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Profiling of Microbiota in Liquid Baby Formula and a Baby Drink Consumed with an Artificial Nipple

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Introduction

- ✓ Liquid baby formula is ready-to-use and safe for infants, and is widely used in many countries.
- ✓ Upon natural disasters, liquid baby formula has been well received as part of overseas relief supplies.
- ✓ Powdered milk has been permitted to use for infants as baby formula for several decades in Japan.
- ✓ In 2019, Japanese food companies have brought Japanese-produced liquid baby formulas to market.

Japan-products

Advantages

- ✓ It can be kept at room temperature, and used immediately after opening.
- ✓ We don't have to spend time to prepare it.
- ✓ **It can reduce burden of childcare, and may provide social advantages.**

Food companies recommend that any remaining liquid baby formula should be discarded after feeding. However some consumers may store the remaining liquid baby formula after feeding and use it later. **Due to its relatively high price in Japan**

It is suspected that oral bacteria can be transferred to liquid baby formula through artificial nipples and that the bacteria can proliferate in the bottles after feeding.

To elucidate the effect of bacteria on liquid baby formula by after feeding,

The present study examined that
①The transfer of oral bacteria through artificial nipples
②Their multiplication and survival in liquid baby formula and baby drinks

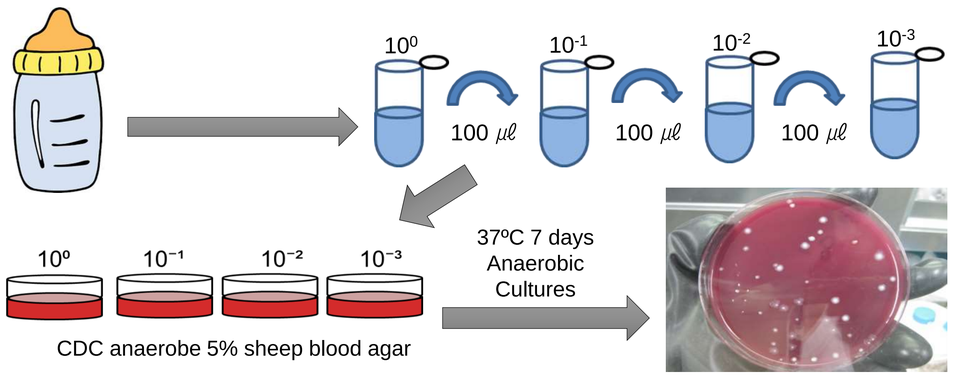
Baby drinks (Bean Stalk®), an infant-specific ion supply drink was examined in this study→

Materials & Methods

Sampling and Culturing of samples

After obtaining informed consent, 8 healthy human subjects (19-23 years old) were asked to drink liquid baby formula (Hohoemi RakuRaku Milk®) or baby-drinks (Bean Stalk®) via the artificial nipples of a baby bottle (ca. 50 mL each).

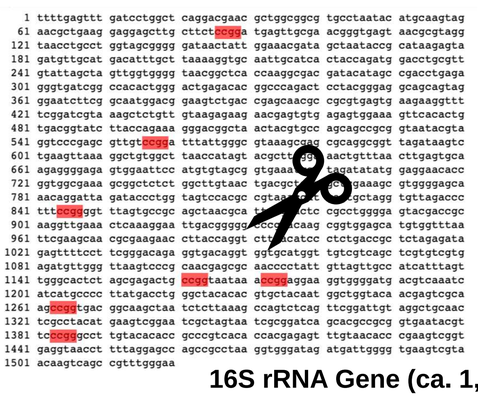
The samples of liquid baby formula immediately after drinking and after storage at 4ºC for 3 h, 12 h, and 24 h were mixed vortexing. The diluted samples were spread onto the surface of CDC blood agar plates, and incubated anaerobically at 37ºC for 7 days.



CDC anaerobe 5% sheep blood agar

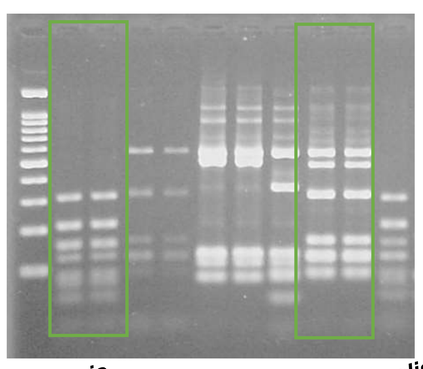
DNA extraction and identification of isolates by DNA sequence analisis

Genomic DNA was then extracted from single colonies using InstaGene Matrix kit.
The 16S rRNA gene were individually digested with *Hpa*II, and digested products were separated on 2% agarose gels.
Isolates were identified according to **PCR-RFLP analysis** and sequence.



16S rRNA Gene (ca. 1,500bp)

Stained with Ethidium Bromide



Actinomyces oris ***Streptococcus mitis/oralis***

◆ Program

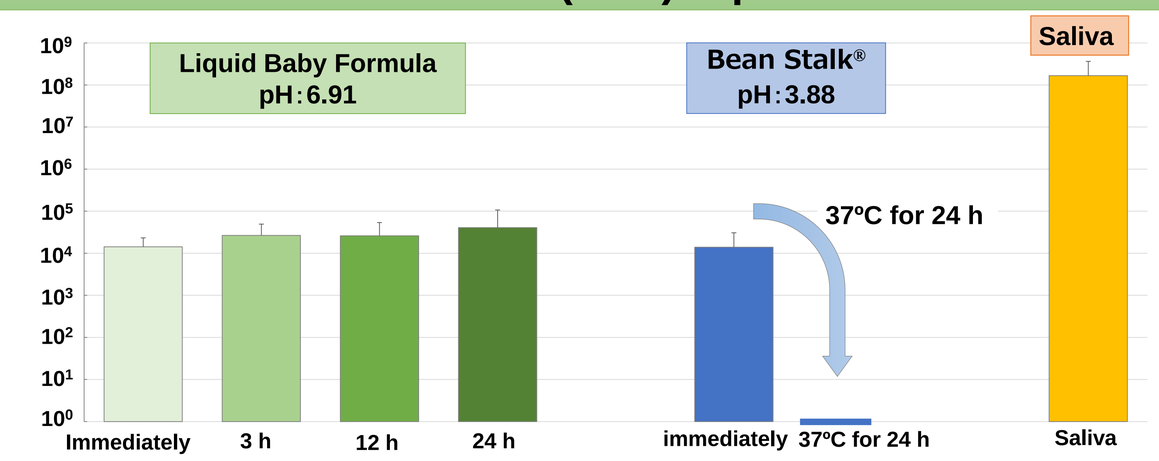
Initial heat activation	95ºC	5min
Denaturation	94ºC	1min
Annealing	55ºC	1min
Extension	72ºC	1.5min
Final extension	72ºC	10min

30 cycles

◆ Universal primers

27F:5'- AGA GTT TGA TCM TGG CTC AG -3'
1492R:5'- TAC GGY TAC CTT GTT ACG ACT T -3'

Results 1:Bacterial amounts (CFU) & pH



The mean concentrations of bacteria in remaining liquid baby formula were 1.4×10^4, 2.7×10^4, 2.6×10^4 and 4.1×10^4 CFU/mL immediately after drinking and after storage at 4ºC for 3h, 12h and 24h, respectively. In baby-drinks (Bean Stalk®), CFU was 1.4×10^4 immediately after drinking. No bacteria were detected in the remaining baby-drink after incubation at 37ºC for 24 h.
The mean concentration of bacteria in salivary samples was 1.7×10^8 CFU/ml.

Results 2:Bacterial Compositions

	Liquid Baby Formula (n=4) Immediately after		Liquid Baby Formula (n=4) Stored at 4ºC for 12 h		Bean Stalk® (n=9)		Saliva (n=10)	
Total (%)	127	(100.0)	160	(100.0)	258	(100.0)	343	(100.0)
Anaerobes	26	(20.5)	27	(16.9)	78	(30.2)	57	(16.6)
Veillonella	13	(10.2)	15	(9.4)	36	(14.0)	18	(5.2)
Prevotella	3	(2.4)	7	(4.4)	13	(5.0)	17	(5.0)
Propionibacterium	1	(0.8)			7	(2.7)	7	(2.0)
Atopobium	1	(0.8)	4	(2.5)	1	(0.4)	4	(1.2)
Porphyromonas	6	(4.7)			3	(1.2)	4	(1.2)
Fusobacterium					3	(1.2)	3	(0.9)
Leptotrichia							1	(0.3)
Megasphaera							1	(0.3)
Selenomonas			1	(0.6)	1	(0.4)	1	(0.3)
Solobacterium							1	(0.3)
Eubacterium					3	(1.2)		
Olsenella					7	(2.7)		
Oribacterium	1	(0.8)						
Peptostreptococcus	1	(0.8)			4	(1.6)		
Aerotorelant anaerobes					6	(2.3)		
Cutibacterium					6	(2.3)		
Facultative anaerobes	99	(78.0)	117	(73.1)	170	(65.9)	278	(81.0)
Streptococcus	56	(44.1)	73	(45.6)	92	(35.7)	172	(50.1)
Schaalia	6	(4.7)	11	(6.9)	60	(23.3)	31	(9.0)
Neisseria	17	(13.4)	5	(3.1)	1	(0.4)	22	(6.4)
Actinomyces	8	(6.3)	11	(6.9)	9	(3.5)	19	(5.5)
Rothia	4	(3.1)	8	(5.0)	1	(0.4)	16	(4.7)
Gemella	4	(3.1)	4	(2.5)	3	(1.2)	10	(2.9)
Staphylococcus							6	(1.7)
Campylobacter	2	(1.6)			2	(0.8)	1	(0.3)
Capnocytophaga	1	(0.8)	2	(1.3)	2	(0.8)	1	(0.3)
Corynebacterium	1	(0.8)	3	(1.9)				
Unknown					4	(1.6)	5	(1.5)
Lost	2	(1.6)	16	(10.0)			3	(0.9)

◆ ***Streptococcus*** **(40.7%),** ***Neisseria*** **(11.3%),** ***Veillonella*** **(10.7%) and** ***Actinomyces*** **(9.3%) species were predominantly recovered from the samples immediately after drinking (n=4), followed by** ***Schaalia*** **(5.3%),** ***Porphyromonas*** **(4.0%),** ***Prevotella*** **(3.3%),** ***Rothia*** **(3.3%) and** ***Gemella*** **(2.7%) species.**

◆ **In contrast,** ***Streptococcus*** **(43.2%),** ***Veillonella*** **(9.3%),** ***Schaalia*** **(8.2%) and** ***Actinomyces*** **(7.1%) species were predominant in the samples after storage at 4ºC for 12 h (n=4), followed by** ***Rothia*** **(4.4%),** ***Prevotella*** **(4.4%),** ***Neisseria*** **(3.3%),** ***Gemella*** **(2.7%) and** ***Atopobium*** **(2.2%) species.**

◆ **In the remaining baby drink (Bean Stalk® ,n=9),** ***Streptococcus*** **(35.7%),** ***Schaalia*** **(23.3%) and** ***Veillonella*** **(14.0%) were predominantly recovered followed by** ***Prevotella*** **(5.0%),** ***Actinomyces*** **(3.5%),** ***Propionibacterium*** **(2.7%) and** ***Cutibacterium*** **(2.3%)**

◆ **From saliva (n=10),** ***Streptococcus*** **(50.1%),** ***Schaalia*** **(9.0%) and** ***Neisseria*** **(6.4%) were predominantly detected, followed by** ***Actinomyces*** **(5.5%),** ***Veillonella*** **(5.2%),** ***Prevotella*** **(5.0%),** ***Rothia*** **(4.7%),** ***Gemella*** **(2.9%) and** ***Propionibacterium*** **(2.0%).**

◆ **The bacterial compositions of the liquid baby formula and baby drink after drinking were similar to those of human saliva and that the predominant species in the saliva infiltrated the liquid baby formula and baby drink via the artificial nipples of baby bottles.**

Discussion

More than ten thousand per mL of oral bacteria such as ***Streptococcus*****,** ***Actinomyces*** **and** ***Veillonella*** **infiltrated into baby drinks after drinking.**
The bacterial compositions of drinks was similar to that of saliva, indicating that the infiltrated bacteria originated from saliva.

The levels of bacteria immediately after drinking and after storage at 4ºC for 12 h were similar, suggesting that remaining liquid baby formula may be preserved safety in a refrigerator for a specified amount of time.

No bacteria were detected in remaining baby drink even after incubation at 37ºC for 24 h; this was likely due to the low pH (3.88 ± 0.01) of the baby drink, when compared to the pH of liquid baby formula (6.91 ± 0.01). This suggests that the remainder of low-pH drinks may be preserved for a longer time period than neutral-pH drinks from the view point of bacterial concentrations.