Supplementary information

Materials and methods

1 Study subjects and clinical screening

Preschool children with complete caries-free primary dentition were recruited from kindergartens in Hangzhou City, Zhejiang Province, China. Children using antibiotics, probiotics, synbiotics, or additional fluoride to fluoridated toothpaste 3 months prior to commencement of the study, as well as those with apparent active infection in any part of their body, those wearing any kind of orthodontic devices and accessories, visually detectable enamel or dentin hypoplasia as well as those without a habit of tooth brushing, were excluded from the study (Wang et al., 2017; Xu et al., 2018).

The experienced dentist was trained before performing oral examination. The training included theoretical information study and diagnostic training with slides, and was evaluated by examinations of 40 extracted teeth which were performed twice within 3 months, according to previous study (Jablonski-Momeni et al., 2014).

2 Sample collection and preparation

Sampling of subjects was performed in the morning, before brushing, gargling, and taking breakfast. Subjects were asked to open their mouths, and stop swallowing to allow saliva to flow into a sterile cryogenic vial (Corning Inc., Corning, NY, USA). A total of 2 ml of non-stimulated saliva was obtained from each subject. Finally, 35 samples were collected and immediately frozen in liquid nitrogen, then stored at -80°C until use in subsequent experiments (Ling *et al.*, 2010; Xu et al., 2018).

3 Extraction of microbial genomic DNA

Microorganisms in saliva samples were first pelleted by centrifugation, at 13 000*g* for 10 min, then resuspended in lysis buffer. The mixture was homogenized with 200 mg of glass beads (0.1 mm) for 2 min using a FastPrep Mini-Beadbeater, with a 1-h of incubation at 56°C, followed by DNA extraction using the QIAamp DNA Mini kit according to the manufacturer's instructions . DNA concentration was measured using a NanoDrop ND-1000 spectrophotometer, after which the DNA samples were stored at -20°C until use in subsequent experiments (Xu et al., 2018).

4 Polymerase chain reaction and sequencing

We used PCR to amplify microbial 16S rRNA genes' V3-V4 regions, using universal primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Briefly, a 20 μl reaction mixture, comprising 4 μl of 5X reaction buffer, 10 ng of microbial genomic DNA, 0.8 μl of each primer, 0.4 μl of DNA polymerase, and 2 μl of 2.5 mM dNTPs, was prepared. PCR amplification was performed in an ABI GeneAmp 9700 instrument, under the following conditions: 95°C for 3 min; 27 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and 72°C for 10 min. The PCR products were resolved on a 2% agarose gel, to reveal fragments of ~450 base pairs (bp). The products were purified using the AxyPrep DNA Gel Extraction Kit, quantified using the QuantiFluor-ST hand-held fluorometer, then sequenced on the Illumina MiSeq platform (Xu et al., 2018).

5 Data processing

Raw fastq files were demultiplexed, and their quality filtered using QIIME v.1.17 (Caporaso et al., 2010) according to the following criteria: (i) reads were truncated at any site at an average quality score <20 over a 50-bp sliding window, while discarding truncated reads that were shorter than 50 bp; (ii) two-nucleotide mismatches, in primer matching, and reads containing ambiguous

characters were removed; and (iii) only sequences overlapping by more than 10 bp were assembled according to their overlap sequence. Reads that could not be assembled were discarded (Xu et al., 2018). Operational taxonomic units (OTUs) were clustered using UPARSE v.7.1 (http://drive5.com/uparse/), with a similarity cutoff set at 97%. Chimeric sequences were also identified and removed using UCHIME. Taxonomy of each 16S rRNA gene sequence was analyzed using RDP Classifier (http://rdp.cme.msu.edu/) against the Silva (SSU115) 16S rRNA gene database, at a 70% confidence level (Amato et al., 2013).

6 Sequence analysis

Demographic data was compared using a Student's *t*-test (age), whereas differences in α diversity indices and microbial relative abundance between male and female subjects were calculated using the non-parametric Mann-Whitney *U* test to reveal the difference in microbial diversity and composition. All analyses were performed using SPSS v.19.0 software (SPSS Inc., Chicago, IL, USA), and $P<0.05$ was considered to be statistically significant. In addition, we performed principle coordinates analysis (PCoA), based on the weighted UniFrac distance, to visualize the similarities in microbial structure between male and female subjects. Information of COG (Cluster of Orthologous Group) and KO (KEGG Orthology) was obtained using the PICRUSt software. Functions and pathways predictions were done by comparing information from EggNOG (Evolutionary genealogy of genes: Non-supervised Orthologous Groups, http://eggnog.embl.de/) and KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) databases, respectively.

References

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