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Original Article

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Salivary Microbiota Associated with Peripheral Microvascular Endothelial Dysfunction

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Aims: Oral health is associated with atherosclerotic cardiovascular disease (ACVD). We previously identified the salivary microbiota characteristics of patients with ACVD. However, whether salivary microbiota is characteristic under impaired vascular endothelial function before ACVD onset remains unclear. Therefore, we aimed to evaluate the characteristics of salivary microbiota associated with peripheral microvascular endothelial dysfunction.

Methods: We collected saliva samples from 172 community-dwelling elderly individuals without a history of ACVD and performed 16S rRNA metagenomic analysis. We assessed the peripheral microvascular endothelial function using reactive hyperemia index (RHI) and compared the salivary microbiota in the groups with normal (RHI ≥ 2.10), borderline, and abnormal (RHI <1.67) peripheral endothelial function. Furthermore, we applied machine learning techniques to evaluate whether salivary microbiota could discriminate between individuals with normal and abnormal endothelial function.

Results: The number of operational taxonomic units (OTUs) was higher in the abnormal group than in the normal group (p=0.037), and differences were found in the overall salivary microbiota structure (unweighted UniFrac distances, p=0.038). The linear discriminant analysis (LDA) effect size (LEfSe) algorithm revealed several significantly differentially abundant bacterial genera between the two groups. An Extra Trees classifier model was built to discriminate between groups with normal and abnormal vascular endothelial function based on the microbial composition at the genus level (AUC=0.810).

Conclusions: The salivary microbiota in individuals with endothelial dysfunction was distinct from that in individuals with normal endothelial function, indicating that the salivary microbiota may be related to endothelial function.

Key words: Oral microbiota, Saliva, Endothelial function, 16S rRNA metagenomic analysis

Introduction

Oral health is associated with atherosclerotic cardiovascular disease (ACVD). Oral infections, especially periodontal disease, are a risk factor for ACVD[1, 2]. Oral bacteria may influence the initiation and progression of ACVD by acting on the vascular system by inducing inflammation[3, 4]. Endothelial

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dysfunction, the initial step in ACVD, is caused by various factors, including hypertension, dyslipidemia, diabetes, smoking, obesity, lack of exercise, excessive salt intake, and menopause. Poor oral hygiene and severe periodontal disease are also associated with endothelial dysfunction, and treatment of periodontal diseases may improve endothelial function[5-7]. In addition, specific oral bacteria such as *Porphyromonas gingivalis* cause disturbances in the vascular endothelial function *in vitro*[8, 9]. Therefore, the oral bacterial environment may have an important influence on vascular endothelial function.

Oral bacteria form a community, namely the microbiota in the oral cavity. In recent years, increasing knowledge of the microbiota has revealed that it plays a symbiotic role in maintaining the physiological functions of the host. Oral microbiota is closely associated with various systemic diseases, such as cancer, diabetes, and autoimmune disorders, suggesting that the salivary microbiota may be a disease-specific biomarker[10-13]. Although individual oral bacterial species associated with vascular endothelial dysfunction have been identified[14, 15], no association between the salivary microbiota as a whole and vascular endothelial function has yet been identified.

We have previously shown that the salivary microbiota of patients with ACVD is clearly different from those without ACVD[16]. However, it remains unclear whether characteristic salivary microbiota is also observed under impaired vascular endothelial function before the onset of ACVD. Vascular endothelial dysfunction is an early sign of atherosclerosis (observed before clinical and laboratory findings of atherosclerosis) and plays an important role in its development[17-19]. Atherosclerosis is commonly diagnosed using tests such as carotid artery ultrasound examination, pulse wave velocity (PWV), and ankle-brachial index (ABI); however, endothelial function tests can detect lesions at an earlier stage than these tests. Vascular endothelial function can be assessed using the reactive hyperemia index (RHI) via either flow-mediated dilation (FMD), which uses ultrasound to measure changes in the dimensions of the brachial artery lumen in response to ischemia, or reactive hyperemia-peripheral artery tonometry (RH-PAT), automated measurement of reactive hyperemia in the finger. The RH-PAT examination, a simple method for assessing peripheral microvascular endothelial function, has been reported to reflect atherosclerotic vascular damage and the presence of cardiovascular disease, and is useful in predicting future cardiovascular events[20, 21]. Therefore, in this study, we focused on RHI using the RH-PAT examination as an indicator of peripheral microvascular endothelial function and evaluated its relationship with oral microbiota.

Aim

This study aimed to characterize the salivary microbiota associated with impaired peripheral microvascular endothelial function by comparing the composition and diversity of salivary microbiota among individuals with normal and abnormal RHI values, an index of peripheral microvascular endothelial function, using bacterial 16S ribosomal RNA (16S rRNA) gene sequencing in community-dwelling elderly individuals without a history of ACVD. In addition, machine learning techniques were used to evaluate whether people with abnormal peripheral vascular endothelial function could be discriminated from those with normal vascular endothelial function based on the salivary microbiota.

Methods

Participants

This study was conducted in accordance with the guidelines of the Helsinki Declaration and its latest amendments. It was approved by the Ethics Committee of Osaka Medical College, Takatsuki City, Japan (approval no. 2145). Furthermore, written informed consent was obtained from all participants.

The study participants included 172 Japanese individuals between 65–87 years of age (mean age: 73 years), selected from 263 community-dwelling individuals who participated in the Takatsuki study from March 2018 to November 2019. Takatsuki study was a prospective cohort study to clarify the relationship between oral health and various systemic disease, frailty, or a condition of need for nursing care in elderly people living in Takatsuki City, Japan. The entry criteria of Takatsuki study were that community-dwelling men and women aged 65 years or older living in Takatsuki city. The participants were volunteer who agreed to be recruited into the study after public lectures held in October 2017 and October 2018, who completed baseline survey including clinical and oral functional examinations, and filling out a self-administered questionnaire. Individuals with self-reported medical history for coronary or other vascular diseases, taking medication of anti-platelets or anti-coagulants, currently on treatment for infectious or autoimmune diseases, renal or hepatic failure, cancer, and use of antibiotics within one month of sample collection, were excluded. The medical history and medication use information were

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collected using a questionnaire.

Peripheral Microvascular Endothelial Function

Peripheral microvascular endothelial function was estimated using the Endo-PAT2000® device (Itamar Medical, Caesarea, Israel) and expressed as an RHI value according to the manufacturer's instructions. RH-PAT examinations were performed on the same day as the oral sample collection in the fasting state in the early morning. The participants were divided into three groups according to the criteria for RHI levels of peripheral vascular dysfunction[21]: normal (RHI ≥ 2.10), borderline (1.67 ≤ RHI < 2.10), and abnormal (RHI < 1.67).

Oral Sample Collection and Oral Examination

Oral sample collection and examination were performed as previously described[12]. Briefly, saliva samples were collected from participants in the morning (at least 2 h after brushing their teeth or consumption of meals) using the cotton swab method, as previously described[22], using the saliva collection system SalivaBio® oral swab and swab storage tube (Salimetrics, Irvine, PA, USA) prior to the oral examination. The samples were frozen immediately after collection and stored at −80℃ before DNA extraction. In addition, all participants underwent a full-mouth clinical examination by certified dentists as previously described[16].

DNA Extraction, 16S rRNA Sequencing, and Taxonomic Classification

DNA extraction, 16S rRNA sequencing, and taxonomic classification were performed as previously described[16], with some modifications. Briefly, the samples were homogenized with 0.1 mm glass beads. Bacterial genomic DNA from homogenized samples was extracted using GENE PREP STAR PI-480 (Kurabo Industries Ltd., Osaka, Japan) according to the manufacturer's instructions. The V1–V2 region of the 16S rRNA gene was amplified, and each library was prepared in accordance with the 16S metagenomic sequencing library preparation protocol (Illumina, San Diego, CA, USA). DNA was sequenced for 500 cycles using the MiSeq Reagent Kit v2 (Illumina). An average of 36,653 sequence reads with 250 bp paired-ends were denoised and quality filtered using DADA2 in Quantitative Insights into Microbial Ecology 2 (QIIME2) version 2020.02. After denoising with DADA2, amplicon sequence variants (ASVs) were generated and regarded as 100% operational taxonomic units (OTUs). A rarefaction minimum depth cut-off was chosen at 10,000, and all samples were retained for downstream analysis. Each OTU was assigned to a curated Greengenes 13_8 reference database.

Statistical Analysis

For participant characteristics, intergroup comparisons were analyzed using the unpaired *t*-test, Mann–Whitney *U* tests, or Chi-square test, as appropriate. The triglyceride and C-reactive protein values had log-normal distributions; therefore, statistical analyses were performed after logarithmic conversion of the values. The statistical software Stata (College Station, TX, USA), version 15 (Stata Corporation), was used for database construction and data analysis. Statistical significance was set as $p < 0.05$.

The within-group alpha diversity of bacterial communities was assessed using Shannon's index and the observed OTU index and was compared among groups using the Kruskal–Wallis test. Between-group beta diversity was assessed based on the unweighted and weighted UniFrac distance metrics. To visualize the overall differences in the microbiome structure in the UniFrac analysis, we performed a principal coordinate analysis (PCoA). In addition, the significance of compositional differences between groups was assessed using permutational multivariate analysis of variance (PERMANOVA). QIIME2 software was used for these analyses.

The linear discriminant analysis (LDA) effect size (LEfSe) algorithm was used to detect differentially abundant genera between the two groups. All analyses were run with the α parameter of LEfSe for pairwise tests set to 0.05, and the threshold of the logarithmic score for the LDA analysis was set to 2.0.

Classification Model

To evaluate whether the salivary microbiota could discriminate between normal and abnormal groups, some classification models based on microbial composition at the genus level were built using a machine learning library in Python, PyCaret (v2.3.10), and their prediction performances were evaluated. The split between the training and test data was 0.9. The prediction performance of the built prediction models was evaluated by 5-fold cross-validation in the training dataset. Finally, receiver operating characteristic (ROC) curves and area under the ROC curves (AUCs) of the predictive models were calculated in the test dataset. The best predictive model, the Extra Trees classifier, was evaluated using the confusion matrix to calculate accuracy, precision, recall, and F1 score metrics. A 2-by-2 matrix with the best cut-off value was obtained using PyCaret (v2.3.10). Accuracy was calculated as correct

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Table 1. Baseline characteristics and laboratory data of the study population (n=172)

Variables	Normal group RHI ≥ 2.10 (n=59)	Borderline group 1.67 ≤ RHI <2.10 (n=47)	Abnormal group RHI <1.67 (n=66)	p value
Characteristic				
Age (years)	73.3 ± 4.7	74.0 ± 5.5	73.8 ± 4.9	0.730
Sex (male, %)	22.0	29.7	28.7	0.597
BMI (kg/m^2)	22.1 ± 3.1	22.4 ± 2.9	22.7 ± 3.5	0.535
Never smoker (%)	88.1	74.4	74.2	0.074
Ex-smoker (%)	11.8	25.5	21.2	
Current smoker (%)	0.0	0.0	4.5	
Systolic blood pressure (mmHg)	131.8 ± 22.6	127.1 ± 14.9	129.9 ± 19.7	0.479
Diastolic blood pressure (mmHg)	72.7 ± 11.0	71.2 ± 8.7	70.8 ± 11.0	0.584
Dyslipidemia (%)[†]	54.2	59.5	56.0	0.857
Hypertension (%)[‡]	55.9	44.6	54.5	0.463
Diabetes (%)[§]	8.4	3.9	13.6	0.242
Oral conditions				
Number of teeth	24 [21 – 27]	25 [21 – 28]	25 [20 – 27]	0.775
Denture wearing (%)	38.9	25.5	42.4	0.166
Severe periodontitis (%)	23.7	23.4	22.7	0.991
Medications				
Anti-diabetes drug (%)	0.0	4.2	6.0	0.173
Statin (%)	20.3	10.6	24.2	0.187
ACE-I/ARB (%)	10.1	6.3	19.6	0.086
Beta-blocker (%)	1.6	2.1	1.5	0.970
Calcium channel blocker (%)	11.8	17.0	27.2	0.084
PPI/H2 blocker (%)	5.0	4.2	13.6	0.114
Laboratory data				
HDL-C (mg/dL)	70.1 ± 17.6	71.1 ± 16.5	68.9 ± 18.0	0.790
LDL-C (mg/dL)	121.8 ± 30.4	121.6 ± 26.6	116.0 ± 26.3	0.433
TG (mg/dL)	81.0 (1.6)	72.6 (1.5)	77.5 (1.6)	0.492
HbA1c (NGSP, %)	5.8 ± 0.4	5.7 ± 0.5	5.8 ± 0.6	0.518
CRP (ng/ml)	328 (3)	332 (3)	342 (3)	0.977

Distributions of continuous variables were presented as mean±standard deviation, geometric mean (geometric standard deviations), or median [inter-quartile range]. Categorical variables were presented using proportions. The p values are the results of comparisons between groups using unpaired t-test, Mann–Whitney U tests, or Chi-square test.
[†]Dyslipidemia is defined as LDL-C ≥ 140 mg/dl, triglycerides ≥ 150 mg/dl, or use of anti-dyslipidemic drugs.
[‡]Hypertension is defined as blood pressure ≥ 140/90 mmHg or use of anti-hypertensive drugs.
[§]Diabetes is defined as HbA1c ≥ 6.5 % (NGSP) or use of oral anti-diabetic drugs or insulin therapy.

predictions (true positives (TP) plus true negatives (TN)) divided by total predictions. Precision, the positive predictive value, was calculated as the number of correctly classified positive classes (TP) divided by the sum of predicted positive classes (TP) plus false-positive classes (FP). Recall (sensitivity) was calculated as TP divided by the sum of TP plus false negatives (FN). Finally, the F1 score, the harmonic means of precision and recall was calculated as $(2 * precision * recall) \div (precision + recall)$.

Results

Characteristics of the Participants

The mean RHI level of the 172 participants was 1.96 ± 0.65, with 59 participants in the normal group (34.3%), 47 participants in the borderline group (27.3%), and 66 participants in the abnormal group (38.4%) (Table 1). The three groups had no significant differences in characteristics, oral status, medications, or laboratory data.

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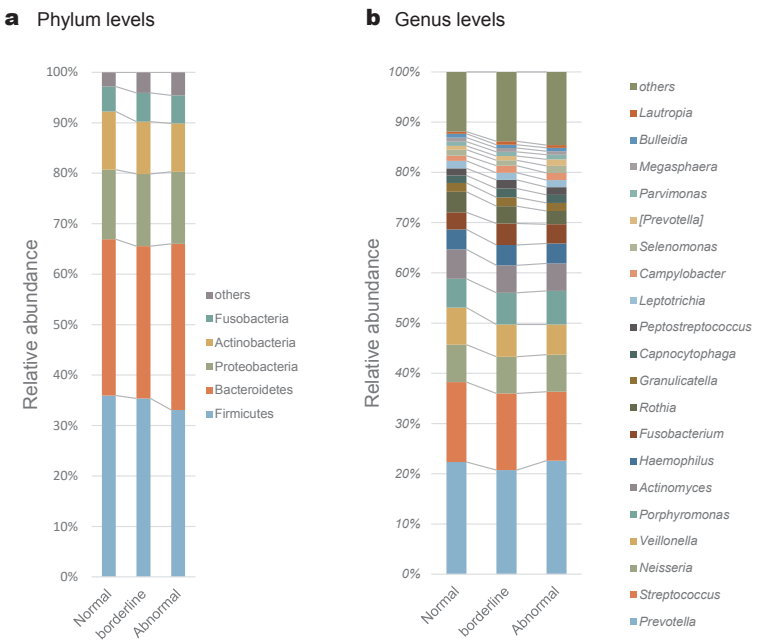

Fig. 1. Taxonomic composition of saliva microbiota

Vertical bar plot showing the relative abundance of bacterial phyla (a) and genus (b) in the normal (RHI ≥ 2.10), borderline (1.67 ≤ RHI < 2.10), and abnormal (RHI < 1.67). (a) Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria are the five most abundant phyla in all the participants, comprising 97.16, 95.91, and 95.45 % of the total bacterial communities in each group, respectively. (b) Bar plots of 20 most abundant genera in each group.

Oral Microbiota Composition

Salivary bacteria with relative abundances > 0.1% were classified into 12 phyla, 20 classes, 36 orders, 65 families, and 117 genera. At the phylum level, Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, and Fusobacteria had more than 5% relative abundances in each group, representing 97.16, 95.91, and 95.45% of the oral microbiota in normal, borderline, and abnormal groups, respectively **(Fig. 1a)**.

At the genus level, the proportions of the 20 most abundant genera in each group **(Fig. 1b)** were 88.08%, 86.19%, and 85.41% in the normal, borderline, and abnormal groups, respectively. The predominant bacteria at the genus level were *Prevotella*, with mean relative abundances of 22.34% and 22.63% in the normal and abnormal groups, and *Streptococcus*, with 15.91% and 13.72%, respectively. The normal group contained 100 genera, of which 8 were absent in the abnormal group, whereas the abnormal group contained 102 genera, of which 10 were absent in the normal group. In over 50% of the participants, there were 38 genera present in all three groups, plus *Aggregatibacter* and *Treponema*, making 40 genera in the abnormal group. Treponema, one of the genera included in the 'others' category in **Fig. 1b**, was present in 56.06% of participants in the abnormal group but only in 25.42% of participants in the normal group.

Microbial Diversity in the Three Groups

The evaluation of alpha diversity among the three groups showed that the observed OTU index was significantly higher in the abnormal group than in the normal group ($p=0.037$; **Fig. 2a**). On average, 260 ± 50, 278 ± 66, and 280 ± 62 OTUs were obtained from the salivary microbiota of the normal, borderline, and abnormal groups, respectively. The Shannon's index showed no significant differences. PCoA was used to compare the overall structure of the microbial communities among the three groups. According to the PCoA plots based on the unweighted UniFrac distance metric, the overall structure of the salivary microbiota of the normal and abnormal groups was distinguished in a 3-dimensional space. This compositional difference between the two groups was verified by PERMANOVA based on the unweighted UniFrac data (999 permutations, $p=0.038$; **Fig. 2b**). No statistically significant differences were observed in the microbial structure among the three groups using the weighted UniFrac distance metric **(Fig. 2c)**.

Since smoking may affect the salivary microbiota and there was a tendency for more smokers in the abnormal group, we compared the salivary microbiota

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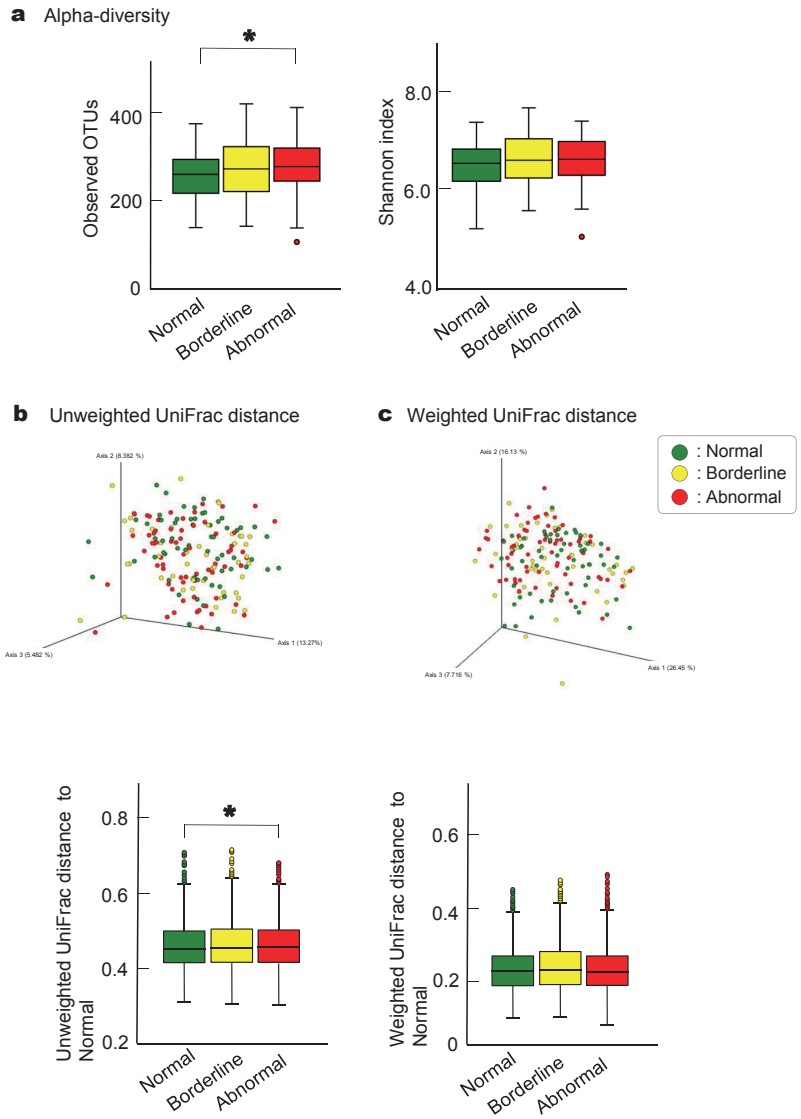

Fig. 2. Alpha and Beta diversity of salivary microbiota

(a) Operational taxonomic unit (OTU) index and Shannon index in the normal (RHI ≥ 2.10), borderline (1.67 ≤ RHI <2.10), and abnormal (RHI <1.67) groups. Normal (n=59, green), borderline (n=47, yellow), and abnormal groups (n=66, red). *p<0.05 compared among groups using Kruskal–Wallis test.
(b) Unweighted UniFrac distances.
(c) Weighted UniFrac distances.
Principal coordinate analysis (PCoA) plot for samples from 59 participants in the normal group (green), 47 participants in the borderline group (yellow), and 66 participants in the abnormal group. Box plots represent UniFrac distances to normal group participants from the normal (green columns), borderline (yellow), and abnormal groups (red columns). *p<0.05, compared between groups using PERMANOVA, 999 permutations.

of the three groups when smokers were excluded. Similar differences were found between the normal and abnormal groups in a comparison of the three groups of 169 individuals, excluding current smokers (observed OTU index: p=0.027, unweighted UniFrac distance: p=0.006, weighted UniFrac: p=0.032; **Supplemental Fig. 1**). Additionally, in a comparison of three groups of 136 never smokers, a similar trend was found between the normal and abnormal groups (unweighted UniFrac distance: p=0.038, weighted UniFrac: p=0.019; **Supplemental Fig. 2**).

Identification of Endothelial Dysfunction-Associated Oral Bacterial Genera Using LEfSe

The cladogram in **Fig. 3a** represents significantly different taxa between the normal and abnormal

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Fig. 3. The differentially abundant bacterial genera between the normal and abnormal groups identified by linear discriminant analysis effect size (LEfSe)

(a) Cladogram of the differentially abundant bacterial taxa where each layer represents different taxonomy. The enriched taxa in the normal (green) or abnormal (red) groups salivary microbiome are presented in the cladogram. The central point represents the root of the tree (bacteria), and each ring represents the next lower taxonomic level (phylum to genus: p, phylum; c, class; o, order; f, family; g, genus). The diameter of each circle represents the relative abundance of the taxon.
(b) Histogram of the linear discriminant analysis (LDA) scores for differentially abundant bacterial taxa between patients in the normal and abnormal groups. LDA scores ≥ 2.0 are shown. Red represents significantly abundant taxa in the abnormal group compared to those in the normal group. Green represents the significantly abundant taxa in the normal group.

groups, with a hierarchy reflecting the taxonomic rank from phylum to genus. We found that the abnormal group was associated with a significantly higher abundance of *Prevotella* and *Campylobacter*, and a lower abundance of *Veillonella* at the genus level over three orders of magnitude (Fig. 3b). These results revealed the presence of specific bacteria in the saliva of participants in the abnormal group.

Furthermore, for the nine genera that revealed significant differences between the normal and abnormal groups by LEfSe with LDA scores ≥ 2.0 in Fig. 3b, the relative abundance was compared among the three groups: normal, borderline, and abnormal (Fig. 4). The results showed that the relative abundance of *Veillonella* was significantly higher in the normal group than in the abnormal group ($p < 0.05$). *Campylobacter*, *Mycoplasma*, *Peptococcus*, *Treponema*, *Desulfovibrio*, and *Hylemonella* were more abundant in

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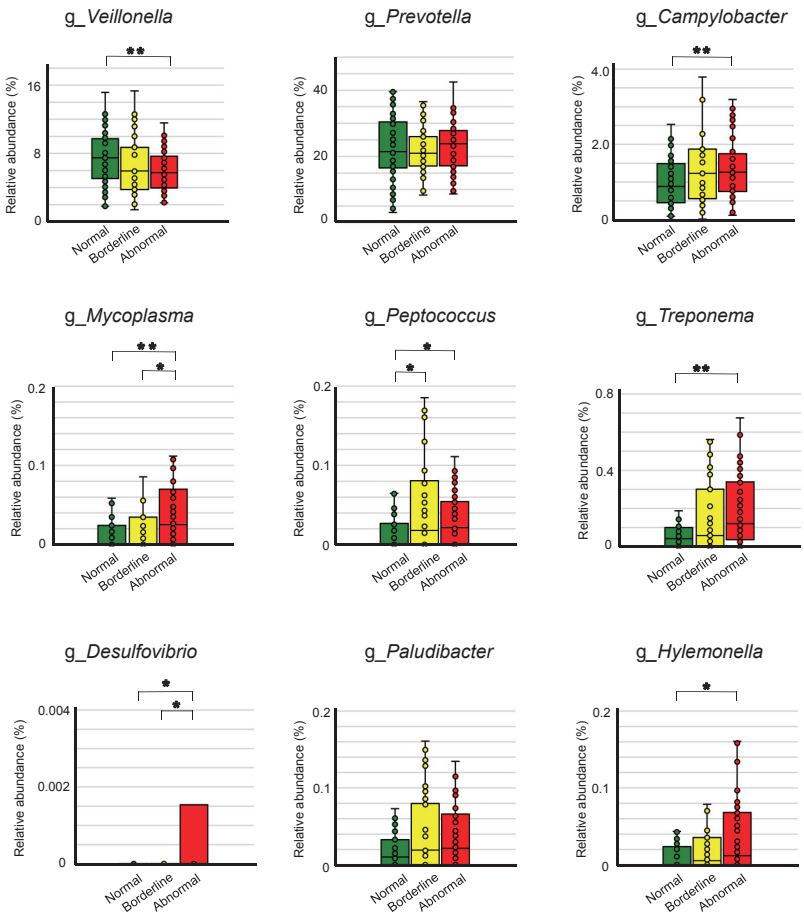

Fig. 4. Comparison of abundance of the selected genera among the normal, borderline, and abnormal groups

Box plot of the relative abundance of the 9 selected genera identified by linear discriminant analysis effect size (LEfSe). Bacterial abundance among the normal (RHI ≥ 2.10, green), borderline (1.67 ≤ RHI < 2.10, yellow), and abnormal groups (RHI < 1.67, red) was compared using the Wilcoxon rank-sum test after multiple comparisons with Kruskal-Wallis test. *$p<0.05$, **$p<0.01$.

the abnormal group than in the normal group ($p<0.05$ for all, **Fig. 4**). The relative abundance of *Veillonella* tended to correlate with RHI values (rS = 0.211, $p<0.05$).

Classification Model for Normal and Abnormal Groups Based on Salivary Microbiota

The best model to discriminate the normal and abnormal groups based on genus-level salivary microbiota was obtained using the Extra Trees classifier. The best predictive model's area under the ROC curves (AUCs) was 0.810. The performance of this classification model, i.e., accuracy, precision, recall, and F1 scores for the abnormal prediction groups were 0.769, 0.833, 0.714, and 0.769, respectively.

Discussion

This study reports the first evidence that the salivary microbiota differs between individuals with poor and normal peripheral microvascular endothelial function based on RH-PAT examination. Additionally, we identified the salivary microbiota characteristics of individuals with peripheral microvascular endothelial dysfunction and showed that the salivary microbiota could discriminate between individuals with poor and normal peripheral endothelial function. These data suggest a relation between peripheral microvascular endothelial function and salivary microbiota and that early vascular endothelial dysfunction could be predicted using salivary microbiota.

In this study, the RHI value obtained from the RH-PAT examination was used as an indicator of peripheral microvascular endothelial function. Peripheral microvascular endothelial dysfunction is reversible at any stage of atherosclerosis and plays a pivotal role in all its phases, from onset to

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atherothrombotic complications[17]. Among the methods used to evaluate vascular endothelial dysfunction, RH-PAT is a noninvasive, simple method to evaluate the peripheral microvascular endothelial function, reflecting the presence of atherovascular damage and cardiovascular diseases[23, 24]. Although RHI values vary with underlying diseases and are affected by medications, they still provide a good estimate of the overall impact of multiple risk factors during the atherosclerotic process[21]. Therefore, the dysbiosis of salivary microbiota in the group with low RHI values (i.e., poor peripheral endothelial function) identified in this study would be associated with the risk of ACVD. FMD is another widely used method to evaluate endothelial function. It has been suggested that the two methods based on reactive hyperemia, FMD and RH-PAT, assess different aspects of vascular function related to atherosclerotic risk factors[25, 26], however, both are useful in predicting the risk of cardiovascular events. Therefore, we believe that the characterization of salivary microbiota associated with abnormal vascular endothelial function as assessed by FMD, as well as RH-PAT, is an important next step in identifying salivary microbiota associated with risk factors for atherosclerosis.

Endothelial dysfunction is related to oral hygiene[5, 27] and periodontal diseases[6, 28, 29]. Moreover, the diversity of the oral microbiota may be reduced by frequent oral hygiene practices[30]. In this study, we revealed that individuals with poor vascular endothelial function had a higher diversity of salivary microbiota and a different overall composition of salivary microbiota compared to those with normal vascular endothelial function. However, there were no differences in oral hygiene and periodontal disease status between individuals with poor and normal vascular endothelial function in this study. Therefore, the salivary microbiota may be related to endothelial dysfunction apart from these oral-related factors. Whether the salivary microbiota can be altered by diet and oral interventions to potentially improve vascular endothelial function remains largely unknown and needs to be evaluated in future studies. It is well known that diet affects the gut microbiota[31, 32], and there are reports that gut microbiota can be altered by dietary intervention to improve vascular endothelial function[33]. However, little is known about the possible effects on the oral microbiota. There are some reports that dietary components are associated with salivary microbiota due to long-term dietary patterns or dietary changes[34, 35]. Therefore, we believe that changes in salivary microbiota due to nutritional intervention are worth investigating in the future. In addition, dental treatments such as antibiotic therapy, periodontal interventional interventions, antimicrobial photodynamic therapy, probiotic therapy, quorum quenching therapy, phage therapy, fluoride nanophase materials, and oral hygiene products based on natural extracts may alter the oral microbiota, but conclusive data is still lacking[36]. Since salivary microbiota is relatively stable[37, 38], the possibility of controlling the salivary microbiota by dental procedures is an issue for future study.

Low diversity of the gut microbiota has been shown to be associated with ACVD[39]. In our previous study, the alpha diversity of salivary microbiota did not differ between groups of ACVD and non-ACVD subjects[16]. These conflicting results indicate that differences in gut and salivary microbiota cannot be explained by diversity alone. The high alpha diversity in the group with peripheral vascular endothelial dysfunction in subjects without ACVD in this study suggests that salivary microbiota characteristic of the stage before the onset of ACVD may be relevant. Recently, several animal model studies have shown that oral bacteria may affect the gut microbiota[40-42]. Since the gut microbiota plays an important role in the balance of vascular homeostasis via nitric oxide production, inflammation, and thrombosis[43, 44], the mechanism of the association between salivary microflora and vascular endothelial function requires studies inclusive of the gut microbiota.

Analysis of LEfSe showed that there were significant characteristics in the groups with poor and good vascular endothelial function. The bacterial genus *Veillonella* was dominant in the group with good vascular endothelial function, and its abundance was correlated with the RHI values. Interestingly, *Veillonella* is one of the oral bacteria associated with nitric oxide homeostasis, and the nitrate (NO_2^-) produced by *Veillonella* is known to be related to the normalization of blood pressure[45, 46]. Nitrate-reducing oral bacteria such as *Veillonella* are associated with cardiometabolic outcomes[47, 48]; therefore, it is conceivable that they are associated with good vascular endothelial function.

In addition, among the bacterial genera that were more common in participants with poor vascular endothelial function, some bacteria have been suggested to be associated with ACVD. For example, among the bacterial genera that were more abundant in the abnormal group, *Mycoplasma* and *Treponema* were more prevalent in patients with ACVD than in normal individuals in our previous study[16]. Therefore, each of these bacteria may be associated with an impaired vascular endothelial function that may develop into ACVD. However, detailed investigations are needed to determine whether the complete

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microbiota community and not just individual bacteria are associated with vascular endothelial function.

In this study, to evaluate whether the whole salivary microbiota can discriminate between normal and abnormal groups of vascular endothelial function, classification models based on microbial composition at the genus level were built using machine learning techniques, and their prediction performances were evaluated. The Extra Trees classifier model obtained was the best, with an AUC value of 0.810. Evaluation of the statistical measures indicated that the salivary microbial composition at the genus level could discriminate between normal and abnormal groups. Thus, the salivary microbiota of individuals with poor vascular endothelial function is characteristic and sufficiently different from those with normal endothelial function. Additionally, saliva is relatively easy to collect and non-invasive and may be useful for screening to predict the status of vascular endothelial dysfunction. Therefore, our data suggest that the use of salivary bacteria for the early diagnosis of ACVD has great potential and merits future validation and optimization.

This study has several limitations. First, it is a case-control observational study and cannot prove causality. The mechanisms by which differences in the composition of salivary microbiota are related to vascular endothelial function should be further explored in future research. Second, all study participants were elderly Japanese living in Takatsuki City; therefore, our results may not be extrapolated to other populations. Participant background, such as age, ethnicity, and food culture, are factors known to affect the salivary microbiota and can confound the study results if not addressed appropriately; thus, large multi-ethnic and multi-center studies are needed to confirm our results. Third, 16S rRNA gene sequencing is limited. In this study, we could only make phylogenetic identifications that did not go beyond the genus level. Since differences in functional capacity among microorganisms can already be seen even at the species or strain level, both 16S rRNA metagenomic analysis and combined analytical methods, such as shotgun sequencing and various omics analyses, should be performed to elucidate, in detail, the changes in microbiota that affect the vascular endothelial function. Nevertheless, this preliminary pilot study should be further validated to confirm our observations, and the causal relationship between vascular endothelial function and salivary microbiota should be thoroughly examined.

Conclusion

In conclusion, we characterized salivary microbiota associated with peripheral microvascular endothelial dysfunction in community-dwelling elderly individuals without a history of ACVD. This study suggests that it may be possible to identify individuals with impaired vascular endothelial function, a prelude to the development of ACVD, based on salivary microbiota. Furthermore, since salivary microbiota may affect endothelial function, and endothelial dysfunction is reversible, further elucidation of these mechanisms may help develop novel diagnostic and therapeutic approaches that may reduce the risk of ACVD.

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Conflicts of Interest

The authors declare no conflict of interest.

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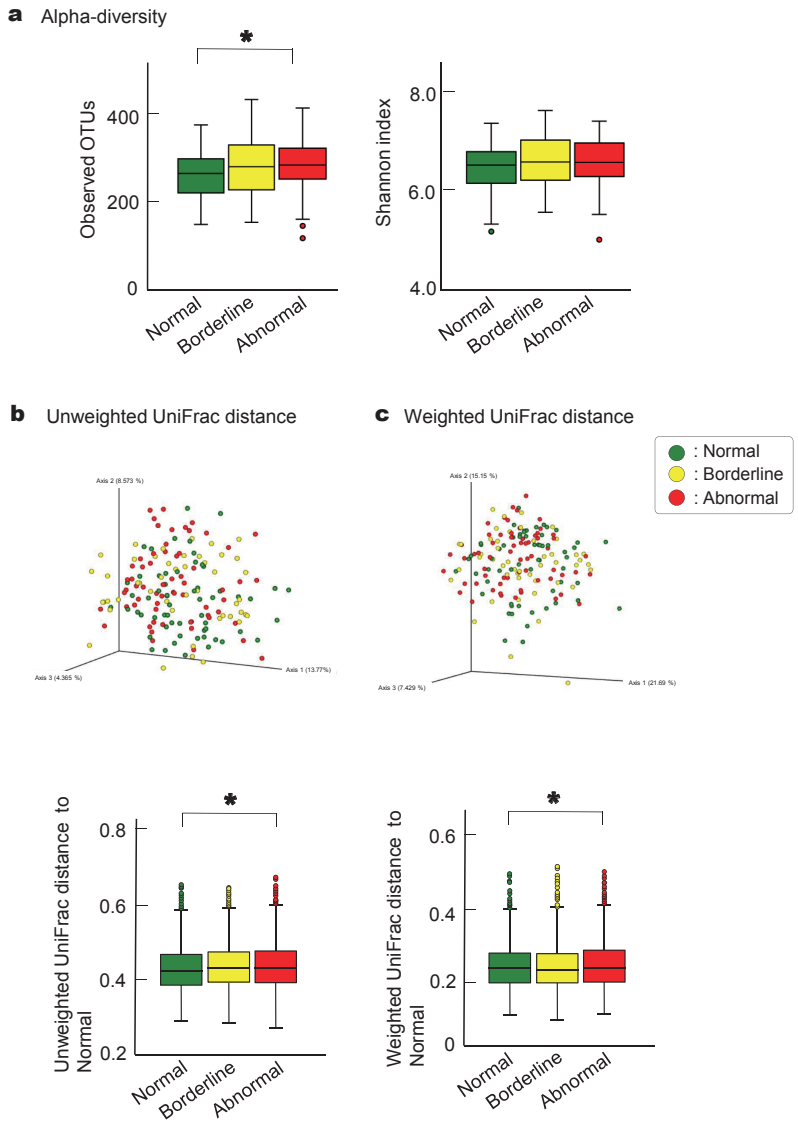

Supplemental Fig. 1. Alpha and Beta diversity of salivary microbiota in participants excluding current smokers

(a) Operational taxonomic unit (OTU) index and Shannon index in the normal (RHI ≥ 2.10), borderline (1.67 ≤ RHI <2.10), and abnormal (RHI <1.67) groups. Normal (n=59, green), borderline (n=47, yellow), and abnormal groups (n=63, red). *p<0.05 compared among groups using Kruskal–Wallis test.
(b) Unweighted UniFrac distances.
(c) Weighted UniFrac distances.
Principal coordinate analysis (PCoA) plot for samples from 59 participants in the normal group (green), 47 participants in the borderline group (yellow), and 63 participants in the abnormal group. Box plots represent UniFrac distances to normal group participants from the normal (green columns), borderline (yellow), and abnormal groups (red columns). *p<0.05, compared between groups using PERMANOVA, 999 permutations.

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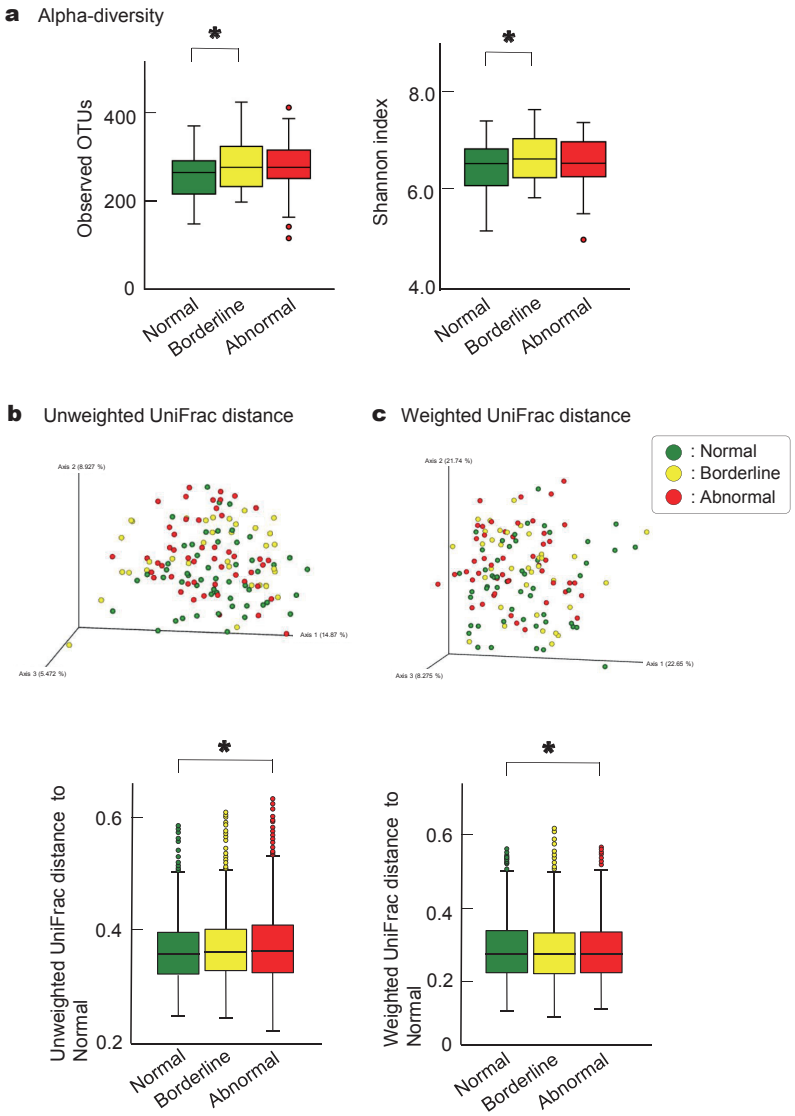

Supplemental Fig. 2. Alpha and Beta diversity of salivary microbiota in participants excluding current smokers and EX-smokers

(a) Operational taxonomic unit (OTU) index and Shannon index in the normal (RHI ≥ 2.10), borderline (1.67 ≤ RHI <2.10), and abnormal (RHI <1.67) groups. Normal (n=52, green), borderline (n=35, yellow), and abnormal groups (n=49, red). *p<0.05 compared among groups using Kruskal–Wallis test.
(b) Unweighted UniFrac distances.
(c) Weighted UniFrac distances.
Principal coordinate analysis (PCoA) plot for samples from 52 participants in the normal group (green), 35 participants in the borderline group (yellow), and 49 participants in the abnormal group. Box plots represent UniFrac distances to normal group participants from the normal (green columns), borderline (yellow), and abnormal groups (red columns). *p<0.05, compared between groups using PERMANOVA, 999 permutations.

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