Abstract: Coffee is a widely consumed beverage containing organic compounds with antibacterial activity. To investigate its possible effect on the growth of oral indigenous microbiota, saliva samples collected from nine young adults were inoculated into brain heart infusion (BHI) medium with or without addition of coffee compounds and cultured at 37°C in 5% CO₂ for 12 h. The total bacterial density and composition after cultivation for 0, 6, and 12 h were determined by quantitative PCR analysis and 16S rRNA gene sequencing, respectively. The increase in total bacterial load was significantly inhibited by addition of the coffee compounds. The microbiota was mostly composed of *Streptococcus* species after culture in BHI medium regardless of the addition of coffee compounds. The proportion of *Streptococcus salivarius* was significantly reduced after addition of coffee relative to that in untreated medium alone, whereas the proportions of *Streptococcus mitis* and *Streptococcus infantis* were increased. These results suggest that exposure to coffee affects the composition of the oral *Streptococcus* population, in addition to inhibiting the overall growth of salivary bacteria. Considered in the light of data from earlier epidemiological studies, it is possible to conclude that coffee consumption contributes to better health.

Keywords: oral microbiota; coffee compounds; *Streptococcus*; 16S rRNA; next-generation sequencing.

**Introduction**

The human oral cavity is a complex ecosystem colonized by a large number of diverse indigenous microorganisms, which are constantly developing and depositing polymicrobial communities on the various intraoral surfaces that are bathed in saliva (1). Among these communities, the microbiota that surrounds the teeth—dental plaque—is responsible for two major oral diseases, dental caries and periodontitis (2), and the microbiota on the tongue dorsum primarily contributes to the production of oral malodor (3). Daily oral hygiene including tooth brushing and tongue scraping is commonly practiced in order to reduce the oral bacterial burden. Nevertheless, additional convenient approaches are still required to ensure inhibition of the oral bacterial population. In this context, the biological activities of natural foods and beverages as well as chemical agents have received attention in recent years (4).

Coffee is a widely consumed beverage containing various bioactive compounds. Its antibacterial activity against potentially pathogenic microorganisms including *Streptococcus mutans*, a common organism associated with dental caries, has been reported previously (5-8). Therefore, although coffee consumption would be expected to have benefits for oral health mainte-
nance, the effect of coffee exposure on the overall oral indigenous microbiota remains unclear. The present investigation demonstrated a shift in salivary bacterial composition after culture were evaluated using comprehensive molecular genetic approaches including bacterial 16S rRNA gene sequencing.

**Materials and Methods**

**Saliva collection**
Unstimulated saliva was collected from nine young adults (five males and four females) aged 28-36 years. All of the subjects were systemically healthy with good oral health, i.e. no active dental caries or periodontitis. The subjects consumed no food or drink, except for water, for 1 h prior to saliva collection. The study was approved by the ethics committee of Kyushu University (No. 28-127) in 2016, and written informed consent was obtained from all participants.

**Coffee compounds and culture medium**
Coffee compounds were prepared by dissolving coffee powder (Nescafe Gold Blend, Nestle Japan, Kobe, Japan) in distilled water and sterilized by filtration through a 0.22-μm membrane filter (Millex-GP, Merck & Co., Inc., Kenilworth, NJ, USA). Saliva was inoculated into brain heart infusion (BHI) broth (BD/Difco, Franklin Lakes, NJ, USA) at 1:50 dilution in the presence or absence of coffee compounds [final coffee powder concentration 1.5% (w/v)] and cultured at 37°C in 5% CO₂ for 12 h. After the 0-, 6-, and 12-hour time points, 500 µL of the cell suspension was collected and DNA was extracted from each sample using a beads-beating method as described previously (9). The DNA samples were stored at −30°C until further analysis.

**Quantitative PCR analysis of bacterial density**
Quantitative PCR analysis of total bacterial density was performed using a QuantiFast SYBR Green PCR kit (Qiagen, Hilden, Germany) using the bacterial universal primers 806F (5'-TTC GAT ACC CYG GTA GTC C-3') and 926R (5'-CCG TCA ATT YCT TTG AGT TT-3') as described previously (10). The 16S rRNA gene of *Porphyromonas pasteri* was inserted into the vector plasmid pBluescript SK II (+) (Stratagene, La Jolla, CA, USA) and used as the real-time PCR control.

**High-throughput 16S rRNA gene amplicon sequencing analysis**
The bacterial composition of each sample was analyzed by high-throughput 16S rRNA gene amplicon sequencing analysis using Ion PGM (Thermo Fisher Scientific, Waltham, MA, USA), a next-generation sequencer. The V1-V2 regions of 16S rRNA genes from each sample was amplified using the following primers: 338R (5'-TGC TGC CTC CCG TAG GAG T-3') with the Ion Torrent trP1 adaptor sequence and 8F (5'-AGA GTT TGA TYM TGG CTC AG-3') with the Ion Torrent adaptor A and the sample-specific 8-base tag sequence. PCR amplification, purification, emulsion PCR and sequencing were performed as described previously (11). Briefly, KOD DNA polymerase (Toyobo, Osaka, Japan) was used for PCR amplification. The pooled PCR amplicons were purified using Wizard SV Gel and a PCR Clean-Up System (Promega, Madison, WI, USA). A KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA, USA) was used for estimation of the purified DNA concentration. The diluted DNA (8 pM) was used as the template DNA for emulsion PCR using an Ion PGM Hi-Q View OT2 Kit (Thermo Fisher Scientific). The template-positive particles obtained by the emulsion PCR were sequenced on the Ion PGM (Thermo Fisher Scientific) using an Ion 318 v2 chip (Thermo Fisher Scientific) and an Ion PGM Hi-Q View Sequencing kit (Thermo Fisher Scientific).

**Sequence data processing**
Raw sequence reads were quality-filtered using a script written in R version 3.3.1. The reads were excluded from the analysis if they were ≤240 bases (not including the tag sequence), had a homopolymer sequence of >7 nucleotides, did not include the correct reverse primer sequence (one mismatch was allowed) and the correct forward primer sequence, or had an average quality score of <25. The remaining reads were assigned to the sample based on the 8-base tag sequence. After trimming the forward primer sequence, similar sequences were clustered into operational taxonomic units (OTUs) using UPARSE (12) at a distance cutoff of 3%. All reads were mapped to each OTU in UPARSE. Chimeric sequences were removed from the representative set after identification using Chimera Slayer (13) in QIIME (14). The taxonomic information for representative sequences was obtained on the basis of BLAST analysis against 16S rRNA gene sequences in the Human Oral Microbiome Database (HOMD 16S rRNA RefSeq version 14.51) (15). Of the named taxa, nearest-neighbor species with 98.5% identity as a candidate for each OTU. If only unnamed species hit the corresponding sequence, the taxon was assigned as a OTU candidate. The taxonomic information for sequences with no hit was further determined.
using Ribosomal Database Project (RDP) classifier with a minimum support threshold of 80%. Two OTUs corresponding to *Pseudomonas fluorescens* HOT-612, which was predominant in some pre-cultured samples with low DNA concentrations, were excluded from the analysis as contaminants, because they were almost undetectable in the other samples including the original saliva samples. The number of OTUs and Shannon diversity index were evaluated following rarefaction to 20,000 reads per sample using R. The sequence data obtained in this study were deposited in the DDBJ Sequencing Read Archive under the accession number DRA007425.

**Statistical analysis**

Statistical analyses were performed using R. The paired Wilcoxon signed rank test was used to assess differences in total bacterial density, the number of identified OTUs, the Shannon diversity index, and the relative abundance of each bacterial taxon at each time point. The *P*-values obtained were adjusted by a Benjamini-Hochberg false discovery rate correction for multiple testing in the evaluation of differences in the relative abundance of bacterial taxa.

**Results**

This study observed a shift of the salivary bacterial populations cultured *in vitro* in the presence or absence of coffee compounds. Saliva samples were collected from nine young adults and cultivated in BHI medium at 37°C in 5% CO₂ for 12 h. The total bacterial density and bacterial composition after culture for 0, 6 and 12 h were determined.

Quantitative PCR analysis demonstrated that the total bacterial density after culture for 6 and 12 h was significantly lower in the presence, than in the absence, of coffee compounds (*P* < 0.001 and *P* < 0.01, respectively; Wilcoxon signed rank test: Fig. 1), suggesting that addition of coffee compounds inhibited the growth of salivary microbiota under these culture conditions.

The relative abundances of predominant bacterial genera in the microbiota at each time point are shown in Fig. 2. While several bacterial genera such as *Streptococcus*, *Neisseria*, *Actinomyces* and *Prevotella* were present at high relative abundances (mean relative abundance >1%) in the microbiota prior to culture, the microbiota comprised mostly *Streptococcus* after culture for 6 h, regardless of the addition of coffee compounds. Nevertheless, the relative abundance of genera such as *Veillonella*, *Prevotella* and *Haemophilus* recovered slightly after culture for 12 h, but became significantly lower when cultured in the presence of coffee extract relative to growth in medium alone (Fig. 2, Table 1). The microbiota cultured in BHI medium with coffee compounds for 12 h showed a greater predominance of *Streptococcus* species (Fig. 2, Table 1) and lower microbial richness (no. of identified OTUs, Fig. 3A) than that in the medium alone.

On the other hand, the Shannon diversity index was higher for microbiota cultured in BHI medium with coffee compounds for 6 h than that in the medium.
alone (Fig. 3B), suggesting that the addition of coffee compounds allowed coexistence of several predominant *Streptococcus* species. Of the 19 species-level OTUs corresponding to genus *Streptococcus* deposited in the Human Oral Microbiome Database (15), OTU1 assigned to *Streptococcus salivarius* accounted for most of the *Streptococcus* populations after culture for both 6 and 12 h in the medium alone (Fig. 4, Table 2). On the other hand, its predominance was considerably lower after culture in the presence of coffee compounds. In contrast, OTU7 and OTU54 assigned to *Streptococcus mitis* and *Streptococcus infantis*, respectively, showed significantly increased abundance in the presence of coffee compounds relative to that in the medium alone (Fig. 4).

**Discussion**

The antimicrobial activity of coffee extract against specific organisms including *Streptococcus mutans* has been demonstrated in previous studies (5-8). However, it appears that no reports have documented the effect of coffee on the growth of polymicrobial communities whose members interact with each other. The present study demonstrated significant differences in salivary bacterial populations after culture for both 6 and 12 h.

**Table 1** Relative abundance of predominant bacterial genera (mean relative abundance >1% before cultivation) after cultivation with and without coffee extract addition

| bacterial genera   | control 85.1 ± 7.4 | coffee addition 87.5 ± 3.1 | control 73.5 ± 12.7 | coffee addition 89.4 ± 3.7*** |
|--------------------|--------------------|----------------------------|--------------------|-------------------------------|
| Streptococcus      | 85.1 ± 7.4         | 87.5 ± 3.1                 | 73.5 ± 12.7        | 89.4 ± 3.7***                 |
| Granulicatella     | 2.4 ± 1.4          | 6.7 ± 3.2*                 | 3.7 ± 0.9          | 6.8 ± 3.3                     |
| Veillonella        | 2.3 ± 4.7          | 0.4 ± 0.3                  | 12.9 ± 8.4**       | 1.9 ± 3.3                     |
| Actinomyces        | 0.6 ± 0.5          | 0.9 ± 0.6                  | 0.9 ± 0.7*         | 0.3 ± 0.4                     |
| Rothia             | 0.9 ± 0.9          | 1.5 ± 0.8                  | 0.8 ± 0.8          | 0.2 ± 0.2                     |
| Prevotella         | 0.9 ± 1.9          | 0.2 ± 0.2                  | 2.2 ± 3.5*         | 0.2 ± 0.3                     |
| Gemella            | 0.5 ± 0.2          | 0.8 ± 0.5                  | 0.4 ± 0.2***       | 0.1 ± 0.1                     |
| Neisseria          | 4.8 ± 2.7***       | 0.3 ± 0.2                  | 2.1 ± 2.1***       | 0.1 ± 0.1                     |
| Porphyromonas      | 0.3 ± 0.2          | 0.3 ± 0.2                  | 0.2 ± 0.2*         | 0.1 ± 0.1                     |
| Leptotrichia       | 0.4 ± 0.3          | 0.2 ± 0.1                  | 0.1 ± 0.2          | 0.1 ± 0.1                     |
| Haemophilus        | 0.8 ± 0.7          | 0.2 ± 0.3                  | 1.4 ± 1.5***       | 0.0 ± 0.0                     |
| Alloprevotella     | 0.2 ± 0.2          | 0.1 ± 0.1                  | 0.3 ± 0.2*         | 0.0 ± 0.1                     |
| Fusobacterium      | 0.1 ± 0.1          | 0.1 ± 0.1                  | 0.5 ± 0.6**        | 0.0 ± 0.0                     |
| Lautropia          | 0.0 ± 0.0          | 0.0 ± 0.0                  | 0.0 ± 0.0          | 0.0 ± 0.0                     |

Significant differences at each time point were assessed by the paired Wilcoxon signed rank test and higher scores are signified by asterisk. ***P < 0.001, **P < 0.01, *P < 0.05 after the Benjamini-Hochberg adjustment for multiple testing.
in the presence of coffee compounds, relative to culture in medium alone. Although the culture conditions in this study differed from those in the actual oral environment, the results suggested that coffee exposure had an impact on the growth of oral indigenous microbiota, especially the predominant species.

A previous epidemiological study has reported that individuals who regularly consume coffee have lower levels of cultivable bacteria in their saliva and dental plaque (16). Consistent with this, total bacterial growth was significantly inhibited by addition of coffee compounds in the present study (Fig. 1). Most of the genera except for Streptococcus and Granulicatella were less predominant when cultured in medium treated with coffee compounds, relative culture in medium alone (Fig. 2, Table 1), suggesting that a wide range of bacteria present in the salivary microbiota are influenced by the antibacterial effect of coffee compounds. Natural coffee
beans contain dozens of bioactive compounds, including caffeine and chlorogenic acid (6). Furthermore, high-molecular-weight compounds such as melanoidins (17) and α-dicarbonyl compounds (6) are formed during the roasting process. These diverse components of brewed coffee would be involved in antimicrobial activity against a wide range of oral bacteria. The characteristic increase in the abundance of Gramulicatella associated with exposure to coffee (Fig. 2, Table 1) is consistent with the results of a recent epidemiological study of individuals who drink coffee frequently (18). Streptococcus and Granulicatella species seem to be relatively adaptable to coffee exposure.

The abundance of Streptococcus species was significantly higher in microbiota grown in coffee-treated medium relative to that in the medium alone (Fig. 2, Table 1), whereas the most predominant Streptococcus species in saliva, S. salivarius, was found to account for an unexpectedly lower proportion in the presence of coffee compounds (Fig. 4). Alternatively, the increased presence of S. mitis and S. infantis in the cultured microbiota (Fig. 4) suggests that exposure to coffee led to a shift in the composition of oral Streptococcus species. A previous Dutch study suggested that the salivary microbiota dominated by S. salivarius/vestibularis represents an early phase of acidogenic adaptation that is prone to a more cariogenic state (19). A previous large-scale population-based study of salivary microbiota also indicated that S. salivarius, along with Prevotella and Veillonella species, comprise a cohabiting bacterial group associated with poorer oral health conditions, whereas S. mitis belongs to a health-associated cohabiting group (11). A similar microbiota composition associated with pneumonia-related death was found in institutionalized elderly subjects (20). The compositional shift in the Streptococcus population induced by coffee exposure observed in this study could be regarded as a favorable one, possibly leading to better oral and systemic health.

As the oral microbiota comprised mostly Streptococcus and Granulicatella after culture, the effects of coffee on the other bacteria were unlikely to be evaluated accurately in this study. Nevertheless, Prevotella and Veillonella species, which are associated with poorer oral health conditions (11), were less predominant in the microbiota cultivated in the presence of coffee compounds, relative to that cultured in medium alone. The lower microbial richness observed after culture for 12 h (Fig. 3A) was also consistent with the salivary microbiota of individuals with better periodontal conditions (11). Although health-associated genera such as Neisseria (11) also showed a decrease (Fig. 2 and Table 1), coffee exposure appears to have a largely beneficial effect on the oral microbiota overall.

The culture conditions used in this study (BHI medium

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**Table 2** Relative abundance of 19 operational taxonomic units (OTUs) corresponding to *Streptococcus* species after cultivation with and without coffee extract addition

| OTU No. | Relative abundance of OTU (%) | After cultivation for 6 h | After cultivation for 12 h | Bacterial candidate corresponding to each OTU |
|---------|--------------------------------|--------------------------|---------------------------|--------------------------------------------|
|         | control | coffee addition | control | coffee addition |
| OTU1    | 59.5 ± 10.1** | 28.9 ± 14.6 | 50.8 ± 12.4* | 25.3 ± 15.6 |
| OTU7    | 4.2 ± 1.7    | 17.2 ± 8.4** | 2.8 ± 1.2    | 28.7 ± 16.7*** |
| OTU54   | 2.2 ± 1.3    | 6.9 ± 3.8*   | 1.7 ± 1.3    | 5.1 ± 2.1* |
| OTU14   | 1.4 ± 1.4    | 7.8 ± 9.5    | 2.5 ± 2.7    | 4.1 ± 4.4 |
| OTU18   | 0.8 ± 0.6    | 3 ± 3.1      | 1 ± 1.1      | 2.1 ± 2.7 |
| OTU111  | 0.3 ± 0.6    | 1.2 ± 2.6    | 0.4 ± 1      | 0.4 ± 0.6 |
| OTU21   | 1.2 ± 1.4    | 1.9 ± 1.9    | 1 ± 1.2      | 3.2 ± 3 |
| OTU12   | 0.9 ± 1.6    | 0.6 ± 0.8    | 1.2 ± 2.9    | 3.3 ± 4.1 |
| OTU552  | 0.1 ± 0.1    | 0.2 ± 0.4    | 0 ± 0        | 0.1 ± 0.1 |
| OTU235  | 0.7 ± 0.7    | 0.4 ± 0.5    | 0.6 ± 0.7    | 0.2 ± 0.3 |
| OTU156  | 0.2 ± 0.3    | 0.5 ± 0.8    | 0.2 ± 0.4    | 0.3 ± 0.5 |
| OTU64   | 0 ± 0        | 0.1 ± 0.1    | 0 ± 0        | 0.3 ± 0.3 |
| OTU97   | 0 ± 0        | 0 ± 0        | 0 ± 0        | 0 ± 0 |
| OTU182  | 0 ± 0        | 0 ± 0        | 0 ± 0        | 0 ± 0 |
| OTU517  | 0 ± 0        | 0.1 ± 0.1    | 0 ± 0        | 0.1 ± 0 |
| OTU107  | 0 ± 0        | 0 ± 0        | 0 ± 0        | 0 ± 0 |
| OTU61   | 0 ± 0        | 0.1 ± 0.3    | 0 ± 0        | 0.2 ± 0.3 |
| OTU168  | 0 ± 0        | 0 ± 0        | 0 ± 0        | 0 ± 0 |
| OTU186  | 0 ± 0        | 0 ± 0        | 0 ± 0        | 0 ± 0 |

Significant differences at each time point were assessed by the paired Wilcoxon signed rank test and higher scores are signified by asterisk. ***P < 0.001, **P < 0.01, *P < 0.05 after the Benjamini-Hochberg adjustment for multiple testing. Str.: *Streptococcus*; sp.: species. Oral taxon IDs in Human Oral Microbiome Database were given in parentheses following bacterial name.
at 37°C in 5% CO₂) are suitable for the growth of bacteria predominant in saliva, such as *Streptococcus, Rothia* and *Neisseria*. However, microbiota members with special nutritional requirements as well as obligate anaerobes are difficult to grow under these conditions. In fact, the bacterial populations become considerably less diverse when cultured in growth medium with or without added coffee (Fig. 3), suggesting that multiple minority members were missed during culture. Therefore, the effects on such species remained unclear. Further analysis using different culture conditions will be required in order to understand how coffee compounds lead to a compositional shift of the oral indigenous microbiota.

The present data suggest that coffee compounds inhibit growth of the total salivary bacterial population in BHI medium, and affect the composition of the oral *Streptococcus* population. It is possible that coffee consumption affects the growth and maintenance of indigenous oral microbiota, resulting in better oral and general health.

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**Conflict of interest**

None.

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