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PROBLEMS & PARADIGMS

Prospects & Overviews

# How does the early life environment influence the oral microbiome and determine oral health outcomes in childhood?

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## Abstract

The first 1000 days of life, from conception to 2 years, are a critical window for the influence of environmental exposures on the assembly of the oral microbiome, which is the precursor to dental caries (decay), one of the most prevalent microbially induced disorders worldwide. While it is known that the human microbiome is susceptible to environmental exposures, there is limited understanding of the impact of prenatal and early childhood exposures on the oral microbiome trajectory and oral health. A barrier has been the lack of technology to directly measure the foetal "exposome", which includes nutritional and toxic exposures crossing the placenta. Another barrier has been the lack of statistical methods to account for the high dimensional data generated by-omic assays. Through identifying which early life exposures influence the oral microbiome and modify oral health, these findings can be translated into interventions to reduce dental decay prevalence.

**KEYWORDS**
dental caries, environment, exposures, -omics, Oral microbiome, postnatal, prenatal

## INTRODUCTION

The oral microbiome is a major determinant of oral health. Consisting of over 700 prevalent bacteria, in addition to fungi, archaea and viruses, the oral microbiome is the second most complex and diverse microbial community in the human body.[1] Dysbiosis between the oral microbiome and host underpins the development of oral diseases such as dental caries[2] and periodontal disease.[3,4] The Global Burden of Health Report (2016) found dental caries was the most prevalent disease worldwide, affecting an estimated 2.44 billion and the majority of adults (80%) in the United States, with severe periodontal disease ranking as the 11th most prevalent disease globally.[5] Both diseases can cause pain, tooth loss, systemic infection and, reduced quality of life and productivity due to days lost from school and work. In addition to impacting the oral environment, dysbiosis in the oral microbiome has been associated with systemic conditions including Alzheimer's

disease,[6] diabetes,[7] mental health issues[8] and gastrointestinal disorders.[9]

Identification of factors driving dysbiosis in the oral microbiome and mediating disease risk have remained elusive. This is particularly concerning for the situation of dental caries given it is the most common chronic disease in children, occurring at five times the rate of asthma.[10] With this comes huge costs and implications on healthcare systems, resulting in enormous burden for both children and their families. Due to this high prevalence, much time is spent in daily dental practice on the prevention and management of dental caries. While it is established that paediatric caries is a multifactorial disease, involving the developing oral microbiome, environmental factors and host genetics,[11] what is yet to be determined is the interaction and relative contribution of these factors to the disease. This information is necessary to design effective prevention strategies to reduce the high prevalence of childhood dental caries.

Recent technological advancements to study oral microbiome and environmental factors have enabled incredible investigative depth, but the high dimensional data generated presents a major challenge to characterising the interaction of these factors. The development of genome-wide or -omic based sequencing to study the oral microbiome has revealed that caries is not caused by a few species and is instead a polymicrobial disease.[12] Adding to the complexity, is the identification of strain level variation of species within the oral microbiome, which are potentially clinically important.[13] Similarly, there has been a paradigm shift in studying environmental factors towards characterizing the "exposome". The exposome describes the totality of all environmental exposures throughout an individual's lifetime.[14,15] This encompasses exposures from the wider external environment (e.g., stress, climate and socioeconomic), specific external environment (e.g., chemicals, diet and infections) and the internal environment (e.g., biological response to exposures). Multiple studies also demonstrate that the timing of exposures can be critical to observed health outcomes and that the prenatal and early childhood periods are particularly vulnerable to environmental insult.[16] Measuring these exposures over the course of a lifetime, particularly during the perinatal period, is a complex challenge but new technological advances are able to measure exposure biomarker mixtures over time, creating another high dimensional data set. The interaction of the environment with the oral microbiome to predispose an individual to caries during childhood has to date focused on population level effects,[17] which are too broad to reveal which exact environmental factors are critical. Emerging evidence indicates that exposures during the foetal and early postnatal environment at the chemical level may affect the trajectory of the oral microbiome and modify an individual's caries risk. To understand the interaction at higher resolution will require application of these new"-omic" technologies as well as new statistical approaches that can handle these high dimensional data sets.

To tackle the problem of the aetiology of childhood dental caries, we will review the current understanding of the disease, with a focus on the role of the developing oral microbiome and its interaction with the environment at an individual level within large-scale studies. We then present the changing paradigms in the area, in particular the mediation of the disease through environmental factors at the chemical rather than population level, and the importance of exposure timing. Lastly, we present how new approaches may resolve the problem, though direct measurements of the microbiome and early environmental exposures, in combination with multivariate statistics to distil information from high dimensional data, to propose a new, more characterized model for caries development.

## CURRENT THINKING: CARIES AS A POLYMICROBIAL DISEASE INFLUENCED BY POPULATION LEVEL FACTORS

### Caries and the oral microbiome

The microbial cause of dental caries has been framed by the long-standing dogma that *Streptococcus mutans* is a keystone species for disease development.[18,19] This model of caries was originally based on findings from animal studies, which demonstrated that rats inoculated with *S. mutans* and fed a diet containing sucrose, developed rampant caries.[20] The role of *S. mutans* in caries was reinforced by culture-based research,[21] which demonstrated the presence of this bacteria in carious lesions in humans. The development of genomic methods has enabled culture-independent identification of bacteria through amplification of the phylogenetically informative 16S rRNA gene, which is present in all bacteria. The 16S approach in combination with next generation sequencing (NGS) has revealed the oral microbiome contains a huge diversity of bacteria, with over 1000 species of which more than 60% are uncultured phenotypes.[1] This finding of greater diversity than previously known in the oral microbiome has been accompanied by an expanding diversity of bacteria being associated with caries. NGS studies of dental caries have revealed there is a gradual shift in the composition of the oral microbiome between health and caries,[22] with no "caries-specific" bacteria found that were completely absent in health. Instead, shifts in the abundance of bacteria, particularly *Prevotella*,[22] *Lactobacillus*[23] and *Bifidobacterium* [24] species, have been observed between health and caries. Currently, there is little consensus regarding the bacterial species enriched in caries, potentially as a result of differing study design. Microbial composition varies with age of participants[25] and by laboratory method, including DNA extraction technique [26] and region of 16S sequenced.[27] In particular; the sample type appears to influence composition. While *S. mutans* was associated with caries when using saliva,[19] studies using oral biofilm or dental plaque often found less of a connection between this bacteria and the disease.[23] Given that caries is a biofilm mediated disease, the species makeup of dental plaque is probably more reflective of the disease process than saliva.

The ecological model of caries, with microbiome-wide species change has been primarily based on assessment of the composition and not function of the oral microbiome. The 16S approach, while inexpensive and straight forward, only provides low-level resolution, at the genus to species level, of the taxonomic makeup of a sample. If caries is caused by microbiome-wide changes, this is likely to be

reflected in changes in the bacterial diversity at a strain level and functional modifications, particularly in metabolism of the bacterial populations. Metagenomic sequencing enables assessment, if done in enough depth, of the totality of genomes of all microbiota.[28] Through assembly of the sequence data and identification of genes, detailed information of bacterial strains and the function of the microbiome as a whole can be predicted. In comparison to the number of 16S-based studies assessing the caries oral microbiome in childhood, to date there have been substantially fewer studies using metagenomics to assess the microbiome in caries.[29–32] All of these studies have a small sample size (e.g., $n < 50$), and many have used saliva[30,31] as opposed to the more clinically relevant biofilm sample. However, these studies have revealed that within species or strain level bacterial diversity and function varies with caries development.[29–31] While bacterial species within a strain may share a "core" genome, they can contain many unique genes which confer virulence to particular strains and hence result in strain level differences within a disease.[33] Metagenomic sequencing of plaque samples from children with caries ($n = 30$) has revealed that strains belonging to the same species had a differential association with caries.[13] For example, *Streptococcus mitis* bv 2 str SK95 showed an association with the caries-affected group, the sister strain showed an association with the caries-free group and there was no association with either health state at the species level. In addition to metagenomic sequencing revealing the role of within species genomic diversity in caries, this method has also revealed that caries was associated with metabolic changes in the oral microbiome. Caries was found to be associated with an enrichment of genes for sugar metabolism, such as the glucose transferase gene (GTF) and increased abundance of pathways for sugar breakdown, including glycolysis and gluconeogenesis. Additionally, caries affected individuals have been found to have an upregulation of genes encoding polyamine production, which is important for biofilm formation.[13] The sole contributors to polyamine synthesis were found to be *Veillonella parvula* and *Veillonella sp*. 612.[13] In caries-free compared with caries-affected individuals, there was an increase in abundance of genes encoding enzyme classes; arginine,[32] threonine, and dCTP deiminases,[13] which are involved in the release of ammonium, which can neutralize acids and prevent enamel demineralization. The species linked to these ammonium producing enzymes included *Neisseria*[32] and *Actinomyces* species (*Actinomyces naeslundii*, *Actinomyces massiliensis*, *Actinomyces johnsonii* and *Actinomyces oris*), in addition to *S. mitis*. In addition, similar to observations of reduced species diversity in caries,[23] the functional diversity[32] of the oral microbiome was also found to decline.

To date the microbiome-wide assessment of caries has been bacterio-centric, given that bacteria dominate the oral environment. If caries is mediated by overall shifts in composition and function of bacteria, these changes would likely impact other members of the oral microbiome, such as fungi and archaea. Fungi are thought to play a role in caries, given the high levels of *Candida albicans* found in children with early childhood caries from culture-based studies,[34–37] and the high acid tolerance of this fungi and ability to excrete organic acid further reducing the oral cavity's pH.[38] NGS studies of the oral mycobiome have supported culture-based findings that *C. albicans* is the dominant fungi in the oral environment, however its role in caries has not been consistently observed.[39,40] Interestingly, studies did find a reduced diversity of fungi in caries.[39,40] In comparison, the role of the hard to culture archaea in caries has been scarcely studied. NGS of caries biofilms have found methanogen archaea to be present.[41] Methanogens were identified in combination with lactobacilli and are known to be able to oxidize acids and alcohols produced by these fermenting bacteria. This small-scale study highlights the interconnectedness of microbial populations within the oral biofilm.

## Early life is a critical period for the assembly of the oral microbiome and establishing caries risk

While a microbial model for caries is yet to be resolved, the preceding of microbial changes before the clinical presentation of caries is generally accepted. NGS studies have revealed microbial changes up to 12 months before the clinical presentation of caries in 3 year old's[42] and potentially even before the emergence of teeth, based on presence of the acidogenic and aciduric *S. mutans*, a species traditionally associated with caries.[43] As such, the development of the oral microbiome in early life and influence of environmental factors during this time has been postulated to be critical for the development of caries in childhood.

Microbial diversity is acquired within the first hours after birth, predominantly shaped by maternal sources, and evolves over time in response to environmental factors, becoming relatively stable in adulthood.[44] The influence of the first 1000 days of life (conception—2 years) on the human microbiome has primarily focused on the assemblage of microbes in the gastrointestinal tract. From the handful of genomic (non-cultivation), longitudinal studies, the assembly of the oral microbiome makeup appears to follow an ordered pattern,[45] which is influenced by birth mode, early feeding practices and antibiotic exposure.[17] Relevant aspects of the oral microbiome trajectory are summarized in Figure 1.

The womb is a sterile environment[46] (unless infection) so the infant oral microbiome is first exposed to microbes through contact with the vagina or uterus during delivery.[47] However, it is primarily inoculated during the first 6 months with early feeding[17,48] and has a narrow diversity dominated by *Streptococcus*, *Veillonella* and *Lactobacillus* species.[17] Diet is a major factor in the microbiome trajectory with breastfed infants having higher abundances of *Streptococcus* and *Veillonella* species compared with formula fed infants.[17,49] From 6 months to 2 years the emergence of teeth provides a non-shedding surface for biofilm maturation and consumption of solid foods increases the variety of nutrients for the microbial community, resulting in increased microbiome diversity.[50,51] This enables other species, such as *Gemella*, *Granulicatella*, *Haemophilus* and *Rothia*, which were present at low abundance from 3 months of age to increase

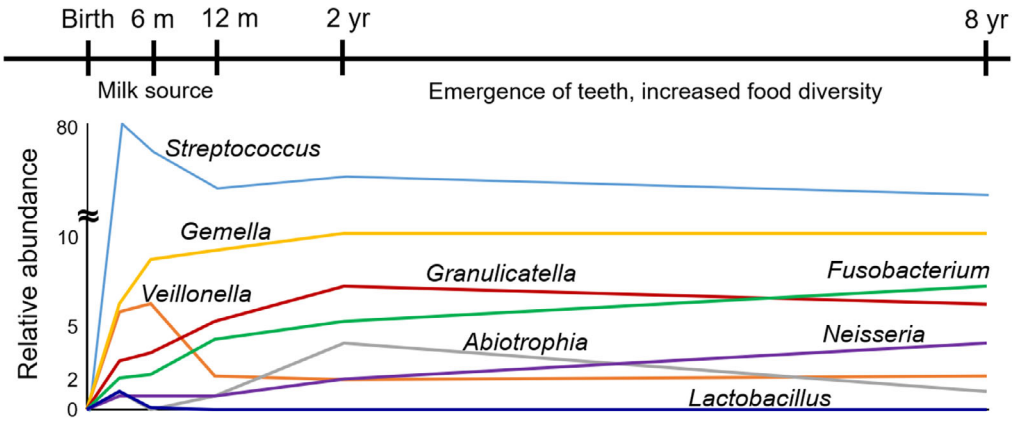


**FIGURE 1** Key aspects and factors effecting the oral microbiome trajectory. Relative abundance plot derived from Dzidic et al[17]

in abundance with time.[17] Later childhood (> 2 years) sees further enrichment and increased abundance of bacterial species, including *Porphymonas*, *Actinomyces* and *Neisseria*,[17] in addition to *Fusobacterium nucleatum*.[25] However, how this assembly relates to health outcomes or functional development of the oral microbiome remains unresolved.

The importance of the environment in modifying the developing oral microbiome to influence caries risk was first identified through strong epidemiological data demonstrating that childhood caries is influenced by population level environmental factors. These factors include poor oral hygiene,[52] a diet containing excess sugar and frequent snacking[53,54] and passive exposure to parental cigarette smoke.[55] In addition, low socioeconomic (SES) status is associated with high paediatric caries risk, due to the correlation between low SES and poor oral hygiene, education and diet. Few studies have evaluated the impact of these factors on the entire oral microbiome, instead focusing on specific bacteria.[56] From the few NGS studies assessing the impact of environmental factors of importance to caries, these have focused on compositional change through analysis of the 16S gene and used this to predict functional shifts. Smoking[55] and excess sugar intake[57] were found to alter the overall predicted function of the oral microbiome, with both displaying microbiome changes similar to caries. This included decreased aerobic respiration and increased anaerobic respiration to enable oxygen independent carbohydrate metabolism.[55,57] Interestingly, the influence of diet on the oral microbiome may be bidirectional, with the composition of the oral microbiome found to influence taste preference and eating habits.[58] To date, studies have yet to interrogate the three-way relationship between environmental factors, the oral microbiome and clinical caries presentation. While reduction of dietary sugar intake and improved oral hygiene are important strategies in preventing caries,[54] these are not the only risk factors.[59] The development of effective prevention strategies for caries requires a better understanding of the complex interactions between multiple risk factors, including other environmental exposures.

## CHANGING PARADIGMS: ENVIRONMENTAL EXPOSURES INFLUENCE THE ORAL MICROBIOME TRAJECTORY AND PEDIATRIC DENTAL CARIES RISK

Past studies of broad environmental effects on the oral microbiome have not reflected the reality of the exposome, which involves complex combinations of multiple exposures over time. In addition, while assembly of the oral microbiota occurs soon after birth, this fact has erroneously been interpreted to mean that only environmental exposures at or after birth can impact the oral microbiome. In fact, there is emerging evidence that prenatal exposures are associated with microbiome composition in childhood[60] and prevalence of caries.[61] Early life exposure to environmental chemical mixtures likely affect the oral microbiome and act in concert to modify caries risk.

### The microbiome is vulnerable to environmental exposures

There is increasing evidence that environmental chemicals interact with the gut microbiome through several pathways that effect composition and function of the microbiota.[62] Several animal studies have demonstrated that exposure to heavy metals (arsenic, cadmium and lead), persistent organic pollutants (polychlorinated biphenyls [PCBs], polycyclic aromatic hydrocarbons [PAHs]), pesticides (diazinon and carbendazim), bisphenol A (BPA) or phthalates, is associated with gut microbiota dysbiosis, leading to adverse disorders of metabolism, nutrient absorption and immune system function.[62–64] Air pollutants like $O_3$ and $NO_2$ are shown to alter compositional and functional profile of gut microbiome[65] while air quality index is shown to affect skin microbiome in healthy women.[66] While there is some overlap between the oral and gut microbiome,[67] interactions between the environment and the gut microbiome are not necessarily directly transferrable to the oral microbiome.[68]

Despite epidemiological evidence showing an association between toxic metal exposures and oral microbiome-mediated diseases, including dental caries[69–71] and gingival diseases,[72] very few studies have investigated the interaction between environmental exposures and the oral microbiome. One such study found that toxic metals antimony, arsenic and mercury in saliva were associated with the oral microbiome composition.[73] Caries was associated with an increased level of antimony and increased abundance of lactobacilli species. Although the impact of smoking on the oral microbiome has been investigated in a handful of studies, results have been largely inconsistent due to differences in study design and small sample sizes.[74,75] In a large study that combined data from two US national cohorts, current smokers showed a significant depletion of Proteobacteria, and enrichment of Firmicutes and Actinobacteria, compared with never smokers.[74] Early studies of the impact of e-cigarette smoking on the oral microbiome suggest a marked difference to cigarette smoking, which may be driven by the different environmental exposures, such as glycerol and propylene glycol, associated with each source.[76]

Studies of how exposure to environmental chemicals in early life effect the assembly and evolution of the oral microbiome are severely lacking. The oral microbiome is the first to come into contact with environmental chemicals via oral exposure. Depletion of pathways involved in the biodegradation of chemicals associated with smoking was observed in smokers compared with non-smokers.[77] Oral bacteria may therefore play an important role in degrading chemicals and thus alter their systemic toxicity. In addition, vulnerability to environmental exposures is heightened during the early life period due to hand-to-mouth activities, greater absorption, underdeveloped mechanisms to metabolize chemicals and subtle disruptions that alter subsequent developmental trajectories.[78] Therefore, the early life period is absolutely critical to study the interaction of environmental exposures and the developing oral microbiome.

## Emerging evidence supports the role of environmental exposures in pediatric dental caries

Just as there is evidence that environmental exposures impact the oral microbiome trajectory, there is also evidence that toxic environmental exposures can increase the risk of dental caries. Since the caries-protective effect of fluoride was first discovered, the role of other trace elements in tooth mineralization and tooth decay have been studied. Several metals, including lead (Pb), cadmium (Cd), copper (Cu), boron (B), molybdenum (Mo) and strontium (Sr) have been linked to caries.[79–85] Importantly, animal studies have shown that exposure to Pb and Cd during tooth development is associated with a greater risk of caries.[79,86,87] Exposure to environmental tobacco smoke (ETS) has also been associated with pediatric caries.[88] Associations between environmental exposures and caries are more consistently reported for deciduous teeth than permanent teeth[70,84,88,89] which indicates deciduous dentitions may be particularly susceptible, possibly due to the evolution of the microbiome which is more stable in adulthood. Possible mechanisms through which environmental exposures could enhance susceptibility to caries on their own include salivary gland function, enamel formation, and interference with saliva formation or oral bacteria.[84,85,88] However, environmental exposures may also alter the acquisition and maturation of the oral microbiome. Therefore, studies of the interaction of the microbiome and environmental factors are critical to understanding how these factors separately and jointly contribute to caries risk. This is because the oral microbiome and environmental factors may act along independent pathways, as well as interact, to modify risk, depending on the different species of these complex mixtures. Importantly, many metals and other environmental chemicals interact and therefore it is vital that studies of the role of environmental exposures encompass multiple exposures and analyze the whole mixture, rather than individual chemicals.

## Host genetics interacts with the environment and oral health

While the environment is a known major modulator of the oral microbiome, host genetics have been shown to significantly influence the composition of select bacterial species from cross-sectional twin studies in later childhood.[44] Host genetics and sex specific differences have also been demonstrated for microbiota changes associated with environmental exposures.[90–93] Furthermore, while exposure to heavy metals has been repeatedly associated with changes in the composition of the gut microbiome in multiple species, the bacterial changes vary across studies. This may be explained by interactions between host genes, environment and the microbiome (G x E x M).[63] This interaction may also explain the variability observed between studies examining the caries microbiome, given that heritability for this phenotype is estimated to range from 30–60%.[94,95] Putative roles for the host genome in mediating environmental influences on the developing microbiome include both direct factors (e.g., immunological; salivary flow, composition and buffering capacity; anatomical variation of the teeth) and indirect factors (e.g., dietary preference; manual dexterity in oral hygiene practices). Environmental factors are shown to affect the host epigenetics which indirectly affect the oral microbiome and oral health status. More light has been shed by studies on twins who despite similar genetic background showed different caries risk,[96] and a possible discordance correlated with methylation profile.[97]

In order to examine the role of the environment in the acquisition and development of the oral microbiome, studies that are nested within a population structure designed to also control for or measure the influence of the host genome are significantly more powerful than those from randomly ascertained population probands. Such studies include traditional twin designs, parent-offspring dyads, and other genetically informative structures.

## FUTURE PERSPECTIVES: OVERCOMING TECHNOLOGICAL CHALLENGES TO STUDY MICROBIOME X ENVIRONMENT DETERMINANTS OF DENTAL CARIES

We hypothesise that environmental exposures during early life alter the acquisition and maturation of the oral microbiome, and in the background of genetic risk, mediate oral health outcomes in childhood. To address this hypothesis and overcome past limitations, studies should assess the developing oral microbiome through metagenomic analysis in longitudinal human studies and combine this data with direct measures of the fetal and early postnatal exposome. Given that the technology to study the exposome is not yet available, early life exposures can be measured through direct assessment of environmental chemicals (external environment) and the metabolome (internal environment). Statistical methods are required that account for the multivariate nature of the data and enable integration of the microbiome, and exposome data, to identify microbiome and environmental features which influence caries, either in synergy or isolation and how these modulate over time in childhood.

### Microbiome data and study design

Resolution of both the microbial cause of caries and disease prediction, through understanding the assembly of the oral microbiome in childhood, requires assessment of the oral microbiome in a longitudinal study design. Repeated assessment of an individual's oral microbiome from birth through to early childhood at regular intervals, preferably using oral biofilm samples, would enable assessment of major life transitions, including tooth emergence, introduction of solids and diet development. While 16S data provides valuable taxonomic information, only metagenomic data can provide the following key pieces of information about the oral microbiome: (1) strain level taxonomic data, (2) functional information, such as metabolic pathways, in addition to (3) other genes, such as those causing antibiotic resistance, and (4) information about the whole microbiome, including bacteria, fungi, archaea and viruses. Importantly, to interrogate the relationship between the microbiome and oral health, clinical assessment of caries is required using validated and widely used methods, such as the Decayed Missing and Filled teeth (DMFT) index or the International Caries Detection and Assessment System (ICDAS II), full code format.

### Direct measures of early life environmental exposures

Undertaking comprehensive studies of the impact of the environment on the assembly and evolution of the oral microbiome and its relation to caries has been limited by: (1) the lack of biomarkers that directly measure fetal (vs. maternal) and childhood environmental exposures across specific developmental periods; (2) the expense and time needed to conduct prospective studies of prenatal exposure and oral health; (3) the expense of conducting large metagenomic studies; and (4) the need for statistical approaches that can handle the high dimensional data generated by"-omic" technologies to study the separate and joint effects on health outcomes. Much of the caries risk is believed to be due to the joint action of environmental factors and the microbiome. It is likely that environmental exposures as early as fetal development, when the primary dentition is developing, may determine the risk of childhood caries. However, no epidemiologic study has undertaken a direct assessment of fetal exposures. Some studies have relied on maternal blood and urine assays, but maternal biomarkers are not always an accurate reflection of fetal exposure due to placental regulation of many environmental chemicals. For postnatal exposures, questionnaires are relied upon, which cannot accurately measure chemical exposures and suffer from misclassification, reporting bias, and recall bias.

Teeth are increasingly used to reconstruct histories of environmental exposure in individuals by measuring biomarkers of chemical exposure, stress, diet and climate.[98–103] Human primary teeth can provide a direct measure of the timing and intensity of chemical exposure from approximately the $14^{th}$ gestational week to early childhood. The method exploits the normal growth pattern of teeth, which is analogous to rings in a tree, and utilizes micro-spatial sampling to measure chemicals archived within growth rings that correspond to specific critical developmental windows. For decades teeth have been used to assay cumulative exposure to metals. In these studies, whole teeth or large fragments were digested and toxicant concentrations reported as a cumulative exposure.[104–106] This type of analysis destroys the temporal information unique to tooth development which provide fine-scale information on timing of exposure. Relevant aspects of tooth development and mineralization are summarized in Figure 2.

The application of laser ablation technology to teeth has enabled the generation of weekly metal exposure profiles over the pre- and postnatal periods.[107–110] This method has been validated against other biomarkers and environmental measures at specific time points.[107,108,110,111] Similar to work on metals, several studies have measured organic compounds in whole teeth or tooth fragments.[112,113] Garcia-Algar et al.[112] undertook early studies to measure cotinine in teeth and showed that children of smoking mothers had higher tooth cotinine levels compared with children whose mothers did not smoke. New innovations in novel high-dimensional analytical methods that combine histological and chemical analyses will move this field beyond metals to sampling of numerous additional markers in tooth growth rings to isolate exposures with serial measurements at precise time points.

The paradigm shift towards the exposome, necessitates technologies and study designs that can measure multiple environmental chemicals and their metabolites (biological response). The field must move past single chemical studies that do not reflect the reality of environmental exposures. No individual is exposed to a single environmental factor in isolation of all other exposures. However, due to technological limitations, very few studies have examined the impact on the microbiome from two or more environmental exposures.[114–116]

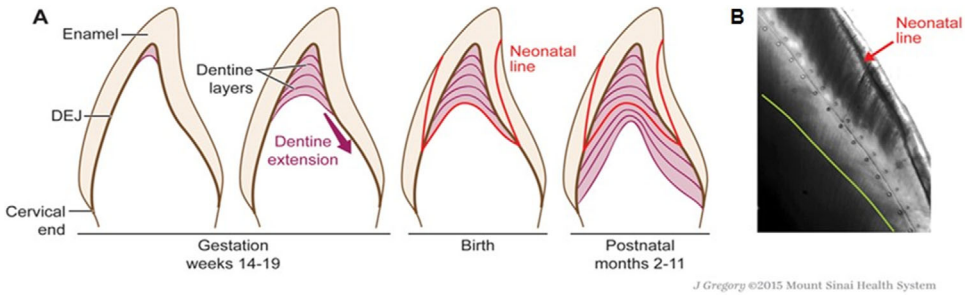


**FIGURE 2** (A) At 14–19 weeks in utero, enamel and dentine begin to mineralize at the future dentine-enamel junction (DEJ) on the cusp tip. Subsequently, enamel and dentine deposition occurs in a rhythmic manner forming incremental lines—akin to growth rings in a tree—in both enamel and dentine.[134] At birth, an accentuated incremental line, the neonatal line, is formed due to disturbances in the secretory cells during matrix deposition.[135] After birth, teeth continue to manifest daily growth lines, which reflect chronological ages at various positions within the tooth. (B) Due to a change in crystal orientation and density, the neonatal line is a clear histological landmark that demarcates pre—and postnatally formed parts of teeth. The neonatal line forms regardless of the type of delivery (C-section vs. vaginal). Reproduced with permission from Hirofumi Morishita and Manish Arora, Tooth-Matrix Biomarkers to Reconstruct Critical Periods of Brain Plasticity, Trends in Neuroscience, 2017, 40 (1), 1–3

Furthermore, it is also important to study the metabolites of external exposures to comprehensively model their health impacts.

Traditionally, studies have either examined organic or inorganic chemical exposures but rarely both concurrently. This separation of scientific inquiry does not reflect the reality that humans are simultaneously exposed to both organics and inorganics from our environment. An important example is tobacco smoke, which contains many chemicals from both these classes.[117] It is not only that metals and organics share common exposure pathways, but they also share common *biochemical* pathways once they enter the human body. For example, phthalates are known for their endocrine disrupting effects and also influence inflammatory pathways.[118] Metals, including Pb, also affect these pathways.[118,119] This is just one of many examples where organics and inorganics can disrupt the homeostasis of the same physiological process. Given the improvements in exposure biology and statistical methods, it is now an appropriate time to jointly study organic and inorganic exposures.

Teeth offer a unique matrix to reconstruct an individual's history of environmental exposure during critical windows of development. In addition, naturally shed primary teeth can be collected around the time of clinical assessment of caries. This enables more cost-effective study designs to be used as participants do not need to be followed prospectively to collect early life exposure measures, significantly reducing the expense and time of studies. The high dimensional data generated from tooth analysis (multiple environmental measures over multiple time points) require novel statistical approaches to investigate associations with the microbiome and oral health outcomes.

## New statistical methods are needed to understand the dynamics of the microbiome and its interaction with the environment

Detection of environmental influences on the oral microbiome and their relationship to caries has proved difficult when using either 16S or metagenomic data. This may be due to the high level of noise within oral microbiome data sets, having great inter-individual variation, the general statistical problems of analysing sparsely distributed microbiome datasets and the lack of methods which identify the influence of factors, both in isolation and their interactions on the oral microbiome. In the oral microbiome field, both univariate and multivariate methods have been applied to examine the relationship of the oral microbiome to caries and other factors, as detailed in Table 1.

Microbiome data analysis currently faces analytical challenges because of the inherent characteristics of the data that are sparse, compositional and multivariate.[120] Existing analyses have mostly been limited to univariate methods, correlation analyses and non-parametric tests. While univariate methods are limited in their interpretation (since they test each taxon independently), multivariate methods including permutational multivariate analysis of variance (PERMANOVA), or analysis of similarities (ANOSIM) only give limited insight on differences between sample groups, rather than particular group of taxa that drive these differences.[121] These methods have been widely used in the oral microbiome field and have highlighted overall compositional differences in the oral microbiome related to caries and early life events,[17] and e-cigarette usage.[76] Emerging multivariate non-parametric methods based on linear discriminant models, such as sparse PLS-DA[122] might be more suitable for analysis and efficient for high-throughput data. This method revealed that the most discriminatory species for caries, from 16S analysis of saliva, was *S. mutans*.[19] However, the latest developments in this area currently do not adjust for covariates or confounders such as gender, medication history, disease activity, sample site, ethnicity, diet, gender or BMI, to list a few.[123] Accounting for the compositionality nature of the data also poses challenges in data analysis.[124] Methods such as ANCOM and variants,[125,126] Aldex[127] or such as PLS-DA use the logratio transformation techniques to convert microbiome data and remove compositionality constraints for better suited analyses. More recent developments have been focusing on ratios of taxa or penalised regression models allowing to identify microbial signatures.[128] These signatures enable to capture interrelated changes of microbial compositions that would be ignored if bacteria are considered

**TABLE 1** Statistical methods applied in the oral microbiome field on NGS produced data

| Statistic/Tool | Description | Statistical model | Multivariable association | Variables assessed on the oral microbiome | References |
|---|---|---|---|---|---|
| Mann-Whitney U test, Kruskal-Wallis test, Wilcoxon signed-rank test | Tests overall differences between groups based on individual taxa/features or summary statistics, such as alpha diversity metrics | Non-parametric | No | Caries, smoking | 13,17,31,32,74 |
| PERMANOVA | Analysis of variance using distance matrices to examine variation in overall microbiome composition | Non-parametric | Yes | Age, breast/formula feeding, caries, delivery mode, electronic cigarettes, education background, height, smoking and sex | 17,31,74,76 |
| DESeq2 | Tests for differentially abundant features using normalized counts between one or more predetermined groups | Negative binomial | Yes | Age, caries, smoking and sex | 39,74 |
| LEfSe | Identifies differentially abundant features and their importance to describing differences between two or more predetermined groups. Combines standard statistical tests with linear discriminant analysis | Non-parametric | No | Caries | 13,17 |
| sPLSDA | Identifies in a multiclass framework differentially abundant features and their importance to describing differences between two or more predetermined groups | Non-parametric | No | Caries | 19 |
| ANOISM | Tests for similarities between groups based on distances matrices | Non-parametric | Yes | Caries, electronic cigarettes | 31,76 |
| DEICODE | Links features with groupings observed in distance matrixes, such as those generated from beta diversity analyses | Non-parametric | Yes | Caries | 32 |
| Random forest | Machine learning algorithm to identify features and their importance to discriminating between two or more groups | Non-parametric | Yes | Electronic cigarettes | 76 |

Abbreviations: NGS, next generation sequencing; PERMANOVA, permutational multivariate analysis of variance; LEfSe, Linear discriminant analysis effect size analysis; sPLSDA, partial least square discriminant analysis; ANOISM, analysis of similarities.

independently in the analysis. However, further causal inference models for longitudinal data are needed to fully understand the dynamics of microbiome.

Data integration of the microbiome with the exposome and other data types can provide a holistic and more complete picture of the oral microbiome compared with the analysis of single 'omics data. As such, this represents a radical paradigm change from the traditional analysis of single microbial markers, or single omics signatures. Analytical challenges include the heterogeneity between data sets which differ in nature and scale and a high risk of overfitting as the number of features is much greater than the number of individuals. In addition, the high correlation structure within a dataset contributes to a decrease in statistical power, especially if one is interested in identifying discriminatory signatures. Different types of data integration methods have been proposed based on dimension reduction using matrix factorisation,[129,130] network based analyses,[131] machine learning techniques or Bayesian approaches to identify multi-omics signatures associated with a phenotypic outcome. However, most approaches have not been designed for microbiome data specifically and require further developments.[132,133] One of the great challenges in data integration is to distinguish causal from correlated changes in the context of disease. Prospective multi-omics as well as time-course studies with novel statistical developments will help address this challenge and refine biomarker candidates.

## CONCLUSION

While there have been decades of research into the microbial and environmental causes of dental caries in childhood, we are still yet to resolve the disease etiology, the interaction between these two major factors in the disease, and how these factors may change during childhood development in their contribution to caries. This inability to elucidate the microbial cause of caries may relate to the diseases complex, polymicrobial nature, which is only during the last decade being revealed with the development of NGS and metagenomic approaches. Our lack of knowledge of the early environments' effects on the microbiome and how this relates to caries outcomes in childhood has been hampered by our inability to directly assess the fetal and early postnatal environment. However, "exposome" analysis of deciduous

teeth may provide a direct window into this early life period. This in-depth approach to describing both the microbial and environmental factors in childhood will only be useful to understanding these factors' role in caries if a multivariate, data integration approach is applied. New methods are required that integrate information about the microbiome, chemicals and metabolome data, to enable identification of microbiome and environmental features which influence caries, either in synergy or isolation and how these modulate over time in childhood. This data integration approach, which is nuanced in relation to the environment and microbiome, is likely to lead to a new model of caries development. This approach and the development of a new model of disease can be translated into actionable and more targeted interventions to reduce caries prevalence, and with further research building upon these findings for other oral diseases.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

Data sharing not applicable—no new data generated.

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