

# Association of Salivary Microbiota with Dental Caries Incidence with Dentine Involvement after 4 Years <sup>S</sup>

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Salivary microbiota alterations can correlate with dental caries development in children, and mechanisms mediating this association need to be studied in further detail. Our study explored salivary microbiota shifts in children and their association with the incidence of dental caries with dentine involvement. Salivary samples were collected from children with caries and their subsequently matched caries-free controls before and after caries development. The microbiota was analyzed by 16S rRNA gene-based high-throughput sequencing. The salivary microbiota was more diverse in caries-free subjects than in those with dental caries with dentine involvement (DC). Although both groups exhibited similar shifts in microbiota composition, an association with caries was found by function prediction. Analysis of potential microbiome functions revealed that *Granulicatella*, *Streptococcus*, *Bulleidia*, and *Staphylococcus* in the DC group could be associated with the bacterial invasion of epithelial cells, phosphotransferase system, and D-alanine metabolism, whereas *Neisseria*, *Lautropia*, and *Leptotrichia* in caries-free subjects could be associated with bacterial motility protein genes, linoleic acid metabolism, and flavonoid biosynthesis, suggesting that functional differences in the salivary microbiota may be associated with caries formation. These results expand the current understanding of the functional significance of the salivary microbiome in caries development, and may facilitate the identification of novel biomarkers and treatment targets.

**Keywords:** Microbiota, dental caries, children, function prediction, biomarkers

## Introduction

Dental caries is one of the most common diseases of the oral cavity. In 2010, the prevalence of untreated caries in permanent teeth was 35.3% [1]. Severely decayed teeth may have an important impact on the general health, nutrition, growth, and body weight [2] of children by causing discomfort, pain, and sleeping problems [3].

Dental caries results from acids produced by commensal microbes within oral biofilms known as plaque [4]. Organic acids, including acetic, lactic, and propionic acids, are produced as fermentation by-products and dissolve the

hydroxyapatite component of enamel and dentine [5], leading to tooth surface breakdown and subsequent cavity formation. In the cavitated lesion, exposed dentine collagen fibers are subject to enzymatic degradation by bacteria [6]. In contrast to most classical medical infections, caries is a polymicrobial infection mediated by commensal oral microbes. The oral environment is home to approximately 700 prokaryote species as determined by the Human Oral Microbiome Database [7]. Clonal analysis of the 16S rRNA gene revealed that diverse bacteria, including *Streptococcus mutans*, non-mutans streptococci, and members of the genera *Actinomyces*, *Bifidobacterium*, *Lactobacillus*, *Propionibacterium*,

*Veillonella*, *Selenomonas*, and *Atopobium*, were associated with different stages of carious lesions [8–10]. Although current reports suggest that the salivary microbiome varies with dental decay [11–13], there is no consensus regarding the bacterial diversity and specific taxa specifically related to decay, possibly because of the differences in the subject age, sample size, cross-sectional comparison, and detection methods used among the different studies. In addition, limited information exists on the functional significance of the microbiota in caries development and longitudinal microbiota shifts associated with lesion progression. Therefore, identification of the association between dental caries progression and the salivary microbiota is necessary to fully understand the role of the microbiota in oral health.

The present study aimed to assess differences in the salivary microbiota of Korean children with or without dental caries, and predict the functional role of the salivary microbiota during caries development. Significantly, these results can improve the present understanding of salivary microbiome shifts and their role in caries development and aging.

## Materials and Methods

### Study Population

The Children of Busan Cohort is a longitudinal, geographically based study designed to examine risk factors for the progression of dental caries. Initial sampling started in 2009, and follow-up exams were conducted in 2012. Study participants were recruited from 13 community child centers since March 2009. We contacted the parents/guardians of 338 eligible children, of which 302 children were enrolled (89.3% response rate). Thirty-six children were excluded because of (i) no salivary flow, (ii) no response or missing data, or (iii) refusal to participate by the children and/or their caregivers [14]. In total, 153 children with no evidence of dental caries were placed in the caries-free (CF) cohort in the 2009 survey. Among them, 27 children were followed until the 2013 survey (follow-up rate 18.3%). Among the follow-up subjects, 12 children developed no dental caries with dentine involvement, and 12 children developed two or more surfaces of dental caries with dentine involvement (DC) in their permanent dentition during the 4 years between the initial sampling and follow-up. Three children were excluded from follow-up subjects, since they had one carious surface in their permanent tooth. Thus, the final sample was composed of 12 CF individuals and 12 DC patients with incidence of two or more surfaces of dental caries (Fig. S1). This study was approved by the Institutional Review Board of Pusan National University Hospital at the Yangsan campus (IRB No. PNUYHIRB-2009016). Written informed consent was obtained from the parents/guardians of all subjects. All experiments were performed in accordance with relevant guidelines and regulations.

### Oral Examination and Questionnaire

The state of dentition and severity of dental caries were assessed by the same investigator with disposable plane mirrors and World Health Organization (WHO) probes. Scoring was based on WHO criteria [15] and the decayed, missing, and filled teeth (DMFT) index. Decay receding into the dentine threshold was considered dental caries. Intra-examiner reliability analysis after a one-week interval revealed *K*-indices of 0.958 ( $n = 42$  children for the baseline survey) and 0.965 ( $n = 21$  children for the follow-up survey), respectively. The salivary flow rate was measured in ml/min. Information on toothbrushing frequency, snack and fresh fruit and/or vegetable consumption, and exposure to second-hand smoke was obtained with a questionnaire.

### Saliva Collection and Preparation

All subjects were instructed to refrain from eating or drinking for a minimum of 2 h before the saliva samples were collected. Individuals rinsed their mouth with water, and stimulated whole-saliva samples were obtained by instructing the participants to chew on wax blocks (Dentocult SM kits; Orion Diagnostica Co. Ltd., Finland) for 5 min in the morning. All samples were collected with 50 ml ice-chilled Falcon screw cap tubes. The samples were centrifuged at 25,000  $\times g$  at 4°C for 10 min. The supernatants of the samples were aliquoted into 1.5 ml Eppendorf tubes to analyze the salivary microbiota, and frozen at  $-80^{\circ}\text{C}$  until analysis.

### DNA Extraction and Pyrosequencing

Metagenomic DNA was extracted from saliva samples using a FastDNA SPIN extraction kit (MP Biomedicals, USA) and used as the template in PCRs to amplify the 16S rRNA gene (target region of V1–V3) with barcoded primers [16, 17] using a C1000 Touch thermal cycler (Bio-Rad, USA). After initial denaturation at 94°C for 5 min, the amplification was performed by 30 cycles of denaturation (94°C for 30 sec), primer annealing (55°C for 30 sec), and extension (72°C for 30 sec), with a final extension step of 7 min at 72°C. The amplified products were purified using a QIAquick PCR purification kit (Qiagen, USA) and quantified using a PicoGreen dsDNA Assay kit (Invitrogen, USA). Equimolar concentrations of samples were pooled and sequenced on a Roche/454 GS Junior system according to the manufacturer's instructions.

### Pyrosequencing Analysis

Sequences were analyzed as previously described [17]. Briefly, sequences for each sample were sorted by a unique barcode, and low-quality reads (average quality score <25 or read length <300 bp) were discarded. Primer sequences were trimmed using the HMM-search program in HMMER 3.0 [18]. Trimmed sequences were clustered by 97% similarity with the USEARCH program [19], and representative sequences in each cluster were selected to identify their taxonomic positions. The taxonomic assignments were conducted according to the RDP classifier against the EzTaxon-e database [20]. Chimera sequences were detected and removed by UCHIME for further analysis [21]. The diversity

**Table 1.** Baseline characteristics of the participants.

		CF <sup>a</sup> (n =12)	DC <sup>b</sup> (n =12)	p Value
Age (years)	Mean (SD)	8.67 (1.87)	9.33 (1.67)	0.368
Salivary flow rate (ml/min)	Mean (SD)	1.07 (0.79)	2.32 (2.49)	0.124
Tooth brushing (times/day)	Mean (SD)	2.42 (1.08)	3.50 (1.83)	0.092
Sex, n (%)	Boy	7 (58.3)	8 (66.7)	0.673
	Girl	5 (41.7)	4 (33.3)	
Snack, n (%)	< 1×/week	7 (58.3)	7 (58.3)	1.000
	≥ 1×/week	5 (41.7)	5 (41.7)	
Fresh fruits and/or vegetables, n (%)	< 1×/day	7 (58.3)	6 (50.0)	0.682
	≥ 1×/day	5 (41.7)	6 (50.0)	
Second-hand smoke, n (%)	No	4 (33.3)	3 (25.0)	0.653
	Yes	8 (66.7)	9 (75.0)	

<sup>a</sup>CF, caries-free group.

<sup>b</sup>DC, dental caries group.

indices were calculated in Mothur [22] after normalization of the read number in each sample. A boxplot was obtained using R software (ver 2.15.2). The functional prediction of saliva microbiota was performed in PICRUSt (ver. 1.0.0) [23]. The *p* value of functional prediction was determined by the Kruskal-Wallis test [24]. Corrections were made using the Benjamini-Hochberg false discovery rate multiple testing correction [25]. All pyrosequencing reads have been deposited in the EMBL SRA database under the study accession number PRJEB19674 (<http://www.ebi.ac.uk/ena/data/view/PRJEB19674>).

### Statistical Analysis

Changes in the saliva microbiota were set as the dependent variable, whereas body measurements and survey results were set as discriminatory variables. Categorical (sex, consumption of snacks, consumption of fresh fruits and/or vegetables, exposure to second-hand smoke) and continuous (age, salivary flow rate, toothbrushing frequency) variables were analyzed using chi-squared and independent *t*-tests, respectively. The statistical differences in predicted functions of microbiota between groups were analyzed using the Statistical Analysis of Metagenomic Profiles (STAMP) software package [26]. The *p* values were determined with Kruskal-Wallis *H* and Mann-Whitney rank sum testing [24]. Multiple test corrections were made using Benjamini-Hochberg false discovery rates [25]. Results with *p* values of <0.05 was considered statistically significant.

## Results

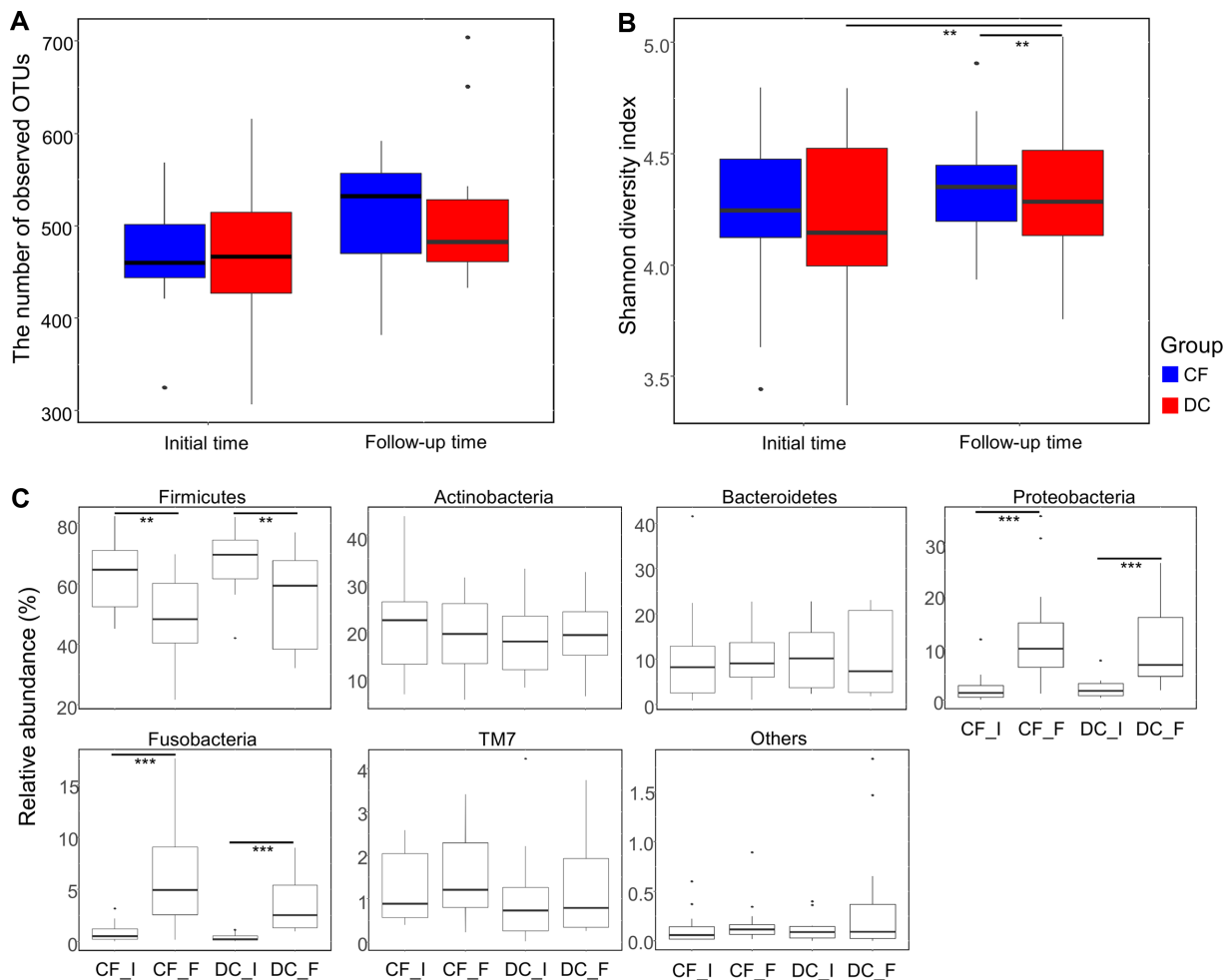
### Subject Characteristics

The baseline characteristics of the DC and CF groups are present in Table 1. The DC group comprised 8 boys and 4 girls (mean age 9.33 years and 95% confidence interval

8.27–10.39 years, age range 6–12 years old) and the CF group comprised 7 boys and 5 girls (mean age 8.67 years and 95% confidence interval 7.48–9.86 years, age range 6–13 years old). Salivary flow rates in the DC and CF groups were  $2.32 \pm 2.49$  ml/min and  $1.07 \pm 0.79$  ml/min, respectively. Toothbrushing frequencies were  $3.50 \pm 1.83$  times/day in the DC group and  $2.42 \pm 1.08$  times/day in the CF controls. Differences in sex, age, salivary flow rate, toothbrushing frequency, consumption of snacks or fresh fruits and/or vegetables, and exposure to second-hand smoke were not significantly different between the groups.

### Comparison of Overall Diversity

Saliva samples were collected from the participants during the initial examination and at a 4 year follow-up. In total, 352,977 reads were analyzed after the quality filtering processes. Diversity indices were compared among samples after normalizing for read numbers using the Mothur program (Table S1). The mean number of observed operational taxonomic units (OTUs) in the initial and follow-up samples was 409.5 and 439.0, respectively. The highest number of observed OTUs (613) was detected in sample #24\_1 in the DC-follow-up group, whereas the lowest number of observed OTUs (265) was detected in sample #16 in the DC-initial group. A comparative analysis revealed an increase in the OTUs and Shannon diversity indices in the follow-up samples of both groups (Figs. 1A and 1B). Moreover, the CF group displayed a significantly higher Shannon diversity index than the DC group in the follow-up samples ( $p < 0.05$ ).



**Fig. 1.** Comparison of microbiota diversity and composition.

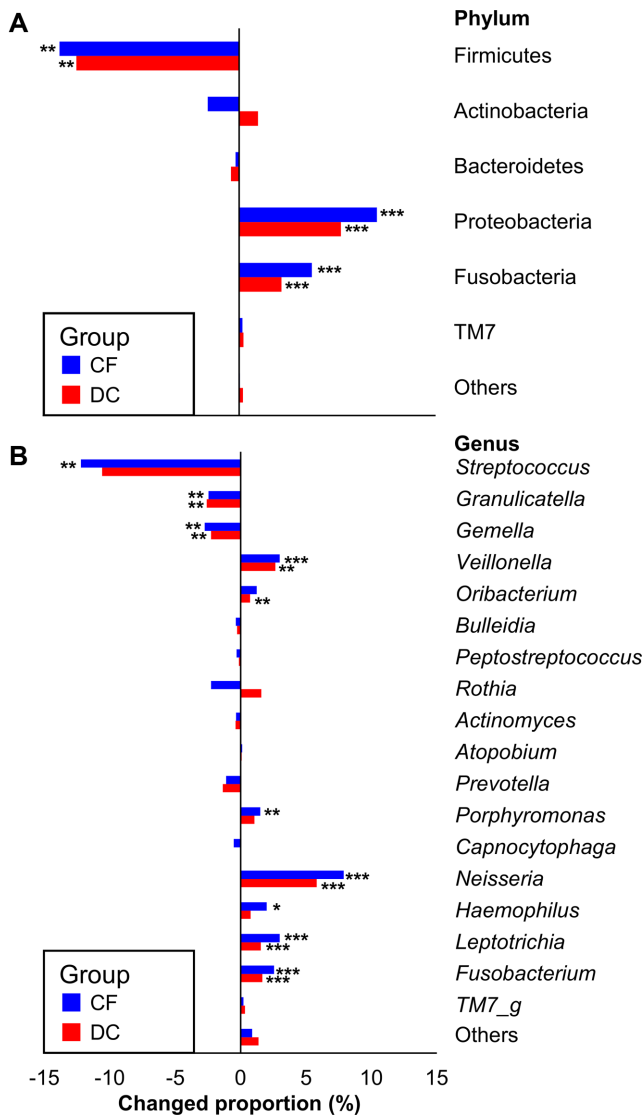
(A) Numbers of observed operational taxonomic units (OTUs) in the initial and follow-up samples were compared between the caries-free (CF) and dental caries (DC) groups. (B) Changes in the Shannon diversity index were compared between groups using boxplot analyses. (C) The phylum compositions in the salivary microbiomes were compared among groups. CF\_I, Caries-free samples at initial time; CF\_F, Caries-free samples at follow-up time; DC\_I, Dental caries samples at initial time; DC\_F, Dental caries samples at follow-up time. \*\* $p < 0.05$ ; \*\*\* $p < 0.01$ .

### Cross-Sectional Differences and Longitudinal Shifts in Microbiota Composition

Phylum composition analysis identified Firmicutes as the predominant phylum in all groups (Fig. 1C). In cross-sectional comparisons, the proportion of Firmicutes was higher in DC samples than in CF samples at both sampling times, whereas Actinobacteria at the initial measurement and Proteobacteria and Fusobacteria at the follow-up measurement were more prevalent in the CF samples than in the DC samples. However, the cross-sectional differences in the phylum composition between the CF and DC groups were not statistically significant ( $p > 0.05$ ). Comparison of the genus composition values (mean value  $>1\%$  of each

group) displayed a trend similar to that observed in the phylum-based analysis (Figs. S2 and S3). *Streptococcus*, *Rothia*, and *Prevotella* were the predominant genera in all groups. The proportions of *Streptococcus* were higher in DC than in CF samples at both sampling times. The proportions of *Oribacterium* were higher in the CF group at the initial time, whereas those of *Actinomyces*, *Leptotrichia*, and *Atopobium* were higher in the CF group at follow-up time than in the DC groups. Although these genera were different between the CF and DC groups, the statistical significances were relatively low ( $p < 0.1$ ).

Shifts in the salivary microbiota composition were compared between the initial and follow-up samples from



**Fig. 2.** Changes in salivary microbiota composition over time between the caries-free (CF) and dental caries (DC) groups. Changes in (A) phylum and (B) genus proportions were compared between the CF and DC groups. \* $p < 0.1$ ; \*\* $p < 0.05$ ; \*\*\* $p < 0.01$ .

each group. At the phylum level, the relative abundance of Firmicutes ( $p < 0.05$ ) and Bacteroidetes was decreased in the follow-up samples of both groups, whereas those of Proteobacteria and Fusobacteria were increased ( $p < 0.01$ ) (Fig. 2A). Although the proportion of Actinobacteria decreased and increased in CF and DC samples, respectively, over time, these differences were not statistically significant ( $p = 0.478$ ).

At the genus level, the relative abundance of *Streptococcus* decreased in both groups with time, and the decrease was more pronounced in the CF group ( $p < 0.05$ ) (Fig. 2B). With

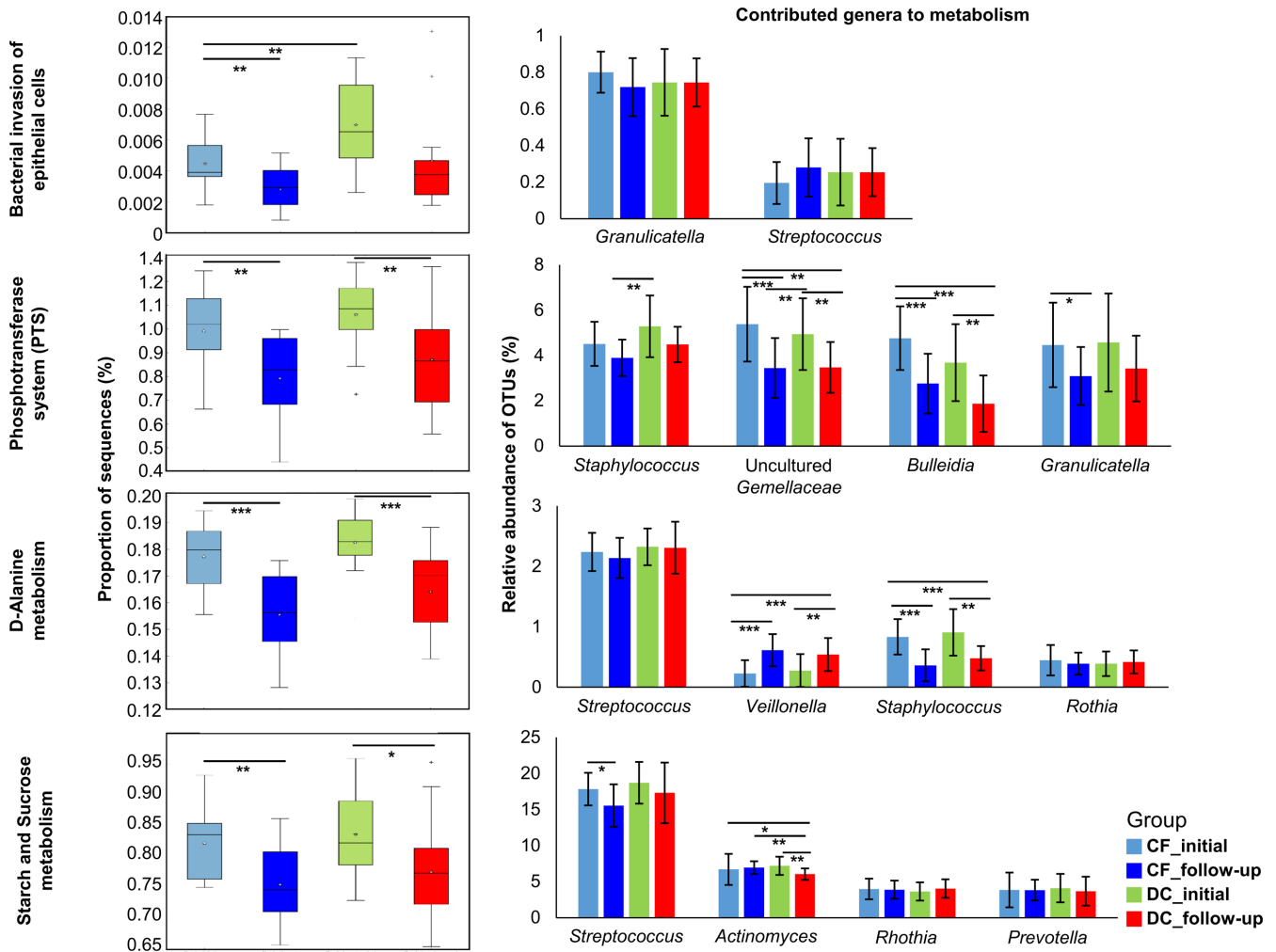
respect to other Firmicutes genera, the proportions of *Granulicatella* and *Gemella* were significantly decreased in both groups ( $p < 0.05$ ), whereas those of *Veillonella* ( $p < 0.05$ ) and *Oribacterium* ( $p < 0.05$  in the CF group) were increased. Moreover, the proportions of the *Neisseria* within Proteobacteria and those of *Leptotrichia* and *Fusobacterium* within Fusobacteria increased in both groups with time ( $p < 0.01$ ). Although the opposing shifts were observed for *Rothia* within Actinobacteria, statistical significance of the difference was not found ( $p > 0.05$ ).

### Differences in the Predicted Functions of the Microbiota Observed in Each Group

The functions of the microbiota were predicted in PICRUSt and the statistical significance was analyzed with STAMP. The KEGG Orthology category for “bacterial invasion of epithelial cells” was different between the groups (Fig. 3). Although this predicted function decreased in both groups over time, the DC group exhibited a higher proportion than the CF group in the initial samples ( $p < 0.05$ ). *Granulicatella* and *Streptococcus* could be the genera contributing to this pathway. However, differences of these genera between groups were not detected ( $p > 0.05$ ). Predicted functions in the “phosphotransferase system” were higher at the initial measurement as compared with follow-up sampling ( $p < 0.05$ ). Diverse genera could be related to this function, including *Staphylococcus*, uncultured Gemellaceae, *Bulleidia*, and *Granulicatella*, which were significantly decreased during follow-up. *Streptococcus*, *Leptotrichia*, *Rothia*, *Haemophilus*, and *Actinomyces* were also related to this pathway (Fig. S4). The predicted relative abundance of “D-alanine metabolism” was also higher in initial samples versus follow-up samples ( $p < 0.01$ ). Notably, this function was predicted most prevalent in the DC group at the initial sampling. *Streptococcus*, *Staphylococcus*, *Veillonella*, and *Rothia* could also be related to this function, and the relative abundance of *Staphylococcus* was mostly associated with the highest proportion of this pathway in the DC group at the initial sampling with significance ( $p < 0.01$ ). The relative abundance of “starch and sucrose metabolism” was predicted to be higher in the initial samples of both groups ( $p < 0.05$  for CF group and  $p < 0.1$  for DC group). *Streptococcus*, *Actinomyces*, *Rothia*, and *Prevotella* could be associated with this metabolism, and the decreased proportions of this predicted metabolism could be related to the decreased contribution of *Streptococcus* ( $p < 0.1$  in CF group) and *Actinomyces* ( $p < 0.05$  in DC group).

The relative abundance of “bacterial motility protein gene families” was predicted to be higher in the CF group



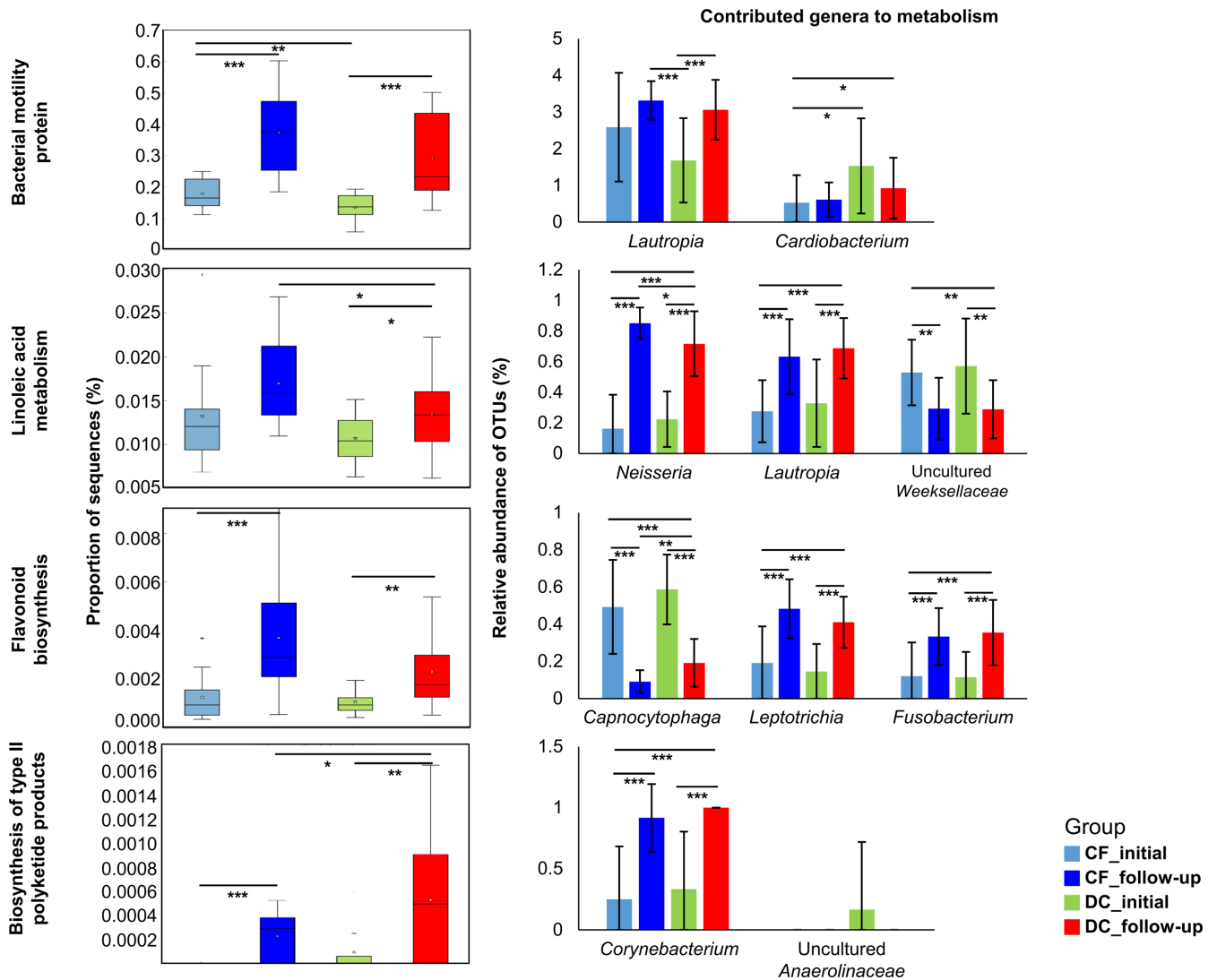


**Fig. 3.** Comparison of higher predicted functions in initial samples than in follow-up samples and their contributing genera between the caries-free (CF) and dental caries (DC) subjects.

Predicted functions of the microbiota were determined in PICRUSt. \* $p < 0.1$ ; \*\* $p < 0.05$ ; \*\*\* $p < 0.01$ .

at both sampling times, and higher at follow-up than at the initial sampling ( $p < 0.01$ ) (Fig. 4). Moreover, the predicted proportion of this category was significantly higher in the CF-follow-up group than in all others. *Lautropia* and *Cardiobacterium* could be contributors to this category, and *Lautropia* was markedly increased in the DC-follow-up group ( $p < 0.01$ ). Furthermore, the proportion of "linoleic acid metabolism" was also predicted to be higher in the CF group at both sampling times, specifically in follow-up samples ( $p < 0.1$ ). *Neisseria*, *Lautropia*, and uncultured Weeksellaceae could be the major contributors of this function. In particular, *Neisseria* and *Lautropia* could be associated with linoleic acid metabolism at follow-up ( $p < 0.01$ ), whereas uncultured Weeksellaceae could be

associated with linoleic acid metabolism at the initial sampling ( $p < 0.05$ ). The relative abundance of "flavonoid biosynthesis" was predicted to be the highest in the CF-follow-up group ( $p < 0.01$  compared with initial time). *Capnocytophaga*, *Leptotrichia*, and *Fusobacterium* could be the contributors to this metabolic pathway, of which *Leptotrichia* and *Fusobacterium* could be the most responsible for this increase at follow-up ( $p < 0.01$ ). "Biosynthesis of type II polyketide products" was predicted to be higher in the DC group than in the CF group at both sampling times ( $p < 0.1$  at follow-up), and higher at follow-up than at the initial sampling ( $p < 0.05$ ). The highest proportion of this category was detected in the DC-follow-up group compared with all others. Although *Corynebacterium* and uncultured



**Fig. 4.** Comparison of higher predicted functions in follow-up samples than in initial samples and their contributing genera between the caries-free (CF) and dental caries (DC) groups. \* $p < 0.1$ ; \*\* $p < 0.05$ ; \*\*\* $p < 0.01$ .

Anaerolinaceae could be related to this metabolism, *Corynebacterium* showed the highest relative abundance in the DC-follow-up group ( $p < 0.01$ ).

### Discussion

The present study analyzed the association between dental caries formation and salivary microbiota in children during a 4-year follow-up period, using high-throughput 16S rRNA gene sequencing. Notably, bacterial diversity was higher in the CF group than in the DC group at follow-up time ( $p < 0.05$ ). The salivary microbiota shifted over

time, and was different between the CF and DC groups for some microbes. Analysis of predicted microbiota functions showed that “bacterial invasion of epithelial cells,” “phosphotransferase system,” “D-alanine metabolism,” and “starch and sucrose metabolism” were higher in the initial time, whereas predicted functions related to “bacterial motility proteins,” “linoleic acid metabolism,” “flavonoid biosynthesis,” and “biosynthesis of type II polyketide products” were higher in the follow-up groups. The cross-sectional differences were predicted in “bacterial invasion of epithelial cells,” “biosynthesis of type II polyketide products,” “bacterial motility proteins,” and

“linoleic acid metabolism.” These differences of predicted functions may be associated with the development and/or progression of dental caries. As such, this information is valuable to the field to prevent dental caries in children in the future.

The diversity of the salivary microbiome was higher in follow-up than in initial samples, and higher in the CF group at follow-up times as compared with the DC group (Fig. 1), suggesting that decreased diversity of the salivary microbiota may be associated with the development of caries in children. This result is consistent with previous reports on the microbiome of saliva and oral biofilm in adults and children, respectively, which showed that caries reduced bacterial community diversity [11, 27]. This reduced diversity could be associated with lower oral pH, a condition associated with increased susceptibility to dental caries. Although some differences in microbiota composition were detected between the CF and DC groups, the overall profile of the microbiota and shifts in trends between sampling times were similar in both groups. This result indicates that age is likely the main factor inducing salivary microbiota shifts in children, particularly because a relatively low significant difference was observed in the cross-sectional samples. Similarly, previous studies also reported that salivary microbiome diversity was higher in adults than in infants, and no difference was detected between subjects with or without caries [28–30]. In addition, some studies suggest that the microbiota composition may be different between these groups and may be responsible for caries development [12, 31], but there is no consensus as to whether the salivary microbiome predisposes individuals to caries formation, or which taxa are associated with decay. This discordance may be related to differences in age, sample size, and detection methods among studies.

The proportions of Proteobacteria, Fusobacteria, and candidate division TM7 appeared to increase with age as previously reported (Figs. 1 and 2) [32]. Specifically, *Streptococcus* was the predominant genus in both CF and DC groups; however, its relative abundance was higher in the DC group than in the CF group at the initial and follow-up sampling (Fig. S3). Although some species of *Streptococcus* have been reported to be associated with tooth decay, *Streptococcus* is very common in the oral cavity of subjects with and without tooth decay [27, 33]. This genus had traditionally been identified by culture-based methods, which exclude various not-yet-cultivated species. Therefore, various *Streptococcus* spp. should be analyzed to determine the association of microbes with the development of caries.

Although differences in *Streptococcus* and shifts in other

microbiota were analyzed, the association of specific bacteria with dental caries remained unclear. Thus, we compared the predicted functions of different salivary microbiotas observed between the CF and DC groups (Figs. 3 and 4). Four functions (bacterial invasion of epithelial cells, phosphotransferase system, D-alanine metabolism, and starch and sucrose metabolism) were predicted to be higher in the initial samples than in the follow-up samples (Fig. 3). Previous studies on host–pathogen interactions at the cellular level demonstrated that bacteria invade host cells and can survive in an intracellular niche to incite periodontal disease [34]. *Streptococcus* and *Granulicatella* could be the potential contributors to bacterial invasion of epithelial cells in the present study, and the association of these bacteria with dental caries was also reported [35, 36]. The phosphotransferase system plays an important role in carbohydrate uptake by *S. mutans* [37, 38]. Although statistical significance was not found, the association of *Streptococcus* with the phosphotransferase system was also predicted in the present study (Fig. S4). Staphylococci were more associated with the phosphotransferase system in the DC group than in the CF group, whereas the opposite result was observed for uncultured Gemellaceae and *Bulleidia*. This result suggests that different bacteria play distinct roles in the oral cavity environment. Moreover, D-alanine metabolism is known to be essential for *S. mutans* growth and biofilm formation, and is a potential target for inhibitors [39]. *Staphylococcus* could be the significant contributor of this function in the DC-initial group ( $p < 0.01$ ), whereas *Veillonella* predominated in follow-up samples ( $p < 0.05$ ). *Veillonella* was also associated with dental caries, and can serve as an acid sink to facilitate the growth of acidogenic bacteria such as *Streptococcus* [8, 35, 40, 41]. Notably, the contribution of *Veillonella* was higher in the CF group than in the DC group at follow-up, whereas the proportion of D-alanine metabolism was lower in the CF group than in the DC group. This suggests that *Veillonella* may be associated with pH regulation in older children, whereas *Staphylococcus* may play a larger role in D-alanine metabolism at the initial sampling.

Alternatively, four functional genes related to bacterial motility protein, linoleic acid metabolism, flavonoid biosynthesis, and biosynthesis of type II polyketide products were predicted to be higher in the follow-up samples than in the initial samples (Fig. 4). Bacterial motility proteins, such as flagellin, can inhibit biofilm formation in the oral cavity. Linoleic acid is an antimicrobial agent that inhibits the growth of oral pathogens, consistent with its increased presence in the CF group. *Neisseria* could be a contributor



to linoleic metabolism in this study, and was previously found to predominate the oral microbiota of CF subjects [29]. Flavonoids are used in dentistry for their antibacterial, anti-inflammatory, and antioxidant properties, as well as their immunoregulatory effects [42]. A recent study suggested that flavonoid glycosides can inhibit sortase A and sortase A-mediated aggregation of *Streptococcus mutans* [43]. In the present study, *Leptotrichia* and *Fusobacterium* could be contributors to flavonoid biosynthesis in the CF group; however, *Leptotrichia* and *Fusobacterium* were also associated with dental caries in previous studies [44, 45]. This is a controversial result, and further identification of the ecological functions of microbes is important for understanding the association of microbes with dental caries. Furthermore, the proportion of biosynthesis of type II polyketide products was higher in the DC follow-up group. In a previous study, *Streptococcus mutans* was found to express the polyketide synthase gene cluster necessary for the biosynthesis of pigments involved in oxygen and H<sub>2</sub>O<sub>2</sub> tolerance [46]. The potential roles of the salivary microbiota in dental caries development are summarized in Fig. S5. However, these results were obtained from in silico prediction, and further studies including metatranscriptomics and metabolomics should be conducted to understand the ecological role of the salivary microbiome in dental caries development.

Most oral diseases are infectious diseases caused by microorganisms in dental plaque. The clinical manifestations of oral infections are related to the activity of these microorganisms and immune responses in the host [4]. Traditional preventive methods for oral infection have focused on mechanical or nonspecific control of dental plaque. Short-term systemic antibiotic therapy, topical antimicrobials, or antiplaque oral care products have been used as adjuvant therapies for the mechanical methods [47]; however, mechanical therapy is far from a targeted treatment for these microorganisms. Pozhitkov *et al.* [48] proposed an alternative approach to replace the microbiome of patients with periodontal disease with the entire healthy oral microbial community. Recent studies have proposed a variety of therapies to target a single species or key virulence factors produced by microorganisms while maintaining resident oral microbiota equilibrium [49].

Although our results could be difficult to generalize owing to the limited number of subjects in each group, this study is valuable because it suggests that the functions of the salivary microbiota can be associated with dental caries development by prediction of longitudinal study. Further comprehensive prospective studies are needed to clarify

the association between the salivary microbiome and dental caries development. In addition, although the salivary microbiome may not be a good indicator for the risk of developing dental caries, some evidence suggests that it may be a good indicator of ongoing functions associated with dental caries [12]. Saliva is an easily acquired, relatively stable, and inexpensively accessible biological fluid that has been thoroughly analyzed for biomarkers of health and disease [50, 51]. In addition, saliva collection is non-invasive and can be performed by an individual without a high level of technical expertise. Therefore, the identification of saliva biomarkers associated with dental caries may facilitate the development of treatments to limit the development and progression of dental caries.

In conclusion, the present study indicates that the salivary microbiota is subject to age-associated shifts and appears to be more diverse in subjects without caries. Moreover, different species may play distinct roles in caries development by participating in the bacterial invasion of epithelial cells, phosphotransferase system, and/or D-alanine metabolism, whereas other species associated with bacterial motility protein expression, linoleic acid metabolism, and flavonoid biosynthesis may block this occurrence. Nevertheless, further studies with a larger sample size are necessary to identify microbial biomarkers sufficient to prevent dental caries.

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## Conflict of Interest

The authors have no financial conflicts of interest to declare.

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