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Effect of Stimulated Salivary Volume on Dysbiosis of the Salivary Microbiome in Children and Young Adults

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ABSTRACT

Objectives: This study aimed to determine the salivary factors that influence salivary bacterial counts and the microbiome composition in children and young adults.

Methods: This cross-sectional study included 382 patients who visited the dental clinic in Hiroshima University Hospital. All participants underwent a saliva test and were divided into high- and low-bacterial-count groups based on the median bacterial count. Salivary factors and clinical variables, including the total number of teeth and plaque control record, were analyzed to determine their association with salivary bacterial counts. Furthermore, a comprehensive analysis of microbiome diversity and composition was performed using 16S rRNA sequencing.

Results: Univariate and multivariate analyses identified stimulated saliva volume (SSV) and plaque control record as independent factors influencing salivary bacterial counts. Principal coordinate analysis revealed a significant decrease in beta diversity in the high-bacterial-count group. LEfSe analysis revealed *Prevotella, Veillonella, Megafaella, Selenomonas*, and *TM7X* as the 5 most abundant bacteria. The relative abundance of the 35 KEGG pathways exhibited significant differences. Furthermore, *Prevotella* and *Veillonella* were strongly associated with 25 functional pathways.

Conclusion: Oral hygiene instruction is necessary even for children and young adults with relatively adequate SSV to maintain a healthy oral microbiome.

Introduction

Similar to the gut, saliva harbors a diverse oral microbiome. Alterations in the human oral microbiome are associated with various diseases.[1] The 2007 Human Microbiome Project[2] results and the Human Oral Microbiome Database[3] indicated that there are currently over 2000 oral reference genomes stored in the database. Over 600 bacterial species are estimated to be commonly associated with oral microbiome,[4] which are involved not only in local diseases, such as dental caries and periodontal disease, but also in aspiration pneumonia in elderly and patients with impaired swallowing and coughing reflexes due to cerebrovascular disease.[5,6] Meanwhile, assessment of oral microbiome in children and young adults is crucial for understanding the subsequent colonization of bacterial species and the formation process of mature oral microbiome. Santacroce et al. have shown that newborns are colonized by new bacteria within minutes and eventually within 10-13 years of birth (intestinal bacteria, oral bacteria, etc.).[7] The factors known to influence the composition of the microbiome include age, diet, pregnancy, alcohol use, medications (e.g., antibiotics), toxic substances, genetic

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predisposition, and socioeconomic status.[7] In children and young adults, oral microbiome development may also be influenced by independent oral hygiene habits, the transition to permanent dentition, and dietary shifts.[8-10] With aging, the oral microbiota changes, and the number of periodontal pathogens increases, elevating susceptibility to oral disease.[8,10,11]

In healthy individuals, oral bacterial count remain stable and infections are rarely caused by the self-cleaning action of saliva and the presence of a systemic immune system.[12] However, failure to maintain oral hygiene due to decreased salivary secretion, weakened immune system, and increased oral commensal bacteria can exert local or systemic effects.[13-15] Aspiration pneumonia is caused by aspiration of indigenous bacteria in the oral cavity at bedtime and can be prevented through oral care.[16,17] The factors that worsen the oral environment and increase the oral bacterial count include low-level awareness, dental caries, tongue lint, and halitosis.[18-20] Sinada et al. also reported that changes in oral conditions due to aging, including tooth loss, denture wear, halitosis, and decreased salivary volume, affect the increase and decrease in oral bacteria.[21] Other factors that allow bacteria to grow in the oral cavity include oxygen, appropriate temperature, and salivary PH and buffer capacity.[7,22-25] Most of the above evidence is based on older adults, and reports on oral bacterial counts in children and young adults are scarce.[26-28]

Many studies have used quantitative polymerase chain reaction (qPCR) to measure salivary bacteria, but this method is labor-intensive and time-consuming, requiring DNA extraction and PCR amplification.[29,30] Furthermore, dead cells are counted. A rapid bacteriometer, commercially available in Japan, can measure the total number of viable bacteria in saliva within approximately 1 min using dielectrophoretic impedance measurement (DEPIM) method and is reportedly useful for evaluating oral hygiene during oral care of hospitalized patients.[31,32]

This study aimed to assess salivary bacterial counts in children and young adults using this rapid device and to examine the salivary and clinical factors affecting bacterial load. Saliva samples were collected under controlled conditions, with restrictions on antibiotics, mouthwash use, and brushing duration as they may affect salivary microbiome. In addition, 16S ribosomal RNA (rRNA) sequencing was conducted using Illumina NovaSeq to compare bacterial diversity and composition between the high- and low-bacterial-count groups.

Methods

Study participants

This study included 384 patients who visited the dental clinic at Hiroshima University Hospital from March 2022 to February 2024. All participants underwent a dental examination, including a plaque control record assessment and a salivary test at baseline. Participants who had taken antibiotics or other medications within 14 days before the salivary test and those with congenital diseases, including oligodontia, were excluded (Figure 1). In addition, 28 participants whose total volume of stimulated saliva was <2 mL in 10 min were excluded owing to insufficient volume for salivary bacterial count measurement (Figure 1).

Stimulated saliva collection

Before sampling, the participants were instructed to refrain from eating, drinking, smoking, and brushing their teeth for at least 2 h. They were also advised to avoid using mouthwash or dentifrices. Stimulated saliva samples were collected while participants were seated comfortably in a dental chair. To evaluate the stimulated saliva volume (SSV), participants

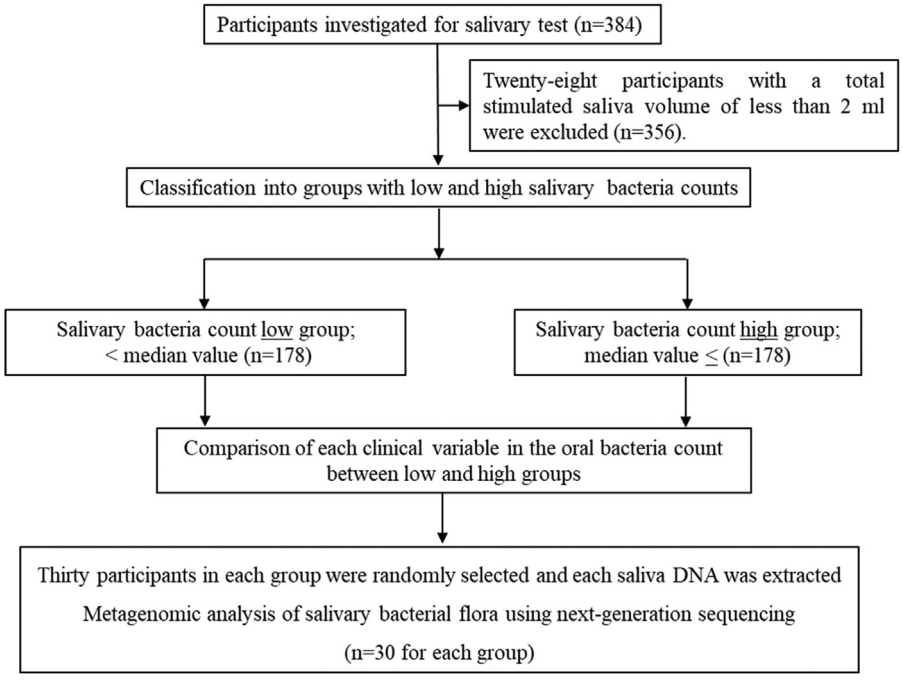

Figure 1 – Flow diagram of the participants analyzed in this study.

were instructed to chew a tasteless gum (HORIBA, Ltd., Kyoto, Japan) and continuously spit into a cup for 5 min. Before chewing the gum, participants were instructed to spit into a dental spittoon to remove any residual saliva. The saliva secreted during the 5-min chewing period was collected in a 15-mL centrifuge tube and sent to the Oral Clinical Examination Center at Hiroshima University Hospital for further examination.

Salivary examinations

Stimulated saliva secretion rate was calculated as the volume of saliva secreted per minute (mL/min). A compact pH meter (LAQUAtwin, HORIBA, Ltd.) was used to measure salivary pH, while CAT21Buf (Morita Co., Osaka, Japan) was used to estimate salivary buffer capacity. A mixture of 1-mL saliva and acid load liquid was vigorously shaken, and the final pH was determined according to the product instructions. To detect *Candida species*, a dentist affiliated with the Oral Clinical Examination Center cultured 0.02 mL of saliva in CHROMagar Candida medium (CHROMagar, Paris, France) for 48 h at 37°C and then conducted semiquantitative evaluation in 2 stages: negative (no colonies detected) and positive (at least 1 colony detected).

Measurement of salivary bacterial count

The bacterial count in the collected saliva samples was measured using the Oral Bacteria Counter (Panasonic Healthcare Co. Ltd., Osaka, Japan), a rapid oral bacteria quantification system, according to the product instructions.[31] This device can quantitatively measures the total number of viable bacteria in saliva within approximately 1 min using DEPIM, allowing for an accurate and objective oral hygiene evaluation.[31] The device has a detection limit ranging from 1×10^5 to 1×10^8 colony-forming units (CFU)/mL. Patients with salivary bacterial counts above and below the median were allocated to the high- and low-bacterial-count groups, respectively.

Calculation of plaque control record and total number of teeth

The plaque control record was calculated using the O'Leary index.[32] A certified dental hygienist stained 6 surfaces along the gum line, namely, the medial, central, and distal surfaces of the cheek and lingual sides of a tooth, to visualize dental plaque deposits. The ratio of stained tooth surfaces to the total number of tooth surfaces was calculated for the plaque control record. The number of erupting teeth was defined as the total number of teeth.

DNA extraction from saliva samples

Thirty participants were randomly selected from each group. After pretreatment and mechanical disruption of the bacterial cell wall, bacterial DNA was isolated from 60 saliva samples using the MagMAX™ Saliva gDNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions and stored at −80°C.

Detection of bacterial 16S rRNA

The extracted DNA was used as a template for PCR amplification with Go TaqR Master Mix (Promega, Madison, WI, USA) and a universal primer pair with Illumina adapter overhang sequences based on specific 16S rRNA (V3-F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′ V4-R: 5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′). PCR was conducted by Macrogen Japan Corp. (Tokyo, Japan).

Next-generation sequencing analysis

Saliva samples with confirmed bacterial presence in the first PCR amplification were subjected to comprehensive next-generation sequencing via amplification of the 16S rRNA V3-4 region by Macrogen Japan Corp. PCR amplicons were pooled and sequenced on an Illumina MiSeq with 300-bp pair-end sequencing.

Sequencing data analysis

Sequencing data were analyzed using the standard Quantitative Insights Into Microbial Ecology (QIIME2; version 2023.02).[33] Low-quality reads and adaptor contaminants were eliminated before analysis. After demultiplexing, paired-end reads of 60 samples collected from the participants were imported into QIIME2. Divisive Amplicon Denoising Algorithm 2 in the QIIME2 platform was used for sequence quality control and feature table construction. After denoising, a pretrained naÿve Bayes classifier was used to examine the taxonomic distribution of the samples. This classifier was trained using the SILVA database version 138.[34] An alpha index (Shannon index) and beta diversity indices (Bray–Curtis and Jaccard distances) were calculated using QIIME2. Furthermore, the Gini–Simpson index was calculated based on the previous report.[35]

Statistical analysis

Statistical analyses were conducted using JMP Pro 16 (SAS Institute Inc., Cary, NC, USA) and QIIME2. Continuous variables were expressed as medians (interquartile range [IQR]), whereas discrete variables were expressed as frequencies and percentages. The Mann–Whitney *U* test (continuous variables) or chi-squared test (discrete variables) was used to assess the statistical significance of intergroup differences, as appropriate. 2-step strategies were adopted to evaluate the relative importance of variables in their association with the efficacy of oral bacterial count. First, univariate analysis was conducted to compare various clinical variables between the high- and low-bacterial-count groups. Second, logistic regression analysis was conducted to estimate odds ratios and 95% confidence intervals (CI) for potential risk factors, including age, gender, SSV, plaque control record, and total number of teeth. Correlations between bacterial count and other clinical variables were determined using Pearson's product–moment correlation coefficients. For all analyses, *P*-values $< .05$ were considered statistically significant.

To analyze the salivary microbiota, the beta diversity index was visualized via principal coordinate analysis (PCoA). Significant differences in the alpha and beta diversity indices between

the groups were calculated using QIIME2 plugins with the Kruskal–Wallis test and pairwise permutational multivariate analysis of variance (PERMANOVA). Furthermore, the alpha and beta diversity indices were compared between the groups with a q-value adjusted for false discovery rate using the Benjamini–Hochberg method to prevent type I error due to multiple comparisons. Results with q-value < 0.05 were considered significant. Microbiome composition was visualized using "MicrobiomeAnalyst" (https://www.microbiomeanalyst.ca/MicrobiomeAnalyst).[36] The linear discriminant analysis effect size (LEfSe) was used to identify taxa that characterized differences between the groups. This algorithm uses the nonparametric factorial Kruskal–Wallis rank-sum test ($\alpha = 0.05$) to detect features with considerable differential abundance and then linear discriminant analysis to estimate the effect size of each differentially abundant taxa.[37] Predictive functional genes were evaluated using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2[38] based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Moreover, statistical analysis of metagenomic profiles[39] was conducted to determine the pathways associated with the high total salivary bacterial count.

Ethical considerations

This case–control study was approved by the Research Ethics Board of Hiroshima University (approval no. epidemiology-2022-0205). Verbal research consent was obtained from the participants, which was noted in the medical record. The study included participants aged below 18 years, and informed consent was obtained from their parents or legal guardians. All methods were performed in accordance with the relevant guidelines and regulations.

Results

A total of 356 participants (200 women [56.2%] and median age 12.5 [IQR, 9-20] years) were analyzed during the enrollment. At baseline, the median values for the SSV, salivary pH, salivary buffer capacity, *Candida species* detection, total number of teeth, plaque control record, and total salivary bacterial count were 1.2 (IQR, 0.9-1.7) mL/min, 7.7 (IQR, 7.5-7.8), 6.7 (IQR, 6.4-6.9), 28 participants, 24 teeth (IQR, 23-28), 56% (IQR, 40%-68%), and 237.6 (IQR, 101.2-477.1) $\times$ 10^4 CFU/mL, respectively (Table 1). The participants were divided into high- and low-bacterial-count groups based on reference values, and clinical variables between the groups were compared via univariate analysis. The results indicated that age, SSV, plaque control record, and total number of teeth were significantly higher in the high-bacterial-count group than in the other group ($P < .05$; Table 1). Logistic regression analysis revealed that SSV and plaque control record were independent factors affecting salivary bacterial count (odds ratio [OR] 13.1, 95% CI 4.33-39.40 and OR 10.6, 95% CI 3.39-33.40, respectively; $P < .05$; Table 2). Next, correlations between bacterial count and clinical variables were assessed. No correlation was observed between the bacterial count and each clinical variable (Figure S1).

From 60 salivary profile data samples, 10,803,843 final reads were generated after filtering, with a mean of 180,064.05 (range, 120,719-203,022) reads per sample. Ultimately, 3724 amplicon sequence variants (ASVs) were detected. Microbiota analysis revealed 18 microbial phyla, 25 classes, 63 orders, 104 families, 234 genera, and 493 species in the saliva samples. Species richness or evenness in a single sample indicates the alpha diversity of an ecosystem. No significant differences were observed in overall species diversity, which was evaluated using the Shannon and Gini-Simpson indices between the high- and low-bacterial-count groups (Figure 2A and B). Moreover, no correlation was found between alpha diversity and salivary bacterial counts (Figure S2). PCoA plots showed distinct clusters representing both groups, indicating a significant difference in Bray–Curtis and Jaccard distances in their salivary microbial communities, as determined by PERMANOVA (Figure 2C and D; $P = .001$ and $P = .002$, respectively). Beta diversity was significantly decreased in the high-bacterial-count group than in the other group.

Table 1 – Characteristics of the study participants.

Variables	n = 356	Bacterial count in saliva		*P* value
		Low[†] (n = 178)	High[‡] (n = 178)	
Age (median, IQR)	12.5, 9-20	11.0, 8.0-19.3	14.0, 10.0-21.0	0.003[§]
Gender (male, female)	156, 200	74/104	82/96	0.45[¶]
Stimulated saliva volume (mL/min; [median, IQR])	1.2, 0.9-1.7	1.1, 0.7-1.6	1.4, 1.0-1.9	<0.001[§]
Salivary pH (median, IQR)	7.7, 7.5-7.8	7.7, 7.5-7.8	7.7, 7.5-7.8	0.85[§]
Salivary buffer capacity (median, IQR)	6.7, 6.4-6.9	6.7, 6.3-6.9	6.7, 6.4-6.9	0.41[§]
Candida species (positive, negative)	28, 328	16, 162	12, 166	0.43[¶]
Plaque control record (%; median, IQR)	56, 40-68	52.0, 36.0-65.0	60.0, 45.0-71.0	<0.001[§]
Total number of teeth (median, IQR)	24, 23-28	24.0, 23.0-28.0	26.0, 24.0-28.0	0.006[§]
Salivary bacterial count ($\times$ 10^4 CFU; [median, IQR])	237.6, 101.2-477.1	101.95, 61.25-156.11	475.3, 330.29-774.20	

$P < 0.05$ (statically significant).
IQR, interquartile range; CFU, colony-forming unit.

[†] The value below the median was defined as low.
[‡] The median value or higher was defined as high.
[§] Mann–Whitney U test.
[¶] Chi-squared test.

Table 2 – Multivariate analysis for exploring the factors associated with salivary bacterial count.

Variables	Reference	Odds ratio	95% CI		P value†
			Lower	Upper	
Age		2.47	0.41	14.82	0.32
Gender	male/female	1.10	0.69	1.74	0.70
Stimulated saliva volume (mL/min)		13.1	4.33	39.40	<0.001
Plaque control record (%)		10.6	3.39	33.40	<0.001
Total number of teeth		2.52	0.44	14.54	0.30

P < 0.05 (statically significant).
CI, confidence interval.
† Logistic multivariate analysis.

The salivary microbial composition in both groups demonstrated dominance of the following genera, each with a relative abundance exceeding 5% (high; low), as shown in Figure 3A: *Streptococcus* (21.71%; 34.66%), *Prevotella* (16.14%; 6.48%), *Veillonella* (14.59%; 5.53%), and *Neisseria* (6.50%; 9.63%). Similarly, the dominance of the following families is presented in Figure 3B (high; low): *Streptococcaceae* (21.71%; 34.24%), *Prevotellaceae* (17.06%; 7.21%), *Veillonellaceae* (15.63%; 5.72%), and *Neisseriaceae* (6.77%; 9.81%).

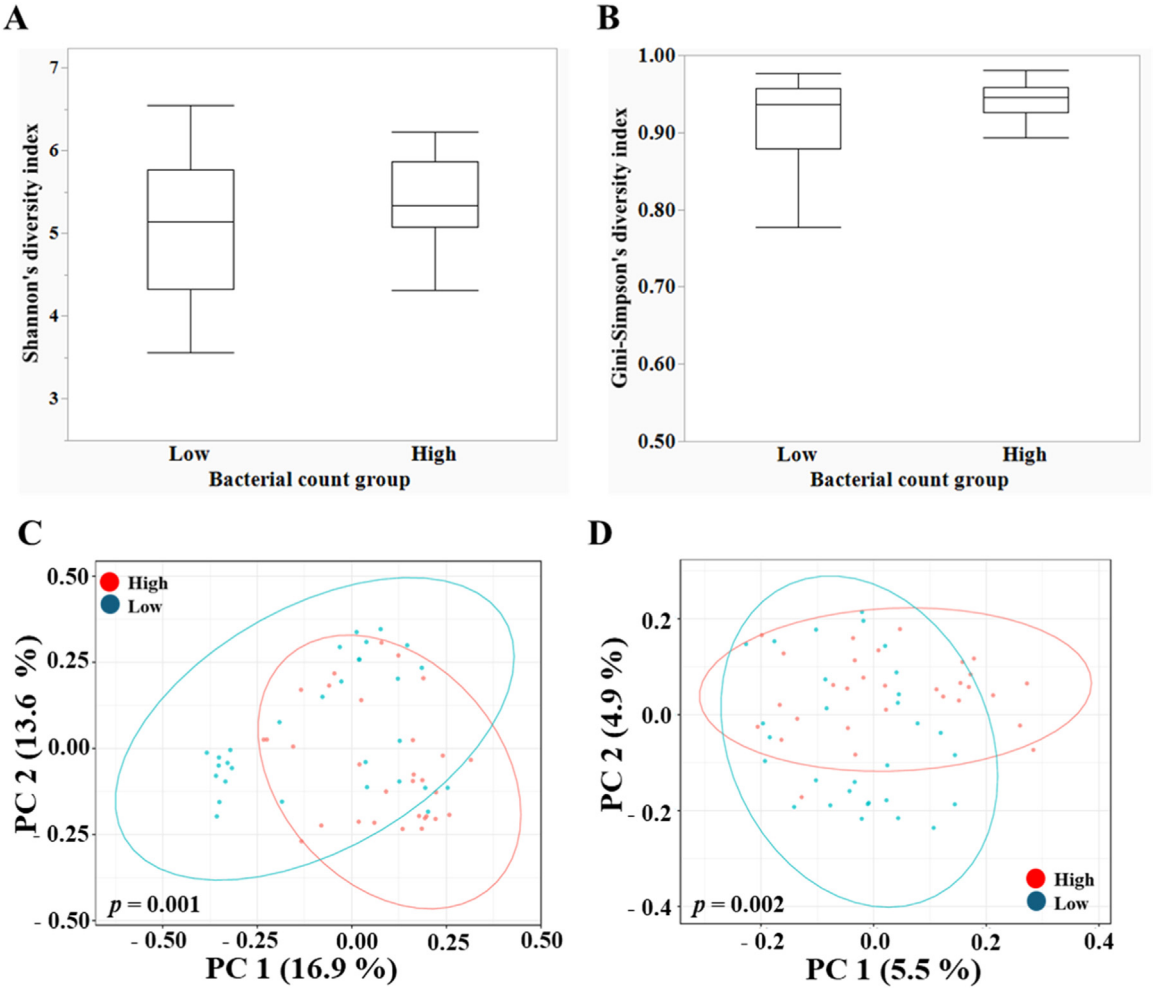

Figure 2 – Comparisons of the salivary microbiota amplicon sequence variants between the high- and low-bacterial-count groups. Alpha diversity of the salivary microbiome between the high- and low-bacterial-count groups. The indices are the Shannon index (A) and the Gini–Simpson index (B). Beta diversity of the salivary microbiota of both groups. PCoA plots indicated a marked distinction between the groups based on the Bray–Curtis (C) and Jaccard (D) distances. P < 0.05 (statically significant).

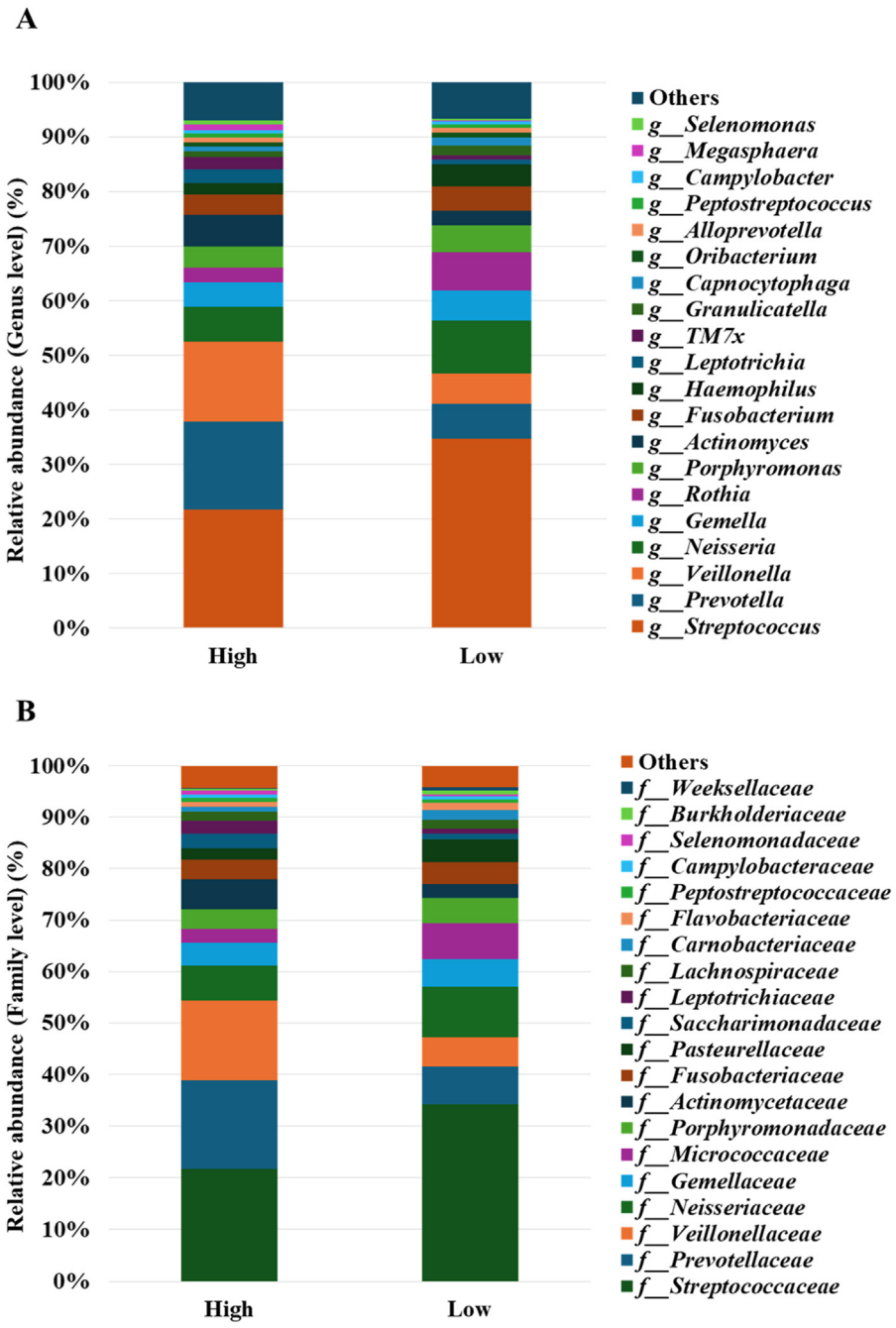

Figure 3 – Relative abundance of salivary microbiota. The percentage relative abundance at the genus (A) and family (B) levels between the high- and low-bacterial-count groups are shown.

To determine differences in salivary bacterial composition between the groups, the relative abundance of salivary microbial communities in the 2 groups was analyzed. Using LEfSe, 13 genera with LDA scores of 2 or higher were identified in the high-bacterial-count group. This group showed considerable increases in the abundance of *Prevotella*, *Veillonella*, *Megasphaera*, *Selenomonas*, *TM7X*, *Actinomyces*, *Leptotrichia*, *Atopobium*, *Clostridia*, *Eubacterium*, *Catonella*, *Scardovia*, and *Dialister* (Figure 4A and B). Meanwhile, the low-bacterial-count group showed considerable increases in the abundance of *Streptococcus*, *Rothia*, *Haemophilus*, *Granulicatella*, *Capnocytophaga*, *Lautropia*, *Bergeyella*, *Cardiobacterium*, *F0332*, *Tannerella*, and *Ralstonia* (Figure 4A and B).

The functional pathways associated with salivary microbiota in the high-bacterial-count group were predicted via PICRUSt analysis of the ASV tables. It showed a functional pathway with a q-value $<2 \times 10^{-5}$ that was differentially expressed in both groups (Figure 5A). Among these pathways, 19 were upregulated, namely, mitochondrial biogenesis, cysteine/methionine metabolism, amino acid metabolism,

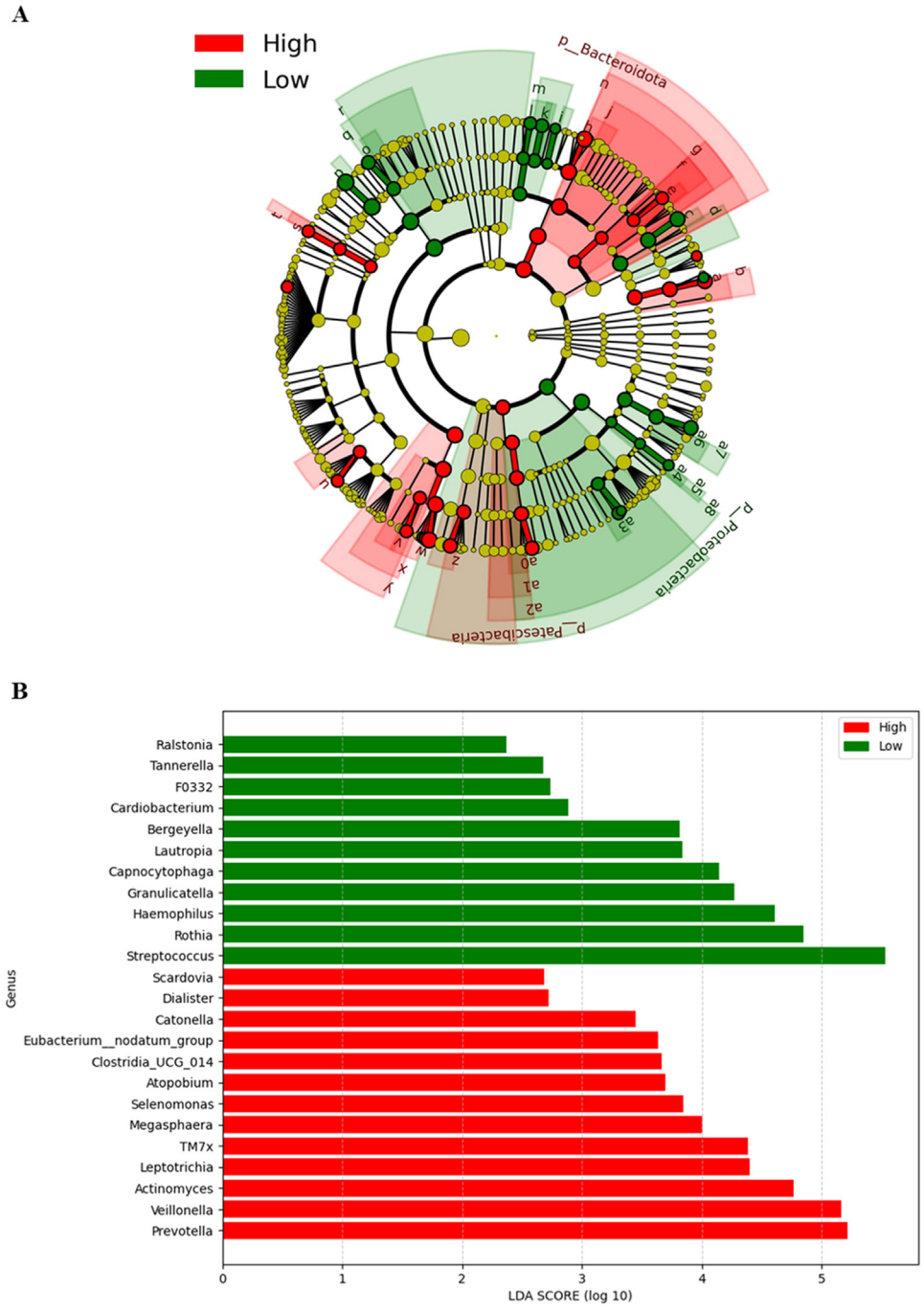

Figure 4 – Differential abundance analysis of the salivary microbiota constituents between the groups. A phylogenetic tree presenting the hierarchical relationships among various taxa, ranging from the phylum to the genus levels based on the LEfSe method (A). Marker microbes in the high- and low-bacterial-count groups with LDA score (log10) > 2 (B).

lipopolysaccharide biosynthesis, metabolic pathways, glycolysis/gluconeogenesis, superoxide reductase, microbial metabolism, carbon-sulfur lyases, flagellar assembly, beta-lactam resistance, carbohydrate metabolism, major facilitator superfamily transporter, protein processing in the endoplasmic reticulum, glycan metabolism, fatty acid biosynthesis, lysine biosynthesis, transcription factors, and glycosyltransferases, whereas 8 were downregulated, namely, microbial metabolism, metabolic pathways, membrane protein, carbohydrate metabolism, cysteine/methionine metabolism, transfer RNA biogenesis, kinase/phosphorylase, and DNA helicases, in the high-bacterial-count group (Figure 5A). The 32 distinct functional pathways and 13 bacterial genera were subjected to Spearman's correlation analysis (Figure 5B). *Prevotella*, *Veillonella*, *Megasphaera*, and *Selenomonas*, whose abundance increased in the high-bacterial-count group, exhibited significant positive correlations with majority of the KEGG pathways, albeit with

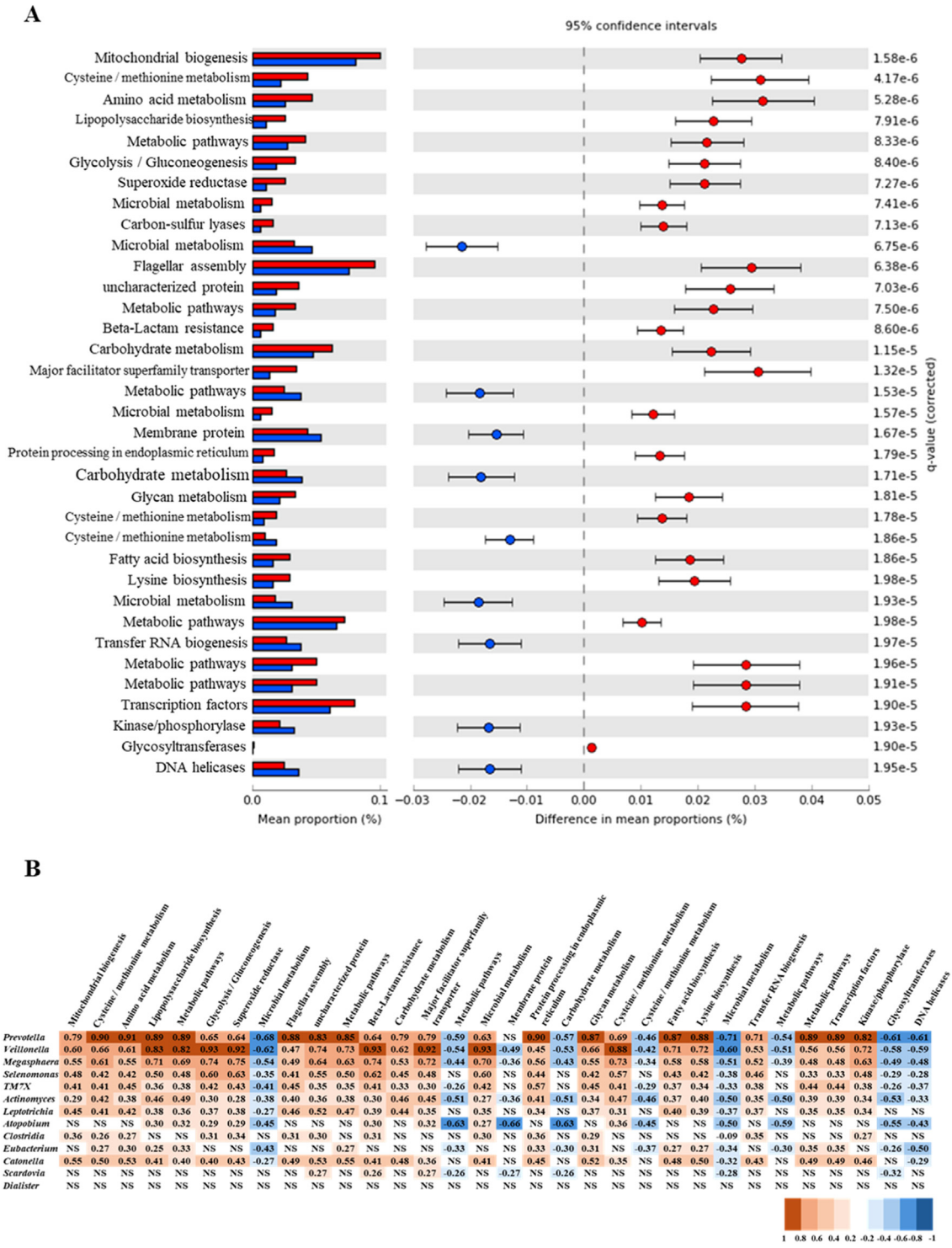

Figure 5 – Functional prediction by PICRUSt2. A total of 35 KEGG pathways with significant differences across the groups according to the Kruskal–Wallis rank-sum test ($P < 0.05$, corrected using the Benjamini–Hochberg) (A). A total of 13 genera were identified as differentially correlated with pathways through the Spearman correlation analysis. Positive correlations between genera and pathways are shown in red, whereas negative correlations are shown in blue. The numbers indicate the Spearman correlation coefficient (B).

similar strengths ($P < .05$). Meanwhile, some functional pathways did not exhibit correlation with *Atopobium*, *Clostridia*, *Eubacterium*, *Scardovia*, and *Dialister*, whose abundance increased in the same group. It suggests that changes in the KEGG metabolic pathway, i.e., activation of intracellular homeostasis and energy production, in the high-bacterial-count group further increase the bacterial count.

Discussion

The causes of increased oral bacteria include inadequate oral hygiene, decreased saliva production, and poor function of the mouth and tongue muscles. An increased oral bacterial count can disrupt the oral microbiome and increase susceptibility to certain diseases.[40] This study first examined the salivary properties associated with increased bacterial counts, followed by 16S rRNA sequencing to analyze the salivary microbiome in children and young adults. In this study, we first examined salivary factors and several clinical variables that increase bacterial counts in saliva in children and young adults. Subsequently, we performed 16S rRNA sequencing to analyze the microbiome between groups with high and low salivary bacterial counts. A whole microbial community approach was used to explore the characteristics of the salivary microbiota and potential changes in functional pathways associated with increased bacterial counts.

To improve the accuracy of the study, the use of antibiotics and antimicrobial mouthwashes was limited, as they may affect the microbiome. D'Ercole et al. used 50×10^4 CFU/mL as the reference value for bacterial counts,[41] which is similar to the value in this study, though the measurement methods differed. The participants were divided into 2 groups based on the median bacterial count. Univariate and multivariate analyses were then conducted to identify the salivary factors or clinical variables influencing salivary bacterial counts. The results indicated that plaque control record and SSV were independent factors contributing to increased bacterial counts. Furthermore, no correlation was observed between SSV and bacterial counts. The results are novel as there have been no previous reports on the association between increased SSV and increased salivary bacterial counts. Next-generation sequencing was used to analyze the microbiome between the groups. It has been reported that the salivary microbiome is mainly supplied by the biological membrane of the tongue.[7] The bacterial species detected in this study were similar to those in previous studies.[42]

No statistical differences were observed in alpha diversity between the groups among the datasets using standard estimates. It is possible that the limited age range and the conditions under which saliva samples were collected have contributed to the lack of change in alpha diversity. We used PERMANOVA to assess beta diversity and found significant associations between the groups. This result was unexpected, as the high-bacterial-count group showed significantly less diversity than the other group. Previous studies have reported that patients with inflammatory bowel disease exhibit decreased intestinal microbiota diversity and changes in the constituent bacterial species (dysbiosis).[43] However, less is known about the reduced diversity of the salivary microbiome in children and young adults.[27,44] The decrease in the diversity of the salivary microbiome in the high-bacterial-count group indicated that the counts of certain bacteria are increasing.

LEfSe analysis revealed that the counts of *Prevotella, Veillonella, Actinomyces*, and *TM7X* increased in the high-bacterial-count group. *Prevotella* is isolated not only from dental infections but also from all the other infections in the maxillofacial region, including postoperative infections. It is known to be highly pathogenic and plays a pivotal role in the development and progression of jaw and oral cavity infections.[45] *Veillonella* spp. are considered to be causative agents of halitosis as they reduce sulfur-containing compounds, such as cysteine, to produce hydrogen sulfide; furthermore, they are closely associated with opportunistic infections. Biofilm formation is thought to be induced by *Veillonella* spp.[46] *Actinomyces* is a major component of the oral commensal community and is proportionally abundant in a healthy mouth; however, it can sometimes cause opportunistic or endogenous infections.[47] The participants in this study were relatively healthy, and the high count of *Actinomyces* is consistent with previous reports.[47] *Saccharibacteria* (*TM7*), a commensal epiphyte that resides on the surface of host bacteria, has been strongly correlated with dysbiosis in inflammatory diseases such as periodontitis, suggesting that it is a putative pathogen. Recent evidence indicates that *TM7* protects mammalian hosts from inflammatory damage induced by host bacteria.[48] Thus, it was suggested that the increased numbers of certain bacteria in the high-bacterial-count groups reduce diversity. As regards the composition of the microbiome, *Streptococcus* spp. (family) accounted for the largest proportion in the low-bacterial-count group. They are also the predominant bacteria in the oral microbiome of young children. *Streptococcus salivarius* spp. have been shown to exert a protective effect against potentially pathogenic species.[7] An important recent finding is the association of the genera *Rothia* and *Hemophilus* with dental health.[42,49] Our results indicated that these bacteria predominated in the low-bacterial-count group.

Functional profiling of all the saliva samples using the KEGG database was performed to describe the functional characteristics of the microbiome in the high-bacterial-count group. Consequently, 35 distinct KEGG pathways were identified, demonstrating a functional landscape associated with increased salivary bacterial counts. It was found that 27 pathways involved in bacterial growth were upregulated in the high-bacterial-count group compared with 8 pathways in the other group. These results indicated that bacterial counts continued to increase in the high-bacterial-count group. Spearman's correlation analysis revealed a strong positive correlation between the increased abundance of *Prevotella, Veillonella, Megasphaera*, and *Selenomonas* spp. and numerous differentially enriched KEGG pathways. These results indicated that a correlation exists between changes in microbial composition and functional changes observed in the high-bacterial-count group. We believe that the upregulation of lipid metabolism, coenzyme and vitamin metabolism, as well as carbohydrate biosynthesis and metabolism in the high-bacterial-count group represents a shift in functional priorities that may reflect changes in energy utilization, signaling pathways, and biosynthetic processes of specific bacteria in the oral environment.[50,51] We demonstrated that increased SSV is an independent factor for increased salivary bacterial counts. It is well known that salivary secretion, along with its oral cleaning effect, antimicrobial activity, and calcium phosphate supersaturation, plays a role in the prevention of dental caries.[28,52] In addition, it has been reported that the higher the flow rate of stimulated saliva, the lower the risk and prevalence of oral bacterial infections such as dental caries and

periodontitis.[53] However, in children and young adults, as seen in this study, increased stimulated saliva flow may create a favorable environment for bacterial colony formation, activating bacterial metabolism, and creating the microbiome with a high bacterial count. Certain environmental conditions are required for microorganism growth in the oral cavity, including oxygen, proper temperature, and pH. In children and young adults, increased SSV was also considered to be a factor increasing the number of oral bacteria.

This study has some limitations. It follows a cross-sectional design, which limits its ability to establish causal relationships. While it identifies associations between SSV, plaque control, and bacterial counts, it cannot determine whether increased salivary volume causes dysbiosis or if other confounding factors are at play. A longitudinal study design would provide more substantial evidence of causality and enable observation of changes in the microbiome over time. The participants were recruited from a dental clinic, potentially introducing selection bias. These patients may have oral health statuses different[54] from those of the general population, potentially limiting the generalizability of the findings. The study identifies associations between specific bacteria, such as *Prevotella* and *Veillonella*, and functional pathways. However, it does not provide mechanistic insights into how these bacteria contribute to the occurrence of dysbiosis or oral disease.[55] Further experimental studies are warranted to explore these mechanisms. In addition, the study focuses on bacterial communities but does not explore other components of the oral microbiome, such as fungi, viruses,[56] or archaea, which could also play a role in the development of dysbiosis. Future studies should look into this aspect to give a holistic picture of the oral microbiome. The functional pathway analysis was based on predictive metagenomics (PICRUSt2), which relies on reference databases and may not fully capture the functional potential of the microbiome. Therefore, metatranscriptomic or metabolomic analyses in future research could provide more direct evidence of functional changes. Furthermore, the salivary microbiome of all the participants should have been analyzed, but due to insufficient research funds, only 60 participants were selected for analysis. In children and young adults, the SSV in the high-bacterial-count group was significantly higher than that in the low-bacterial-count group. The diversity of the salivary microbiome was significantly lower in the former than in the latter group. The structure and composition of the salivary microbiome in the high-bacterial-count group was significantly different from that in the other group. The increase in specific bacterial abundance observed in the high-bacterial-count group was closely associated with distinct functional pathways. Therefore, the instruction of oral hygiene is considered necessary, even for children and young adults with relatively adequate SSV.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Tomoaki Shintani: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Nami Obayashi:** Conceptualization, Methodology, Formal analysis, Investigation. **Tetsuya Yoshimoto:** Writing – original draft, Writing – review & editing. **Toshinori Ando:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Nagisa Morihara:** Formal analysis, Investigation. **Nanako Kataoka:** Methodology. **Rie Miyata:** Methodology. **Mai Yoshino:** Methodology. **Hiroki Yoshii:** Methodology. **Shin Morimoto:** Methodology. **Yuka Hayashi:** Methodology. **Masayuki Suzuki:** Methodology. **Kotaro Tanimoto:** Methodology, Supervision. **Mikihito Kajiya:** Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Supervision.

Study approval statement

This study protocol was reviewed and approved by the Research Ethics Board of Hiroshima University, approval number epidemiology-2022-0205.

Consent to participate statement

Verbal informed consent was obtained from all subjects.

This study included participants under the age of 18, in which case informed consent was obtained from the parent or legal guardian.

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Supplementary materials

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