday, March 11, 1998	9:00 AM	C-F 24 Pc
Wednesday, March 11, 1998	9:00 AM	C-F 36 Mn
Wednesday, March 11, 1998	9:00 AM	C-F 48 Mc & Bl
Wednesday, March 11, 1998	9:00 PM	C-F 36 Pc
Wednesday, March 11, 1998	9:00 PM	C-F 48 Mn
Thursday, March 12, 1998	9:00 AM	C-F 48 Pc

Key

A, B, C, D, E, F = Participants
0, 12, 24, 36, 48 = Storage Time in Refrigerator
Pc = plate count agar, Mn = mannitol salt agar
Mc = MacConkey agar, Bl = blood agar

Appendix E - Colony Counts and t-test Calculations (Averages per Time)

Counts	**0 hr-E**	**0 hr-C**	**E - C**	**12 hr-E**	**12 hr-C**	**E - C**
Participant A	860	770	90	1400	790	610
Participant B	1,160	880	280	850	750	100
Participant C	1,400	1,350	50	1,180	750	430
Participant D	13,300	10,500	2,800	14,000	15,500	-1,500
Participant E	22,200	29,000	-6,800	24,300	18,300	6,000
Participant F	33,900	28,900	5,000	38,400	27,900	10,500
Calculations						
Σ	72,820	71,400	1,420	80,130	63,990	16,140
mean	12,137	11,900	237	13,355	10,665	2,690
Σ Differene^2			56,011			7,236,100
Standard Deviation (Sx)			3,971			4,597
Sx / Square Root of n			1,621			1,877
t-test=Average Difference / Sx / Square root of n			**0.1460**			**1.4334**
degrees of freedom			5			5
critical value α=(.05)			2.02			2.02

Counts	**24 hr-E**	**24 hr-C**	**E - C**	**36 hr-E**	**36 hr-C**	**E - C**
Participant A	980	610	370	870	710	160
Participant B	1,080	1560	-480	1,140	1,120	20
Participant C	700	570	130	420	340	80
Participant D	13,500	9350	4,150	7,000	7,850	-850
Participant E	27,000	22400	4,600	28,500	30,500	-2,000
Participant F	38,500	37700	800	36,900	31,800	5,100
Calculations						
Σ	81,760	72,190	9,570	74,830	72,320	2,510
mean	13,627	12,032	1,595	12,472	12,053	418
Σ Differences^2			2544025			175,003
Standard Deviation (Sx)			2197			2,438
Sx / Square Root of n			897			995
t-test=Average Difference / Sx / Square root of n			**1.7780**			**0.4203**
degrees of freedom			5			5
critical value α=(.05)			2.02			2.02

Counts	**48 hr-E**	**48 hr-C**	**E - C**
Participant A	890	1170	-280
Participant B	670	830	-160
Participant C	110	80	30
Participant D	10,800	16,400	-5,600
Participant E	27,400	30,700	-3,300
Participant F	32,500	24,900	7,600
Calculations			
Σ	72,370	74,080	-1,710
mean	12,062	12,347	-285
Σ Differences^2			81,225
Standard Deviation (Ss)			4,464
Sx / Square Root of n			1,822
t-test=Average Difference / Sx / Square root of n			**-0.1564**
degrees of freedom			5
critical value α=(.05)			2.02

Appendix F - Colony Counts and t-test Calculations (Averages per Participant)

Time (hours)	A After	Participant A Before	Difference	B After	Participant B Before	Difference
0	860	770	90	1160	880	280
12	1400	790	610	850	750	100
24	980	610	370	1080	1560	-480
36	870	710	160	1140	1120	20
48	890	1170	-280	670	830	-160
Sum	5000	4050	950	4900	5140	-240
Average	1000	810	190	980	1028	-48
Sum of Differences Squared			36100			2304
Standard Deviation			332			289
S.D. / Square Root of n			148			129
t=Av Dif / S.D. / Sq rt of n			**1.28**			**0.37**
degrees of freedom			4			4
critical value alpha=(.05)			2.13			2.13

Time (hours)	C After	Participant C Before	Difference	D After	Participant D Before	Difference
	1400	1350	50	13300	10500	2800
	1180	750	430	14000	15500	-1500
	700	570	130	13500	9350	4150
	420	340	80	7000	7850	-850
	110	80	30	10800	16400	-5600
Sum	3810	3090	720	58600	59600	-1000
Average	762	618	144	11720	11920	-200
Sum of Differences Squared			20736			40000
Standard Deviation			164			3847
S.D. / Square Root of n			73			1720
t=Av Dif / S.D. / Sq rt of n			**1.96**			**0.12**
degrees of freedom			4			4
critical value alpha=(.05)			2.13			2.13

Time (hours)	E After	Participant E Before	Difference	F After	Participant F Before	Difference
0	22200	29000	-6800	33900	28900	5000
12	24300	18300	6000	38400	27900	10500
24	27000	22400	4600	38500	37700	800
36	28500	30500	-2000	36900	31800	5100
48	27400	30700	-3300	32500	24900	7600
Sum	129400	130900	-1500	180200	151200	29000
Average	25880	26180	-300	36040	30240	5800
Sum of Differences Squared			90000			33640000
Standard Deviation			5428			3587
S.D. / Square Root of n			2427			1604
t=Av Dif / S.D. / Sq rt of n			**-0.12**			**3.62**
degrees of freedom			4			4
critical value alpha=(.05)			2.13			2.13

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