Polymicrobial detection and salivary metabolomics of children with early childhood caries

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Abstract

Background: Early childhood caries (ECC) has been proposed to be associated with various microorganisms and metabolites. This study aims to compare the prevalence of specific microbial species and salivary metabolomics profile in children with and without ECC, and to explore the correlation between salivary metabolites and targeted microbes.

Method: Five-ml of unstimulated saliva was collected from 32 ECC and 22 caries-free children. Clinical indexed were recorded and questionnaires regarding oral health and dietary habits were obtained from the guardians. Presence of 8 specific microbial species were examined using species-specific quantitative PCR (qPCR)qPCR. Untargeted metabolomics was analysed to identify key differential metabolites and pathways. Correlations among clinical, microbial, and metabolomic data were further explored.

Results: The prevalence of *Scardovia wiggsiae* (90.6%, *P* <0.001), *Streptococcus mutans* (43.8%, *P* =0.006), *Streptococcus sobrinus* (62.5%, *P* <0.001), *Ligilactobacillus salivarius* (93.6%, *P* =0.01) and *Candida albicans* (56.3%, *P* <0.001) were significantly higher in ECC group. Prevalence of ECC was higher in children with two targeted species present compared with children with one targeted species. Histidine metabolism and branched-chain amino acids degradation were activated in ECC group, while glyoxylate and dicarboxylate metabolism, purine and pyrimidine metabolism were inhibited. Histidine and glutathione metabolism was activated with enrichment of targeted microbial species, while linoleic acid metabolism and biotin metabolism was inhibited. The duration of each toothbrushing was a significant risk factor for ECC experience.

Conclusion: The prevalence of *Scardovia wiggsiae, Streptococcus mutans, Streptococcus sobrinus* and *Candida albicans* is higher in ECC children compared to caries-free children. Oral habits and salivary metabolites also vary between ECC and caries-free children.ECC is potentially associated with specific microorganisms. Co-detection of two targeted microbial species indicates higher possibility of caries. Oral habits and salivary metabolic activities vary between ECC and caries-free children. Selected microbes can well present the salivary metabolic status of ECC.

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Introduction

Dental caries remains one of the most common chronic disease of the human oral cavity and can occur at any age. Children with deciduous teeth are more susceptible to caries because of the risk factors including higher organic content of primary teeth, higher frequency of sugary intake, and relatively poor maintenance of personal oral hygiene. Children under 6 years of age who have one or more caries (cavities or non-cavities), missing (due to caries), or filled surfaces in any deciduous tooth are referred to as early childhood caries (ECC) (Deng, Zhang & Zou, 2020). ECC is a multifactorial disease influenced by several factors, including diet, oral hygiene practices, the oral microbiome, and genetic predispositions (Reisine & Douglass, 1998; Park & Choi, 2022; de Jesus et al., 2022; Kateeb et al., 2023). (Pattanaporn et al., 2013)(Felsypremila et al., 2024) Socioeconomic factors such as place of residence and mode of delivery have also been associated with ECC experiences (Khan et al., 2024). Infants born vaginally had significantly higher intraoral colonization of Streptococcus mutans compared to those born by caesarean section (Pattanaporn et al., 2013). A cross-sectional study found that children delivered by caesarean section have a higher caries risk compared to those delivered vaginally (Felsypremila et al., 2024). The results of the third National Dental Epidemiologic Survey in China showed that the caries prevalence among 3-year-olds and 5-year-olds reached 34.5% and 71.9%, respectively (DU et al., 2018). The etiology of caries has confirmed that microorganisms play an important role in the initiation and progression of caries. Examination of supragingival plaques from preschool-age children revealed that *Streptococcus mutans*, *Selenomonas sputigena*, *Pseudomonas salivae*, and *Lactobacillus wadei* were significantly associated with ECC (Cho et al., 2023). A large number of previous studies have focused on a classical cariogenic bacterium, *Streptococcus mutans* (*S. mutans*) (Krzyściak et al., 2014). However, it has been found that *S. mutans* cannot be isolated from some children with ECC (Tanner et al., 2011). Moreover, topical application of fluoride, a commonly accepted caries-preventive agent, is less effective in preventing caries in children, especially children with high caries risks (Manchanda et al., 2023). With the development of microbiology and the diversification of detection methods for oral microorganisms, a variety of other bacteria have been found to be potentially correlated with ECC, including but not limited to *Scardovia wiggsiae* (*S. wiggsiae*), *Streptococcus salivarius* (*S. salivarius*), *Streptococcus sobrinus* (*S. sobrinus*), *Streptococcus parasanguinis* (*S. parasanguinis*), *Ligilactobacillus salivarius* (*L. salivarius*), *Veillonella parvula* (*V. parvula*) and *Candida albicans* (*C. albicans*) (Tanner et al., 2011; Xiao et al., 2018; Zhang, Chu & Yu, 2022). An investigation of these novel caries-associated microorganisms in Chinese ECC children may help understandexplain the high prevalence in the population and contribute to targeted prevention and treatment strategies. However, there have been no reports of prevalence of these novel caries-associated microorganisms in the Chinese ECC community (Xiao et al., 2019).

More and more researchers have come to realize the importance of taking host-microbe interaction into consideration when exploring oral infectious diseases (Lamont, Koo & Hajishengallis, 2018). Metabolites in saliva, which is the key environment where oral bacteria / fungi meet host cells, have been analyzed in several studies on adult caries. Several biomarkers, including salivary mucins, glycoproteins (sCD14), interleukins (IL-2RA, 4,-13), urease, carbonic anhydrase VI, and urea, has been associated with adult caries (Antonelli et al., 2024). A previous study on carbohydrate metabolites of adolescents with caries found significantly different clustering of organic acids in caries-active subjects (Havsed et al., 2021). Histamine, L-histidine and succinate emerges are considered to be the major salivary metabolites associated with caries status, reflecting significant alterations in metabolic pathways in caries-active children (Li et al.,

2023b)-. In addition, salivary metabolites were significantly altered in children with dental caries studied, including amino acid metabolism, pyrimidine metabolism, purine metabolism (Li et al., 2023a). The application and development of untargeted metabolomic technologies, represented by Liquid Chromatograph Mass Spectrometer (LC-MS), allows for detection of metabolites involved in carbohydrate metabolism, amino acid metabolism, lipid metabolism and many other metabolic pathways. A metabolomic study on children with ECC would benefit our understanding of this disease and help identify potential biomarkers for diagnostic and risk prediction purposes.

Thus, the aim of the present study was to explore the prevalence of several targeted microbial species and differential salivary metabolites in children with and without ECC children in Nanjing District of China. Additionally, the study seeks to explore the correlation between salivary metabolites and targeted microorganisms associated with ECC. and identify metabolites and metabolomic pathways potentially associated with ECC. The hypothesis was that novel microbial species other than *S. mutans* would be more prevalent in saliva of ECC children compared to caries-free children. Salivary metabolic activities as well as behavioural risk factors were also hypothesized to differ in ECC and caries-free children.

Materials & Methods

Study population and Sample collection

The study is a case-control observational study. Subjects were selected from patients who came to the department of pediatric dentistry, Nanjing Stomatological Hospital (Ethical approval: NJSH-2023NL-020-1). A total of 32 children with ECC and 22 caries-free (CF) children were included in this study. All the study participants were required to provide informed consent from the parent or guardian of the children. Additionally, for children who were developmentally capable of understanding the nature of the study, assent was sought in accordance with ethical guidelines for pediatric research. Inclusion criteria for this study: 1) Aged 6 years or below; 2) No antibiotics were used for 3 months systemic disease; 3) Consent of the parent or guardian to the child's clinical examination and microbiological sampling. No antibiotics were used for 3 months; 4) Consent of the parent or guardian to the child's clinical examination and microbiological sampling. Exclusion criteria: 1) children who had systemic disease or congenital disease; 2) children unable to cooperate with sampling or refuse to sample; 3) Bacteria or severe infections in other parts of the body. 1) Above 6 years old; 2) children who had systemic diseases; 3) Antibiotics were used in the past 3 month; 4) Any parent or guardian declined to participate. Two examiners with at least 5 years of experience in the department of pediatric dentistry performed dental examinations and dmft (decay, missing, filling teeth) index according to the World Health Organization (WHO) criteria were recorded (World Health Organization, 2013). Examinaer realiability was determined using the weighted kappa coefficient based on images of tooth teeth with or without caries. The mean kappa value was 0.73. Subjects were then divided into the CF group (dmft = 0) and ECC group (dmft =13.16 ± 3.39). The salivary collection time was between 7:00 and 8:00 AM, with no brushing of teeth on the evening before and the morning of the collection.

All participants were provided with a sterile saliva collection tube, and were instructed to spit 5 mL whole unstimulated saliva. Collected saliva were immediately placed on ice and transferred to laboratory. Saliva was centrifuged at 4˚C for 10 min and the pellets and supernatant were separately stored at −80 ◦C. The pellets were used for DNA extraction, while the supernatant was used for untargeted metabolomic analysis (Zhang et al., 2016).

Meanwhile, the questionnaires were completed by the parents or guardians. The questionnaire used in our study was designed and modified based on the questionnaire used in the national oral health survey (DU et al., 2018). The questionnaire from one child in the caries-free group was excluded in later statistical analysis due to poor quality. General and oral status-associated information was collected: (1) General general information of the children; (2) mother-related factors: a) mode of delivery; b) feeding patterns within 6 months of birth; c) whether mother has caries experience; (3) oral health habits: a) age of starting to brush teeth; b) whether brush teeth everyday; c) frequency of brushing everyday; d) duration of each brushing; e) use of fluoride toothpaste; (4) dietary habits: a) frequency of sugary drinks or food; b) frequency of eating / drinking before bedtime.

DNA extraction and quantitative PCR (qPCR)

Genomic DNA from the saliva was extracted by DNA extraction kits (TIAamp Bacteria DNA Kit, TIANGEN, China). DNA concentration and purity was examined using Nano-Drop spectrophotometer using A260/A280 (Nano-Drop one/one^c, Thermo Fisher Scientific, USA). The specific primers used for qPCR are detailed in Table 1. Universal 16s rDNA was used as positive control. A 10-µL reaction protocol was set up for this qPCR screening which consisted of 5 µL ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China), 0.5 µL forward and reverse primers and 4 µL template DNA, performed in triplicate.. Processing was performed using pre-denaturation at 95℃ for 15 min, followed by 50 cycles denaturation (95℃ for 15 s and 60℃ for 30s). The last step was melting curve analysis at 95℃ for 15 s, 65℃ for 60s and 95℃ for 15 s (Huo et al., 2022).

Untargeted metabolomics

LC-MS analysis was used to perform untargeted metabolomics on supernatant of saliva which was collected from ECC and CF children. Two mobile phase condition was used to improve the metabolite coverage, including both positive ion mode and negative ion mode. Detection and identification of metabolites were performed using MS-dial (ver.5.1.230912) by searching against online database (MoNA, GNPS, HMDB and MS-dial database) and in-house database. Untargeted metabolomic analysis was performed at Cosmos Wisdom company (Hangzhou, China). Detailed methodology is described in the attachment.

Statistical analysis

Clinical data Analysis

Children with missing data were excluded from the analysis. The normality of the data was tested through the Shapiro-Wilk test. Nonparametric test was used for continuous data of skew distribution. Demographic variables were summarized and descriptive statistics were reported.

176   The Chi-square test was used to analze variables including gender, mode of delivery, whether
177 the mother has caries experience, age of starting to brush teeth, whether brushing teeth daily,
178 frequency of daily brushing, use of fluoride toothpaste and frequency of sugary food or drink
179 intake. Fisher's exact test was used to analyze variables including feeding patterns within 6
180 months of birth, duration of brushing and whether intake of foods before bedtime after brushing.
181 ~~or Fisher's exact test was used to compare the categorical variables and the detection of bacteria~~
182 ~~between two groups.~~ Only the variables that showed significant relation to dental caries
183 experience were studied by multiple logistic regression analysis. All conclusions were drawn
184 considering the level of significance of 5%. Data analysis was performed using Statistical
185 Package for Social Science version 29.0 (SPSS Inc., Chicago, IL, USA).
186

187 *Bioinformatics Analysis*
188 R software package was employed to test for significant differences between two groups of
189 samples. T-test, Variable Importance in Projection (VIP) obtained from the Orthogonal partial
190 least squares-discriminant analysis (OPLS-DA) model (biological replicates ≥ 3) and the P /FDR
191 (biological replicates ≥ 2) or fold change (FC) values from univariate were used together to
192 screen differential metabolites between two groups. Generally, variables meeting both criteria of
193 $P < 0.05$ and VIP > 1.0 were considered as differential metabolites. The Fisher's exact test was
194 used to find out whether the differentially expressed metabolites had significant enrichment
195 trends in specific functional types. The differential metabolites identified in the previous step
196 were then subjected to Kyoto Encyclopedia of Genes and Genome (KEGG) pathway. Metabolite
197 Set Enrichment Analysis (MSEA) was performed based on all identified metabolites and KEGG
198 pathway databases using the R package corto (version 1.2.4). Based on the MSEA enrichment
199 results, we screened for significantly enriched pathways and differential metabolites in the
200 pathways by setting a p value threshold ($P < 0.05$) and mapped the metabolic regulatory network
201 using the R package graph (version 2.1.0). Spearman's correlation analysis as well as KEGG
202 pathway enrichment analysis was performed to analyze the correlation between delta Cq
203 ($Cq_{species}$-$Cq_{universal\ 16s}$) of targeted species and metabolites to screen for significantly up-regulated
204 and down-regulated metabolites associated with the targeted microbes.
205

206 **Results**

207 **General and clinical characterization**

208 A total of 53 children were included in the study. General and clinical informational data of the
209 subjects are shown in ~~the~~ Table 2. Age and gender were not statistically different between the CF
210 and ECC groups.

211 **Questionnaire results**

212 Notably, age of starting to brush teeth, whether brush teeth everyday, frequency of brushing,
213 duration of each brushing, use of fluoride toothpaste and frequency of sugary drinks or food were
214 significantly different in CF and ECC groups (Table 3).

215 The significant variables that were incorporated into the multiple logistic regression model are as
216 follows: age of starting to brush teeth, brushing teeth every day, frequency of brushing, duration
217 of each brushing, use of fluoride toothpaste, and frequency of sugary drinks or food. ~~The~~
218 ~~significant variables were included in the multiple logistic regression model.~~ The
219 multicollinearity was first determined by testing the tolerance and the variance inflation factor
220 (VIF) (Table 4). Factors with tolerance greater than 0.1 or ~~VIP~~ VIF less than 10 were further
221 examined in the multiple logistic regression analysis. As shown in Table 5, the duration of each
222 brushing was a significant risk factor associated with ECC experience. ~~Brushing teeth for more~~
223 ~~than 3 minutes can significantly reduce the risk of caries.~~
224

Detection and co-detection of specific microbial species

226 The detection rate of specific microbial species was shown in ~~the~~ Table 6. The prevalence of *S.*
227 *wiggsiae* ($P < 0.001$), *S. mutans* ($P = 0.006$), *S. sobrinus* ($P < 0.001$), *L. salivarius* ($P = 0.01$) and
228 *C. albicans* ($P < 0.001$) were significantly higher in the ECC group when compared to CF.
229 Co-detection of *S. wiggsiae*, *S. mutans*, *S. sobrinus*, *L.salivarius* and *C. albicans* in pooled and
230 groupwise samples is shown in Fig. 1. All children with presence of the combination of *C.*
231 *albicans* and *S. mutans* or *C. albicans* and *S. sobrinus* had ECC (Fig. 1A). Prevalence of ECC
232 was higher in children with two targeted species present compared to that in children with only
233 one targeted species present. Co-detection was more prevalent in ECC group compared with CF
234 group (Fig. 1B).
235 ~~Positive detection of *S. wiggsiae*, *S. mutans*, *S. sobrinus*, *L.salivarius* and *C. albicans* and of their~~
236 ~~combinations in saliva samples from CF and ECC children is shown in Figure 1. All children~~
237 ~~with presence of the combination of *C. albicans* and *S. mutans* or *C. albicans* and *S. sobrinus*~~
238 ~~had ECC. Prevalence of ECC was higher in children with two targeted species present compared~~
239 ~~to that in children with only one targeted species present.~~
240

Metabolomic results

242 In the present study, a total of 442 metabolites were detected by untargeted metabolomics.
243 Differential metabolites between ECC group and CF group are shown in Figure 2. A total of 132
244 differential metabolites were identified, including 97 up-regulated metabolites and 35 down-
245 regulated metabolites. The top 10 up/down-regulated differential metabolites are shown in Table
246 7 and Table 8 respectively.
247 KEGG enrichment analysis and MSEA were performed to determine functional pathways and
248 metabolite sets with major differences between two groups. Figure 3 showed results from KEGG
249 enrichment analysis. Nitrogen metabolism was mostly enriched. D-amino acid metabolism and
250 pyrimidine metabolism contains the most numbers of differential metabolites. MSEA revealed
251 that 8 metabolite sets were significantly different between ECC and CF groups (Fig. 4),
252 including caffeine metabolism, glyoxylate and dicarboxylate metabolism, citrate cycle (TCA
253 cycle), pyrimidine metabolism, histidine metabolism, valine, leucine and isoleucine degradation,
254 pyruvate metabolism and purine metabolism.
255 Based on the MSEA enrichment results, we screened the pathways with $P < 0.05$ and the
256 different metabolites in the pathway to construct the metabolic regulatory network (Fig. 5).
257 Histidine metabolism and valine, leucine and isoleucine degradation were activated in the ECC

258 group. Conversely, glyoxylate and dicarboxylate metabolism, purine metabolism and pyrimidine
259 metabolism were inhibited in saliva of children with ECC.
260

261 **Correlation between microorganisms and metabolomics**

262 Based on the results of the correlation analysis between the Cq ($Cq_{species}$-$Cq_{universal\ 16s}$) values of
263 the targeted specific microbial species and the metabolites, we screened the metabolites with
264 significant correlation with the microbial species content and performed KEGG pathway
265 analysis. Histidine metabolism and glutathione metabolism was activated with enrichment of
266 targeted microbial species, while linoleic acid metabolism and biotin metabolism was inhibited
267 (Fig. 6).
268

269 # Discussion

270 Results from this current study about the detection of multiple acid-producing microorganisms
271 are consistent with the polymicrobial etiology of dental caries suggested by a number of
272 researchers (Simón-Soro & Mira, 2015; Lamont, Koo & Hajishengallis, 2018; Shao et al., 2023).
273 The dominant species in early childhood caries seems to differ from that in adolescent and adult
274 caries. While the detection of *Scardovia wiggsiae* has been reported in children with ECC from
275 different populations (Tanner et al., 2011; Simón-Soro & Mira, 2015; Chandna et al., 2018;
276 Lamont, Koo & Hajishengallis, 2018; McDaniel et al., 2021; Tantikalchan & Mitrakul, 2022;
277 Shao et al., 2023), the extremely high prevalence of this species in Chinese ECC children
278 (90.6%, Table 6) is firstly reported and suggests that it may play a role in population-specific
279 caries etiology. Notably, *Scardovis wiggsiae* has been found to be more tolerant to fluoride
280 inhibition when compared to *S. mutans* (Kameda et al., 2020), indicating a poorer effect of
281 fluoride prevention on dental caries with *Scardovia wiggsiae* being dominant in the oral cavity.
282 While there is no water fluoridation in China, high concentrations of fluoride in groundwater
283 have been reported in different regions of China (Cao et al., 2022; Huang et al., 2023). It can be
284 hypothesized that the predominance of *Scardovia wiggsiae* could be a result of natural selection.
285 This predominance could also partly explain the relatively compromised protective effect of
286 topical fluoride, which is regularly applied to pre-school children in most areas of China.
287 Except for *Scardovia wiggsiae*, other microbial species including *S. mutans*, *Streptococcus*
288 *sobrinus*, *Ligilactobacillus salivarius* and *Candida albicans* were also found to be more
289 prevalent in children with ECC. Among them, *S. mutans* and *S. sobrinus* has been long studied as
290 main pathogens of dental caries and have synergistic effect (Rupf et al., 2006; Li, Wyllie &
291 Jensen, 2021). High prevalence of *Candida albicans* in ECC has also been reported in numerous
292 studies (De Carvalho et al., 2006; Xiao et al., 2018; Sridhar et al., 2020). The co-existence could
293 greatly promote metabolic activities and biofilm formation of *Candida* and bacteria through
294 direct interaction and indirect signal molecules, thus facilitating caries progression (Xiao et al.,
295 2018). In our study, we also noticed a notably higher prevalence of caries when two targeted
296 species were detected in salivary samples, indicating synergistic interactions between different
297 microbes (Fig. 1). Future studies could explore the potential physical / chemical / metabolic
298 interactions and synergies between these targeted species as this may provide deeper insights
299 into their role in caries development. The high detection rate of *Ligilactobacillus salivarius* in

children with ECC was somewhat surprising as this species has long been regarded as a probiotic which can inhibit biofilm formation of *S. mutans* and *Candida albicans* (Krzyściak et al., 2017; Wasfi et al., 2018). Caries incidence and prevalence was reported to be decreased after a short-term intervention with *Ligilactobacillus salivarius* probiotic (Staszczyk et al., 2022). However, controversial results were also reported by other researchers, who found higher level of *Ligilactobacillus salivarius* in adolescents with dental caries (Shimada et al., 2015). One explanation for this controversy may due to the genomic diversity of this species and the variety of strains existed in nature (Neville & O'Toole, 2010; Raftis et al., 2011). More and more researchers have come to realize that virulence of caries-associated bacteria could be strain-specific (Tanner et al., 2018; Al-Hebshi et al., 2019). A previous study identified two significantly different groups of *Ligilactobacillus salivarius* based on randomly amplified polymorphic DNA fingerprinting (Švec, Kukletová & Sedláček, 2010), implicating potentially different metabolic phenotypes. Strain-based study is required to further unveil the cariogenic strains of *Ligilactobacillus salivarius* identified in our current study.

Metabolomic analysis revealed 132 differential metabolites in two groups of children, indicating significantly different salivary status due to microbial activity and host conditions. Amino acid metabolism, especially histidine metabolism, was found to be activated in ECC group (Fig. 5), which is consistent with a previous metabolomic study in children with mixed-dentition (Li et al., 2023b).Interestingly, when analyzing metabolomic data based on enrichment of the targeted microbial species, activation of histidine metabolism was also noticed in samples with enriched targeted species (Fig. 6), indicating that microbes selected in the current study can well represent the metabolic status of the ECC oral microcosm. Histidine is an essential amino acid in the human body and plays a central role in metabolism of histidine-containing proteins (Moro et al., 2020). Previous studies have emphasized the significance of salivary histidine-rich proteins, represented by histatin 5, which are important components of the non-specific immune system in the oral cavity (Jurczak et al., 2015; Munther, 2020). Antimicrobial peptides derived from histatin 5 have been proved to impose killing effect on oral pathogens, including candida and streptococci (Stewart et al., 2023; Skog et al., 2023). Besides, histatin 5 can inhibit the aggregation of *S. mutans* with other microbiota and thus biofilm formation (Huo et al., 2011; Krzyściak et al., 2015). In this current study, we detected activated histidine metabolism and higher levels of histamine in ECC group, which is a hydrolytic product of histidine. This result could be associated with lower histatin 5 levels in children with ECC found in other studies (Jurczak et al., 2015; Munther, 2020). Based on the current result, supplement of histidine, especially histatin 5, should be suggested for children with ECC to improve the host defense against cariogenic microorganisms.

This study also addresses distinct activities of purine and pyrimidine metabolism in children with ECC, correlating with several previous reports which suggested use of purine and pyrimidine-related metabolites as caries biomarkers (He et al., 2018; Li et al., 2023b). The role of purine and pyrimidine metabolism in caries etiology is still unknown as it has not been found until recent years due to advances in sequencing and metabolomic technologies. Purine and pyrimidine metabolism has been associated with biological interconversion of DNA, RNA, lipids and carbohydrates (Garavito, Narváez-Ortiz & Zimmermann, 2015). Pathogens could manipulate pyridine metabolism in host cells to generate an optimal host cellular environment for them to proliferate (Garavito, Narváez-Ortiz & Zimmermann, 2015) . Interestingly, a recent study also reported decreased purine and pyrimidine synthesis in a periodontitis microbial model and the decrease has been hypothesized to be associated with the environmental oxygen gradient caused

> **Commented [CC2]:** Given the noted associations between metabolites and specific bacteria with biofilm formation, the authors may want to comment on the lack of biofilm measures in the current study and perhaps recommend inclusion in future studies confirming impacts on biofilm.

346 by bacteria itself (Yamaguchi-Kuroda et al., 2023). The recurrent discovery of purine and
347 pyrimidine metabolism change in oral infectious diseases is worth deeper explorations.
348 The inhibition of glyoxylate and dicarboxylate metabolism in children with ECC is novel in this
349 study. Glyoxylate and dicarboxylate metabolism is well conserved in the entire biosphere and
350 serves as important bypass of the citric acid cycle (TCA) (Dolan & Welch, 2018). The central
351 metabolite, glyoxylate, is closely associated with glyoxylate shunt which is involved in oxidative
352 stress, antibiotic stress and host infection in various pathogenic microorganisms (Xu et al.,
353 2022). Also, the use of glyoxylate metabolism indicates the ability of the microorganisms to take
354 advantage of carbon sources such as acetate, fatty acids, or ketogenic amino acids (Dolan &
355 Welch, 2018). Inhibition of glyoxylate and dicarboxylate metabolism may lead to compromised
356 growth and metabolic potential and competitiveness of the microorganisms in the host
357 environment. Studies about glyoxylate and dicarboxylate metabolism in oral microbes are still
358 rare, suggesting future efforts to understand its role in pathogenesis of caries, especially in ECC.
359
360 The current study also included behavioral risk factors of ECC. Factors including age of starting
361 to brush teeth, frequency of brushing, duration of brushing, use of fluoride toothpaste and
362 frequency of sugary intake significantly varied between ECC and CF group, emphasizing the
363 importance of introducing oral health knowledge and habits to parents (SUN, ZHANG & ZHOU,
364 2017; Manchanda et al., 2023). Among the studied factors, brushing teeth for more than 3
365 minutes can significantly reduce the risk of caries~~longer toothbrushing time showed protective~~
366 ~~effect against caries~~ (Table 5). Very limited number of studies looked into toothbrushing time in
367 children. This issue may reflect the fact that while parents have been told to brush teeth for their
368 children, more specific toothbrushing techniques have not been taught. A previous study on oral
369 health knowledge, attitudes and behavior of parents of preschool children found that parents have
370 different habits and opinions about how to help their children brush their teeth (Naidu & Nunn,
371 2020). In order to enhance the caries preventive effect, more community toothbrushing programs
372 should be addressed to teach parents or guardians to correctly supervise toothbrushing.
373 This current study locates novel microbial species and salivary metabolites associated with ECC
374 in China, which can be further explored to better understand the disease mechanism and develop
375 targeted preventive agent. Due to the limited sample size, other confounding factors including
376 socioeconomic status and genetics are not strictly controlled in this current study. Also, the
377 association found between ECC and microbial / metabolic factors does not gurantee a causal
378 relationship, which should be noted when interpreting the data.
379

Conclusions

381 In conclusion, this current study found higher prevalence of *Scardovia wiggsiae, Streptococcus*
382 *mutans, Streptococcus sobrinus* and *Candida albicans* in ECC children compared to caries-free
383 children. Co-detection of two targeted microbial species may indicate higher possibility of caries
384 in children. Oral habits and salivary metabolites also vary between ECC and caries-free children.
385 ~~In conclusion, this current study found high prevalence of microbial species other than classical~~
386 ~~*S. mutans* in ECC children. Co-detection of two targeted microbial species may indicate higher~~
387 ~~possibility of caries in children. Oral habits and salivary metabolic activities vary between ECC~~
388 ~~and caries-free children. Selected microbes can well present the metabolic status of ECC oral~~
389 ~~microcosm.~~ ==Metabolomic pathways such as histidine metabolism, purine and pyrimidine==

390 metabolism and glyoxylate and dicarboxylate metabolism may reflect host-microbe interaction in
391 ECC status. Key metabolites may be further identified to predict caries risks in children.
392

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