Bacterial Concentration and Composition in Human Breast Milk before and after Feeding with an Artificial Nipple

Anna WAKUI

Division of Clinical Chemistry,

Niigata University Graduate School of Health Sciences,

Niigata, Japan

哺乳瓶乳首を介して授乳する前後の搾乳母乳の細菌叢解析

涌井杏奈

新潟大学　大学院保健学研究科 検査技術科学分野

臨床化学研究室

ABSTRACT

Objectives: Breast milk is a valuable and useful source of nutrition; however, surplus milk is routinely discarded for hygiene reasons despite an unclear scientific basis. Here, we profiled the microbiota of expressed breast milk before and after feeding with an artificial nipple and examined the bacterial survival in breast milk stored at 4°C.

Methods: Eleven mother–baby pairs were included in the study. Samples of expressed breast milk were collected before and after feeding with an artificial nipple and examined both immediately (0 h) and after storage for 3 and 12 h at 4°C. Each sample was inoculated onto a blood agar plate and incubated anaerobically and aerobically at 37°C. Genomic DNA was extracted from individual bacterial colonies, which were identified by 16S rRNA gene sequencing.

Results: Before feeding, the bacterial counts at 0 and 12 h were $(1.4 \pm 1.6) \times 10^5$ colony-forming units (CFU)/mL and $(1.4 \pm 0.6) \times 10^5$ CFU/mL, respectively. *Staphylococcus* (47.7% and 41.9%, respectively), *Cutibacterium* (20.7% and 36.0%, respectively), and *Streptococcus* (16.1% and 6.6%, respectively) were identified among the samples. In contrast, after feeding, the bacterial counts at 0 and 12 h were $(2.7 \pm 1.7) \times 10^5$ CFU/mL and $(2.1 \pm 2.5) \times 10^5$ CFU/mL, respectively. *Staphylococcus* (30.1% and

37.4%, respectively), *Cutibacterium* (11.7% and 31.7%, respectively), and *Streptococcus* (41.5% and 25.2%, respectively), were identified among the samples.

Conclusions: Bacteria were present in the breast milk before feeding. Although the main component of the microbiota shifted from *Staphylococcus* to *Streptococcus* species after feeding, these results suggest that surplus expressed breast milk may be preserved safely in a refrigerator for at least 12 h after feeding with an artificial nipple.

Keywords: Bottle feeding, Breast milk, Microbiota

1. Introduction

Breast milk is widely known to be nutritionally and immunologically valuable and useful. However, in obstetric and gynecological wards, leftover expressed milk is routinely discarded for hygienic reasons, partly due to concerns of bacterial contamination from the oral cavity of infants, and the possible health risks to infants if they are fed the leftover expressed milk, but the scientific basis for this is unclear. It has been reported that freshly expressed milk can be stored in the refrigerator for up to 4 days [1], and that the levels of bacteria in freshly expressed breast milk collected before and after feeding remained constant during storage at 4°C for at least 6 days [2], suggesting that breast milk, even after feeding, can be safely preserved in terms of the bacterial content. However, the microbiota of leftover breast milk after feeding has not yet been profiled in detail.

In the present study, 16S rRNA sequence analysis was performed to profile the microbiota of breast milk before and after feeding with an artificial nipple. We also examined the survival of oral bacteria in breast milk immediately before and after feeding, and after storage to examine whether leftover breast milk can be safely stored in a refrigerator in terms of the bacterial content.

2. Materials and methods

2.1. Subjects

Eleven pairs of mothers (19 to 39 years old) and their babies (3 to 7 days old) were included in this study. All of the mothers and babies were considered to be healthy based on their medical history, and none of them had received antibiotics during the 3 months prior to sampling. Informed consent was obtained from all subjects and this study including bacteria sampled from human subjects was approved by the Research Ethics Committee of Niigata University, and Tohoku University Graduate School of Dentistry, Japan.

2.2. Sampling of expressed breast milk before feeding

The 11 mothers were asked to express milk from the breast using a breast pump. Samples (1.0 mL each) of the breast milk were collected immediately after expression (0 h) and after storage at 4°C for 3 and 12 h, and dispersed by vortexing.

2.3. Sampling of expressed breast milk after feeding

The 11 infants were fed the expressed breast milk of their own mother through an artificial nipple of a baby bottle. Samples of the remaining breast milk were collected

immediately after feeding (0 h) and after storage at 4°C for 3 and 12 h.

2.4. Sampling of infant saliva

Oral swabs were collected from the 11 infants as previously reported [3, 4]. Each sample was dispersed by vortexing.

2.5. Culturing of samples

Serial 10-fold dilutions (0.1 mL each) of the samples were prepared in sterilized 40 mM potassium phosphate buffer, and the diluted samples were spread onto the surfaces of CDC Anaerobe 5% Sheep Blood agar plates (BD, Franklin Lakes, NJ, USA) in duplicate, and incubated anaerobically (Anaero Pack anaerobic cultivation sets; Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) for 7 days, or in air for 3 days at 37°C. After incubation, the colony-forming units (CFU) were counted. Because the total CFU were higher after incubation under the anaerobic condition than under the aerobic condition, colonies for further inspection were selected only from plates that were incubated anaerobically. All colonies from suitably diluted plates containing <100 colonies (mean, 37.5; range, 4 to 48 colonies) were subcultured.

2.6. DNA extraction and identification of isolates by DNA sequence analysis

Genomic DNA was extracted from single colonies using the InstaGene Matrix Kit (Bio-Rad Laboratories, Richmond, CA, USA) according to the manufacturer's instructions. The 16S rRNA gene sequences were amplified by polymerase chain reaction (PCR) using universal primers 27F and 1492R, and *Taq* DNA polymerase (HotStar*Taq* Plus Master Mix Kit; Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The primer sequences were: 27F, 5'-AGA GTT TGA TCM TGG CTC AG-3'; and 1492R, 5'-TAC GGY TAC CTT GTT ACG ACT T-3' [3–8]. Amplification was conducted using a PCR Thermal Cycler MP (Takara Bio, Otsu, Japan) programmed as follows: 5 min at 95°C for the initial heat activation, then 30 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1.5 min at 72°C for extension, followed by 10 min at 72°C for the final extension. The PCR products were separated on 1% agarose gels (High Strength Analytical Grade Agarose; Bio-Rad Laboratories) in Tris-borate EDTA buffer (100 mM Tris, 90 mM borate, and 1 mM EDTA; pH 8.4), stained with ethidium bromide, and photographed under ultraviolet light. The sizes of the bands (approximately 1466 bp) were determined in comparison to molecular size markers (ExcelBand 100 bp DNA Ladder; Cosmo Bio, Tokyo, Japan).

The 16S rRNA genes were individually digested with *Hpa*II (FastDigest,

Fermentas; Cosmo Bio) according to the manufacturer's instructions. The digestion products were separated on 2% agarose gels as described above.

The isolates were tentatively identified according to restriction fragment length polymorphism analysis [6] as well as morphological data, *e.g.*, the colony appearance and Gram-staining results. Then, representative isolates were conclusively identified by sequence analysis [8, 11] as described below. The PCR products were purified with the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK), then sequenced at Fasmac (Atsugi, Japan) using a BigDye Terminator Cycle Sequencing Kit and an automated DNA sequencer (PRISM-3100; Applied Biosystems Japan, Tokyo, Japan). The primer 1492R was used for sequencing (at least 700 bp), and the partial 16S rRNA gene sequences were then compared by using the Basic Local Alignment Search Tool (BLAST) for searching the GenBank database on the National Center for Biotechnology Information website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Bacterial species were identified by the percent sequence similarity (>99%).

2.7. Statistical analysis

Dunn's test was employed to determine the statistical significance of the bacterial

amounts in the samples using statistical software (StatFlex, Ver. 6; Artech Co., Ltd., Osaka, Japan). *P*-values <0.05 were considered to be statistically significant.

3. Results

In the expressed breast milk collected before feeding at 0 h, the amount of bacteria was slightly higher after anaerobic incubation [$(1.4 \pm 1.6) \times 10^5$ CFU/mL] than after aerobic incubation [$(7.3 \pm 7.3) \times 10^4$ CFU/mL], although there was no significant difference (Table 1).

Facultative anaerobes comprised 69.9% and 80.0% of the breast milk microbiota at 0 h before and after feeding, respectively (Tables 2 and 3). Among the facultative anaerobes, *Staphylococcus* (47.7%) was predominant in the breast milk before feeding. In contrast, *Streptococcus* (41.5%) was predominant in the breast milk after feeding, followed by *Staphylococcus* (30.1%).

The mean amount of bacteria in the breast milk before feeding was $(1.4 \pm 1.6) \times 10^5$ CFU/mL at 0 h, and $(7.4 \pm 11.1) \times 10^4$ CFU/mL and $(1.4 \pm 0.6) \times 10^5$ CFU/mL after storage at 4°C for 3 h and 12 h, respectively (Table 1). *Staphylococcus* (47.7%), *Cutibacterium* (20.7%), and *Streptococcus* (16.1%) species were predominantly recovered from the samples before feeding at 0 h, followed by *Corynebacterium* (3.9%), *Prevotella* (3.4%), *Bifidobacterium* (2.6%), and *Actinomyces* (2.1%) species (Table 2).

In contrast, *Staphylococcus* (41.9%), *Cutibacterium* (36.0%), *Gemella* (9.6%), and *Streptococcus* (6.6%) species were predominantly recovered from the samples after storage at 4°C for 12 h (Table 5).

The mean amount of bacteria in the breast milk after feeding was (2.7 ± 1.7) × 10^5 CFU/mL at 0 h, and (3.0 ± 1.5) × 10^5 CFU/mL and (2.1 ± 2.5) × 10^5 CFU/mL after storage at 4°C for 3 h and 12 h, respectively (Table 1). *Streptococcus* (41.5%), *Staphylococcus* (30.1%), and *Cutibacterium* (11.7%) species were predominantly recovered from the samples after feeding at 0 h, followed by *Gemella* (3.3%), *Actinomyces* (2.4%), and *Prevotella* (2.1%) species (Table 4). In contrast, *Staphylococcus* (37.4%), *Cutibacterium* (31.7%), *Streptococcus* (25.2%), and *Neisseria* (0.8%) species were predominantly recovered from the samples after storage at 4°C for 12 h (Table 5).

The mean concentration of bacteria in the oral swabs was (4.1 ± 3.8) × 10^7 CFU/mL (Table 1). *Streptococcus* (57.3%), *Staphylococcus* (19.3%), and *Neisseria* (11.1%) species were predominantly detected from the oral swabs, followed by *Gemella* (4.0%), *Rothia* (3.3%), and *Cutibacterium* (2.0%) species (Table 4).

4. Discussion

In general, in obstetric and gynecological wards in hospitals, it is recommended that any leftover expressed breast milk be discarded for hygienic reasons; thus, little information has been reported to date on the bacteria in residual breast milk after feeding. Although human breast milk can be stored in the refrigerator for up to 4 days [1] or 6 days [2], the detailed microbiota profiles have not yet been clarified. In the present study, to obtain scientific data on the characteristics of the bacteria in human breast milk, analyses on the bacteria in breast milk were performed under aerobic and anaerobic culture conditions. The results showed that the bacterial levels in breast milk before feeding were similar immediately before feeding (at 0 h) and after storage at 4°C for 3 and 12 h (Table 1 and Figure 1). Similar findings were obtained in a study of liquid baby formula and a baby drink after storage at 4°C for 3, 12, and 24 h [3]. These findings suggest that human breast milk, as well as baby drinks, may be safely preserved in refrigerators at home for a certain period of time. The findings of the present study are in accord with those of previous studies on the preservation of bottled drinks, such as Japanese tea [7], and a sports drink and orange juice [8].

It has been reported that human breast milk contains substantial amounts of bacteria, and that the composition of the breast milk microbiota is similar over time (from shortly after delivery to 6 months after delivery) [9–11]. *Streptococcus,*

Staphylococcus, Gemella, Rothia, and *Veillonella* are the most predominant genera in human breast milk as well as in infant oral samples [12, 13]. The oral microbiota, especially *Streptococcus* species, of breast-fed infants has previously been characterized [14], and it has been reported that oral bacteria are transmitted from mothers to infants [15].

The concentration of bacteria was relatively higher in the oral swabs (Table 1) than in the breast milk samples collected after feeding, both immediately after feeding (0 h) and after storage at 4°C for 12 h (Table 1). Regarding the composition of the microbiota of breast milk (Tables 2 and 3), the proportion of *Streptococcus* species increased from 16.1% before feeding to 41.5% after feeding; this was likely due to the infiltration of oral (salivary) bacteria from the infant (Table 4, and Figure 1) [3, 14]. These results suggest that the oral microbiota of the infant may influence the composition of the microbiota of remaining human breast milk after feeding.

5. Conclusions

Bacteria, including *Staphylococcus*, *Cutibacterium*, and *Streptococcus* species, were found at a concentration of more than 10^4 cells/mL in the expressed breast milk before feeding. The main bacteria in the human breast milk samples shifted from

Staphylococcus to *Streptococcus* species after feeding. Nevertheless, the concentrations of bacteria after feeding were similar before (at 0 h) and after storage at 4°C for 3 h and 12 h, suggesting that leftover human breast milk after feeding may be preserved safely in a refrigerator at least for 12 h.

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Figure legends

Fig. 1 Comparison of the bacteria isolated from expressed human breast milk before and after feeding with an artificial nipple, and from infant oral swabs.

Table 1
Subjects and the bacterial concentration (CFU/mL) of each sample in this study

Subjects		1	2	3	4	5	6	7	8	9	10	11	Mean ± SD
Anaerobic culture													
Breast milk before feeding	At 0 h	1.4×10^5	3.1×10^5	1.4×10^5	5.3×10^5	1.4×10^4	1.1×10^5	2.6×10^5	8.0×10^3	1.6×10^4	5.4×10^3	5.4×10^4	$(1.4 \pm 1.6) \times 10^5$
	After storage at 4°C for 3 h	NT	NT	NT	NT	1.7×10^4	NT	2.4×10^5	1.4×10^4	2.5×10^4	NT	NT	$(7.4 \pm 11.1) \times 10^4$
	After storage at 4°C for 12 h	9.9×10^4	2.3×10^5	9.9×10^4	NT	NT	NT	NT	NT	NT	NT	NT	$(1.4 \pm 0.6) \times 10^5$
Breast milk after feeding	At 0 h	2.6×10^5	3.8×10^5	2.9×10^4	5.4×10^5	4.8×10^5	1.5×10^5	3.5×10^5	2.9×10^5	3.2×10^5	3.9×10^4	1.4×10^5	$(2.7 \pm 1.7) \times 10^5$
	After storage at 4°C for 3 h	NT	NT	NT	NT	5.1×10^5	NT	2.5×10^5	1.6×10^5	2.8×10^5	NT	NT	$(3.0 \pm 1.5) \times 10^5$
	After storage at 4°C for 12 h	1.0×10^5	5.0×10^5	3.8×10^4	NT	NT	NT	NT	NT	NT	NT	NT	$(2.1 \pm 2.5) \times 10^5$
Infant oral swab		5.0×10^7	4.1×10^7	2.0×10^7	6.1×10^7	5.9×10^7	2.1×10^7	7.2×10^6	2.0×10^5	3.0×10^7	1.4×10^8	2.1×10^7	$(4.1 \pm 3.8) \times 10^7$
Aerobic culture													
Breast milk before feeding	At 0 h	5.2×10^4	1.7×10^5	1.1×10^4	2.0×10^5	1.6×10^4	1.2×10^5	1.5×10^5	1.0×10^4	1.9×10^4	3.3×10^3	4.9×10^4	$(7.3 \pm 7.3) \times 10^4$
	After storage at 4°C for 3 h	NT	NT	NT	NT	2.0×10^4	NT	1.3×10^5	1.1×10^4	1.7×10^4	NT	NT	$(4.5 \pm 5.7) \times 10^4$
	After storage at 4°C for 12 h	4.6×10^4	1.8×10^5	1.6×10^4	NT	NT	NT	NT	NT	NT	NT	NT	$(8.1 \pm 8.7) \times 10^4$
Breast milk after feeding	At 0 h	8.1×10^4	3.0×10^5	8.0×10^4	1.6×10^5	4.7×10^5	1.2×10^5	3.3×10^5	1.9×10^5	4.2×10^5	7.2×10^4	1.4×10^5	$(2.2 \pm 1.4) \times 10^5$
	After storage at 4°C for 3 h	NT	NT	NT	NT	5.2×10^5	NT	2.4×10^5	1.3×10^5	5.3×10^5	NT	NT	$(3.6 \pm 2.0) \times 10^5$
	After storage at 4°C for 12 h	7.1×10^4	3.0×10^5	2.4×10^4	NT	NT	NT	NT	NT	NT	NT	NT	$(1.3 \pm 1.5) \times 10^5$
Infant oral swab		2.3×10^7	2.3×10^7	2.1×10^6	2.3×10^7	4.0×10^7	1.5×10^7	4.3×10^6	7.0×10^5	1.3×10^7	3.0×10^7	1.0×10^7	$(1.7 \pm 1.2) \times 10^7$

NT, not tested.

Table 2
Number of bacterial isolates from the human breast milk samples collected before feeding

Subjects	1	2	3	4	5	6	7	8	9	10	11	Total	(%)
CFU/mL	1.4×10^5	3.1×10^5	1.4×10^5	5.3×10^5	1.4×10^4	1.1×10^5	2.6×10^5	8.0×10^3	1.6×10^4	5.4×10^3	5.4×10^4	$(1.4 \pm 1.6) \times 10^5$	
Total number of isolates	39	48	48	46	14	46	26	11	16	48	44	386	100.0%
Anaerobes				23			1					24	6.2%
Prevotella												13	3.4%
P. nanceiensis				12									
P. jejuni				1									
Bifidobacterium												10	2.6%
B. longum				10									
Finegoldia												1	0.3%
F. magna							1						
Aerotolerant anaerobes	36		5	8		13		1		15	2	80	20.7%
Cutibacterium												80	20.7%
C. acnes	36		5	8		13		1		15	2		
Facultative anaerobes	3	48	40	14	13	28	25	10	16	32	41	270	69.9%
Staphylococcus												184	47.7%
S. aureus/epidermidis	3	48	8	11	10	14	17	10	16	7	40		
Streptococcus												62	16.1%
S. mitis/oralis/sanguinis/infantis			21		2	5				2			
S. parasanguinis/salivalius			11			8				13			
Gemella												1	0.3%
G. haemolysans/parahaemolysans						1							
Corynebacterium												15	3.9%
C. kroppenstedii				2			2				1		
C. tuberculostearicum										10			
Actinomyces												8	2.1%
A. neuii				1	1		4						
A. ihuae							2						
Unknown			3	1	1	5				1	1	12	3.1%

Table 3
Number of bacterial isolates from the human breast milk samples collected immediately after feeding.

Subjects No.	1	2	3	4	5	6	7	8	9	10	11	Total	(%)
CFU/mL	2.6×10^5	3.8×10^5	2.9×10^4	5.4×10^5	4.8×10^5	1.5×10^5	3.5×10^5	2.9×10^5	3.2×10^5	3.9×10^4	1.4×10^5	$(2.7 \pm 1.7) \times 10^5$	
Total number of isolates	44	48	31	48	45	33	35	28	28	39	40	419	100.0%
Anaerobes				9								9	2.1%
Prevotella												9	2.1%
P. nanceiensis				9									
Aerotolerant anaerobes	32			4	1	11					1	49	11.7%
Cutibacterium												49	11.7%
C. acnes	32			4	1	11					1		
Facultative anaerobes	12	47	23	26	42	22	34	28	27	38	36	335	80.0%
Streptococcus												174	41.5%
S. mitis/oralis/sanguinis/infantis	9		15		32	5				13	13		
S. parasanguinis/salivalius			4	4		11	6	17	16	22	2		
S. australis									5				
Staphylococcus												126	30.1%
S. aureus/epidermidis	3	43	4	16	7	6	18		6	3	20		
Gemella												14	3.3%
G. haemolysans/parahaemolysans					3			11					
Actinomyces												10	2.4%
A. neuii				3			5						
A. ihuae							2						
Corynebacterium												8	1.9%
C. kroppenstedii		1		3			2				1		
C. pyruviciproducens							1						
Neisseria												3	0.7%
N. perflava		3											
Unknown		1	8	9	2		1		1	1	3	26	6.2%

Table 4

Number of bacterial isolates from the oral swabs of the infants in this study

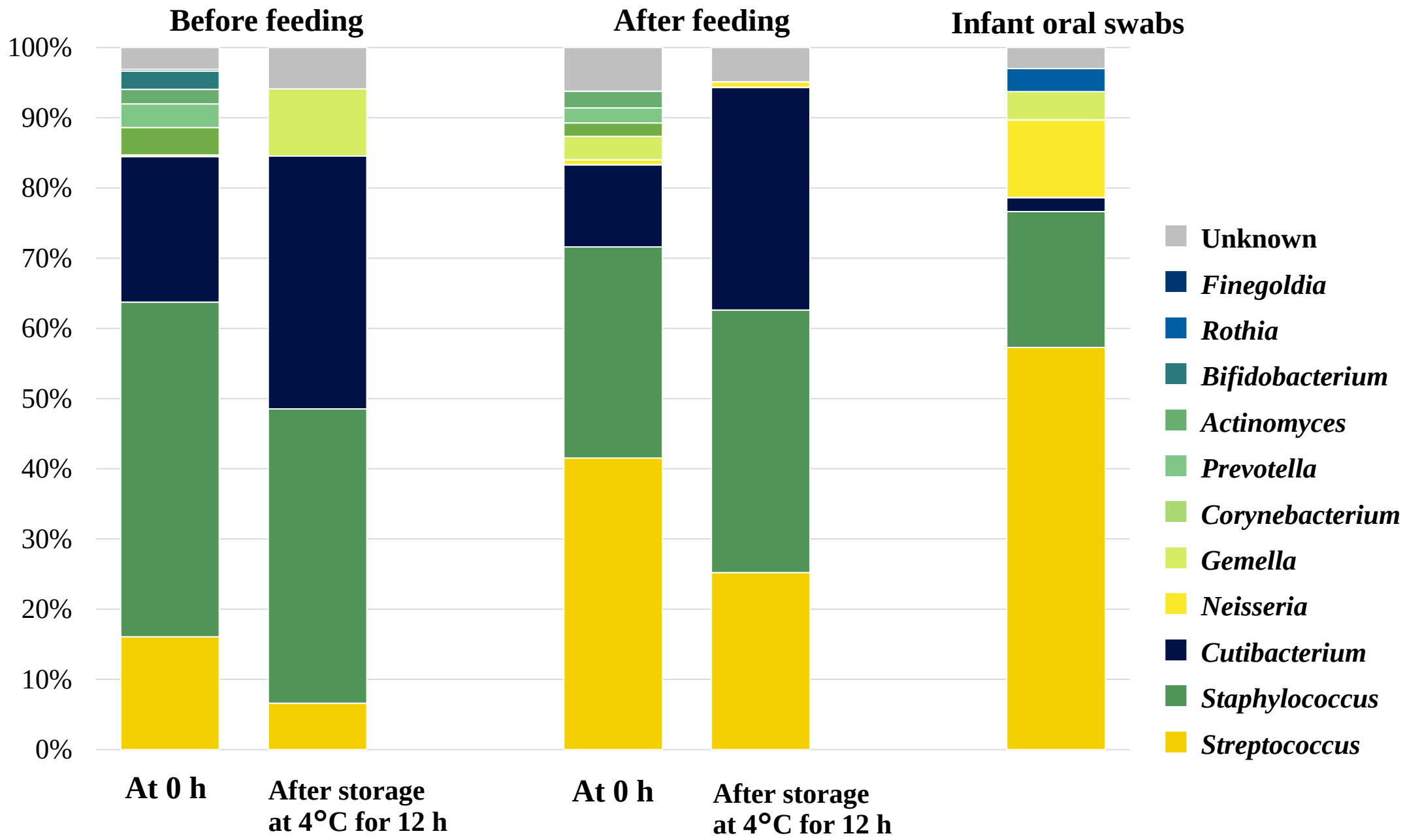
Subjects No.	1	2	3	4	5	6	7	8	9	10	11	Total	(%)
CFU/mL	5.0×10^7	4.1×10^7	2.0×10^7	6.1×10^7	5.9×10^7	2.1×10^7	7.2×10^6	2.0×10^5	3.0×10^7	1.4×10^8	2.1×10^7	$(4.1 \pm 3.8) \times 10^7$	
Total number of isolates	45	44	47	31	48	38	32	4	31	34	44	398	100.0%
Aerotolerant anaerobes			7					1				8	2.0%
Cutibacterium												8	2.0%
C. acnes			7					1					
Facultative anaerobes	44	43	39	31	48	36	31	3	30	31	42	378	95.0%
Streptococcus												228	57.3%
S. mitis/oralis/sanguinis/infantis	1		7	19	44	23		1		26	23		
S. parasanguinis/salivalius	17		28				12		21		6		
Staphylococcus												77	19.3%
S. aureus/epidermidis	2	22	1	8	4	3	9	1	9	5	13		
Neisseria												44	11.1%
N. perflava	23	21											
Gemella												16	4.0%
G. haemolysans/parahaemolysans	1			4		10		1					
Rothia												13	3.3%
R. mucilaginosa			3										
R. dentocariosa							10						
Unknown	1	1	1			2	1		1	3	2	12	3.0%

Table 5

The predominat bacterial genera isolated from human breast milk before and after feeding, and from infant oral swabs

	Breast milk before feeding				Breast milk after feeding				Infant oral swabs	
	At 0 h		After storage at 4°C for 12 h		At 0 h		After storage at 4°C for 12 h			
Total number and %	386	100.0%	136	100.0%	419	100.0%	123	100.0%	398	100.0%
Anaerobes	24	6.2%			9	2.1%				
Prevotella	13	3.4%			9	2.1%				
Finegoldia	1	0.3%								
Bifidobacterium	10	2.6%								
Aerotolerant anaerobes	80	20.7%	49	36.0%	49	11.7%	39	31.7%	8	2.0%
Cutibacterium	80	20.7%	49	36.0%	49	11.7%	39	31.7%	8	2.0%
Facultative anaerobes	270	69.9%	79	58.1%	335	80.0%	78	63.4%	378	95.0%
Streptococcus	62	16.1%	9	6.6%	174	41.5%	31	25.2%	228	57.3%
Actinomyces	8	2.1%			10	2.4%				
Gemella	1	0.3%	13	9.6%	14	3.3%			16	4.0%
Staphylococcus	184	47.7%	57	41.9%	126	30.1%	46	37.4%	77	19.3%
Corynebacterium	15	3.9%			8	1.9%				
Neisseria					3	0.7%	1	0.8%	44	11.1%
Rothia									13	3.3%
Unknown	12	3.1%	8	5.9%	25	6.0%	6	4.9%	12	3.0%

Fig. 1


Before feeding
After feeding
Infant oral swabs
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
At 0 h
After storage at 4°C for 12 h
At 0 h
After storage at 4°C for 12 h
Unknown
Finegoldia
Rothia
Bifidobacterium
Actinomyces
Prevotella
Corynebacterium
Gemella
Neisseria
Cutibacterium
Staphylococcus
Streptococcus