Sucrose promotes caries progression by disrupting the microecological balance in oral biofilms: an in vitro study

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Sucrose has long been regarded as the most cariogenic carbohydrate. However, why sucrose causes severer dental caries than other sugars is largely unknown. Considering that caries is a polymicrobial infection resulting from dysbiosis of oral biofilms, we hypothesized that sucrose can introduce a microbiota imbalance favoring caries to a greater degree than other sugars. To test this hypothesis, an in vitro saliva-derived multispecies biofilm model was established, and by comparing caries lesions on enamel blocks cocultured with biofilms treated with sucrose, glucose and lactose, we confirmed that this model can reproduce the in vivo finding that sucrose has the strongest cariogenic potential.

In parallel, compared to a control treatment, sucrose treatment led to significant changes within the microbial structure and assembly of oral microflora, while no significant difference was detected between the lactose/glucose treatment group and the control. Specifically, sucrose supplementation disrupted the homeostasis between acid-producing and alkali-producing bacteria. Consistent with microbial dysbiosis, we observed the most significant disequilibrium between acid and alkali metabolism in sucrose-treated biofilms. Taken together, our data indicate that the cariogenicity of sugars is closely related to their ability to regulate the oral microecology. These findings advance our understanding of caries etiology from an ecological perspective.

Dental caries, one of the most prevalent diseases occurring on tooth hard tissues, is driven by a disequilibrium in the oral microbial community that is termed dental biofilm1–3. Dental biofilm is a highly organized polymicrobial structure on tooth surfaces4 and is enmeshed in an extracellular matrix whose major component is extracellular exopolysaccharides (EPS)5. By metabolizing dietary fermentable carbohydrates, microorganisms within the dental biofilm generate organic acids (e.g., lactic acid). When acid production exceeds the neutralizing capacity of both alkali-producing bacteria and saliva, the low pH caused by acid accumulation within the dental biofilm initiates demineralization of tooth hard tissues4,6–11. Meanwhile, the acidic environment favors the growth of acidic/aciduric species but not alkali-producing bacteria, which in return prompts the progression of dental caries and the formation of tooth cavities7,10,11.

There is a consensus that carbohydrates, especially dietary sugars, determine whether caries develops or not12. Three variables of sugar consumption, the amount, frequency and sugar type, are closely related to caries progression13,14, as studies showed that individuals frequently taking large amounts of specific sugars experienced greater caries severity relative to those with a lower intake15,16. In addition to serving as bacterial metabolism substrates for energy production, sugars also affect the formation and properties of dental biofilms. For example, oral bacteria use sugars to synthesize EPS17, while EPS enhance the adherence of biofilm to tooth surfaces18,19, mediate biofilm three-dimensional organization and structural integrity20, and increase biofilm porosity, thereby facilitating...

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Sucrose but not glucose or lactose influences the microbial structure and assembly of the oral microbiota. The genomic DNA of biofilms was isolated and sequenced to investigate the microbiome shift introduced by sugar supplementation. After preprocessing and filtering, the average amplicon length of the remaining high-quality reads was 518 bp. These reads were assigned to 22 operational taxonomic units (OTUs) at 97% similarity. Microbial richness as revealed by OTU number showed no statistically significant difference introduced by sugar supplementation. After preprocessing and filtering, the average amplicon length of the remaining high-quality reads was 518 bp. These reads were assigned to 22 operational taxonomic units (OTUs) at 97% similarity. Microbial richness as revealed by OTU number showed no statistically significant differences among groups (p > 0.05, Fig. 3a). Venn diagram analysis showed that 18 out of 22 OTUs were shared by all groups and that there was no sugar-specific OTU that was exclusively detected in one group (Fig. 3b). By applying weighted UniFrac principal coordinates analysis (PCoA) based on the Bray-Curtis distance (Fig. 3c).
and dissimilarity tests, including Adonis and ANOSIM, we found that the overall microbial structure of sucrose-treated biofilms was significantly different from that of biofilms of the control group (Adonis: \( R^2 = 0.47, p = 0.006 \); Adonis: \( R^2 = 0.825, p = 0.005 \)). However, both weighted PCoA and dissimilarity tests detected no significant differences in the microbial community structure between the lactose/glucose treatment group and the SHI medium control (Fig. 3d,e). Ecological network analyses at the OTU level were also constructed to better show how microbial community assembly changed after sucrose, glucose and lactose treatments. Although the networks of all sugar treated groups contained 15 nodes (16 nodes in SHI medium group), and all networks were dominated by OTUs belonging to Firmicutes, there were more intermodular connections in the network of the sucrose group than in the that of either the glucose or lactose group. More importantly, the antagonistic relationship indicated by the green line between OTUs in the sucrose group was totally lost (Fig. 3f).

Sucrose introduces the most severe homeostasis disruption between acid-producing and alkali-producing bacteria. From the sequencing data, we pinpointed the genera that were distributed differently between dietary sugar-treated biofilms and the control biofilm. The distributions of 4 genera were disturbed by sucrose, while only 2 genera showed different distribution patterns between glucose-treated biofilms and the control biofilm (Fig. 4a). Lactose imposed the least pressure on the microbial community, as only 1 genus demonstrated a change in relative abundance (Fig. 4a). Among the 4 genera (Atopobium, Granulicatella, Gemella and Veillonella) that showed different relative abundances between the sucrose group and the control, Veillonella is of particular interest because species belonging to this genus can metabolize lactic acid produced by cariogenic bacteria by converting lactic acid to acetic acid and propionic acid, which are less acidic. The genus Veillonella changed its distribution in only sucrose-treated biofilms (Fig. 4a). The distributions of species belonging to Veillonella were further compared, and we found that the relative abundance of Veillonella atypica and an uncultured Veillonella spp. significantly decreased in the sucrose-treated group based on sequencing data (Fig. 4b,c). The different distribution pattern of another Veillonella species, that is, V. parvula, was also confirmed by qPCR since we did not detect this species from the sequencing data, possibly due to low abundance, and the colonization of V. parvula showed a similar tendency as other Veillonella species (Fig. 4d). Although the
bacterial load of *V. parvula* in the glucose-treated biofilms also decreased compared to that of the control biofilm, it decreased less than that of the sucrose group.

We further quantified the acid-producing bacteria *S. mutans* and alkali-producing bacteria, including *S. sanguinis* and *Streptococcus gordonii*, within the biofilms using qPCR to determine the homeostasis disruption between acid-producing and alkali-producing bacteria. Sucrose exposure increased the concentrations of *S. mutans* and *S. gordonii* but slightly decreased the concentration of *S. sanguinis*. Biofilms receiving glucose treatment also had higher concentrations of *S. mutans* and *S. gordonii* than the control biofilm, whereas the concentration of *S. sanguinis* was unaffected. Interestingly, lactose had a contrasting effect to glucose, as the levels of *S. mutans* and *S. gordonii* were unchanged; however, the level of *S. sanguinis* decreased (Fig. 5a–c). Since the *S. mutans/S. sanguinis* or *S. mutans/S. gordonii* ratio is believed to be more closely correlated with dental caries than the load of single acid-producing or alkali-producing bacteria, we further compared these ratios (summarized in Fig. 5d,e). Although both sucrose and glucose treatments increased the ratio of acid-producing/alkali-producing bacteria, the *S. mutans/S. sanguinis* and *S. mutans/S. gordonii* ratios within sucrose-treated biofilms were higher than those within the glucose-treated biofilms. In addition, no significant difference was observed between lactose-treated biofilms and the control group biofilm (Fig. 5d,e).
Dysbiosis of acid-producing and alkali-producing bacteria results in disequilibrium between acid and alkali metabolism, directly contributing to caries progression. The dysbiosis between acid-producing and alkali-producing bacteria raised the question of whether the acid and alkali metabolism of oral biofilms could be changed more significantly by sucrose than by other sugars. By quantifying the lactic acid and ammonia generation of biofilms receiving different sugar treatments, we found that the most significant disequilibrium between acid and alkali production was observed in sucrose-treated biofilms. The pH of the control group decreased to plateau at approximately 4.18; however, the pH values continuously decreased to 4.15 in the lactose group, 4.08 in the glucose group and 4.05 in the sucrose group (Fig. 6a). A similar trend was detected in lactic acid production on the 6th day, as sucrose significantly enhanced the lactic acid production of the saliva-derived biofilm, while glucose and lactose did not (Fig. 6b). In addition, NH₃ production was significantly suppressed by sucrose and to a lesser degree by glucose. However, no difference was observed in the lactose treatment group compared to the control (Fig. 6c).

**Discussion**
Sucrose has long been considered the most cariogenic carbohydrate. In past decades, numerous studies have been carried out to understand the cariogenicity of sucrose, and these studies mainly focused on the effects of sucrose on the biochemical and physiological characteristics of dental biofilms favoring caries initiation and progression [12,28,31–33]. According to the ecological plaque hypothesis, caries results from an unfavorable shift in the resident oral microbial community (that is, a dysbiotic oral ecosystem) driven by environmental factors [3]. However, to the best of our knowledge, few studies have attempted to understand the microecological regulatory effects of sucrose on dental biofilms, and it is still unknown whether the prominent cariogenicity of sucrose is due to this macromolecule imposing higher ecological pressure on oral biofilms than other dietary sugars.
In the present study, we used the human salivary microbiome of healthy donors and SHI medium to prepare an in vitro multispecies biofilm model to test whether sucrose can introduce microecology imbalance favoring caries progression to a greater degree than the other sugars. Although this in vitro biofilm model system has been proven to maintain a highly reproducible species and metabolic diversity approaching that of the human oral microbiome, we admitted that there is still some difference between our biofilm model and the in vivo dental biofilm because the diversity of the salivary microbiome may be reduced during centrifugation and some of the salivary bacteria are uncultivable even in SHI medium. The saliva-derived biofilm was cultured with SHI medium for 3 days, and then different sugars at the same concentration (2%) were added into the SHI medium to simulate the ecological challenges that the oral microbial community of healthy individuals faces. We noticed that, demineralization can be also detected in the control group (SHI medium alone), probably due to the fact that SHI medium contained 0.5% sucrose (which is an important ingredient to achieve a better recovery of the streptococci from the salivary samples) and that the biofilms were cultured in a closed system (i.e., a 24-well plate) which did not allow transfer of acid generated by bacteria through sugar metabolization out of the system. Nonetheless, we found that, all dietary sugars we supplemented into SHI medium enhanced the demineralization of the tooth enamel in comparison to SHI medium alone, among which sucrose caused the greatest demineralization lesion depth and most obvious mineral loss on enamel blocks, followed by glucose and then lactose. This result was consistent with the data of previous in vivo animal studies and epidemiologic studies showing that consumption of sucrose strongly influenced the incidence of dental caries and suggested that the microbiota changes we observed in the in vitro biofilm model here provided us with the chance to understand what happened in vivo.

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There seemed to be no linear dose–response relationship between sucrose supplementation and demineralization, as when 4 times more sucrose was added to the SHI medium than it was present in the control, only approximately 50% additional demineralization occurred. Potential reasons included, first, sugars were supplemented into SHI medium with high dosage. Since SHI medium contains 0.5% sucrose, the extra added sugars cannot be metabolized completely by bacteria. Therefore, we did not detect linear dose/response relationship between sucrose supplementation and demineralization. Second, previously published epidemiological data showed that neither a linear nor a log-linear relationship existed between sugar consumption and caries. In that case, this

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Sugar disrupts the equilibrium between acid- and alkali-producing bacteria. qPCR quantification of *S. mutans* (a), *S. sanguinis* (b), *S. gordonii* (c), and the ratios of *S. mutans*/*S. sanguinis* (d) and *S. mutans*/*S. gordonii* (e) in biofilm treated without or with sucrose/lactose/glucose. Results were presented as mean ± standard deviation. (n = 5; *p* < 0.05; **p** < 0.01; ***p*** < 0.001; ns, not significant; S. *m* = *S. mutans*; S. *s* = *S. sanguinis*; S. *g* = *S. gordonii*).
finding from our in vitro experiment reflected what happened in vivo. Only a pooled salivary microbiome from healthy subjects was used to prepare biofilms in this study, and further studies using the salivary microflora from both healthy and caries-active subjects to establish an in vitro multispecies model and to compare whether cariogenic and noncariogenic dental biofilms have similar ecological responses to sugar challenge will be helpful for us to gain additional information about the etiology of caries.

To comprehensively understand the impact of different dietary sugars on the oral microecology, we profiled taxa within biofilms by using 16S rRNA sequencing. Interestingly, no difference was detected in the microbial richness (represented by the number of OTUs) between sugar-treated groups and the control group, and we did not find sugar-specific OTUs, suggesting that if microbial structure shifts occurred after sugar exposure, they mainly resulted from the changed microbial relative abundances. Previously published in vivo data also showed that sugars mainly influence the microbial relative abundance but not the presence of the microflora, which is highly consistent with our findings and the ecological plaque hypothesis emphasizing the synergy of multiple bacterial species in the development of caries. Therefore, we used weighted PCoA instead of unweighted PCoA analysis to compare the overall microbial structure between groups. As we expected, sucrose was the only tested sugar that significantly reshaped the overall microbial structure of oral biofilms. Ecological network analysis, a system-level method to identify species interactions within an ecosystem that cannot be directly observed, further showed that sucrose-treated biofilms had no subnetworks, but there were more inter-modular connections in the sucrose network than in the glucose/lactose/control networks. Since the modules in the ecological networks could be regarded as putative microbial ecological niches or subcommunities, the ecological network analysis suggested that sucrose treatment strengthened relationships between different oral bacterial community “niches”. In addition, the interspecies antagonism and synergy among microbiota within a microecological region is among the major mechanisms maintaining microbial homeostasis in dental biofilms. Through ecological network analysis, we also found that the antagonistic relationships, which could be detected in the glucose/lactose/control groups, were absent from the microbial network of sucrose-treated biofilms, suggesting that sucrose influenced the interspecies interactions within the biofilms.

To look closely at how the microbial distribution was changed, we pinpointed the differently distributed microbiota between biofilms treated with sugars and the control. Again, we found that sucrose imposed the
highest pressure on microbial taxa distribution. Some of the genera whose relative abundance was increased by sugar treatment in the present study, particularly Gemella and Granulicatella, were reported to be dominant in the saliva and dental biofilms of caries-active subjects\(^2\). Interestingly, Veillonella, one of the most prevalent genera in the human oral cavity\(^2\), showed lower relative abundance in only the sucrose-treated biofilms in comparison to the control, and the relative abundance of all species belonging to this genus detected in this study was significantly decreased by sucrose treatment. It was reported that the load of Veillonella in the caries-active adult population is lower than that of caries-free adults\(^2\) and that Veillonella reduces the risk of caries by converting lactate to less-acidic acids\(^4\). These data suggest that sucrose is more efficient at reducing the level of bacteria contributing to acid reduction within biofilms than glucose and lactose are. Considering that some species with very low abundance are hard to capture by 16S rRNA sequencing, we further used qPCR to test the distribution of some well-known bacteria highly associated with caries (including the acid-producing species S. mutans and the alkali-producing species S. sanguinis and S. gordonii) within the biofilms to assess the homeostasis disruption between acid-producing and alkali-producing bacteria, as the enrichment of acidogenic pathogens and depletion of alkali-generating commensal residents within the biofilm is the core of dental caries pathogenesis\(^7\). We found that sucrose changed not only the bacterial load of all three species but also the ratio between acid-producing and alkali-producing bacteria. Sucrose enhanced both S. mutans/S. sanguinis and S. mutans/S. gordonii ratios, breaking the balance between acid-producing and alkali-producing bacteria within biofilms. Parallel to the dysbiosis between acid-producing and alkali-producing bacteria, the most significant disequilibrium between acid and alkali metabolism was detected in the sucrose group, as enhanced lactic acid production and suppressed ammonia production, which contributes to an environment with low pH that directly contributes to caries progression, were observed.

Taken together, our data demonstrated that the potential of sucrose in promoting the cariogenicity of oral biofilms was attributed to its ability to influence the microbial structure and assembly of the oral microecology and to disrupt the homeostasis between acid-producing and alkali-producing bacteria. These findings advance our knowledge of the mechanisms by which sucrose promotes dental caries formation from an ecological perspective.

**Materials and Methods**

**Saliva collection.** The present study was reviewed and approved by the Institutional Review Board of West China Hospital of Stomatology, Sichuan University (WCHSIRB-D-2017-095). Written informed consent was obtained according to the Ethical Guidelines of the Declaration of Helsinki (2014) before sampling\(^9\). Dental examination was carried out by one experienced dentist, and all enrolled volunteers (n = 10, aged 25–30) had no clinical symptoms of either oral or systematic diseases. Exclusion criteria also included body mass index ≥35 or ≤18, blood pressure >160/100 mmHg, oral temperature >37.8 °C, pulse >100 times/min, systemic medicine use in the last six months, local oral medicine use or treatment within 7 days, irregular eating within 3 months, and pregnant and lactating women. Subjects were asked to refrain from any food or drink 2 h before donating saliva. Approximately 5 ml of spontaneous, unstimulated whole saliva was expectorated into a sterile 15 ml cryogenic vial. Saliva samples were pooled together and then centrifuged at 2600 g for 10 min to spin down large debris and eukaryotic cells. The supernatant, referred to as pooled saliva, was diluted in sterile glycerol (final concentration of 25%), aliquoted and stored at −80 °C\(^41\). Some of the pooled saliva was further filtered using 0.22 μm filters. Filtered cell-free saliva was used to coat enamel blocks before growing in vitro multispecies biofilms\(^41,42\).

**Enamel block preparation.** Molars were collected from patients who underwent third molar extraction surgery in the Department of Oral and Maxillofacial Surgery, West China Hospital of Stomatology, Sichuan University. Written informed consent was obtained before molar collection. Dental examination was carried out by one experienced dentist, and twenty-five molars without white spots, cracks or other defects were collected and stored with 0.05% thymol solution at 4 °C. Crowns were separated from roots and then cut into four sections (5 mm × 5 mm × 3 mm). The enamels were embedded in polymethylmethacrylate and then polished with water-poor SiC abrasive papers (800~2400 grit; Struers, Struers Co., Ltd., Copenhagen, Denmark). The surface microhardness (SMH) of the sound enamel was determined with a microhardness tester (Duramin-1/−; 2; Struers), and five indentations spaced 100 μm apart were made on every exposed enamel surface at a 200 g load for 15 s. Enamel blocks with SMH between 320 and 400 Knoop hardness were selected for further study. After microhardness testing, the surfaces were slightly polished again. Each enamel surface was painted with two layers of acid-resistant enamel blocks were sterilized in an ethylene oxide sterilizer.

**SHI medium preparation.** To support the growth of different subpopulations within the pooled saliva, we prepared SHI medium and used it to culture biofilms. The composition of SHI medium was 10 g/l proteose peptone (Difco, Becton Dickinson, Sparks, MD, USA), 5.0 g/l trypticae peptone (Difco), 5.0 g/l yeast extract (Difco), 2.5 g/l mucin (type III, porcine, gastric; Sigma-Aldrich, St. Louis, MO, USA), 10 mg/l N-acetyl muramic acid (NAM, Sigma-Aldrich), 5% sheep blood, 2.5 g/l KCl, 5.0 g/l sucrose, 5.0 mg/l hemin, 1.0 mg/l vitamin K, 0.06 g/l urea and 0.174 g/l arginine\(^41\).

**In vitro saliva-derived multispecies biofilm culturing.** Enamel blocks were put into 24-well plates, and 200 μl of filtered cell-free saliva was added to each well, allowing attached pellicle growth, and incubated at 37 °C with the lid open for 1 h to dry the saliva coating. The plates were then sterilized under UV light for 1 h before adding SHI medium (1.5 ml per well) and pooled saliva (30 μl per well). The plates were incubated at 37 °C without disturbance for the first 24 h under anaerobic conditions to allow initial biofilm formation. After 24 h, the culture medium was changed daily.
Dietary sugar treatment. Starting at 72 h, the biofilms were transferred to SHI medium containing different dietary sugars (i.e., 2% sucrose, 2% glucose and 2% lactose) to simulate a cariogenic challenge until the endpoint (144 h). Figure 1 gives a brief introduction to the experimental design.

Acid and alkali production measurement. The pH value of spent medium was measured with a pH meter (Thermo Fisher Scientific, Waltham, MA, USA), and the NH₃ level in the medium was determined with an ammonia assay kit (Jiancheng, Jiangcheng Bioengineering Institute, Nanjing, China). To measure the production of lactic acid in the 144 h biofilms, samples were rinsed in cysteine peptone water first and then transferred into new plates. Buffered peptone water (BPW) with 0.2% sucrose was added into each well and anaerobically incubated at 37 °C for 3 h. Lactic acid was determined by an enzymatic method as described before. Briefly, the BPW solution absorbance was measured at 340 nm, and the lactic acid content was calculated based on a standard curve. The data are reported as the mean of 5 separate tests.

Caries lesion depth and mineral loss assay. The caries lesion depth and mineral loss were measured by transversal microradiography (TMR). After removing the biofilms on the surface, enamel blocks were ultrasonically cleaned for 10 min. Then, blocks were cut into sections approximately 300 μm thick vertically to a window on the surface. Sections were then polished to a thickness of 100 μm. The polished slices were then fixed on a TMR sample holder with an aluminum calibration step wedge serving as a standard curve and microradiographed using a monochromatic CuK X-ray source (Philips, VitalAir/Comcare, Eindhoven, the Netherlands) at 20 kV and 20 mA for 30 s. The lesion depth and mineral loss at the selected area were analyzed by imaging software (Transversal Microradiography Software 2006, Inspektor Research Systems BV, Amsterdam, the Netherlands). Five TMR traces were measured on each slice. Mineral loss of the lesions was calculated by a computer program using a step wedge scale to compare the lesion to sound tissue. The lesion depth was defined as the length from the enamel surface to the point where the mineral content reached 95% of that of sound enamel. The data are reported as the mean of 5 separate tests.

DNA extraction and 16S rRNA sequencing. The biofilms were scraped with a sterile knife from enamel blocks into 1 ml of phosphate-buffered saline (PBS) and then sonicated. Genomic DNA was isolated using a QIAamp DNA micro kit (Qiagen, Valencia, CA, USA) according to the instructions but with additional lysozyme treatment (3 mg/ml, 1.5 h). The DNA quality was evaluated with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the NH₃ level in the medium was determined with an ammonia assay kit (Jiancheng, Jiangcheng Bioengineering Institute, Nanjing, China). To measure the production of lactic acid in the 144 h biofilms, samples were rinsed in cysteine peptone water first and then transferred into new plates. Buffered peptone water (BPW) with 0.2% sucrose was added into each well and anaerobically incubated at 37 °C for 3 h. Lactic acid was determined by an enzymatic method as described before. Briefly, the BPW solution absorbance was measured at 340 nm, and the lactic acid content was calculated based on a standard curve. The data are reported as the mean of 5 separate tests, and representative pictures are shown.

Bioinformatic analysis of sequencing data. The sequences were clustered into 22 OTUs at the 97% similarity level. Bioinformatics was performed by mothur and QIIME 2.0 software, including quality control of raw data and taxonomic annotation according to the Silva database. The data were further analyzed as follows: (1) The microbial richness revealed by OTU number was analyzed with one-way ANOVA, followed by Dunnett’s t test to compare the means of all other groups with that of the control group. (2) A Venn diagram was constructed to compare the shared and different OTUs among groups. (3) Weighted PCoA and dissimilarity tests, including Adonis and ANOSIM, were used to examine the community differences between groups. (4) Microbe-microbe networks were constructed by the MENA pipeline. A Pearson correlation cutoff of 0.7 was determined using the random matrix theory approach by observing the transition point of the nearest-neighbor spacing distribution of eigenvalues from a Gaussian to a Poisson distribution, two universally extreme distributions. Then, Cytoscape 3.2.0 was performed with a force-directed algorithm to visualize networks and compute network topological parameters with NetworkAnalyzer. (5) The relative abundances of bacterial taxa at the genus and species levels were calculated. The relative abundance difference between groups was compared by the Kruskal-Wallis test at both the genus and species levels.

**Microbial quantification by qPCR.** S. mutans, S. sanguinis, S. gordonii and V. parvula were quantified using qPCR with standard curves as previously described. Primers were chosen based on the bacterial 16S rRNA sequences (Table 1). qPCR amplification was performed on the Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific). The reaction mixture (20 μl) contained 10 μl of 2 × SYBR Premix Ex Taq II (TaKaRa, Takara Bio Inc., Shiga, Japan), 2 μl of template DNA, 0.8 μl of forward and reverse primers (10 mM

| Species       | Primer name | Sequence(5′→3′)       |
|---------------|-------------|-----------------------|
| **S. mutans** | S. mutans-F | GATAATGATTGAAAGATGCAAGC |
|               | S. mutans-R | ATTCCCTACTGCTGCCCTCCC  |
| **S. sanguinis** | S. sanguinis-F | AGTGGCCATCGTTGAGTTG |
|               | S. sanguinis-R | GTACGGCCATTGTAACAC |
| **S. gordonii** | S. gordonii-F | GCTTGCTACACCATAGACT |
|               | S. gordonii-R | CGCTTACCTGACCTACTAG |
| **V. parvula** | V. parvula-F | TGCTAAATACCAGCATTAGCATAAAC |
|               | V. parvula-R | GCTTATAAATAGAGCGCCACCCTTCA |

**Table 1.** Oligonucleotide primers used in qPCR for microbial quantification.
each), 0.4 μl of ROX Reference Dye II and 6 μl of PCR-grade water. Thermal cycling conditions were as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. An additional step, 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s, was performed to establish a melting curve. Threshold cycle values (CT) were determined, and the copy number was calculated based on the standard curve (log copies/μl versus CT values). Each sample was examined in triplicate. The data are reported as the mean of 5 separate tests.

Statistical analysis. Data other than those obtained from microbiome sequencing were compared with SPSS (version 16.0 for Windows). Data were analyzed with one-way ANOVA, followed by Student-Newman-Keuls test to compare means of each group, and Dunnett’s t test to compare the mean of all other groups with the control group. The difference was statistically significant if the 2-tailed p value < 0.05.

Data availability All data generated or analyzed during this study are included in this published article. The 16S rRNA sequencing raw data have been deposited in the public database Sequence Read Archive (http://www.ncbi.nlm.nih.gov/Traces/sra) under accession number PRJNA550154.

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Qian Du: performed the majority of the experiments, analyzed the data and drafted the manuscript; Min Fu: performed the majority of the experiments, analyzed the data, writing-review and editing; Yuan Zhou, Yangpei Cao, Tingwei Guo, Zhou Zhou: helped the bioinformatics on 16S rRNA sequencing data and provided suggestions for the project; Mingyun Li, Xian Peng, Xin Zheng, Yan Li: helped to design the study and draft the manuscript, provided suggestions for the project and critically reviewed the manuscript; Xin Xu: funding acquisition, writing-review and editing; Jinzhi He, Xuedong Zhou: conceptualization, writing-review and editing, supervision, funding acquisition. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

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