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Original Article

Microbial Profiling of Bottle Mouths and Residual Tea Following Direct Consumption from Plastic Tea Bottles

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ABSTRACT

It has been hypothesized that when individuals drink tea directly from plastic bottles, oral microorganisms may be transferred to the bottle surface and subsequently proliferate in the leftover tea. This study investigated the transfer of oral bacteria to the bottle mouth and their persistence in residual tea immediately after consumption and following 24 hours of incubation at 37 °C. Twelve healthy participants aged 19–23 years each consumed approximately 50 mL of unsweetened tea directly from a plastic bottle. Sterile cotton swabs were used to collect samples from the bottle mouths, while the remaining tea was analyzed using anaerobic culture and 16S rRNA gene sequencing, including metagenomic profiling. The mean bacterial counts at the bottle mouth were (1.8 ± 1.7) × 10^4 CFU/mL immediately after drinking and (1.4 ± 1.5) × 10^4 CFU/mL after 24 hours. In the residual tea, bacterial levels were (0.8 ± 1.6) × 10^4 CFU/mL right after drinking and increased markedly to (2.5 ± 2.6) × 10^6 CFU/mL after 24 hours. Streptococcus (59.9%) was the dominant genus detected at the bottle mouth immediately after consumption, with smaller proportions of Schaalia (5.5 percent), Gemella (5.5 percent), Actinomyces (4.9 percent), Cutibacterium (4.9 percent), and Veillonella (3.6 percent). Culture and metagenomic analyses revealed comparable bacterial profiles, highlighting Streptococcus (59.9 percent and 10.711 percent), Neisseria (1.6 percent and 24.245 percent), Haemophilus (0.6 percent and 15.658 percent), Gemella (5.5 percent and 0.381 percent), Cutibacterium (4.9 percent and 0.041 percent), Rothia (2.6 percent and 4.170 percent), Veillonella (3.6 percent and 1.130 percent), Actinomyces (4.9 percent and 0.406 percent), Prevotella (1.6 percent and 0.442 percent), Fusobacterium (1.0 percent and 0.461 percent), Capnocytophaga (0.3 percent and 0.028 percent), and Porphyromonas (1.0 percent and 0.060 percent), respectively. Streptococcus remained the predominant bacterial genus even after 24 hours of storage. Overall, the findings confirmed that oral bacteria were transferred to both the bottle mouth and the residual tea following direct consumption.

Keywords: Unsweetened tea, Oral microbiota, Profiling, PET bottle

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Introduction

In Japan, the habit of drinking tea from polyethylene terephthalate (PET) bottles is widespread, particularly as a convenient way to stay hydrated. It has been suggested that when individuals drink straight from these bottles, microorganisms from their mouths can enter the tea and continue multiplying inside the bottle. Although health authorities recommend discarding any leftover beverage after direct drinking [1, 2], there is still little research addressing how much and what kinds of bacteria persist in the residual tea. Nonetheless, many people tend to keep the unfinished drink—especially during hot weather—to prevent dehydration. While earlier investigations have confirmed that some bacteria can remain inside PET

bottles after consumption [1, 2], most of these focused on common environmental species such as Escherichia coli, and detailed microbial profiling of leftover tea has not been performed.

To fill this knowledge gap, the current work investigated bacterial transfer from the oral cavity to both the mouth of PET bottles and the remaining tea, immediately after consumption and after 24 hours of storage at 37 °C. Because these microbes were presumed to originate from the mouth, they were cultured under anaerobic conditions favorable for oral flora growth. In addition, 16S rRNA gene-based metagenomic sequencing was applied to validate and expand the bacterial identification results.

Materials and Methods

Sampling procedure

Twelve volunteers (aged 19–23 years), confirmed healthy and free of antibiotic use for at least three months prior to the experiment, were recruited. Each participant drank roughly 50 mL of unsweetened tea (Sokenbicha®; Coca-Cola (Japan) Company, Ltd., Tokyo, Japan) directly from a PET bottle. The bottle mouth was immediately swabbed with a sterile cotton applicator both right after consumption and again after the bottles had been stored for 24 hours at 37 °C. Each swab was placed in 1.0 mL of sterile 40 mM potassium phosphate buffer (pH 7.0) and mixed thoroughly by vortexing. A 1.0 mL portion of the remaining tea was treated in the same manner. From each preparation, 0.1 mL aliquots of serial 10-fold dilutions were spread onto CDC Anaerobe 5% Sheep Blood Agar (BD, Franklin Lakes, NJ, USA) and incubated anaerobically at 37 °C for seven days. The number of colony-forming units (CFU) was then calculated, and colonies from plates containing fewer than 100 colonies (mean 21.1; range 4–38) were isolated for further study. As bacterial growth was significantly greater under anaerobic conditions (~10^4 CFU/mL) than aerobic ones (~10^3 CFU/mL), only anaerobic isolates were subjected to identification analyses.

Determination of pH

Tea pH values were recorded using a calibrated digital pH meter (HM-25R; DKK-TOA Corporation, Tokyo, Japan).

Bacterial identification through DNA sequencing

Genomic DNA was isolated from individual bacterial colonies using the InstaGene Matrix Kit (Bio-Rad Laboratories, Richmond, CA, USA). Universal primers 27F and 1492R were employed to amplify the 16S rRNA gene by PCR with HotStarTaq Plus Master Mix (Qiagen GmbH, Hilden, Germany), following previously published protocols [3, 4]. Amplified DNA fragments were visualized on 2% agarose gels (High Strength Analytical Grade Agarose; Bio-Rad Laboratories) under established electrophoresis conditions [3, 4].

Preliminary classification of bacterial isolates was achieved through PCR–restriction fragment length polymorphism (PCR-RFLP) analysis [3, 4], and representative strains were subsequently confirmed by direct sequencing, as detailed in prior methodologies [3, 4].

Metagenomic sequencing and analysis

DNA from swab suspensions was extracted using the Mora-Extract Kit (Cosmo Bio) according to the manufacturer's guidelines. Amplification of the 16S rRNA gene, library construction, and sequencing were conducted at the Oral Microbiome Center (Takamatsu, Japan) using an Illumina MiSeq system with a MiSeq Reagent Kit (Illumina, San Diego, CA, USA), following the Illumina 16S Metagenomics Sequencing Library Preparation protocol. The primers used were 341F (5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3′) and 806R (5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGG ACT ACH VGG GTW TCT AAT-3′). Data analysis was performed using QIIME software (version 1.9.1; http://qiime.org/, accessed April 24, 2021), and bacterial taxa were identified using the Human Oral Microbiome Database (version 14.51; http://www.homd.org/, accessed April 24, 2021).

Statistical analysis

Differences in bacterial counts were analyzed using Dunn's test with StatFlex software (version 6; Artech Co., Ltd., Osaka, Japan). A p-value of less than 0.05 was regarded as statistically significant.

Results and Discussion

The average bacterial count detected at the bottle mouth was (1.8 ± 1.7) × 10^4 CFU/mL immediately after consumption and (1.4 ± 1.5) × 10^4 CFU/mL after 24 hours **(Table 1)**. In contrast, bacterial concentrations in the remaining tea were (0.8 ± 1.6) × 10^4 CFU/mL right after drinking and markedly increased to (2.5 ± 2.6) × 10^6 CFU/mL after 24 hours of incubation **(Table 1)**, showing a statistically significant difference ($p < 0.05$). The bacterial load exhibited a roughly 100-fold increase following 24-hour storage at 37 °C, which may be attributed to the near-neutral pH of the tea (ranging from 5.77 to 6.08). These findings emphasize

the importance of keeping leftover beverages such as tea refrigerated to limit microbial proliferation. In supplementary experiments, bacterial growth remained minimal at lower temperatures: tea stored at 4 degrees Celsius and 24 degrees Celsius showed bacterial counts of $(4.2 \pm 5.2) \times 10^3$ and $(2.5 \pm 3.7) \times 10^4$ CFU/mL, respectively. Moreover, unopened bottles contained no detectable bacteria prior to drinking.

Table 1. Bacteria isolated from plastic bottles of tea immediately after drinking and 24 h after drinking.

	Tea Drink of Plastic Bottles				**At the Mouth of Plastic Bottles**			
	Immediately after Drinking (n = 10)		**24 h after Drinking (n = 12)**		**Immediately after Drinking (n = 11)**		**24 h after Drinking (n = 8)**	
Mean ± SD (CFU/mL)	$(0.8 \pm 1.6) \times 10^4$		$(2.5 \pm 2.6) \times 10^6$		$(1.8 \pm 1.7) \times 10^4$		$(1.4 \pm 1.5) \times 10^4$	
Total	201	(%)	213	(%)	309	(%)	141	(%)
Anaerobes	28	(13.9)	27	(12.7)	41	(13.3)	0	(0)
Cutibacterium	9	(4.5)	27	(12.7)	15	(4.9)		
C. acnes	9		27		15			
Veillonella	12	(6.0)			11	(3.6)		
V. parvula/tobetsuensis	12				11			
Prevotella	7	(3.5)			5	(1.6)		
P. loescheii	1				4			
P. melaninogenica	6				1			
Fusobacterium					3	(1.0)		
F. periodonticum					3			
Porphyromonas					3	(1.0)		
P. pasteri/bronchialis					3			
Selenomonas					1	(0.3)		
S. noxia					1			
Leptotrichia					1	(0.3)		
L. sp					1			
Solobacterium					1	(0.3)		
S. moorei					1			
Facultative anaerobes	162	(80.6)	178	(83.6)	259	(83.8)	137	(97.2)
Streptococcus	132	(65.7)	183	(85.9)	185	(59.9)	136	(96.5)
S. mitis/oralis/infantis	102		172		129		120	
S. parasanguinis	12		5		30		7	
S. australis	8		5		8		1	
S. salivarius	5				13			
S. cristatus	5		1		5		8	
Actinomyces	29	(14.4)			15	(4.9)		
A. oris/naeslundi	28				15			
A. johnsonii	1							
Schaalia	6	(3.0)			17	(5.5)		
S. odontolytica	6				17			
Gemella	3	(1.5)			17	(5.5)		
G. sanguinis					10			

G. haemolyssans/parahaemolyssans	3				7			
Staphylococcus			2	(0.9)	7	(2.3)	1	(0.7)
S. epidermidis			2		5		1	
S. warneri/pasteuri					2			
Neisseria	1	(0.5)	1	(0.5)	5	(1.6)		
N. mucosa					2			
N. perflava	1		1		2			
N. oralis					1			
Rothia	2	(1.0)			8	(2.6)		
R. aeria	1				3			
R. mucilaginosa					5			
R. dentocariosa	1							
Haemophilus					2	(0.6)		
H. parainfluenzae					2			
Capnocytophaga					1	(0.3)		
C. gingivalis					1			
Abiotrophia					1	(0.3)		
A. defectiva					1			
Unknown	8	(4.0)	2	(0.9)	4	(1.3)	2	(1.4)
Lost	3	(1.5)	6	(2.8)	5	(1.6)	2	(1.4)

Analysis of bacterial cultures **(Table 1)** showed that immediately after drinking, the bottle mouths were primarily colonized by Streptococcus species, representing 59.9% of the isolates, with smaller proportions of Schaalia (5.5 percent), Gemella (5.5 percent), Actinomyces (4.9 percent), Cutibacterium (4.9 percent), and Veillonella (3.6 percent). In contrast, metagenomic sequencing **(Table 2)** indicated a broader bacterial diversity, with Delftia dominating at 34.506%, followed by Neisseria (24.245 percent), Haemophilus (15.658 percent), Streptococcus (10.711 percent), Rothia (4.170 percent), Lautropia (3.083 percent), Sphingomonas (1.745 percent), Ralstonia (1.436 percent), and Veillonella (1.130 percent), alongside minor genera including Fusobacterium (0.461 percent), Prevotella (0.442 percent), Actinomyces (0.406 percent), Agrobacterium (0.394 percent), and Gemella (0.381 percent). Despite differences in relative abundances between culture and sequencing methods, both approaches consistently identified the main bacterial genera present in the residual tea, namely Streptococcus, Neisseria, Haemophilus, Gemella, Cutibacterium, Rothia, Veillonella, Actinomyces, Prevotella, Fusobacterium, Capnocytophaga, and Porphyromonas **(Table 2)**.

Table 2. Culture and metagenomic analyses of the bacterial compositions (percentages) at the mouth of plastic bottles immediately after and 24 h after drinking.

	Immediately after Drinking		**24 h after Drinking**	
	Culture (n = 11)	**Metagenomic (n = 3)**	**Culture (n = 8)**	**Metagenomic (n = 3)**
Streptococcus	59.870	10.711	96.454	46.586
Delftia		34.506		17.907
Neisseria	1.618	24.245		14.608
Haemophilus	0.647	15.658		8.926
Schaalia	5.502			
Gemella	5.502	0.381		0.320
Cutibacterium	4.854	0.041		0.308

Rothia	2.589	4.170	4.509
Veillonella	3.560	1.130	0.651
Actinomyces	4.854	0.406	0.351
Lautropia		3.083	0.328
Sphingomonas		1.745	2.374
Prevotella	1.618	0.442	0.352
Ralstonia		1.436	1.090
Fusobacterium	0.971	0.461	0.214
Capnocytophaga	0.324	0.028	
Agrobacterium		0.394	0.185
Porphyromonas	0.971	0.060	0.114
Abiotrophia	0.324		
Bergeyella		0.344	0.142
Burkholderia			0.320
Corynebacterium		0.289	0.074
Ochrobactrum		0.208	0.123
TM7 [G-1]		0.031	0.142
Granulicatella		0.010	0.126
Bradyrhizobium		0.099	0.098
Bosea		0.026	0.076
Bacillus			0.072
Peptostreptococcus		0.019	
Alloprevotella		0.019	
Ruminococcaceae [G-1]		0.012	
Aggregatibacter		0.012	
Tannerella		0.011	
Campylobacter		0.009	0.002
Escherichia		0.009	
Catonella		0.005	
Cardiobacterium		0.003	
TM7 [G-3]			0.002
Fretibacterium			0.002
Selenomonas		0.001	
Peptostreptococcaceae [XI] [G-1]			0.001
Unknown	1.294	1.418	
Lost	1.618	1.418	

Both culture-based and metagenomic analyses indicated that Streptococcus species remained the most frequently detected bacteria at the bottle mouths 24 hours after drinking **(Tables 1 and 2)**, while metagenomic sequencing also revealed the presence of Delftia, Neisseria, Haemophilus, Rothia, Sphingomonas, and Ralstonia **(Table 2)**. In the residual tea immediately after consumption **(Table 1)**, the dominant bacteria were Streptococcus (65.7 percent) and Actinomyces (14.4 percent), followed by Veillonella (6.0 percent), Prevotella (3.5 percent), Schaalia (3.0 percent), Gemella (1.5 percent), and Rothia (1.0 percent). After 24 hours, Streptococcus (85.9 percent) and Cutibacterium (12.7 percent) became the primary species in the remaining tea **(Table 1)**.

Previous studies have reported bacterial contamination in foods [5, 6] and beverages in PET bottles following drinking [1, 2], although these investigations largely focused on common waterborne bacteria, such as E.

coli. Little is known about bacterial presence at the mouths of plastic bottles after direct drinking, as leftover beverages are typically recommended to be discarded. To address this gap, the present study examined the bacteria at the bottle openings and in the remaining tea under culture conditions tailored to oral microorganisms, including appropriate media, incubation temperatures, and anaerobic environments, assuming an oral origin of the isolates. The results obtained from culture analyses were further validated through 16S rRNA-based metagenomic sequencing **(Table 2)**. In addition, the study evaluated bacterial growth in the residual tea after 24 hours of storage at 37 °C, mimicking summer or hot-weather storage conditions.

The findings demonstrated that oral bacteria were consistently present both at the bottle mouth and in the leftover tea, with Streptococcus species dominating after 24 hours. Although the unsweetened tea used in this study contained minimal catechins and caffeine, previous reports suggest that green tea rich in catechins can suppress bacterial growth over 8 hours at 23.4–25.6 °C [7]. Immediately after drinking, the predominant genera included Streptococcus, Actinomyces, Veillonella, Cutibacterium, Prevotella, Schaalia, Gemella, Rothia, and Neisseria, yet the reason why Streptococcus persisted so robustly for 24 hours remains unclear.

Ongoing research in our laboratory is analyzing the microbiota of other beverages, including a sweetened sports drink (pH < 4.0) and orange juice (pH < 4.0). Preliminary results show that the dominant bacteria in the sports drink included Streptococcus, Actinomyces, Rothia, Prevotella, Neisseria, Veillonella, Schaalia, and Gemella, while orange juice contained Streptococcus, Actinomyces, Gemella, Veillonella, and Prevotella, mirroring the patterns observed in the current study.

Conclusion

In conclusion, approximately 10,000 bacterial cells per milliliter, including Streptococcus, Actinomyces, Schaalia, Gemella, Cutibacterium, and Veillonella, were detected at the bottle mouths and in the remaining tea immediately after drinking, and these findings were confirmed by metagenomic analysis. Bacterial counts increased roughly 100-fold after 24 hours at 37 °C, with Streptococcus species remaining predominant, highlighting the importance of either discarding leftover tea or storing it in a refrigerator after drinking directly from PET bottles.

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