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龋病儿童唾液菌群动态变化及功能分析*

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【摘要】 目的 观察龋病和无龋儿童唾液菌群的动态变化，同时对二者口腔微生态在糖代谢及多种氨基酸合成转运过程中的功能差异进行分析。**方法** 采用宏基因组学的方法分别对10例无龋儿童和10例龋病儿童唾液菌群进行组成及功能分析，6个月后，进一步利用PacBio SMRT测序技术，分析两组儿童口腔菌群变化，探索疾病和健康状态下，口腔菌群变化规律，从组成到功能对儿童口腔微生态进行全面解读。**结果** 随时间推移，两组儿童口腔微生态组成发生显著改变，在门水平上，菌群变化趋势一致，*Firmicutes*比例增加，而*Actinobacteria*和*Bacteroidetes*比例降低。在属水平上，两组儿童唾液菌群的变化出现差异，其中，*Lactobacillus*、*Methylobacterium*和*Megasphaera*在龋病组儿童中的丰度呈上升趋势，而在无龋组儿童中呈下降趋势。在种水平上，隶属于乳杆菌属的*L. fermentum*、*L. gasseri*、*L.oris*、*S. downei*等菌株在龋病儿童中呈上升趋势，但在无龋儿童中始终处于较低水平。*S. gordonii*以及*S. mutans*在龋病儿童中有一定程度下降，但在无龋儿童中始终处于低水平。*S. mutans*和*C. gracilis*等菌种与龋失补牙数（decayed, missing and filled teeth, dmft）呈正相关，而*N. flavescens*与dmft呈负相关。三羧酸循环关键节点相关基因，*gltA*、*icd*以及*mqo*，谷氨酸生成相关基因*gudB*以及精氨酸生成相关基因*argAB*/*C*/*J*在无龋儿童中丰度显著增加。同时，电子传递链中的NADH脱氢酶相关基因*nuoB*/*C*/*D*/*E*/*H*/*I*/*J*/*K*/*L*/*M*在无龋儿童中的丰度显著增加。**结论** 儿童唾液菌群是动态变化的，其变化趋势与口腔健康状态有关，健康的口腔微生态中有更广泛的氧化磷酸化，谷氨酸以及精氨酸等氨基酸生成和转运也更为活跃。

【关键词】 龋病 唾液菌群 高通量测序 氨基酸生成

Dynamic Microbial Shifts and Functional Analysis of Saliva Microbial Communities with Caries Children

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【Abstract】 Objective To observe the dynamic changes in the salivary microbiota of children with dental caries and those who were caries-free and to analyze the functional differences in the oral microecology of the two groups during the course of sugar metabolism and the synthesis and transport of multiple amino acids. **Methods** Ten children with dental caries and 10 caries-free children were enrolled. We employed Illumina metagenomics technology to analyze the composition and function of salivary microbiome in children with and without caries. Six months later, PacBio single-molecule long-read sequencing technology was used to analyze the changes over time in the oral microbial communities of the two groups. We studied the patterns of change in the oral microbial communities under diseased or healthy conditions and attempted to offer a comprehensive interpretation of children's oral microbiota in terms of its composition and functions. **Results** The composition of the oral microbiota of children with or without dental caries changed significantly over time. At the phylum level, changing trends in the salivary microbial communities of children with dental caries were in line with those in caries-free children. In these microbial communities, increased proportions of *Firmicutes* and decreased proportions of *Actinobacteria* and *Bacteroidetes* were found in the two groups. At the genus level, however, the two groups showed significantly different changes of the salivary microbial communities. Upward trends in the abundance of *Lactobacillus*, *Methylobacterium*, and *Megasphaera* were found in the caries group, while the abundance of these genera in the caries-free group showed downward trends. At the species level, *L. fermentum*, *L. gasseri*, *L. oris*, *S. downei*, and some other species belonging to the genus *Lactobacillus* showed upward trends in the saliva of children with caries, while they consistently stayed at a relative low level of abundance in caries-free children. The abundance of *S. gordonii* and *S. mutans* decreased to a certain extent in children with dental caries, but the abundance of *S. gordonii* and *S.*

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mutans in caries-free children were always at a low level. Species such as *S. mutans* and *C. gracilis* were positively correlated to the sum of decayed, missing and filled teeth (dmft), while *N. flavescens* was negatively correlated to dmft. *gltA*, *icd*, and *mqo*, the key genes related to tricarboxylic acid (TCA) cycle, *gudB*, a glutamate synthesis-related gene, and *argAB*/*C*/*J*, arginine synthesis-related genes, were significantly increased in caries-free children. In addition, the abundance of the NADH dehydrogenase-related gene *nuoB*/*C*/*D*/*E*/*H*/*I*/*J*/*K*/*L*/*M* in the electron transport chain increased significantly in caries-free children. **Conclusion**　Dynamic changes were found in the oral microbiota of children with or without caries. The trends of microbial shifts over time were associated with the oral health status. Oxidative phosphorylation and the synthesis and transport of amino acids such as glutamate and arginine in the oral microecology were more active in caries-free children.

【Key words】　Dental caries　Salivary microbial community　High-throughput sequencing　Amino acid synthesis

作为人体微生物集聚地之一, 口腔菌群失衡可导致多种疾病。龋病是其中最常见的一种感染性疾病, 2019年, 柳叶刀杂志将龋病定义为一项全球性的公共卫生挑战[1]。口腔菌群被认为是龋病发生发展的始动因素。不仅如此, 很多系统性疾病[2-3]都能追溯到口腔来源的细菌, 有学者提出: 可将口腔菌群作为多种疾病的辅助诊断标志[4]。因此, 探究疾病和健康状态下口腔微生态的多样性和动态变化, 能帮助我们更好地认识口腔菌群, 以此为基础, 判断口腔菌群作为生物学标记物预测和诊断相关疾病的精确度和可靠性, 具有重要的临床意义。

口腔微生态结构受宿主基因、饮食和多种疾病影响[5], 其在全生命周期中不断发生变化[6-7]。因此, 不同年龄的个体可能会存在不同的生态型。在唾液菌群演替过程中, 特定功能菌群间的互作决定了口腔菌群结构[8-9]。课题组前期研究发现: 龋病与菌群失调密切相关[10-11]。前期研究是基于两个时点的横断面研究, 主要对龋病和健康儿童口腔微生态的组成, 结构和功能进行分析, 然而, 龋病和健康儿童口腔微生态随时间的变化, 尚无明确结论。为明确健康和疾病状态下口腔微生态的变化规律, 本实验拟探究龋病和无龋儿童唾液菌群的动态变化, 同时对两组儿童口腔微生态在糖代谢及氨基酸合成和转运中的功能差异进行分析, 是对之前研究的补充, 能够从组成到功能对儿童口腔微生态进行全面探索。

1　材料和方法

1.1　研究对象

本研究纳入2014年4–11月在浙江省口腔健康流行病学调查中筛选的龋病和无龋儿童各10例, 研究对象均来自浙江临安的一所幼儿园, 年龄为4 ~ 6岁。纳入标准: ①龋病儿童: 龋失补牙数(decayed, missing and filled teeth, dmft)≥7。龋病的诊断标准为: 国际龋病诊断和评估标准Ⅱ(International Caries Detection and Assessment System Ⅱ, ICDAS-Ⅱ)评分为3 ~ 6[12-13]。②无龋对照儿童: 年龄、性别与龋病儿童相匹配, dmft= 0。排除标准: 牙齿少于18 颗, 佩戴正畸矫治器, 3个月内使用过抗生素、微生态调节剂或氟化物, 伴有全身细菌或病毒感染, 患有先天性疾病或系统性疾病。

本研究经浙江大学口腔医学院伦理委员会批准(伦审研2013年第8号), 告知监护人详细的采样流程和研究计划, 签署知情同意书。研究对象在初次检查时, 收集口腔唾液样本, 进行测序分析。6个月后回访, 由同一位口腔医生进行检查, 再次收集唾液样本并进行测序分析。

1.2　研究方法

1.2.1　*唾液样本采集*　样本采集参考NIH人类微生物组计划的标准采样流程, 样本采集前24 h内不刷牙, 取样前2 h禁食。用无菌一次性移液管从口底采集非刺激性唾液2 mL, 转移至无菌冻存管内, –80 ℃冰箱冻存。

1.2.2　*唾液DNA抽提*　①使用QIAamp DNA Mini Kit(Qiagen, Hilden, Germany)试剂盒, 根据标准流程提取唾液样本全基因组DNA[14]。②为减少人类DNA污染, 使用NEB Next Microbiome DNA Enrichment Kit(New England Biolabs. Inc, Ipswich, the United States)试剂盒, 富集微生物DNA。

1.2.3　*宏基因组测序*　构建宏基因组DNA文库, 随后进行HiSeq 2000(Illumina, San Diego)测序。

1.2.4　*PacBio*　SMRT测序 6个月后采集的样本, 进行PacBio SMRT测序。利用27F和1492R作为引物, 特异性扩增细菌16S rRNA基因V1 ~ V9区。

1.3　生物信息学分析

1.3.1　*两组样本物种组成分析*　利用SOAP align 2.21软件, 将测序得到的序列与参考基因组(NCBI, http://ncbi.nlm.nih.gov)和人类微生物组计划(HMP, http://www.hmpdacc.org/resources/data_browser.php)进行比对, 分析物种组成。

1.3.2　*物种丰度计算及差异物种分析*　根据与数据库匹配的序列数量, 计算物种丰度, 分别在不同时间和分类学水平上进行分析。

1.3.3 计算基因丰度，寻找差异基因 将有效序列与参考基因组比对，获得功能基因丰度[10]。

1.4 统计学方法

研究结果采用$\bar{x}\pm s$表示。两组间计量资料比较采用独立样本Mann-Whiteny *U*检验以及Wilcoxon秩和检验；dmft与物种的相关性采用Spearman相关系数分析；$P<0.05$为差异有统计学意义。

2 结果

2.1 原始测序序列统计

Illumina宏基因组测序共产生89.18 Gb原始数据。PacbioSMRT测序共产生540 Mb原始数据。原始数据上传存入NCBI数据库，检索号分别为(SRP103050, SRP108162)。

2.2 唾液微生态群落组成分析

宏基因组测序，共得到10门、144属和745种细菌，PacbioSMRT测序共得到10门、114属和739种细菌。研究发现，随时间推移，两组儿童口腔微生态的组成出现变化，与是否患龋相比，时间因素对菌群的影响更大。

2.3 唾液微生物群落的动态变化分析

在门水平上(图1)，两组儿童唾液菌群的变化趋势保持一致，*Firmicutes*比例增加，*Actinobacteria*和*Bacteroidetes*比例降低。

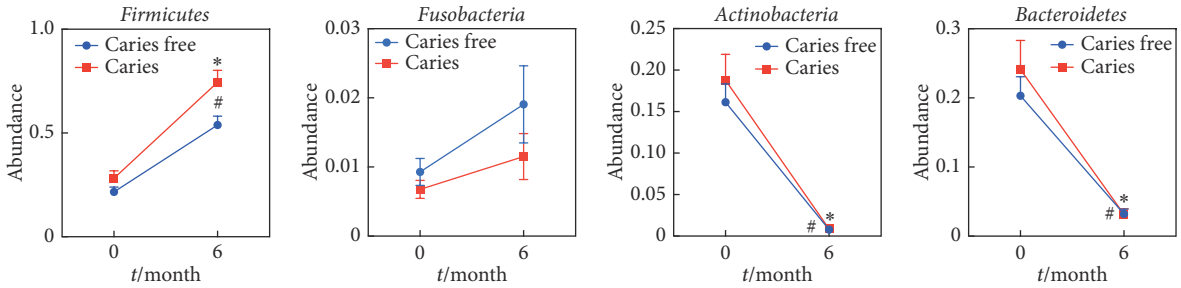

图 1 门水平上两组儿童口腔细菌随时间的动态变化

Fig 1 The dynamic changes of oral bacteria in children with or without dental caries at the phylum level over time

*$P<0.05$, vs. 0 month caries group; #$P<0.05$, vs. 0 month caries free group. $n=10$.

在属水平上(图2)，两组儿童唾液菌群的变化出现差异，其中，*Lactobacillus*、*Methylobacterium*和*Megasphaera*在龋病组儿童中的丰度呈上升趋势，而在无龋组儿童中丰度呈下降趋势。*Lactobacillus*和*Methylobacterium*在无龋儿童口腔中的丰度很低，仅占其口腔微生态细菌总量的0.009%和0.000 04%。*Dialister*、*Veillonella*和*Streptococcus*在两组儿童中均呈上升趋势。其中，*Dialister*在龋病儿童中的丰度始终高于无龋儿童($P<0.05$)。*Rothia*、*Shuttleworthia*和*Lautropia*等细菌在两组儿童中均呈下降趋势，其中，*Shuttleworthia*在无龋儿童中始终处于低丰度水平。

在种水平上(图3)，隶属于乳杆菌属的*Lactobacillus fermentum*、*Lactobacillus gasseri*、*Lactobacillus oris*、*Lactobacillus vaginalis*和*Streptococcus downei*等菌株在龋病儿童唾液中呈上升趋势，但在无龋儿童中始终处于较低水平丰度，这与属水平上的分析结果保持一致。*Streptococcus gordonii*、*Dialister invisus*和*Streptococcus mutans*在龋病儿童中丰度有一定程度下降，但在无龋儿童中始终处于低水平丰度。*Neisseria polysaccharea*在两组儿童中的丰度，随时间呈下降趋势，且无龋儿童丰度始终高于龋病儿童($P<0.05$)。*Capnocytophaga gingivalis*和*Neisseria flavescens*在无龋组中丰度逐渐上升，龋病组中丰度逐渐降低，且无龋组中丰度始终高于龋病组($P<0.05$)。*Streptococcus australis*在龋病儿童中呈上升趋势，而在无龋儿童中呈下降趋势，且无龋儿童丰度始终高于龋病儿童($P<0.05$)。

2.4 dmft与口腔菌群种水平的相关性分析

通过对口腔菌群与dmft进行Spearman关联分析发现：在两个时间点，*Streptococcus mutans*和*Campylobacter gracilis*菌种的丰度始终与dmft呈正相关，在0个月时，相关系数*r*分别为0.559($P=0.01$)和0.644($P=0.002$)，在第6个月时，相关系数*r*分别为0.785($P=0.00$)和0.563($P=0.01$)。而*Neisseria flavescens*的丰度始终与dmft呈负相关，在0个月和第6个月的相关系数*r*分别为−0.528($P=0.017$)和−0.463($P=0.04$)。

2.5 龋病和健康儿童在糖代谢及氨基酸合成过程中关键基因分析

在0个月时，三羧酸循环关键节点相关基因，*gltA*(编码柠檬酸合酶)、*icd*(编码异柠檬酸脱氢酶)以及*mqo*(编码苹果酸醌氧化还原酶)，谷氨酸生成相关基因*gudB*(编码谷氨酸脱氢酶)以及精氨酸生成相关基因*argAB/C/J*在无龋儿童中丰度显著增加。同时，三羧酸循环电子传递链中的NADH脱氢酶相关基因*nuoB/C/D/E/H/I/J/K/L/M*

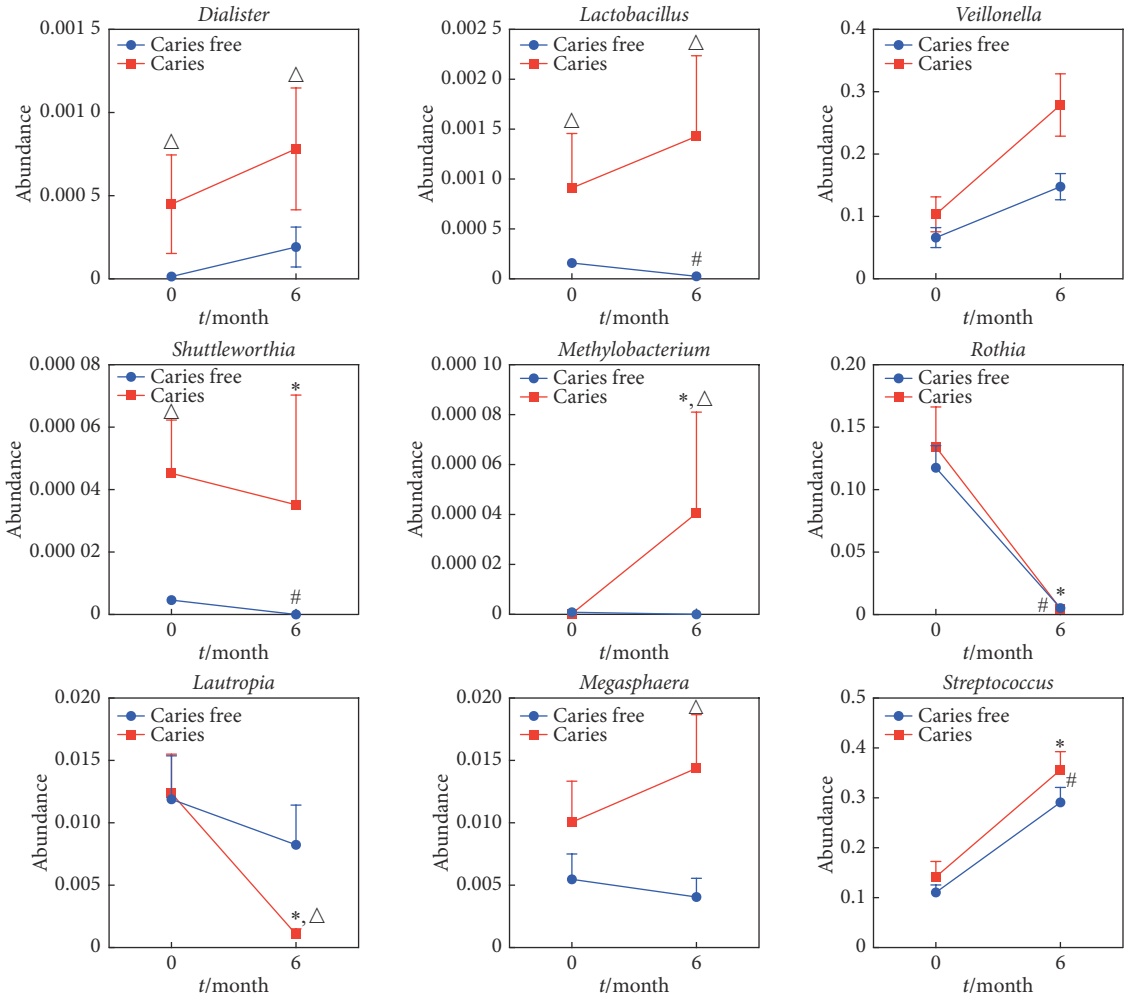

图 2 属水平上两组儿童口腔细菌随时间的动态变化

Fig 2 The dynamic changes of oral bacteria at the genus level in children with or without dental caries over time

*P<0.05, vs. 0 month caries group; # P<0.05, vs. 0 month caries free group. △ P<0.05, vs. caries free group at the same time point. n=10.

在无龋儿童中的丰度显著增加(图4)。腐胺运输相关基因在无龋儿童中丰度更高。其中, *potF*/*I*/*H*/*G*基因丰度高于龋病儿童(P< 0.05)。同时, 谷氨酸/丙氨酸/甘氨酸等氨基酸转运蛋白家族的基因丰度值在无龋儿童中上调(P< 0.05)(图5)。

3　讨论

一项为期7年的纵向研究显示: 口腔微生态中的“早期定植菌”包括链球菌属、韦荣菌属和乳杆菌属[15]。本研究发现: 在属、种水平上, 两组儿童的口腔菌群随时间推移存在不同的变化趋势。可见, 口腔微生态的组成和结构不仅与患龋与否相关, 也会随着时间的推移不断发生变化。其中, 隶属于乳杆菌属的多个菌株在龋病儿童口腔中逐渐上升, 而在无龋儿童中, 始终处于较低水平。另外, 研究发现*Streptococcus mutans*、*Streptococcus gordonii*、*Streptococcus downei*和*Streptococcus australis*同隶属于链球菌属, 然而, 在相隔6个月的两个时间点, *Streptococcus mutans*、*Streptococcus gordonii*和*Streptococcus downei*在龋病组的丰度较高, 而*Streptococcus australis*在无龋组的丰度较高。这提示我们, 相对于属水平的研究, 在种或者菌株水平的研究更为准确。

口腔菌群与dmft关联分析的结果提示我们: *Streptococcus mutans*和*Campylobacter gracilis*等菌种与龋病的严重程度密切相关, 而*Neisseria flavescens*等菌种与维持健康的口腔微生态息息相关, 这与其他学者的研究结果一致[16]。

2015年, TENG等[17]发现口腔菌群与儿童生理年龄存在关联性, 并提出“口腔菌群年龄”的假说, 以期利用这一关联, 纵向追踪群体和个体龋病的发生。该假说主要基于口腔菌群的组成和结构变化, 而本研究进一步对龋病和无龋儿童口腔菌群的功能差异进行分析。研究结果显示, 三羧酸循环相关基因在无龋儿童中显著增加。*gltA*可

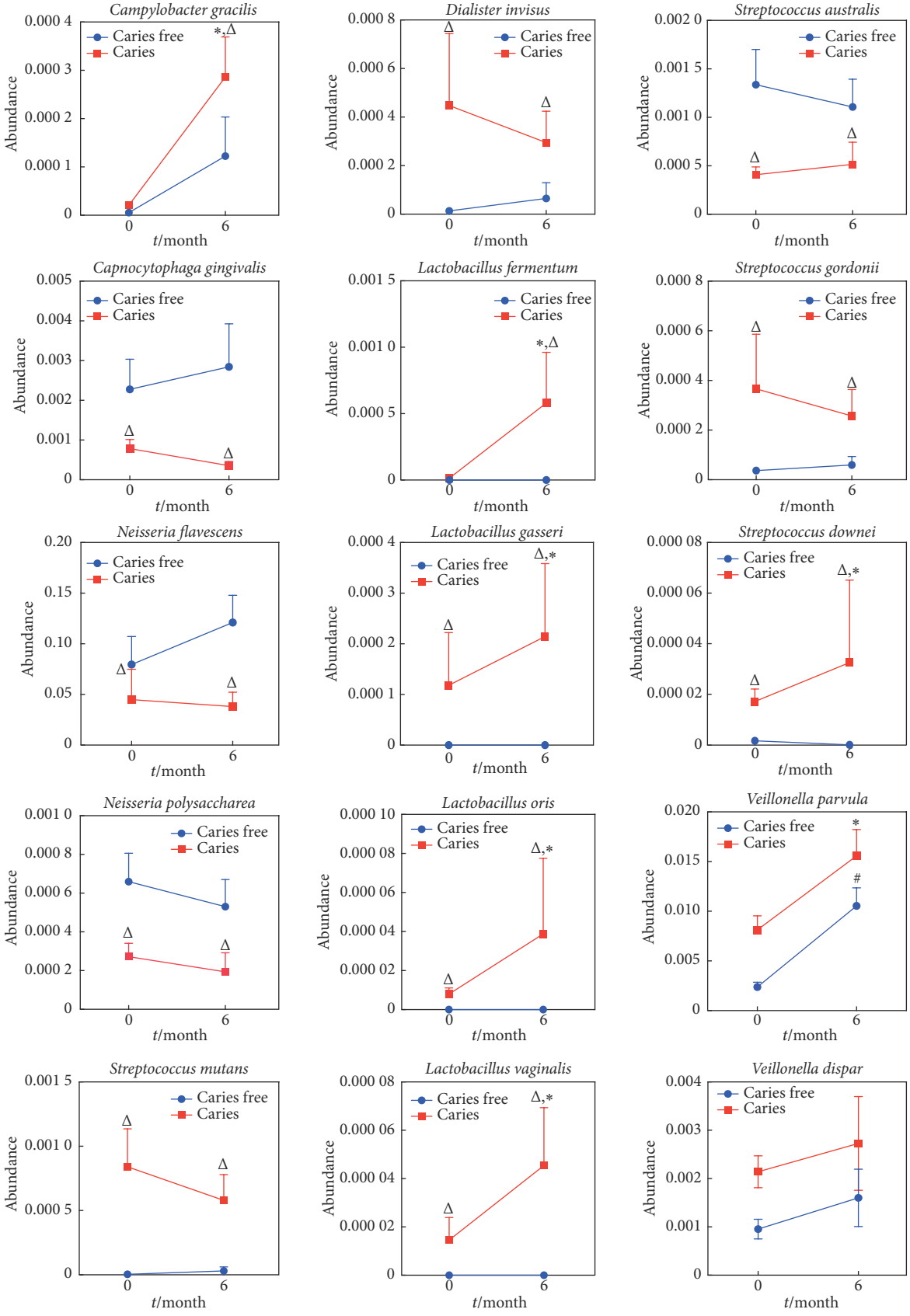

图 3 种水平上两组儿童口腔细菌随时间的动态变化

Fig 3 The dynamic changes of oral bacteria at the species level in children with or without dental caries over time

*P<0.05, vs. 0 month caries group; # P<0.05, vs. 0 month caries free group; Δ P<0.05, vs. caries free group at the same time point. n=10.

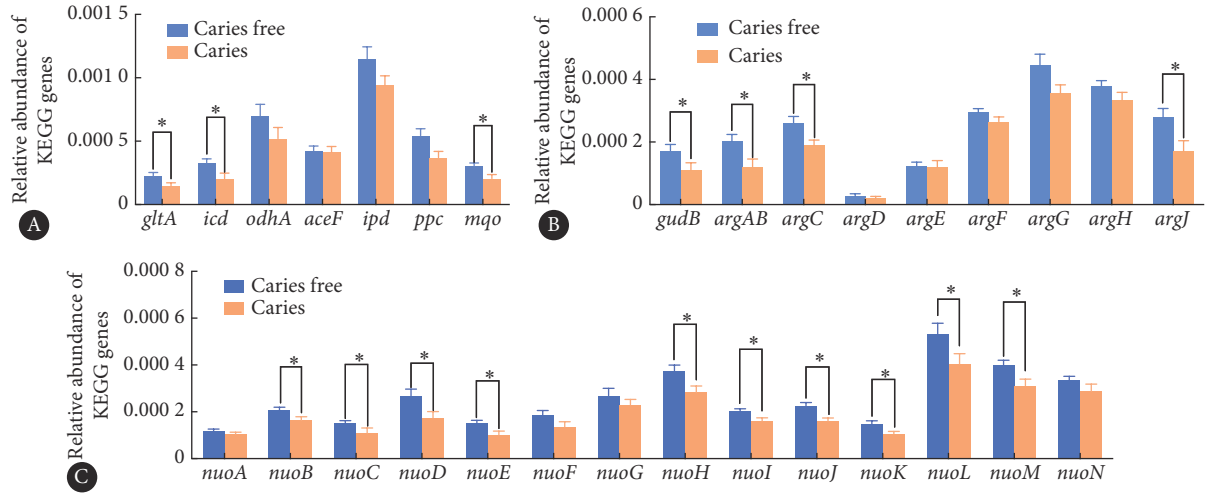

图 4 糖代谢及氨基酸合成过程中关键基因差异分析

Fig 4 Analysis of differences in key genes involved in glucose metabolism and amino acid synthesis

KEGG: Kyoto Encyclopedia of Genes and Genomes database. A: Key gene abundance for TCA cycle; B: Key gene abundance for glutamate and arginine cycle key genes synthesis; C: Abundance of NADH dehydrogenase related genes. *P<0.05. n=10.

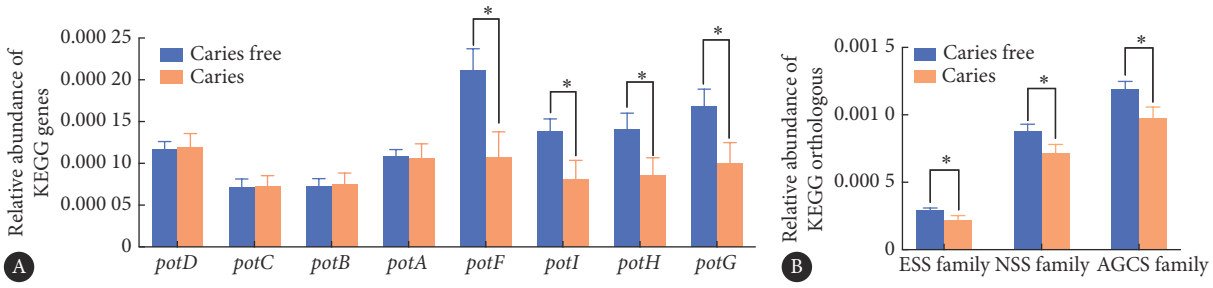

图 5 腐胺及氨基酸运输过程关键基因差异分析

Fig 5 Analysis of differences in key genes involved in putrescine and amino acid transportation

ESS: Electrochemical sodium symporter; NSS: Neurotransmitter sodium symporter; AGCS: Alanine or glycine cation symporter. A: Key gene abundance for putrescine transport system; B: The ESS family represents glutamate: Na^+ symporter; The NSS family represents neurotransmitter: Na^+ symporter; The AGCS family represents alanine or glycine: cation symporter. *P<0.05. n=10.

以编码柠檬酸合酶, 同时过表达的*gltA*能将更多的碳定向到三羧酸循环[18]。*icd*编码异柠檬酸脱氢酶, *icd*过表达会使更多的碳流向α-酮戊二酸[19], α-酮戊二酸参与三羧酸循环, 也是谷氨酸合成重要的前体物质[20]。*mqo*编码醌氧化还原酶, 可增加草酰乙酸的供应。草酰乙酸不仅是三羧酸循环关键的中间物质, 也是多种氨基酸生成的前体物质[21]。因此, *gltA*、*icd*以及*mqo*在无龋儿童的丰度增加, 有效地加速了无龋儿童口腔微生态中的三羧酸循环速度, 同时是多种氨基酸生成的基础。NADH脱氢酶是电子传递链中最大的复合物, 对于细菌胞内氧化还原反应至关重要[22]。本研究发现, 三羧酸循环电子传递链中的NADH脱氢酶相关基因在无龋儿童中显著增加, 因此, 我们推测, 健康的口腔微生态菌群中心代谢更为活跃。

本研究中与谷氨酸和精氨酸合成相关的基因在无龋儿童中增加。精氨酸为功能性氨基酸的代表, 能通过多种代谢途径调节机体的生理功能。精氨酸脱亚胺酶系统, 作为口腔微生态重要的产碱来源, 可提高口腔环境的pH值[23], 有学者将精氨酸视为对抗牙菌斑生物膜的武器[24]。不仅如此, 精氨酸还可以刺激机体分泌胰岛素和生长激素[25-27], 参与生物体的特异性免疫和非特异性免疫[28], 能有效的活化T淋巴细胞[29-31], 在维持健康的口腔微生态中发挥重要的作用。

本研究中健康儿童多种氨基酸转运蛋白基因明显增加, 口腔中氨基酸的循环代谢, 对于调控氨基酸的稳态至关重要[24, 32]。谷氨酰胺、精氨酸等可以通过合成鸟氨酸, 进而生成多胺, 而多胺对口腔黏膜上皮细胞的生长、分化、迁移以及成熟和修复发挥重要的作用[33]。本研究中, 腐胺运输相关基因的丰度在无龋儿童中增加, 进一步验证了多胺对维持健康的口腔微生态具有积极的作用。

综上所述, 本研究表明: 在不同口腔健康状况下, 口

腔微生态的组成以及动态变化趋势不同。同时，通过对两组儿童口腔微生态的功能基因进行差异分析，对我们了解健康口腔微生态的功能具有重要的意义。课题组后续将继续探究氨基酸在调节口腔免疫中的作用，通过人群随访，结合多种手段，揭示口腔微生态中氨基酸的合成和代谢在机体健康中的重要作用。

* * *

利益冲突 所有作者均声明不存在利益冲突

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