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Microbiome interaction with sugar plays an important role in relapse of childhood caries

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ABSTRACT

Childhood caries have a high relapse rate after full mouth therapy. This study aimed to elucidate the relationship between the microbiome, sugar, and the relapse of childhood caries after therapy. A total of 24 children aged 2–4 years who underwent one caries treatment session participated in this study. Supragingival plaque was collected before therapy and 1 and 7 months after therapy, then sequenced using the 16S rRNA high-throughput approach. We found 11 phyla, 140 genera, and 444 species in 72 samples. The children were divided into relapse-free (n = 13) and relapse (n = 11) groups according to whether they relapsed 7 months after therapy. The bacterial community richness, diversity, structure, and relative abundance of bacterial taxa were significantly different between the two groups 7 months after therapy. The two groups also differed in the relative abundance of bacterial taxa, both before and 1 month after therapy. Bacterial community richness and diversity were lower in the relapse-free group 1 month after therapy. Using different operational taxonomic units between the relapse-free and relapse groups 1 month after therapy, a relapse-risk assessment model was built with 75% accuracy, 0.1905 out-of-bag error, and 66.67% validation accuracy. Patients in the relapse group had higher sugar intake frequencies than those in the relapse-free group during follow-up. Interactions between the microbiome and sugar intake frequency were found through co-occurrence networks. We conclude that the microbiome is significantly different between the relapse-free and relapse groups at the time of relapse. Supragingival plaque collected immediately after therapy can be used to predict the risk of relapse. Furthermore, the correlation between sugar intake frequency and microbiome is associated with the relapse.

1. Introduction

Childhood caries are highly prevalent in China. The third national oral health epidemiological survey (2008) reported that 66% of children develop caries by 5 years of age and that 79.3% of these caries occur in one-third of all children. A recent survey reported that the prevalence of caries was 31.09% among children 2 years of age, 48.33% among those 3 years of age, and 59.18% among those 4 years of age in Haidian District, Beijing [1]. These findings suggest that caries in 2- to 4-year-old children are very prevalent in a subset of children. Because of their young age and the extensive and rampant form of caries they often develop, children frequently need to be treated under general anesthesia. However, despite thorough treatment under general anesthesia, the relapse rate is very high globally, ranging from 37% to 79% [2–4]. Many children require a second procedure under general anesthesia [5]. Children and parents will benefit from determining the factors associated with relapse and ways to prevent it.

The microbiome associated with childhood caries has been well studied [6–8], but risk assessment for childhood caries in the general population is different from predicting relapse in children who have already developed extensive caries [9]. Previous studies have found that the numbers of *Streptococcus mutans*, *Lactobacillus* sp., and some acidic bacteria decrease after therapy, especially in relapse-free children, but this decrease is not permanent unless

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factors promoting caries are controlled [10,11]. The microbiome of relapse-free children changes significantly after therapy, but the modified microbiome is not found in relapsed children. This suggests that pretreatment of microbiota might have an impact on the progression of caries after therapy [9].

These studies mainly focused on specific pathogenic bacteria using culture-based or Human Oral Microbe Identification Microarray (HOMIM) assays. Many studies have revealed that caries are infectious and caused by bacterial community structure shifts rather than specific pathogenic bacteria [12,13]. The 16S rRNA high-throughput sequencing method can detect the broad spectrum of both culturable and non-culturable microbiota, providing insight into the diversity and community structure of the microbiome [14]. Over the next several years, next-generation sequencing is anticipated to transition from basic genomic research to clinical applications [15]. A recent study identified microbiome shifts during the onset of caries using the 16S rRNA high-throughput sequencing method and successfully predicted the onset of caries [16]. Changes in the microbiome during relapse of caries relapse are not very clear. As a multifactor disease, the association between diet and relapse of caries has not been well elucidated.

The present study attempts to answer following questions: Does the pretherapy microbiome and the microbiome at the time of relapse differ between children with and without relapse? Do dietary and oral care habits differ between these two groups of children during follow-up? If so, how does diet interact with the microbiome in children during shifts from healthy status to relapse?

2. Materials and methods

2.1. Patient recruitment and sampling

The Ethics Committee of Peking University Health Science Center approved this study (PKUSSIRB-2012056), and written informed consent was obtained from the children's parents or caregivers. Twenty-four children aged 2–4 years who had undergone caries treatment at Peking University School and Hospital of Stomatology were recruited. All children were healthy, had not used antibiotics within the preceding 1 month, had primary dentition with more than 16 teeth, had at least 7 decayed teeth ($dt \geq 7$), and had two or fewer filled teeth ($ft \leq 2$).

Caries therapy was performed under general anesthesia in one session and included comprehensive restoration and extraction (extracted $\leq$2 teeth). The children underwent two follow-up examinations at 1 and 7 months after therapy. During the follow-up, fluoride varnish was applied to all children, and the relapsed caries were treated. The children's parents or caregivers completed a questionnaire about dietary and oral care habits before and 7 months after therapy.

Supragingival dental plaque was sampled from the intact enamel before therapy and 1 and 7 months after therapy. Samples were collected by a sterile excavating spoon and immediately placed in sodium thiosulfate solution, then washed twice with TE buffer (10 mM Tris–HCl, 1 M ED-TA, pH 8.0) and stored at −80 °C for further use [17].

2.2. DNA extraction and sequencing

Genomic DNA was extracted using a Wizard Genomic DNA Purification Kit according to the manufacturer's instructions (Promega, Madison, WI, USA). The final quantity and quality of the DNA was evaluated using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Bacterial 16S rRNA gene amplification and sequencing were performed at the BGI (Huada Gene Institute). PCR amplification of the V3–V4 region of bacterial 16S rRNA was performed using universal primers (338F 5′GTACTCC TACGGGAGGCAGCA-3′ and 806R 5′-GTGGACTACHVGGGTWTCTAA T-3′). All PCR products were sequenced by an Illumina Miseq Sequencing platform according to standard protocols.

2.3. Bioinformatic and statistical analysis

The multiplexed samples were divided based on unique barcodes assigned to each sample. The barcodes and primers were then trimmed off. Low-quality sequences with an average quality score of less than Q20 were removed. Pair-end sequences were joined together and chimeras were removed. Joined sequences were clustered into operational taxonomic units (OTUs) at 97% sequence similarity, and singletons were excluded from the analysis [18]. The Human Oral Microbiome Database (HOMD) ([19] was used for taxonomic assignment. Bioinformatics data were analyzed by QIIME pipeline [20].

The children were separated into relapse-free ($n = 13$) and relapse ($n = 11$) groups according to whether they relapsed 7 months after therapy. A p value of <0.05 was considered statistically significant. Mean differences in age and clinical characteristics between the relapse-free and relapse groups were calculated with the Mann–Whitney U test, using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). The proportional differences in sex, diet, and oral care habits were compared by the chi-square test. Statistically significant tests of alpha diversity between the relapse-free and relapse groups were performed by a nonparametric two-sample t-test using QIIME with default parameters. Within-group comparisons were performed by repeated-measures ANOVA using SPSS 22.0. Dissimilarity analysis of beta diversity (principal coordinates analysis [PCoA]) between the relapse-free and relapse groups was performed by non-parametric multivariate ANOVA with the Adonis function using QIIME. Mean differences in the relative abundance of phylum, class, order, family, genus, and species between and within groups were evaluated using LEfSe [21], with an alpha value of 0.05 for the Kruskal–Wallis test and a threshold of 2.0 for logarithmic linear discriminant analysis scores. Relapse-risk assessment models were generated by random forests [22] using Weka 3.7.12 (University of Waikato, New Zealand). Co-occurrence networks were calculated with the Spearman test [23], using R software and visualized with Cytoscape 3.2.1 [24].

3. Results

There were no significant differences in the children's age, sex, or clinical characteristics (i.e., dt, ds, number of stainless steel crowns, or number of extracted teeth) between the relapse-free and relapse groups. The relapse group had higher frequencies of sugar intake than the relapse-free group 7 months after therapy ($p = 0.013$), although this difference was not present before therapy (Table S1). Other dietary and oral care habits were not significantly different between the two groups before or 7 months after therapy (Table S1).

After quality control and removal of chimeras and singletons, this study covers a total sample reads of 1,141,561, ranging from 8567 (minimum) to 22,435 (maximum) per sample. In total, 23,262 OTUs were clustered at 97% similarity, with 1825 ± 401 in each sample. After taxonomic assignment, we identified 11 phyla, 22 classes, 39 orders, 75 families, 140 genera, and 444 species from the 72 samples.

Bacterial community richness (Chao1) and diversity (Shannon) were not significantly different between the relapse-free and relapse groups before therapy. However, at 1 and 7 months after therapy, the relapse-free group had significantly lower richness and

diversity than the relapse group ($p = 0.032$ for Chao1 and $p = 0.019$ for Shannon). Within-group analysis revealed that in the relapse-free group, community richness and diversity were significantly lower at 1 month ($p = 0.013$ for Chao1, $p = 0.011$ for Shannon) and 7 months ($p = 0.037$ for Chao1, $p = 0.022$ for Shannon) after therapy than before therapy. However, within-group analysis of the relapse group revealed no significant differences (Fig. 1).

The bacterial community structures were not significantly different between the relapse-free and relapse groups before therapy or 1 month after therapy ($p = 0.465$ and $p = 0.428$, Adonis) (Fig. 2A, B). However, at 7 months after therapy, the relapse-free and relapse groups separated into two different clusters on the PCoA plot ($p = 0.004$, Adonis), indicating that the bacterial community structure was significantly different between the two groups at the time of relapse (Fig. 2C).

LEfSe between the relapse-free and relapse groups 7 months after therapy indicated significant differences between the relapse-free and relapse state microbiomes for many bacterial taxa (Fig. 3C). The relapse-free group had a higher relative abundance of bacterial taxa that mainly belonged to the Firmicutes phylum, while the relapse group had a higher relative abundance of bacterial taxa that mainly belonged to the Bacteroidetes phylum. LEfSe of relative abundances before and 1 month after therapy showed that these two groups of children already had differences in some bacterial taxa before and immediately after therapy (Fig. 3A, B). Within-group analysis of differences in bacterial taxa between 7 months after therapy and before therapy in the relapse-free and relapse groups was performed using linear discriminant analysis. The relative abundance of species is shown in Figure S3.

Co-occurrence networks of major species (relative abundance of >0.01%) that were significantly different between the relapse-free and relapse groups 7 months after therapy are presented in Fig. 4A ($p < 0.05$, LEfSe). Relapse-free enriched species and relapse enriched species were negatively correlated with each other. Compared with relapse-free enriched species, relapse enriched species were more closely associated with each other. The second networks showed the co-occurrence of a relationship between sugar intake frequencies and major species (relative abundance of >0.01%) in the 24 children 7 months after therapy (Fig. 4B). *Cardiobacterium hominis* was enriched in the relapse-free group and was negatively correlated with sugar intake frequency. *Campylobacter rectus*, *Capnocytophaga* sp. Human Oral Taxon (HOT) 323, and *Treponema* sp. were enriched in the relapse group and positively correlated with sugar intake frequency.

A cross-validated random forest model was constructed based on relative abundance of 29 OTUs that were significantly different between the relapse-free and relapse groups 1 month after therapy using the random forest and cross-validation algorithms. The accuracy and out-of-bag error of this model were 75% and 0.1905, respectively (Fig. S1). This model used 5-fold of cross-validation and generated five decision trees to assess the relapse risk 7 months after therapy. We further confirmed the power of this relapse-risk assessment model by nine additional supragingival microbiomes using the five decision trees, and the validation accuracy was 66.67%. These nine independent children met the inclusive criteria and were sampled, followed-up, and sequenced in the same way as the previous 24 children, but supragingival samples were only collected 1 month after therapy. The samples were

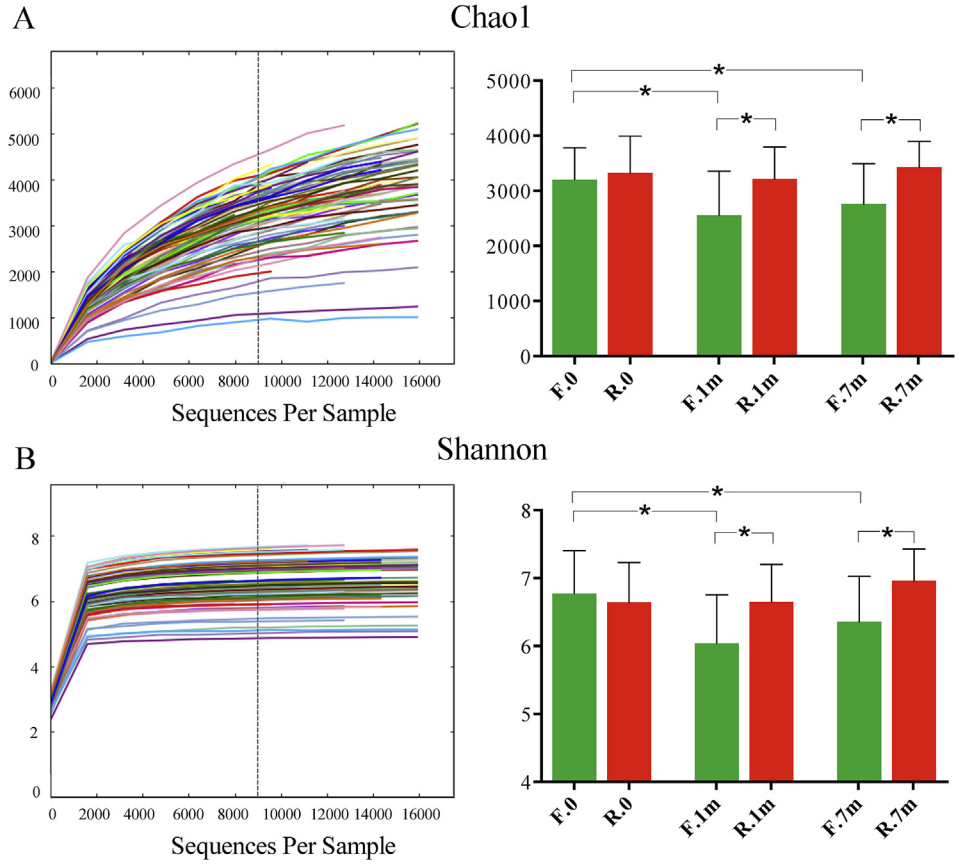

Fig. 1. Alpha diversity index. 0 = pretherapy, 1 m = 1 month after therapy, 7 m = 7 months after therapy. *$p < 0.05$. Alpha-diversity metrics were calculated after subsampling 8567 sequences from each sample. (A) Chao1 index shows bacterial community richness. (B) Shannon index shows bacterial community diversity.

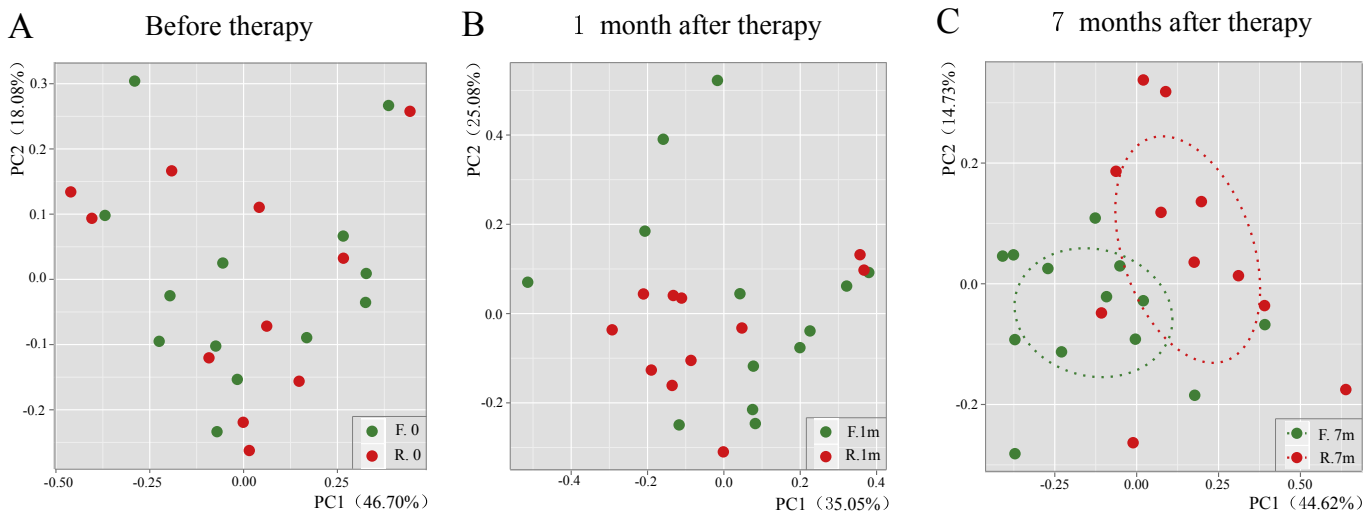

Fig. 2. Principal coordinates analysis (PCoA) plot of community structure based on weighted UniFrac distance. (A) Community membership of relapse-free and relapse groups before therapy. Dissimilarity analysis between the two groups by Adonis shows p = 0.465. (B) Community membership of the two groups 1 month after therapy. Dissimilarity analysis between the two groups by Adonis shows p = 0.428. (C) Community membership of the two groups 7 months after therapy. Dissimilarity analysis between the two groups by Adonis shows p = 0.004.

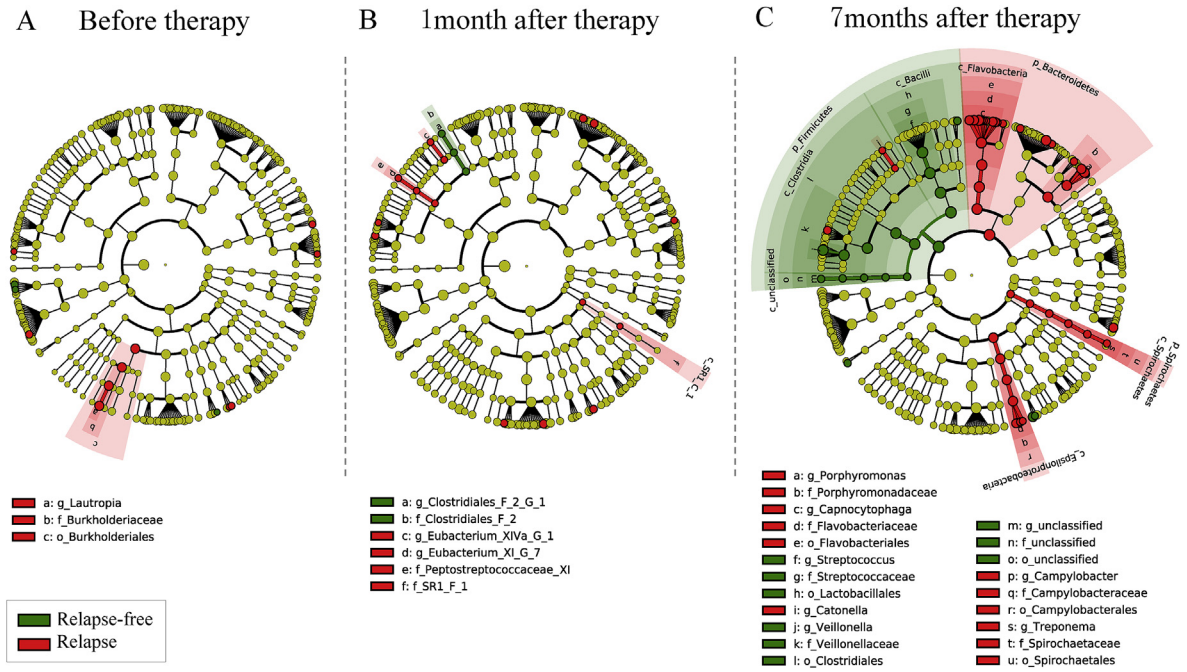

Fig. 3. Cladogram representing the taxonomic hierarchical structure of bacterial taxa (A) before therapy, (B) 1 month after therapy, and (C) 7 months after therapy, generated using LEfSe. Bacterial taxa that appear in green had a higher relative abundance in the relapse-free group, while bacterial taxa that appear in red had a higher relative abundance in the relapse group. The names of the species with different relative abundances in the two groups are not shown here (Fig. S2) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

sequenced and used for validation.

4. Discussion

In this study, relapse-free and relapse states 7 months after therapy differed significantly in bacterial community richness, diversity (Fig. 1), structure (Fig. 2C), and relative abundance of bacterial taxa (Fig. 3C). Previous studies revealed that community structure shifts, not the presence or absence of particular microbes, are more closely associated with cariogenesis [12,13]. Concordantly, our co-occurrence networks, which were based on bacterial species (Fig. 4A), revealed that relapse-enriched species had much tighter and more complicated correlations with each other than did relapse-free enriched species. Within-group comparisons between 7 months after therapy and before therapy revealed that at the species level, *Campylobacter concisus* decreased in the relapse-free group after treatment but was elevated in the relapse group, *Lautropia mirabilis* decreased in the relapse group, and both *Campylobacter gracilis* and *Selenomonas artemidis* increased in the relapse group (Fig. S3). Similar to our observations, a previous report

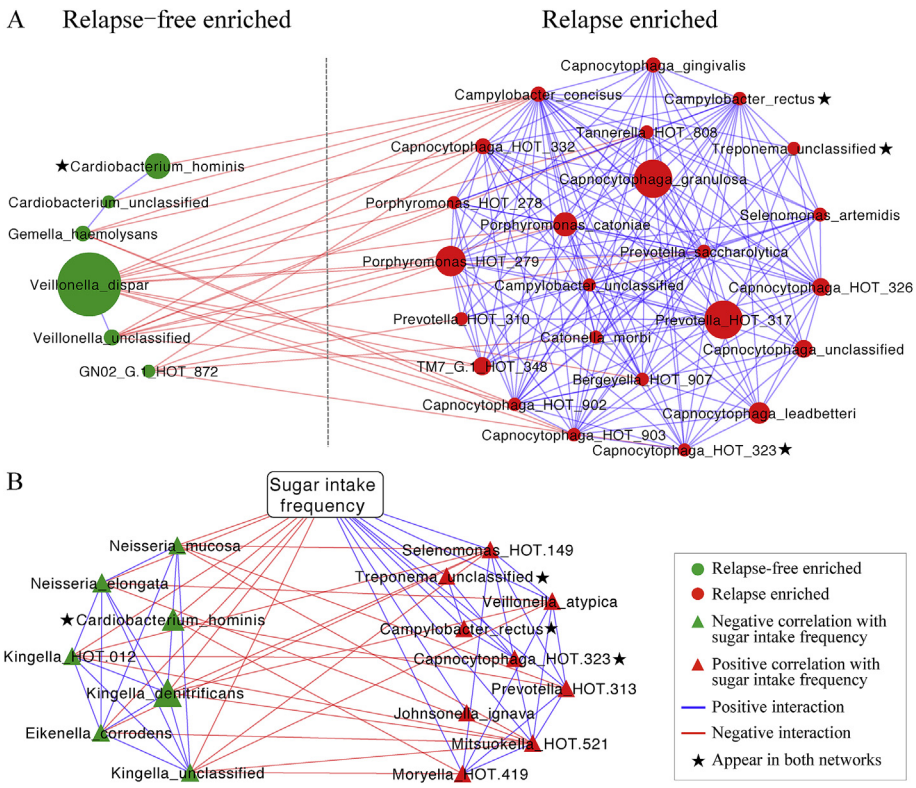

Fig. 4. Co-occurrence networks. (A) Shown here are the co-occurrence networks of major species (relative abundance of >0.01%) that were significantly different in the relapse-free and relapse groups 7 months after therapy. (B) Shown here are the co-occurrence networks of sugar intake frequencies and major species (relative abundance of >0.01%) from the 24 children 7 months after therapy. Blue edges, Spearman's correlation coefficient >0.4, $p < 0.05$; red edges, Spearman's correlation coefficient < −0.4, $p < 0.05$ (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

showed that the detection rate of *C. concisus*, *C. gracilis*, and *S. artemidis* decreased and increased for *L. mirabilis* in the relapse-free group using an HOMIM assay [9].

We observed that 13 bacterial taxa differed between the relapse-free and relapse groups before therapy (Fig. 3A) and that 18 bacterial taxa differed between the two groups 1 month after therapy (Fig. 3B). Moreover, we found that the community richness and diversity decreased in the relapse-free group 1 month after therapy (Fig. 1). These results suggest that microbiome differences between the relapse-free and relapse groups before treatment became greater as a result of therapy. A previous study suggested that the persistence of the microbiota before therapy was responsible for caries progression after therapy [9]. Random forest and cross-validation algorithms were proven effective in a previous study for establishment of both a prediction model [16] and the classification model [23]. We used the microbiome differences between the relapse-free and relapse group 1 month after therapy to build a relapse-risk assessment model with 75% accuracy by the random forest and cross-validation algorithms (Fig. S1). Prediction and classification of host states based on human microbiota are key goals of human microbiome projects worldwide [25]. Our study indicates that the supragingival plaque microbiome collected from children immediately after therapy can be used to assess their relapse risk in the future. This finding has clinical significance as an assessment model to facilitate recognizing children who have a high relapse risk.

However, the assessment model based merely on the microbiome could only partially assess (75% accuracy) the relapse risk. Caries are a complicated multifactor disease not only caused by selected bacterial infection, but also associated with dietary and host factors. The pivotal role of sugars in the occurrence of caries was re-emphasized in a previous study [26]. The authors also demonstrated that cariogenesis is sensitive to even a very low sugar intake. We surveyed the dietary habits of children during the follow-up and observed that sugar intake frequencies were higher in the relapse than relapse-free group (Table 1). We observed that both the microbiome and sugar intake frequency play important

Table 1
Demographic and clinical characteristics and dietary habits of participants.

	Relapse-free n = 13	Relapse n = 11	p value
Age (months) ± SD	36.54 ± 6.78	40.64 ± 8.13	0.134[a]
Male sex	10 (76.9%)	5 (45.5%)	0.206[b]
Clinical characteristics			
dt ± SD	14.15 ± 2.76	12.09 ± 3.36	0.207[a]
ds ± SD	31.08 ± 9.92	23.91 ± 10.81	0.167[a]
Stainless steel crowns ± SD	2.77 ± 1.83	1.36 ± 1.50	0.063[a]
Extracted teeth ± SD	0.46 ± 0.78	0.45 ± 0.82	0.955[a]
Sugar intake frequency			
Before therapy			0.625[b]
≤2 times/week	1/13	0/11	
3–4 times/week	4/13	4/11	
1–2times/day	7/13	7/11	
≥2times/day	1/13	0/11	
7 months after treatment*			0.011[b]
≤2 times/week	6/13	1/11	
3–4 times/week	2/13	0/11	
1–2times/day	3/13	10/11	
≥2times/day	2/13	0/11	

SD = standard deviation, dt = decayed teeth, ds = decayed surfaces.
Sugar indicates sweetened cookies, bread, ice cream, yogurt, beverage, etc.
*$p < 0.05$.
[a] Mann–Whitney U test.
[b] Chi-square test.

roles in childhood caries relapse.

A previous study proved that aseptic mice do not develop caries despite having been treated with high-sugar foods [27]. This finding suggests that sugar causes caries via the microbiome. Associations between cariogenic food intake frequencies and cariogenic bacteria have been observed, but they have mainly focused on *S. mutans*, *Streptococcus sobrinus*, and *Bifidobacterium* species [28]. Interestingly, using co-occurrence networks, we found correlations between sugar intake frequencies and several bacteria species (Fig. 4B). Among the nine species that had positive correlations with sugar intake frequencies in the networks, *Selenomonas*, *Treponema*, *Prevotella*, *Capnocytophaga*, and *Mitsuokella* can metabolize sugar and generate acid. *C. rectus*, *Capnocytophaga* sp. HOT 323, and *Treponema* sp. and also enriched in the relapse state (Fig. 4A). The Ecological Plaque Hypothesis [29–31] proposed that environmental changes in oral conditions, such as diet, drug habits, and smoking [32], were responsible for a population balance shift of the resident microbiota and resulted in caries. In the present study, when we dynamically observed the microbiome changes over time, the differences between the relapse-free and relapse groups became increasingly more significant until relapse occurred. The interaction between the sugar intake frequency and the microbiome might play an important role during the shift from the healthy to relapse state in children.

In summary, this study demonstrated that the microbiome was significantly different between children with and without relapse at the time of relapse. A relapse-risk assessment model based on the microbiome 1 month after therapy could help to predict the relapse risk. However, we cannot neglect the effect of sugar on relapse. The sugar intake frequency is associated with the microbiome during caries relapse, suggesting that prevention of relapse involves multiple factors.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.10.110.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.10.110.

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