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Profiling of microbiota in liquid baby formula consumed with an artificial nipple

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ABSTRACT

It is suspected that oral bacteria are transferred to the liquid baby formula through the artificial nipple and multiply in the bottle after feeding. In the present study, in order to understand the influence of bacteria on liquid baby formula after feeding, the transfer of oral bacteria through artificial nipples and their survival in liquid baby formula were examined immediately after drinking as well as after storage at 4°C for 3 h. Four healthy human subjects (20–23 years old) were asked to drink liquid baby formula (Aptamil®, ca. 50 mL) from baby bottles using artificial nipples. Samples of the liquid baby formula (immediately after drinking and 3 h later) were inoculated onto blood agar plates and incubated anaerobically at 37°C for 7 days. Salivary samples from each subject and 6 newborn infants were also cultured. Genomic DNA was extracted from individual colonies, and bacterial species were identified by 16S rRNA gene sequencing. The mean amounts of bacteria (CFU/mL) were $(3.2 \pm 3.0) \times 10^4$ and $(3.4 \pm 3.3) \times 10^4$ immediately after drinking and 3 h later, respectively. *Streptococcus* (41.6 and 40.5%), *Actinomyces* (24.3 and 21.5%) and *Veillonella* (16.2 and 11.0%) were recovered from the samples immediately after drinking and 3 h later, respectively. On the other hand, *Streptococcus* (38.9%), *Actinomyces* (17.1%), *Neisseria* (9.1%), *Prevotella* (6.9%), *Rothia* (6.9%) and *Gemella* (5.1%) were predominant in the saliva of adult subjects, and *Streptococcus* (65.2%), *Staphylococcus* (18.5%), *Gemella* (8.2%) and *Rothia* (5.4%) were predominant in the saliva of infant subjects. From these findings, oral bacteria, *e.g.*, *Streptococcus*, *Gemella* and *Rothia*, were found to transfer into the liquid baby formula through artificial nipples, and the bacterial composition in the remaining liquid baby formula was found to resemble that of human saliva. The bacterial levels were similar between immediately after drinking and when stored at 4°C for 3 h, suggesting that the remaining liquid baby formula may be preserved in a refrigerator for a specified amount of time.

Liquid baby formula is ready-to-use and safe for infants, and is widely used in many countries (6, 14) other than in Japan. Upon natural disasters, liquid baby formula has been well received as part of overseas relief supplies to Japan. Powdered milk has been permitted to use for infants as baby formula for several decades in Japan. Moreover, very recent-

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ly, liquid baby formula was approved by the Consumer Affairs Agency, Government of Japan, and two Japanese food companies have brought Japanese-produced liquid baby formulas to market (3).

It is suspected that oral bacteria can be transferred to liquid baby formula through the artificial nipple and can multiply in the bottle after feeding; however, definite proof for this hypothesis is currently lacking. Generally, food companies recommend that any remaining liquid baby formula be discarded after feeding (11); thus, no information has been reported to date on the influence of bacteria on residual liquid baby formula after feeding. However, some consumers may store and use the remaining liquid baby formula after feeding, due to its relatively high price in Japan (3).

Importantly, some bacteria have been reported to remain in PET bottles after drinking (1, 10, 12, 15); however, detailed microbiota profiles have not been clarified at present. Furthermore, differences between infants and adults regarding bacterial infiltration to liquid baby formula are unknown, since minimal data is available comparing the oral (saliva) microbiota between infants and adults.

Therefore, in order to elucidate the effect of bacteria on liquid baby formula after feeding, the present study examined the transfer of oral bacteria through artificial nipples and their survival in liquid baby formula immediately after drinking and when stored at 4°C for 3 h. In addition, the salivary microbiota of newborn infants and adults was compared in order to speculate on the main infiltrating bacteria to liquid baby formula.

MATERIALS AND METHODS

Sampling. After obtaining informed consent, whole resting saliva was collected from 4 healthy subjects (20–23 years old) (2). Subsequently, the subjects were asked to drink ca. 50 mL of liquid baby formula (Aptamil, 200 mL; Nutricia Ltd., Dublin, UK) via the artificial nipple of a baby bottle. All subjects were considered healthy based on their medical history, and none had received antibiotics for the 3 months before sampling. Saliva of newborn infants was collected using a sterilized cotton swab from 6 healthy subjects (less than one week old). This study, which included bacteria sampled from human subjects, was approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry, Sendai, Japan.

The samples (1.0 mL each) from the liquid baby formula (immediately after drinking and storage at 4°C for 3 h) and whole saliva of each subject were dispersed by vortexing. The cotton swab from each infant was suspended in 1.0 mL of sterilized 40 mM potassium phosphate buffer (pH 7.0) and dispersed by vortexing. Serial 10-fold dilutions (0.1 mL each) of the samples in sterilized buffer were spread onto the surface of CDC anaerobe 5% sheep blood agar (BD, Franklin Lakes, NJ, USA) plates (duplicate) and incubated anaerobically (Anaero Pack, Anaerobic cultivation sets; Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) at 37°C for 7 days. After incubation, colony-forming units (CFU) were counted, and all colonies from suitably diluted plates having <100 colonies (mean 42.6; range 22–66 colonies) were sub-cultured.

DNA extraction and identification of isolates by DNA sequence analysis. Genomic DNA was extracted from single colonies using an InstaGene Matrix Kit (Bio-Rad Laboratories, Richmond, CA, USA) according to the manufacturer's instructions.

The 16S rRNA gene sequences were amplified PCR by 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. 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' (2, 7, 8, 13). Amplification proceeded using a PCR Thermal Cycler MP (TaKaRa Biomedicals, Ohtsu, Shiga, Japan) programmed as follow: 5 min at 95°C for initial heat activation and 30 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, 1.5 min at 72°C for extension and 10 min at 72°C for final extension. 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, 1 mM EDTA, pH 8.4), stained with ethidium bromide and photographed under UV light, and their sizes (ca. 1466 bp) were confirmed in comparison with 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, Tokyo, Japan) according to the manufacturer's instructions. Digestion products were separated on 2% agarose gels as described above.

Isolates were tentatively identified according to RFLP analysis (13) as well as morphological data, *e.g.*, colony appearance and Gram staining. Then, representative isolates were conclusively identified by sequence analysis (2, 13) as described below. The

PCR products were purified with illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK), and then sequenced at Fasmac (Atsugi, Kanagawa, Japan) using a BigDye Terminator Cycle Sequencing Kit and an automated DNA sequencer (PRISM-3100; Applied Biosystems Japan, Tokyo, Japan). Primer 1492R was used for sequencing (at least 700 bp), and partial 16S rRNA gene sequences were then compared by BLAST searching of the GenBank database via the National Center for Biotechnology Information website. Bacterial species were determined by percent sequence similarity (>99%).

Statistical analysis. Dunn's test was employed to determine the statistical significance of bacterial amounts from the samples using statistical software (StatFlex, Ver. 6; Artech Co., Ltd., Osaka, Japan). *P*-values of <0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

The mean amounts of bacteria (CFU/mL) in the remaining liquid baby formula were $(3.2 \pm 3.0) \times 10^4$ and $(3.4 \pm 3.3) \times 10^4$ immediately after drinking and when stored at 4°C for 3 h, respectively (Table 1). Meanwhile, higher bacterial levels, $(3.0 \pm 2.6) \times 10^7$ and $(1.9 \pm 2.7) \times 10^8$ CFU, were recovered from the saliva of newborn infants and adults, respectively, and these levels were significantly greater than those in the remaining liquid baby formula ($P < 0.05$). In a preliminary study, no bacteria were detected in the liquid baby formula before drinking (data not shown).

Streptococcus (41.6%), *Actinomyces* (24.3%) and *Veillonella* (16.2%) were predominantly recovered from the adult samples immediately after drinking, followed by *Atopobium* (2.9%), *Prevotella* (2.3%), *Oribacterium* (2.3%), *Gemella* (1.7%), *Megasphaera* (1.7%), *Leptotrichia* (1.2%), *Porphyromonas* (1.2%) and *Selenomonas* (1.2%). On the other hand, *Streptococcus* (40.5%), *Actinomyces* (21.5%) and *Veillonella* (11.0%) were predominant in the samples of when stored at 4°C for 3 h, followed by *Prevotella* (8.6%), *Atopobium* (3.7%), *Gemella* (3.1%), *Neisseria* (1.8%), *Peptostreptococcus* (1.8%), *Eubacterium* (1.2%), *Fusobacterium* (1.2%) and *Solobacterium* (1.2%), suggesting that the major constituents of the microbiota were similar to each other, and no particular influence was observed for the residual liquid baby formula when stored at 4°C for 3 h (Table 1).

Meanwhile, *Streptococcus* (38.9%), *Actinomyces* (17.1%), *Neisseria* (9.1%), *Prevotella* (6.9%), *Rothia* (6.9%) and *Veillonella* (2.9%) were predominant in the saliva of adult subjects. These results suggest that the bacterial composition of the liquid baby formula after drinking was similar to that of human saliva (Table 2), and that the predominant species in the saliva infiltrated into the liquid baby formula.

Bacterial contamination of foods consumed by infants/babies and children has been reported (4, 5). However, detailed microbiota profiles have not been clarified at present. It has been reported that some bacteria remain in PET bottles after drinking (1, 10, 12, 15), although most of these studies focused mainly on *Escherichia coli* and general (water resources-related) bacteria. In regards to liquid baby formula after feeding, no information is available on the impact of bacteria, as food companies recommend that any remaining liquid baby formula be discarded (11). In the present study, in order to obtain scientific evidence of bacterial effects, analyses on bacteria in liquid baby formula were performed under suitable culture conditions for oral microbiota, *i.e.*, appropriate culture media, incubation temperature and gaseous environment, since the obtained bacteria are assumed to originate from the oral cavity. As a result (Table 1), bacterial levels were found to be similar between immediately after drinking and following storage of the infant formula at 4°C for 3 h, suggesting that the remaining liquid baby formula may be preserved in the home refrigerator for a certain time period.

The genus *Streptococcus* was predominant in the salivary samples of both newborn infants and adults in the present study (Table 2). Further studies focusing on infants are required to assess bacterial infiltration to liquid baby formula through artificial nipples. Nevertheless, the findings of the present study, which targeted adult subjects, could potentially provide useful scientific viewpoints, since the use of powdered milk for adults (9) and feeding cups for the elderly is increasing in Japan at present. In the future, liquid formula for adults, using a feeding cup, may be employed in Japan.

In the present study, we applied molecular biology techniques, such as PCR-RFLP and sequencing, for bacterial identification. The combination of culture and molecular techniques seems to be a comparatively rapid, low-cost as well as accurate approach to the identification of microbiota, compared to the use of culture methods, including conventional biochemical tests, alone. Further examination of the biological characteristics of individual isolates may be necessary to analyze the influence of bacteria in liq-

Table 1 *Bacterial isolates obtained from liquid baby formula after drinking with an artificial nipple*

	After drinking liquid baby formula									
	Immediately					When stored at 4°C for 3 h				
Subjects	1	2	3	4	Total (%)	1	2	3	4	Total (%)
CFU/mL	3.6×10^3	5.3×10^4	6.2×10^4	7.4×10^3	$(3.2 \pm 3.0) \times 10^{4a}$	5.1×10^3	4.8×10^4	7.5×10^4	8.7×10^3	$(3.4 \pm 3.3) \times 10^{4a}$
Total	28	66	41	38	173 (100)	35	44	46	38	163 (100)
Anaerobes	4	28	11	12	55 (31.8)	2	17	13	20	52 (31.9)
Veillonella					28 (16.2)					18 (11.0)
V. parvula	2	15	2	9			6	3	9	
Atopobium					5 (2.9)					6 (3.7)
A. parvulum		1	2	2			1		5	
Prevotella					4 (2.3)					14 (8.6)
P. melaninogenica	1	1				2	1	3	4	
P. jejuni				1				1	1	
P. niqrescens			1							
P. nanceiensis							1			
P. salivae							1			
Oribacterium					4 (2.3)					1 (0.6)
O. parvum		2								
O. asaccharolyticum		1								
Oribacterium sp.			1						1	
Megasphaera					3 (1.7)					
M. micronuciformis		3								
Leptotrichia					2 (1.2)					1 (0.6)
L. wadei		1								
Leptotrichia sp.		1					1			
Porphyromonas					2 (1.2)					1 (0.6)
P. pasteri			2				1			
Selenomonas					2 (1.2)					1 (0.6)
S. noxia			2				1			
Eubacterium					1 (0.6)					2 (1.2)
E. sulci/infirmum		1					2			
Lachnoanaerobaculum					1 (0.6)					
L. orale		1								
Mogibacterium					1 (0.6)					
M. neglectum			1							
Propoinibacterium					1 (0.6)					
P. acnes	1									
Solobacterium					1 (0.6)					2 (1.2)
S. moorei		1						2		
Peptostreptococcus										3 (1.8)
P. stomatis								3		
Fusobacterium										2 (1.2)
F. necrophorum							2			
Parvimonas										1 (0.6)
P. micra								1		
Facultative anaerobes	24	38	30	26	118 (68.2)	33	27	33	18	111 (68.1)
Streptococcus					72 (41.6)					66 (40.5)
S. parasanguinis/salivarius	10	14	12	12		22	8	13	7	
S. mitis/oralis/infantis	4	3	7	1		3	3	5	1	
S. australis		5	1				1	2		
S. cristatus		3								
S. mutans						1				
Actinomyces					42 (24.3)					35 (21.5)
A. odontolyticus	3	11	10	6		5	9	5	3	
A. oris/naeslundii	4			4			1	5	4	
A. graevenitzii		1							3	
Actinomyces sp.				3						
Gemella					3 (1.7)					5 (3.1)
G. haemolyssans / parahaemolyssans	2	1				2	2	1		
Corynebacterium					1 (0.6)					
C. durum	1									
Neisseria										3 (1.8)
N. perflava							1	1		
N. macacae							1			
Campylobacter										1 (0.6)
C. concisus								1		
Rothia										1 (0.6)
R. aeria							1			

[a]Mean CFU/mL are given in parentheses.

Table 2 *Comparison between bacterial isolates obtained from saliva and liquid baby formula after drinking with an artificial nipple*

	Saliva		After drinking liquid baby formula ($n = 4$)	
	Infants ($n = 6$)	Adults ($n = 4$)	Immediately	When stored at 4°C for 3 h
Total	184 (100)[a]	175 (100)	173 (100)	163 (100)
Facultative anaerobes	179 (97.3)	139 (79.4)	118 (68.2)	111 (68.1)
Streptococcus	120 (65.2)	68 (38.9)	72 (41.6)	66 (40.5)
Actinomyces		30 (17.1)	42 (24.3)	35 (21.5)
Gemella	15 (8.2)	9 (5.1)	3 (1.7)	5 (3.1)
Corynebacterium			1 (0.6)	
Neisseria		16 (9.1)		3 (1.8)
Campylobacter		1 (0.6)		1 (0.6)
Rothia	10 (5.4)	12 (6.9)		1 (0.6)
Staphylococcus	34 (18.5)	2 (1.1)		
Capnocytophaga		1 (0.6)		
Obligate anaerobes	0 (0.0)	34 (19.4)	55 (31.8)	52 (31.9)
Veillonella		6 (3.4)	28 (16.2)	18 (11.0)
Atopobium			5 (2.9)	6 (3.7)
Prevotella		12 (6.9)	4 (2.3)	14 (8.6)
Oribacterium			4 (2.3)	1 (0.6)
Megasphaera			3 (1.7)	
Leptotrichia		2 (1.1)	2 (1.2)	1 (0.6)
Porphyromonas		4 (2.3)	2 (1.2)	1 (0.6)
Selenomonas			2 (1.2)	1 (0.6)
Eubacterium			1 (0.6)	2 (1.2)
Lachnoanaerobaculum			1 (0.6)	
Mogibacterium			1 (0.6)	
Propoinibacterium		7 (4.0)	1 (0.6)	
Solobacterium		1 (0.6)	1 (0.6)	2 (1.2)
Peptostreptococcus				3 (1.8)
Fusobacterium		2 (1.1)		2 (1.2)
Parvimonas				1 (0.6)
Unknown	1 (0.5)	1 (0.6)		
Lost	4 (2.2)	1 (0.6)		

[a]Percentages are given in parentheses.

uid baby formula, although these characteristics might be speculated upon based on the general characteristics of reference-type strains.

In summary, more than ten thousand per mL of oral bacteria such as *Streptococcus*, *Gemella* and *Rothia* infiltrated into the liquid baby formula after drinking. The bacterial composition of the liquid baby formula after drinking was similar to that of saliva, indicating that the infiltrated bacteria originated from the saliva. The bacterial levels were found to be similar between immediately after drinking and when stored at 4°C for 3 h, suggesting that the remaining liquid baby formula may be preserved in a refrigerator for a specific time period. Further studies focusing on infants/babies are clearly required prior to applying the findings of this study directly to infants/babies.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

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