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The predictive power of saliva electrolytes exceeds that of saliva microbiomes in diagnosing early childhood caries

Ying Zhang[a], Shi Huang 🔟[c,d], Songbo Jia[f], Zheng Sun 🔟[b], Shanshan Li[a], Fan Li[a,e], Lijuan Zhang[g], Jie Lu[e], Kaixuan Tan[e], Fei Teng[b] and Fang Yang 🔟[a]

[a]School of Stomatology, Qingdao University, Qingdao, Shandong, China; [b]Single-Cell Center, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao, Shandong, China; [c]Centre of Microbiome Innovation, Jacobs School of Engineering, University of California, San Diego, La Jolla, California, 92093, USA; [d]UCSD Health Department of Pediatrics, University of California, San Diego, La Jolla, California, 92093, USA; [e]Stomatology Centre, Qingdao Municipal Hospital, Qingdao, Shandong, 266071 China; [f]Department of Stomatology, Tianjin Children's Hospital, Tianjin, 300400 China; [g]Department of Stomatology, Women & Children's Health Care Hospital of Linyi, Linyi, Shandong, 276000 China

ABSTRACT

Early childhood caries (ECC) is one of the most prevalent chronic diseases affecting children worldwide, and thus its etiology, diagnosis, and prognosis are of particular clinical significance. This study aims to test the ability of salivary microbiome and electrolytes in diagnosing ECC, and their interplays within the same population. We here simultaneously profiled salivary microbiome and biochemical components of 331 children (166 caries-free (H group) and 165 caries-active children (C group)) aged 4-6 years. We identified both salivary microbial and biochemical dysbiosis associated with ECC. Remarkably, K^+, Cl^-, NH_4^+, Na^+, SO_4^{2-}, Ca^{2+}, Mg^{2+}, and Br^- were enriched while pH and NO_3^- were depleted in ECC. Moreover, the dmft index (ECC severity) positively correlated with Cl^-, NH_4^+, Ca^{2+}, Mg^{2+}, Br^-, while negatively with pH and NO_3^-. Furthermore, machine-learning classification models were constructed based on these biomarkers from saliva microbiota, or electrolytes (and pH). Unexpectedly, the electrolyte-based classifier (AUROC = 0.94) outperformed microbiome-based (AUROC = 0.70) one and the composite-based one (with both microbial and biochemical data; AUC = 0.89) in predicting ECC. Collectively, these findings indicate ECC-associated alterations and interplays in the oral microbiota, electrolytes and pH, underscoring the necessity of developing diagnostic models with predictors from salivary electrolytes.

Introduction

Early childhood caries (EEC) is one of the most prevalent chronic diseases of children worldwide [1]. Severe EEC, an aggressive form of dental caries, can lead to acute pain, sepsis, and potential tooth loss and even interfere with the children's quality of life, nutrition, and school participation [2]. Most importantly, once started, the damage to teeth is irreversible, with ECC-affected children continuing to suffer from a higher risk for caries onsets and even tooth loss over their entire lifespan [3]. In addition, ECC incurs enormous societal costs [4,5]. For instance, some of the children who suffer from dental treatment-related anxiety or are challenging to treat successfully in the dental chair require general anesthesia for the treatment of caries [6]. Therefore, the early diagnosis and prognosis of ECC and preventive intervention are of particular clinical significance.

For the assessment and early detection of ECC, visual or visual-tactile detection combining with bite-wing radiography has been commonly employed [7,8]. A commonly used method is the International Caries Detection and Assessment System (ICDAS), which has shown to be an accurate and reproducible method to detect early lesions based on clinical visual appearance [9]. Radiographs are more sensitive than

clinical inspection for detecting approximal lesions, estimating lesion depth, and monitoring lesion behavior [10,11]. However, this chair-side check by a dental explorer might not be friendly to children due to the discomfort of intraoral instruments. Moreover, radiography cannot distinguish active lesions from arrested ones [12]. Additionally, limitations of other useful tools aiding in caries detection are also not insignificant [13,14], such as Optical Coherence Tomography (OCT), Fibre-optic Transillumination (FOTI), and Electrical Caries Monitor (ECM). The principal issue of OCT is the lack of commercial availability due to its high cost and, moreover, determination of the lesion depth in relation to the pulp is prevented because the pulp chambers do not appear clearly in the OCT images [15]. Although FOTI offers dentists and patients a three-dimensional image of carious lesions, it is not useful for the determination of lesion size, depth, volume, and mineral content [16]. ECM has shown superior performance to FOTI and radiography in the early lesion [11], but the inconsistent probe contact with the tooth surface could result in variations in producibility [17]. The lesion activity assessment will assist in the treatment decision, particularly when preventive options should be implemented [18]. Therefore, the development of risk-assessment methods that are objective, accurate, reproducible, patient-friendly, and readily accessible has become a top priority in this field.

ECC has been widely associated with oral microbiome dysbiosis. Salivary microbiome dysbiosis, allowing the promotion of cariogenic bacteria that lead to sustained demineralization of tooth tissue, has been implicated in dental caries [19–22]. Evidence from our and other studies demonstrated that the saliva microbial biomarkers could serve as a non-invasive and host-friendly proxy for risk assessment of ECC [22,23]. However, these organismal markers were tested in relatively small cohorts (e.g. sample sizes ranging from 25 to 50), and only exhibited a moderate performance (e.g. area under the receiver operating characteristic curve (AUROC) ranging from 68% to 78%) in predicting dental caries status [20,22,23]. Surveys and validation in larger children cohorts that include more inter-individual and biogeographic heterogeneity in the oral microbiome thus are urgently needed to refine the landscape of critical dysbiotic microbiota underlying ECC.

ECC is recognized as a multifactorial disease mainly caused by the complex metabolic interactions between the oral microbiome, diet, and many host factors [24]. The dynamic changes of saliva electrolytes reflect the variation of microenvironment along with the occurrence of ECC. In the development of caries, the relationship between demineralization and remineralization is affected by the presence of saliva, which promotes the transport of electrolytes, bacteria, and fermentable carbohydrates to the tooth surface [25]. Among them, Ca^{2+} and PO_4^{3-} are contained in enamel hydroxyapatite. Once the enamel is demineralized, these electrolytes are released to saliva [26]. F^- incorporated with calcium and phosphate, which is taken up by demineralized tooth substrate, forms a fluorapatite crystalline structure by remineralization, thus increasing resistance to the next acid challenge [27]. Na^+, K^+, Mg^{2+} and Cl^- can substitute for calcium and phosphate in the crystal lattice of the various mineral phases present in the teeth, thereby potentially affecting tooth demineralization and remineralization interaction [28,29]. NO_3^- is mainly transported to the salivary glands, through the action of nitrate reductase in the oral cavity, then it is quickly reduced to NO_2^-. These salts are rapidly acidified when encountering some bacteria such as *Lactobacillus, Actinomyces*, and *Streptococcus mutans*, which are related to cariogenic effects [30]. Br- has important functions in the formation of collagen IV and in the activation of α-amylase in saliva [31]. Urea and ammonia, which are produced by arginine-rich protein, play a role in maintaining a neutral pH in the oral cavity [32]. Thus, a comprehensive understanding of the dynamics of salivary constituents and properties in the larger children study is required for assessing its potential for better diagnosis and prediction of ECC.

Given that both oral microbiome and salivary biochemical environments can contribute to the etiology of ECC, here we further address three critical questions in a 331-member Chinese cross-sectional study consisting of both ECC and healthy individuals. (*i*) Do saliva microbial communities assemble differentially at different host disease states in a large-scale study? (*ii*) Do various saliva electrolytes, pH, and total protein differentially distribute across disease states? Does ECC feature a co-occurrence network of saliva microbiota and electrolyte components? (*iii*) What is the predictive power of the salivary microbiota and electrolyte biomarkers distinguishing the caries children from the healthy ones?

Materials and methods

Study design

Our study involved 4 to 6 years old children enrolled in 22 kindergartens in China. According to the 4th National Oral Health Survey, the mean caries prevalence for 4- to 6-year-old children is 70% in China. In a pilot study, 70% of the 4- to 6-year-old children in

China had at least one tooth with untreated caries [33]. We further estimated the sample size of the study employing the following formulate [34].

$$N = (1.96)^2 \times p \times (1 - p)/d^2$$

where N = sample size, p = prevalence = 70%, d = margin of error = 15%. Therefore, the calculated minimum sample size was 36 children per group. The sample size of our study was 331 (165 caries-active and 166 caries-free children), far exceeding the calculated minimum sample size.

Inclusion criteria were as follows: (1) Both genders, aged from around 4 to 6 years; (2) Participants (or legal parents or other guardians for children) provided informed consent; (3) Children could cooperate with the sampling. The exclusion criteria considered individuals: (1) No orthodontic treatment or malocclusion or severe gingival or periodontal diseases; (2) Absence of any systemic or congenital diseases, emotional or intellectual disabilities, developmental malformations, bacterial or severe infections in other parts of the body; (3) No emergency dental recall in the last 3 months; (4) No antibiotics, probiotics, professionally applied fluorine vanish and orthodontic appliance within the past 3 months. The fluorine excluded in this study refers to the professionally applied fluoride vanish within 3 months, while we cannot exclude the use of fluoride in water and dentifrice.

Local authorities and children's parents were informed of the study objectives and procedures. Parents who agreed to the participation of their children signed the informed consent form according to the approval of the ethical committee of Qingdao University. Then, a clinical oral examination was performed on those children in 22 kindergartens in Qingdao, China. Information about children's age, gender, and whether they had used antibiotics, probiotics, fluoride, and dental appliances in the past 3 months was collected using questionnaires. According to the eligibility criteria, 331 children were selected for saliva sample collection, including 165 children (94 boys and 71 girls) diagnosed with active caries (dmft ≥ 4, C group) and 166 healthy children (81 boys and 85 girls; dmft = 0, H group).

All teeth were evaluated according to the criteria recommended by the World Health Organization (WHO) using dmft index (the number of decayed, missing, and filled teeth in deciduous dentition for primary teeth) [35]. The clinical examination was carried out using a visual-tactile method with a dental mirror and WHO probe 5 s per dental surface under artificial light use [36]. In our study, the healthy group consisted of children with intact healthy teeth surfaces. Children with clinical signs of early caries or white spots were not included in the H group. White spot lesions were differentiated from developmental enamel defects simply on clinical grounds based on the association of the lesions with the area of mature plaque and location on the tooth, i.e. white lesion appearing to occur adjacent to the gingival margin, and extending along the buccal or lingual surfaces [37], combined with the aspect of the lesion when dry (white chalk looking versus dry).

Prior to the clinical data collection, the calibration procedures were conducted for the diagnosis of dental caries in a pilot study. These procedures sequentially involved theoretical training with the examination of photographs of different dental conditions, clinical examinations, and the determination of intraexaminer and interexaminer agreement. The process was conducted by a researcher with experience in epidemiological studies involving the indices to be employed (gold standard). Kappa coefficients were calculated, with an interval of 7 days between two examinations for the determination of intraexaminer agreement. The calibration procedures were performed on 20 children in the same age group until achieving excellent intraexaminer and interexaminer agreement (>0.80).

Saliva sample collection

Saliva samples were collected at the same period, in the morning from 8:00 to 10:00. Children were required not to brush their teeth in the morning before sampling. No drinking or eating was allowed for at least 90 min before collection. Subjects sat on ordinary chairs and were placed in Coachman's posture at the time of sampling, with their heads slightly downward, hands palmed on their thighs, and slightly forward bending. These subjects were asked to slowly spit natural, unstimulated saliva into a 50 mL centrifugal tube for 5 min. Samples were recorded and transferred to 4°C until the end of the collection. The saliva samples were centrifuged at 3,000 rpm at room temperature for 10 min, and the supernatant was then stored in a refrigerator of −80°C.

Biochemistry components analysis

The stored saliva was thawed naturally. For each individual, 2 mL saliva samples were taken for biochemistry components analysis. Saliva was centrifuged at 13,000 rpm for 10 min and the clear supernatant was obtained for analysis. The pH values of saliva were measured by a digital pH meter. First, a pH 7.0 buffer was used to calibrate. Once the pH

electrode was immersed in one saliva samples, the electrode should be infiltrated into distilled water prior to the next test. The infiltration part of the electrode was wiped off with filter paper and corrected with the buffer of pH 7.0. An automatic enzyme labeling instrument determined the concentrations of total protein in saliva. Absorbance was measured at 595 nm to evaluate the level of the total saliva protein. As for the determination of salivary ions, a chromatography unit was used to determine concentrations using specific columns. The chromatography unit was calibrated with the use of a standard eluent. Before analysis, all samples were filtered through 0.45 μM membrane filters to remove suspended particles. The treated samples were injected into the ion chromatography system. The anions we tested included sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), ammonium salt (NH_4^+), magnesium (Mg^{2+}), and cations included chlorine (Cl^-), phosphate (PO_4^{3-}), nitrate (NO_3^-), nitrite (NO_2^-), sulfate (SO_4^{2-}), fluoride (F^-) and bromine (Br^-). Chromatographic data were acquired and processed by software (Peaknet 6.2) integrated in the ion chromatography system. Calibration and linearity checks were performed according to a previous protocol [38]. All of the above tests were performed in triplicate.

DNA extraction, PCR amplification, and sequencing of the salivary microbiome

Salivary genomic DNA was extracted by the Qiagen DNeasy Blood & Tissue DNA kit (Qiagen Valencia, CA), according to the manufacturer's instructions [39,40]. Two milliliters of unstimulated saliva were taken into a microcentrifuge tube by centrifugation for 10 min at 13,000 rpm. The precipitate was resuspended by buffer ALT and the mixture was incubated for 30 min at 37°C. Then, 20 μL of proteinase K was added and the mixture was incubated overnight at 56°C. Afterward, 200 μL buffer and 100 μL of ethanol were added. DNA yield and DNA purity were determined using NanoDrop ND-100 (NanoDrop Technologies, DE, USA), A 260/280 nm was used for protein contamination, and A260/230 nm for salt and phenol contamination. The V3-V4 region of the 16S ribosomal RNA (rRNA) gene was amplified using universal primers for pyrosequencing for all 331 volunteers. PCR amplification reaction mixtures in triplicate for each sample were pooled at approximately equal amounts and sequenced. The quality of the amplified PCR product was verified by electrophoresis (2% agarose gel) and PCR products were sequenced using Roche 454 FLX Titanium (GS-Titanium; 454 Life Sciences, Branford, CT, USA).

Sequence analyses, OTU clustering and taxonomy assignment

The raw sequences were filtered based on sequence length, quality, primer with Trimmomatic (v0.36), Pear (v0.9.6), and Flash (v1.20), and the low-quality or chimeric sequences were removed with Vsearch (v2.7.1). Downstream bioinformatics analysis was performed using the Parallel-Meta 3 software package. Both binary and source code packages are available at http://bioinfo.single-cell.cn/parallel-meta.html. Parallel-Meta 3 is popular used for 16S rRNA copy number calibration, diversity statistics, taxonomical comparison of microbial communities, comprehensive biomarkers selection and interaction network construction [41]. Clustering of the operational taxonomic unit (OTU) was conducted at 97% similarity level using the GreenGenes database [42]. Based on the results of OTUs, the microbial richness index (Chao1) and species diversity indices (Shannon index and Simpson index) were calculated to evaluate α diversity of the oral salivary microbiome. The distance between each pair of microbiota was computed based on the weighted and unweighted Meta-Storms algorithm [43], which was used to quantify the differences between any two samples.

S. mutans and Prevotella pallens were selected species for technical verification. S. mutans was found to be related with a variety of electrolytes in our study. P. pallens was not found to have a significant association with ECC in this study, yet their genus Prevetolla was associated with ECC in our previous study [22]. The absolute amounts of S. mutans were assessed by the specific qPCR primer Sm-F2 and Sm-R2. The primer sequence (5′→3′) was GCAGTCA AGGGGTGGAAATCG and TGGACGGCTTG TTGCAGGAATAC respectively, and the amplicon size was 188 bp [44]. Another primer pair for P. pallens was 5′ AGCCTGAACCAGCCAAGTAG and 3′ CATAGCATACTTA-TTCCTGGCCG. The gene copy number was calculated based on the standard curve of each primer system with the LightCycler 480 software 1.5 (Roche). The relative abundance of S. mutans and P. pallens identified via qPCR was described in the following table. The correlation results showed that the relative abundance of S. mutans and P. pallens was consistent with the result of 16S rRNA sequencing (r = 0.64 for S. mutans and r = 0.72 for P. pallens, p < 0.05).

Statistical analyses

The statistical analyses were carried out via R (Version 2.15.0), including the chi-squared test,

Wilcoxon rank sum test, permutational multivariate analysis of variance (PERMANOVA), and Spearman correlation. Biochemical statistics were presented as mean ± standard deviation, and the chi-squared test compares these two groups. Indices of Shannon, Simpson, and Chao1 between status and gender were compared by the Wilcoxon rank-sum test and further, the correlation of the above indices and the dmft index were estimated using the Spearman correlation coefficient. The influence of disease status and gender on the microbial community was calculated with PERMANOVA. The relative abundance of taxonomic groups at six phylogenetic levels between the C and H groups was evaluated with Wilcoxon rank-sum test. 'ECC-enriched' represents those microbial/biochemical indices with significantly increased relative abundance/concentration level in ECC children compared with healthy controls, while 'ECC-depleted' means those microbial/biochemical indices with significantly decreased relative abundance/concentration level in ECC children compared with healthy controls. Spearman correlation coefficients were used to evaluate the correlations among saliva biochemical components, caries activity (dmft index), and bacterial abundances. A *P*-value less than 0.05 was considered as statistically significant.

Establishing the ECC diagnostic model using a machine learning technique

Random Forest models were trained to identify disease status using the default parameters of the R implementation of algorithm (randomForest package in R, ntree = 5,000, using default 'mtry' of p/3, where p is the number of input taxa). We used random sampling to divide the dataset into a training set (90%) and a testing set (10%) so that to balance each dataset. To construct and optimize the diagnostic model, models were built based on biochemical indices alone, microbiomes alone, and both biochemical and microbial indices collectively. The 'rfcv' function estimated the minimal number of top-ranking discrimination taxa required for classifying over 100 iterations. The results were evaluated with a 10-fold cross-validation method and this process was repeated ten times. Then, the average of probability was reported as a result. In the 100 iterations of the algorithm, the sorting list of taxa was determined according to the order of feature importance reported by Random Forests. The performances of models were assessed by AUROC (area under the receiver operating characteristic curve), and a high area under the curve represents both high recall and high precision.

Data availability

The sequencing data in this study have been submitted to the Microbiome Search Engine (MSE) [45] and NCBI Sequence Read Archive, and can be accessed through the project ID P_SCC0005 (http://mse.single-cell.cn/index.php/mse/get_by_project/P_SCC0005) and BioProject numbers PRJNA717886.

Results

Profound dysbiosis of salivary microbiota in ECC

We assigned 331 children into the C group (dmft = 9.01 ± 2.8; n = 165) and H group (dmft = 0; n = 166) according to their caries states determined by the dmft index [35]. There was a balanced gender and age distribution between the H and C groups (chi-squared test, $p > 0.05$). We qualitatively and quantitatively measured the differences of the salivary microbiota with and without ECC in microbial diversity and composition. Firstly, alpha diversity indices, including Shannon index, Simpson index, and Chao1, were significantly higher in the C group as compared with the H group ($p = 0.0062, 0.0048, 0.0074$, respectively), yet not associated with gender ($p = 0.28, 0.35, 0.72$, respectively; Figure 1). Next, to assess microbial structure alterations (i.e. beta diversity) in ECC, we calculated the Meta-Storm distance between all pairs of samples and compared the community structures within and between disease states. We found that the disease state was the most dominant factor explaining the variation in the salivary microbiomes ($p = 0.001$, F = 3.22). Moreover, the degree of variations in the C group was significantly less than that in the H group, suggesting that ECC microbiomes were significantly relatively similar and conserved, whereas the healthy ones were relatively variable ($p = 0.03$; Figure S1). Lastly, we further identified differentially abundant taxa (i.e. 'ECC-enriched' and 'ECC-depleted' taxa) between two host groups at each of the six phylogenetic levels (i.e. phylum, class, order, family, genus, and species levels; Figure 2, S2). At the genus level, ECC children exhibited an increased relative abundance of *Prevotella*, *Mogibacterium*, or *Atopobium* ($p = 8.40e-4, 8.81e-3, 1.57e-4$, respectively; Figure 2A). Moreover, a few bacterial species under these genera, including *Prevotella melaninogenica*, *Atopobium rimae* and *Atopobium parvulum*, *S. mutans* and *Streptococcus* AY020 were found to be enriched in the C group ($p = 2.08e-4, 4.01e-4, 7.96e-4, 9.20e-20, 5.32e-3$, respectively; Figure 2B). Conversely, at the genus level, *Porphyromonas* and
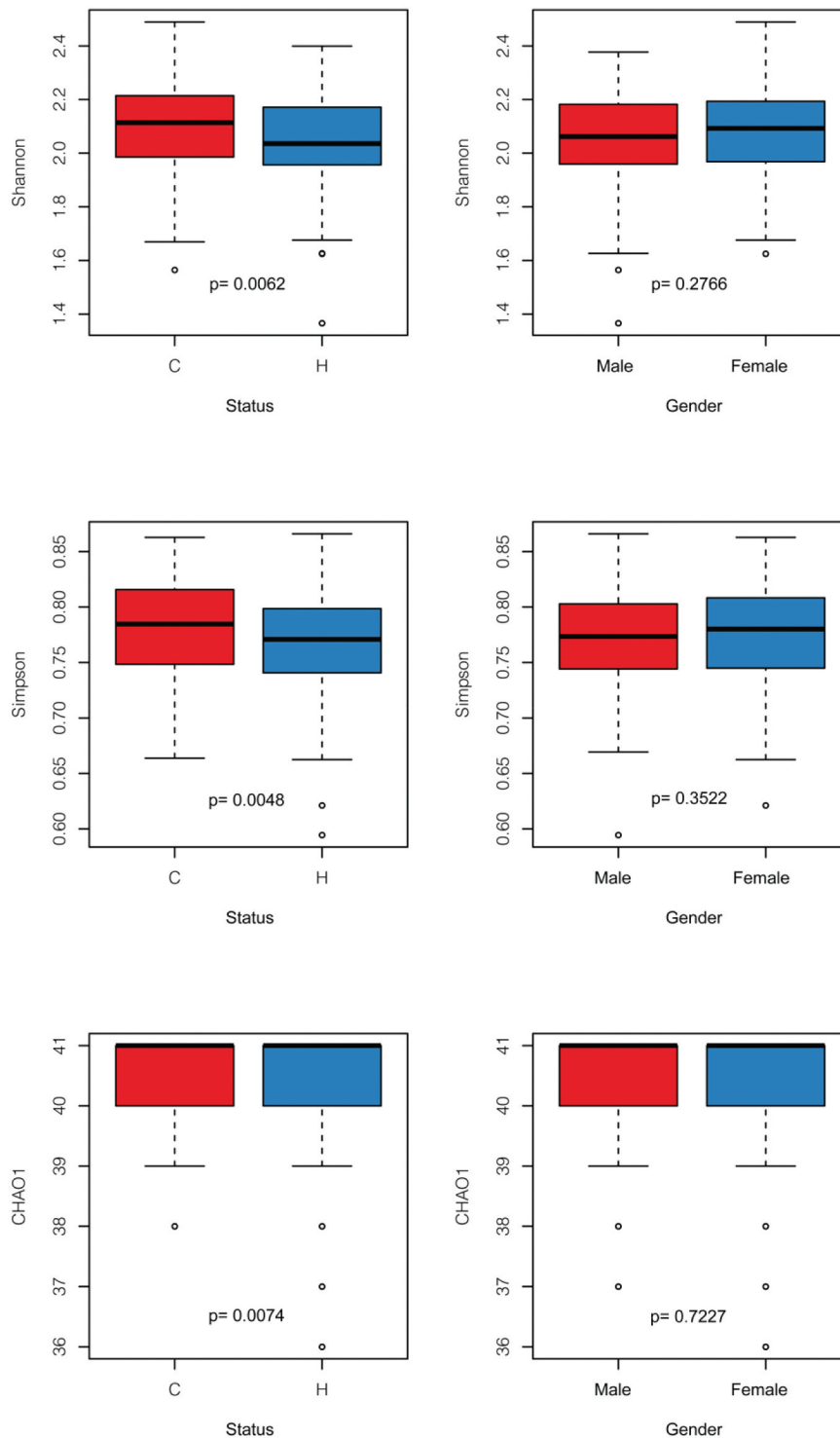
Figure 1. Comparison of indices of α diversity between status and gender. The p value for each comparion was present within the boxplot. The Shannon, Simpson, and Chao1 indices were significantly higher in the C group (*p* = 0.0062, 0.0048, 0.0074), while none of these indices were significantly different between gender (*p* > 0.05).

Capnocytophaga (their representative species: *Porphyromonas* CW034 and *Capnocytophaga sputigena*) were less abundant in the C group than in the H group (ECC-depleted; *p* = 2.67e-4, 6.37e-3, 1.28e-5, 1.05e-3, respectively; Figure 2C) while *Streptococcus australis/sanguinis* depleted in ECC as well (*p* = 6.68e-3; Figure 2D). Furthermore, we found that the dmft index positively correlated with the relative abundance of *S. mutans* (ECC-enriched; *p* = 5.37e-22, rho = 0.52; Figure 3), while negatively correlated with *Porphyromonas* CW034 (ECC-depleted; *p* = 0.006, rho = −0.24; Figure 3).
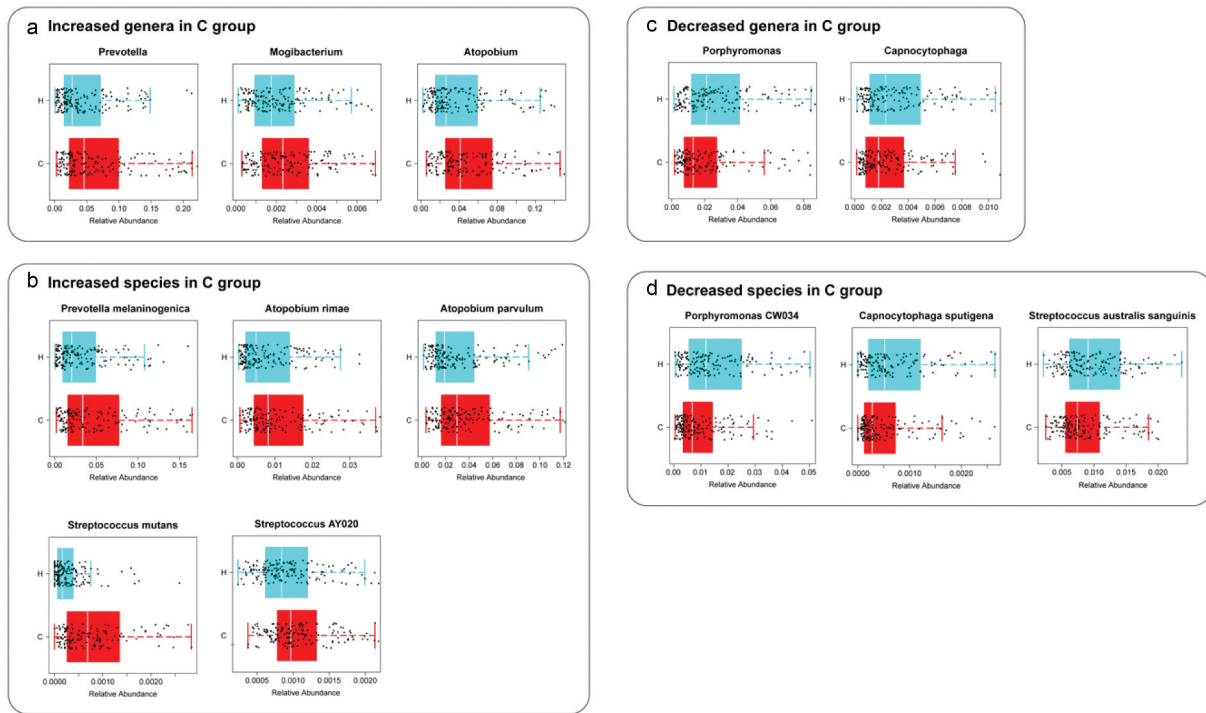
Figure 2. Relative abundance of significantly diverse genera and species in the C group and the H group. Each dot represented a sample. The H group in the blue box (n = 166), while the C group in the red one (n = 165). The ECC-enriched taxa were presented at the genus (**A**) and species (**B**) level, and the ECC-depleted taxa were shown at the genus (**C**) and species (**D**) level as well. PRINTER: Can the figures be increased to make them more readable?.

Key saliva biochemical components underlying ECC pathogenesis

Next, we pinpointed a wide array of ECC-associated biochemical markers (i.e. the 'ECC-enriched' and 'ECC-depleted'). The electrolytic concentrations of K^+, Cl^-, NH_4^+, Na^+, SO_4^{2-}, Ca^{2+}, Mg^{2+}, and Br^- were significantly higher in the C group (p = 6.63e-4, 1.49e-11, 1.63e-4, 1.63e-3, 1.31e-3, 5.68e-6, 4.98e-7, 1.29e-8 respectively; Table 1), while levels of pH and NO_3^- were significantly higher in the H group (p = 9.95e-5, 2.72e-5, respectively; Table 1). Among them, we identified positive correlations between levels of certain ECC-enriched electrolytes, including Cl^-, Br^-, Mg^{2+}, Ca^{2+} and NH_4^+ and the dmft index (p = 2.66e-8, 2.74e-6, 2.43e-4, 3.98e-3, 1.59e-2, respectively; rho = 0.33, 0.29, 0.24, 0.20, 0.18, respectively; Figure 4A). In contrast, changes in the ECC-depleted electrolyte (NO_3^-) level and pH value were negatively correlated with the dmft index (p = 4.62e-4, 2.21e-2, respectively; rho = −0.23 for pH value, −0.17 for NO_3^- level; Figure 4B). Besides, no difference between the two groups was noted for the concentration of total protein. The relative abundance of *S. mutans* (caries-enriched taxon) showed a positive correlation with the concentration of Cl^-, Br^-, K^+, and Mg^{2+} respectively (ECC-enriched electrolytes; p = 2.25e-8, 4.76e-5, 1.41e-2, 2.87e-2, respectively, rho = 0.35, 0.29, 0.23, 0.22; Figure 4C), whereas *Lautropia mirabillis* positively correlated with NO_3^- (ECC-depleted electrolyte; rho = 0.24, p = 6.66e-3; Figure 4C). The abundance of *L. mirabillis* was not statistically significantly different

between the two groups but trended to be lower in ECC samples.

The predictive power of the salivary electrolyte pool is superior to that of microbiota in discriminating ECC

In this study, we observed the substantial alterations in the saliva microbiota and the biochemical compositions of the ECC group relative to those of the healthy controls. These findings raised three key questions: (*i*) whether we can develop the diagnostic model of ECC based on the biochemical profile as well as, if not better than, the oral microbiota; (*ii*) if yes, which feature set could offer a better prediction performance; (*iii*) whether a combined feature set, including both the salivary microbiomes and biochemical constituents, can further improve the model performance. To address these questions, we trained three sample classifiers for predicting ECC states using Random Forests (RF) algorithm based on features from oral microbiota alone, biochemical components alone, and a combined set to distinguish the ECC from the healthy control group, respectively. The model performance was evaluated via the area under the Receiver Operator Curve (AUROC) using a 10-fold cross-validation approach (Materials and methods). Then, we ranked the features by built-in RF importance scores in each predictive model for feature selection.

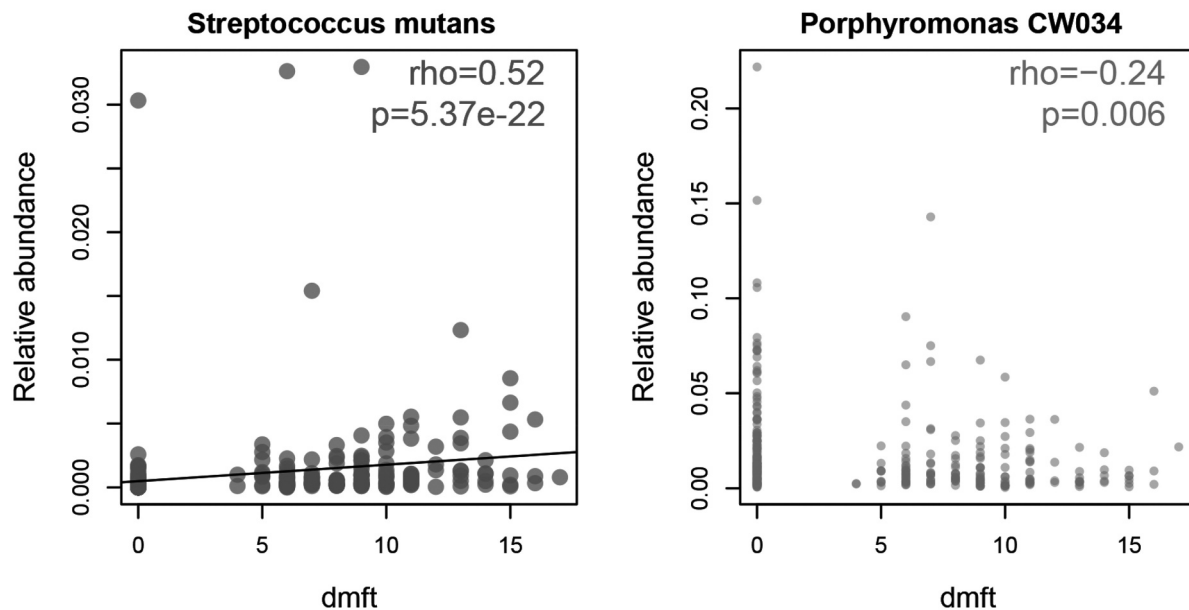## Correlations between species and the dmft index

Figure 3. Correlations between microbiome at the species level and the host's ECC severity (i.e. dmft index). In ECC, an abundance of *Streptococcus mutans* showed a positive correlation with the dmft index; while an abundance of *Porphyromonas* CW034 showed a negative correlation with the dmft index. rho: Spearman correlation coefficient.

Table 1. Comparisons of biochemical parameters between the H and C group. (**: $p < 0.01$; ***: $p < 0.001$).

Saliva components	C group Mean ± SD	H group Mean ± SD	p value (Chi-squared test)
K^+ (mg/L)	813.29 ± 224.38	748.11 ± 202.95	6.63e-4***
Cl^- (mg/L)	621.68 ± 180.91	497.26 ± 120.99	1.49e-11***
NH_4^+ (mg/L)	191.04 ± 63.38	165.65 ± 55.81	1.63e-4***
Na^+ (mg/L)	106.24 ± 68.25	79.36 ± 38.88	1.63e-3**
SO_4^{2-} (mg/L)	21.68 ± 9.15	18.60 ± 6.04	1.31e-3**
Ca^{2+} (mg/L)	14.47 ± 6.01	11.48 ± 6.26	5.68e-6***
Mg^{2+} (mg/L)	3.86 ± 2.12	3.15 ± 2 .40	4.98e-7***
Br^- (mg/L)	2.19 ± 1.52	1.47 ± 0.89	1.29e-8***
TP (mg/L)	1219.26 ± 381.93	1133.45 ± 319.02	0.24 NS
PO_4^{3-} (mg/L)	472.60 ± 173.37	454.85 ± 145.24	0.24 NS
F^- (mg/L)	33.43 ± 18.24	30.71 ± 15.01	0.11 NS
NO_2^- (mg/L)	3.00 ± 4.66	3.47 ± 4.77	0.83 NS
pH value	6.83 ± 0.19	6.88 ± 0.20	9.95e-5***
NO_3^- (mg/L)	13.41 ± 17.72	30.75 ± 66.27	2.72e-5***

TP, total protein

As for the salivary microbiome data, we trained an RF classifier based on the genus-level profile for which AUROC reached 0.70 (95% CI: 63.86%-75.15%, accuracy = 0.65; Figure 5A). The performance improvement was maximized by employing the top-32 most discriminatory genera, many of which (e.g. *Atopobium*, *Prevotella*, *Mogibacterium*, and *Neisseria*) have been reported to be associated with dental caries (Figure 5B) [46–49]. Intriguingly, as compared with the oral-microbiota-based classifier, the ECC classifier performed better based on the biochemical indices, including 12 salivary electrolytes, pH, and total protein (AUROC = 0.94, 95% CI: 91.50%-96.91%, accuracy = 0.89; Figure 5C). Consequently, 11 salivary electrolytes were selected as the optimal marker set, offering the best classification performance in the reduced model (Figure 5D). Unexpectedly, we found that the composite model only exhibited a moderate classification and did not outperform the electrolyte-based model (AUROC = 0.89, 95% CI: 85.81%-92.80%, accuracy = 0.82; Figure 5E), suggesting that the two sets of salivary features could not be complementary in predicting ECC using the RF algorithm. In the feature selection, the performance in the reduced models was optimized when only the top six most discriminatory variables were employed, all of which were derived from salivary electrolytes (i.e. Cl^-, Mg^{2+}, Na^+, NO_3^-, Br^-, NO_2^-; Figure 5F).

Discussion

Saliva is considered the most crucial host-related factor that could affect dental caries development and play a key role in maintaining children's health [50,51]. This study aimed to explore the value of both the salivary microbiome and biochemical components for ECC etiology and diagnosis. Firstly, we evaluated the salivary microbiome diversity and found that the C group exhibited a reduced microbial diversity compared to healthy children, reported in both caries children and adults [52–54]. With the development of caries, an ecological disruption of the normal, healthy bacterial community occurs
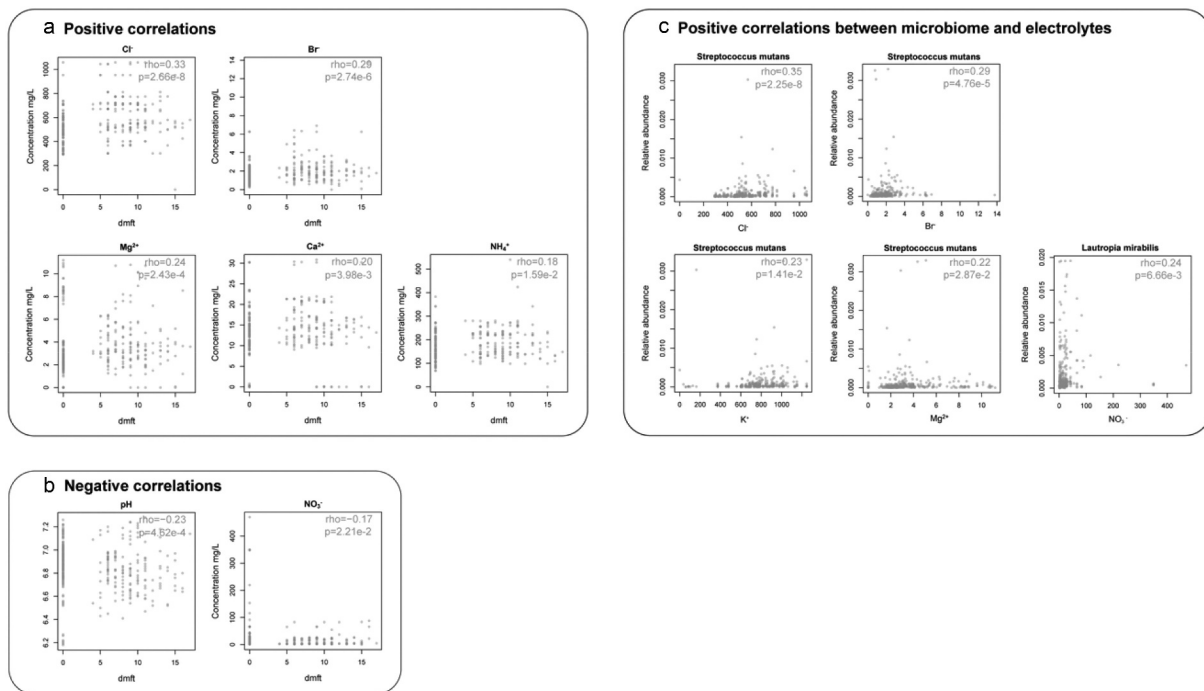
Figure 4. Correlations between biochemical components and the host's ECC severity (i.e. dmft index), as well as microbiome at the species level. Each point represented a sample. In ECC, **(A)** the concentrations of Cl^-, Br^-, Mg^{2+}, Ca^{2+}, and NH_4^+ showed positive correlations with the dmft index, while in **(B)** pH, the concentration of NO_3^- showed negative correlations with the dmft index. **(C)** Correlations between species and electrolytes were present. The relative abundance of *Streptococcus mutans* abundance was positively correlated with the levels of Cl^-, Br^-, K^+, Mg^{2+}; *Lautropia mirabilis* was positively correlated with the NO_3^- level. rho: Spearman correlation coefficient. PRINTER: Can these figures be increased to make them more readable?.

through glycolytic activity. Some dominant bacteria such as acidogenic and aciduric taxa can better adapt to the highly dynamic environment, thus resulting in the reduction of biodiversity in the oral environment. Moreover, we observed significant shifts in the saliva bacterial composition of the ECC relative to health, and identified both ECC-enriched taxa and ECC-depleted taxa. In addition to the classic cariogenic model of *S. mutans* [55,56], our study also identified a few novel bacteria positively associated with dental caries, such as *Atopobium* spp., *Prevotella* spp., and *Neisseria* spp [46,49,57,58]. *Prevotella* spp., which were consistently the main predictors of ECC reported in our previous studies [22]. They may play key roles in dentin caries progression, mainly involved in proteolysis and infiltrating the degradation of organic matrix in dentin [59]. In contrast, the ECC-depleted bacterial taxa, such as *Porphyromonas* CW034 and *S. sanguinis* may provide a protective function against dental caries [55,60]. Especially, in the *in vitro* models, *S. sanguinis* clearly exhibited an antagonistic interaction with *S. mutans* [61]. Taken together, these results indicated the global alterations in the oral microbiota accompanying the shift from health to caries and highlighted the necessity of employing differentially abundant taxa as the diagnostic and predictive markers.

Next, we characterized salivary constituents and properties, including inorganic ions (electrolytes),

organic molecules (i.e. total protein), and physico-chemical property (i.e. pH), associated with ECC. Overall, ECC-affected saliva exhibited a distinct electrolytic pattern compared to healthy ones. For example, our results confirmed that salivary pH significantly decreased in caries patients and even negatively correlated with ECC severity [34]. It has been documented that, in a caries circumstance, acid-producing and acid-resistant bacteria thrive through the glycolytic activity and culminate in demineralization events of tooth tissue after a cariogenic challenge with frequent carbohydrates [1,22]. From this perspective, it is plausible that the promotion of hydrogen ions can be developed as a qualitative and quantitative indicator of the ECC state. Moreover, the nitrate concentration in saliva decreased significantly in the caries state and negatively correlated with ECC severity. It was reported that in the caries condition, nitrate tends to be converted to nitrite by the over-abundance anaerobic and facultative anaerobic bacteria. Nitrite can be further transformed into nitrous acid, which was inherently unstable and easily decomposed to nitric oxide (NO) [62] especially in the acidic pH environment [63]. It has been suggested that an individual is in the progress of preventing cariogenic acidogenic bacteria with the ability of reducing large amounts of nitrate to nitrite [62]. Interestingly, it is widely accepted that ammonium can neutralize the acid and decrease the H^+ level of the oral the microenvironment
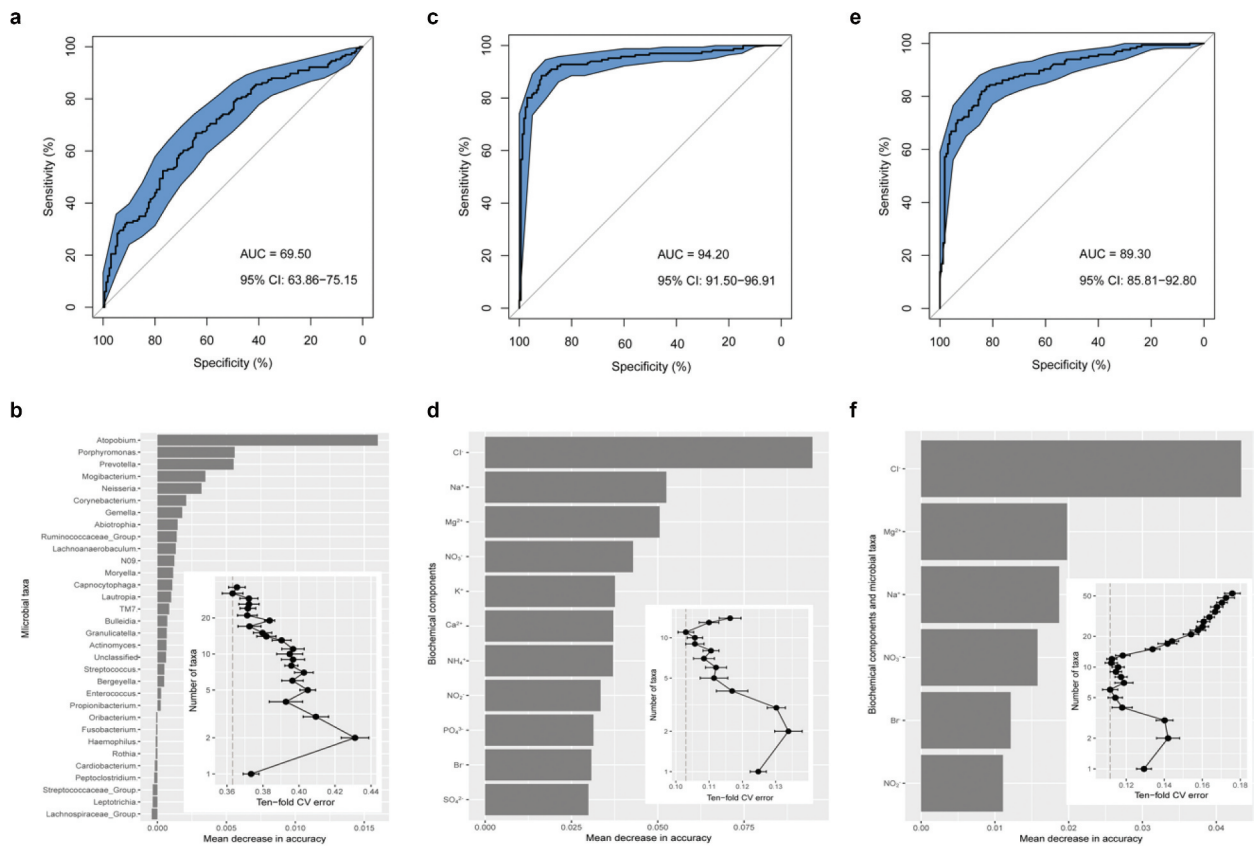
Figure 5. Disease classification based on salivary profiles. (**A**) Classification performance of Random Forest model using the microbial profile, (**C**) biochemical components, and (**E**) all of the variables of biochemical and microbial indices, assessed by the area under the receiver operating a characteristic curve with the distribution of sensitivity and specificity. (**B**) Microbial taxa, (**D**) biochemical components, and (**F**) all profiles of salivary indices with most important were ranked in descending order by their discriminating power to the accuracy of the model. The bar length at each row indicated the relative contribution of the taxa to the RF model. The inset of (**B**), (**D**) and (**F**) showed the relationship between the numbers of variables included in the model based on the saliva indices and the corresponding model performance. PRINTER: Can Figure B, D and F be increased to make them more readable?.

to promote remineralization processes [64]. As a result of a compensatory response, we found that caries patients had an enrichment in the electrolytic concentration of NH_4^+ compared to healthy controls, which was consistent with the relevant research [65].

Saliva acts as a transporter of many ions, such as calcium, phosphate and fluoride, which are essential for the promotion of remineralization. At a normal pH, the calcium and phosphate in saliva are supersaturated, so demineralization will not occur. Acids of bacterial origin and acids in food or beverages tend to shift the balance towards mineral loss [28]. On the one hand, the acid-induced demineralization of enamel can promote a more significant mobilization of saliva electrolytes due to the increased mineral dissolution and the release of inorganic elements (e.g. Na^+, Ca^{2+}, K^+, Mg^{2+}, and Cl^-) [66,67]. On the other hand, saliva is also considered as a buffering pool that could shift the dynamic equilibrium between demineralization and remineralization for controlling the dental caries activity [68–70]. Inorganic components in saliva can participate in the remineralization of the apatite crystal

structure, which directly contributes to the maintenance of enamel integrity and mitigating the damage caused by acid-producing bacteria [67,71]. Ion substitutions that may occur in bioapatite include substitution of magnesium and sodium for calcium, substitution of fluorides and chlorides for hydroxyl sites, and carbonates for phosphate and hydroxyl sites [28]. These biochemical processes can raise an environmental supersaturation of electrolytes (e.g. calcium and fluoride) around and inside the biofilm structure of the tooth surface [71], which can be highly indicative of the oral health status. Collectively, identification of these biochemical markers may permit a more refined understanding of the caries pathogenesis and clarify significant etiologic factors in the shift from health to disease.

Finally, we assessed the predictive power of the salivary electrolytes, pH and total protein as well as oral microbiota for discriminating ECC status using machine-learning analyses. Similar to our recent studies [22], the machine-learning model based on saliva bacterial profiles can classify disease states with

a moderate performance. Interestingly, the predictive power of disease classifier based on salivary electrolytes outperformed that based on the oral microbiota. The seemingly unexpected findings provide novel insights into developing ECC diagnostic tools using saliva samples. (*i*) The underexplored host variables may confound the oral microbiome association with disease and limited its predictive power in discriminating the disease status in a relatively large study. Despite the great potentials in predictive modeling of human diseases, studies also clearly demonstrated that many under-sampled factors (e.g. individual's genetics, diet, lifestyle, physiological state, etc.) can contribute to the variation of the oral microbiome among individuals [72,73]. Among these, oral disease states can only explain less than 10% of the variation in the oral microbiome. Thus, how to detrend the effect of other confounding factors on saliva microbiota for developing microbiome-based diagnostic and predictive tools for dental caries remains particularly challenging and merits further investigation [74–76]. (*ii*) Salivary electrolytes and pH can be more sensitive to the physiological changes on the tooth surfaces than oral microbiota. ECC is essentially an outcome of the continuous demineralization of the crystalline mineral structure of the dental hard issues [77,78]. From this perspective, saliva electrolytes should be highly indicative of the speed and direction of the cariogenic pathways in the disease development (i.e. de- and re-mineralization dynamic process) [78,79]. Moreover, electrolytes are more stable in salivary fluid and relate to the ultimate products of complex metabolic interactions between host and microbiome [80]. From the perspective of clinical practice, the electrolyte-based diagnostic model should merit more attention in the future study. Finally, considering the simplicity, efficiency and cost-effectiveness of a diagnostic or prediction tool of ECC in the clinical practice, oral microbiome profiling still have apparent limitations. For example, it heavily relies on DNA sequencing, which is subject to time-consuming and cost issues. These limitations hamper its broad applications to the on-site disease diagnosis or prediction in the clinical settings. In contrast, the ECC diagnostic model based on salivary biochemical indices is a more patient- and doctor-friendly method and holds the potential to be developed as a remote and automated approach [81].

However, there are also limitations that should be emphasized. First, we only analyzed saliva samples, but we have no idea of plaque samples, which could provide different results. Second, we compared pH values, total protein, and some specific electrolytes, but the metabolites were not measured. Third, functional information of the microbiome was unrevealed. Lastly, our research is a preliminary exploratory experiment to understand the ability of saliva biochemical indicators and microbiome to distinguish caries and health and provide ideas and new biomarkers for risk assessment. In the subsequent work, we will conduct follow-up studies including more indices and following up the survey to evaluate the predictive power of these markers on caries.

In summary, our study showed that the microbial abundance and electrolytic concentration differed between ECC and healthy individuals and these biomarkers also exhibited strong correlations with disease severity. The ECC diagnostic models were established and compared based on the quantitative profiles of saliva microbial and biochemical compositions. Saliva samples are host-friendly and straightforward for monitoring and evaluating caries risk regularly and effectively. The classifier based on biochemical features outperformed the salivary microbiomes-based and composite-based one (both microbial and biochemical data) in predicting the ECC status. The salivary electrolyte levels can reflect the balance of demineralization and remineralization in the micro-environment that is positively associated with the onset and progression of dental caries, thus providing an essential proxy for developing a better diagnostic model.

Abbreviations

AUROC: area under the receiver operating characteristic curve; Br^-: bromine; Ca^{2+}: calcium; Cl^-: chlorine; dmft: the number of decayed, missing, and filled teeth in the deciduous dentition; ECC: Early childhood caries; ECM: electronic caries monitor; F^-: fluoride; ICDAS: International Caries Detection and Assessment System; K^+: potassium; Mg^{2+}: magnesium; Na^+: sodium; NH_4^+: ammonium salt; NO_2^-: nitrite; NO_3^-: nitrate; OTU: operational taxonomic unit; PERMANOVA: permutational multivariate analysis of variance; PO_4^{3-}: phosphate; PTR-LUM: photothermal radiometry and modulated luminescence; RF: Random Forests; SO_4^{2-}: sulfate; TP: total protein.

Contributions

YZ, SH, SJ, FT, and FY conceived and designed the research, YZ, SJ, SL, LZ, and FT collected samples. SL, FL, LZ, JL and KT prepared samples. YZ, SH and SJ carried out the experiments with the supervision of ZS, SL, FL, LZ, JL, and KT. SH, YZ and ZS analyzed data and drew figures. YZ, SH, FT and FY wrote the paper. All authors have reviewed and approved the final version of the manuscript.

Disclosure statement

Funding

ORCID

Shi Huang ⓘ http://orcid.org/0000-0002-7529-2269
Zheng Sun ⓘ http://orcid.org/0000-0002-8537-8576
Fang Yang ⓘ http://orcid.org/0000-0001-8639-5856

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