

Evolution of the Oral Microbiome and Dental Caries

Christina J Adler¹ · Gina V Browne^{1,2} · Smitha Sukumar¹ · Toby Hughes³

Published online: 7 July 2017
© Springer International Publishing AG 2017

Abstract

Purpose of Review Our paper reviews how dietary changes during human evolution have contributed to the increased incidence and prevalence of dental caries in modern populations by shifting the oral microbiome from a “healthy” to “cariou” state. We addressed two questions: (1) what is the microbial difference between the states of health and caries, and (2) how has the development of an agricultural diet impacted the oral microbiome?

Recent Findings The application of next-generation sequencing has revealed the complexity of the oral microbiome and lack of a simple compositional difference between health and caries. Genetic analysis of the oral microbiome from contemporary and ancient humans has shown that the introduction of agriculture was associated with increased frequency of caries-related bacteria and the evolution of the key cariou pathogen, *Streptococcus mutans*.

Summary The adoption of an agricultural diet has contributed to a modern rise in caries by changing the oral microbiome ecology. Identification of evolutionary factors that have influenced the oral microbiome in health and caries, and how the two states differ functionally, as opposed to compositionally, may help to reduce the current burden of caries.

Keywords Oral microbiome · Dental caries · Evolution · Diet · Agriculture · Hunter-gatherer

Introduction

Dental caries is one of the most common diseases in humans. Worldwide, caries affects nearly all adults during their lifetime, and among children, it occurs at five times the frequency of the second most prevalent disease, asthma [1]. Dental caries is a polymicrobial infection, primarily caused by acidogenic oral bacteria [2]. Caries-causing bacteria are members of the human oral microbiome, which refers to the diverse community of microbes that live on our teeth, tongue, oral mucosa, and in the saliva [3]. This microbial community encompasses both commensal and pathogenic microorganisms, including archaea, bacteria, fungi, and viruses, which exist in both planktonic and biofilm forms [4]. The oral microbiome plays an important role in both oral and systemic health. For example, associations have been found between oral microbially mediated diseases, such as periodontal disease, and cardiovascular disease [5], diabetes [6], and pre-term births [7].

In humans, the ubiquitous nature of oral diseases caused by the oral microbiome, such as caries, is a “modern” phenomenon, postulated to be associated with the introduction of an agricultural diet. The development of agriculture, which began approximately 10,000 years ago in the Near East, resulted in humans consuming a diet of domesticated plants and animals [8], with greater carbohydrate content compared to an earlier hunter-gatherer diet [9]. The adoption of an agricultural lifestyle had significant and largely negative effects on human health [10], with increased levels of malnutrition [11] and infectious diseases [12] observed among agriculturalists compared to hunter-gatherers. This decline in health was also observed in the oral environment, with an increased prevalence

This article is part of the Topical Collection on *Microbiology*

✉ Christina J Adler
christina.adler@sydney.edu.au

¹ Institute of Dental Research, The Westmead Millennium Institute for Medical Research, Faculty of Dentistry, The University of Sydney, Westmead, NSW, Australia

² Westmead Centre for Oral Health, Westmead, NSW, Australia

³ School of Dentistry, The University of Adelaide, Adelaide, South Australia, Australia

of pathologies such as caries among prehistoric human remains from agricultural societies [13]. Caries has been observed only rarely among hunter-gatherer populations [13] and early hominids, such as *Australopithecus* [14]. Recent genetic analysis of the human oral microbiome [15, 16, 17–19] has suggested that the hunter-gatherer to agriculturalist dietary transition altered the composition of the oral microbial community, making it more prone to causing the development of caries.

This review discusses recent genetic research examining how diet has been a driving evolutionary force on the oral microbiome, shifting it towards a caries-promoting state and contributing to the ubiquitous nature of caries among modern humans. In this review, we sought to address two questions: (1) what is the microbial difference between health and caries, and (2) how has the development of an agricultural diet impacted the oral microbiome?

Oral Microbiome in Health and Caries

Oral Microbiome

The oral microbiome incorporates a huge range of microbes, second only in complexity to the gut [20]. Molecular methods have been chiefly responsible for revealing this high level of diversity in the oral microbiome [20]. Culture-based methods were unable to provide a comprehensive picture of the oral microbiome, as approximately 50% of oral bacteria are yet to be cultured [3]. Culture-independent, molecular methods for microbial identification involve sequencing microbial DNA either directly or which has been amplified with a barcoding gene, such as 16S for bacteria and archaea, and ITS for fungi [21]. In particular, the advent of high-throughput sequencing, collectively termed next-generation sequencing (NGS), has enabled in-depth analysis of raw or amplified DNA from microbial communities such as the oral microbiome. NGS has shown that the oral microbiome is dominated in load and diversity by bacteria, with over 1000 bacterial species identified to date [3]. In-depth sequencing has also revealed that the oral microbiome houses a diverse array of over 50 fungi [22] and a vast assortment of viruses, dominated by bacteriophages [23]. Archaea make up a small proportion of the oral microbiome and are primarily methanogens [24]. Given the dominance of bacteria in the oral microbiome and their known role in caries, this review will focus on recent genetic findings about the role of bacteria in the caries microbiome, and how this has changed during human evolution.

Health–Caries Microbiome Transition

Information about the composition of the oral microbiome in health and caries provides a basis for interpreting how this

microbial community has evolved and contributed to the current high prevalence of caries. The identified microbiota associated with caries continues to change as we obtain greater depth and breadth of microbial information. Traditional techniques (culture and culture-independent) have provided low-level-resolution information about the microbiota associated with caries. Culture studies found *Streptococcus mutans* to be the chief pathogen associated with caries [25, 26], in addition to *Lactobacillus* and *Actinomyces* species [26, 27]. Culture-independent research (e.g., clonal analysis of the 16S gene) has supported the findings from culture studies [2, 28, 29], revealing, however, a greater diversity of bacteria associated with caries [2, 28, 29]. These included *S. mutans*, non-*mutans* streptococci, and members of the genera *Actinomyces*, *Bifidobacterium*, *Lactobacillus*, *Propionibacterium*, *Veillonella*, and *Atopobium* [2]. In comparison to traditional techniques, NGS studies of the oral microbiome have found that caries is more complicated than being caused by the enrichment of a small selection of species [30–32]. Yang and colleagues examined the oral microbiome in healthy ($n = 26$) and caries-active ($n = 19$) adults by pyrosequencing the 16S gene [30]. There were no “caries-specific” bacteria found that were completely absent in health. Instead, the study demonstrated that there were shifts in the abundance of bacteria, particularly *Prevotella* species, between health and caries.

Revelations that the microbial difference between health and caries represents a gradual change across a spectrum has been supported by NGS studies examining the oral microbiome at different stages of the disease. Dental caries begins as a white spot lesion on the enamel surface, which can progress to a cavitated enamel lesion, further extending into the dentine and eventually the pulp. In a study revealing the shifting microbial profile between health and caries, the microbiome was compared, using a 16S–NGS approach, between caries-free ($n = 30$) and caries-active ($n = 30$) children [33]. The caries-active children included individuals with white spot lesions, and enamel and dentine cavities. Bacterial genera were found to reduce in diversity from health to caries, and as caries progressed. The healthy individuals had a significant enrichment of a range of bacterial genera, including *Capnocytophaga*, *Fusobacterium*, *Porphyromonas*, *Abiotrophia*, *Comamonas*, *Tannerella*, *Eikenella*, *Paludibacter*, *Treponema*, *Actinobaculum*, *Stenotrophomonas*, *Aestuariimicrobium*, and *Peptococcus*. In comparison, caries-affected individuals had a smaller number of enriched bacteria, which changed as the lesion progressed, *Actinomyces* dominating white spot lesions, *Streptococcus* dominating enamel lesions, and *Lactobacilli* being abundant among dentine lesions. The dominance of *Lactobacilli* species in dentine lesions has also been replicated in NGS studies of carious dentine in adults [31, 32]. These in-depth sequencing studies have revealed there is a gradual change in the oral microbiome makeup between health and caries as opposed to a simple compositional change.

Composition Versus Function

The studies above all examined changes in the composition of the oral microbiome, taking a “who’s there?” approach. Recent genetic research of the oral microbiome has indicated that the “what are they doing?” approach of assessing functional differences between health and caries may be more clinically relevant [34••, 35]. The function of the oral microbiome can be assessed by directly sequencing the majority of DNA present in a sample, capturing the vast array of different genes from the different bacterial species. This is called metagenomic sequencing. Identification of the captured genes can be used to predict the functional ability of the oral microbiome sample being assessed. To date, only a few small-scale studies have been performed to assess the metagenomic difference between health and caries [34••, 35], due to the order of magnitude greater cost of metagenomic sequencing compared to NGS of the 16S gene. However, these limited numbers of studies have revealed promising results. Belda-Ferre and colleagues [34••] compared the oral metagenome in two healthy and four caries-affected individuals. They found the metagenome of the healthy individuals to be enriched with genes involved in anti-bacterial functions relative to that of the caries-affected individuals. This difference in the health and caries metagenome was also found in a study of four individuals [35]. These metagenomic studies, due to their small sample size, cannot determine the degree of inter-individual variation, nor predict whether it will be large, as seen in 16S analyses. Hence, larger-scale studies examining the metagenome in health and caries are required to assess the functional difference between health states, and whether individual variation is significant.

Evolutionary Forces Acting on the Oral Microbiome

The transition from a “health” to “caries”-associated oral microbiome is influenced by a range of factors that can be broadly separated into those related to an individual’s genetic makeup and those related to an individual’s environment, or lifestyle. Lifestyle factors play a substantial role in caries development, estimated to account for between 40 and 70% of variation in the phenotype from twin studies [36, 37]. Some lifestyle factors influencing the development of a caries-inducing oral microbiome include an individual’s diet [38, 39], oral hygiene, and medication usage [39]. In terms of human evolution, the adoption of an agricultural lifestyle, with a cereal-based diet, approximately 10,000 years ago is thought to have contributed to the ubiquitous state of caries in the modern human population. It has been observed that the prevalence of caries has increased among humans living an agricultural, compared to hunter-gatherer, lifestyle [13, 14]. If the change from a hunter-gatherer to agriculturalist diet has contributed to the high caries rate observed

among contemporary humans, we would expect to see a shift in the composition and function of the oral microbiome from a “health” to “caries”-promoting state. The next section will review evidence regarding how the hunter-gatherer to agriculturalist lifestyle transition has impacted the oral microbiome, and how this relates to caries development.

Hunter-Gatherer to Agriculturalist Dietary Transition

The composition of the oral microbiome has been found to vary between humans living a hunter-gatherer lifestyle compared to those living an agricultural lifestyle. A number of studies have been undertaken, using the 16S-NGS approach, to compare the oral microbiome makeup of hunter-gatherers to agriculturalists from both prehistoric [15•] and modern [17–19••] populations. The oral microbiome of prehistoric humans has been examined through the genetic analysis of dental calculus from European hunter-gatherer and agriculturalist populations [15•]. The oral microbiome of modern hunter-gatherers examined includes the Batwa Africans [17] and South American, Amerindian [18, 19••] groups. Both prehistoric and modern hunter-gatherers were found to differ from humans living an agricultural or western lifestyle in the overall composition of the oral microbiome, in terms of phylogenetic structure. In the prehistoric European [15•] and modern Batwa [17] and Amerindian [19••] hunter-gatherers, the varied phylogenetic structure in the oral microbiome was indicated by a distinct clustering of these groups away from agriculturalists on principal component analysis (PCoA) plots of Unifrac analyses, which displays the degree of shared bacterial sequences between individuals as a distance. Of interest is which bacteria are causing the overall change in the oral microbiome composition of hunter-gatherers compared to agriculturalists, and what does this mean for modern oral health and dental caries development?

The compositional change from hunter-gatherer to agriculturalists has been found to be associated with a decline in abundance of “health”-associated oral bacteria, and an increase in taxa associated with caries development. For example, prehistoric European agriculturalists were discriminated from prehistoric hunter-gatherers by an increased abundance of bacteria associated with caries [15•]. Random forest analysis was used to identify the taxa that discriminate the prehistoric groups living different lifestyles. This analysis showed that *Clostridia* taxa, such as *Clostridiales* and the non-pathogenic oral microbial family *Ruminococcaceae*, were predictive of hunter-gatherer microbial communities compared to early agriculturalists. In comparison, ancient farming groups were discriminated by both non-pathogenic taxa, such as *Clostridiales Incertae Sedis*, and decay-associated *Veillonellaceae*. A shift from more “health” to “caries”-associated oral bacteria was also observed when comparing modern hunter-gatherers to populations living an agricultural or western lifestyle. For example, Amerindian

hunter-gatherers were found to be discriminated from individuals from the USA by an increased abundance of non-caries-associated bacteria, including *Fusobacterium*, *Leptotrichiaceae*, and *Porphyromonas* [19••]. In comparison, US subjects had a higher abundance of bacteria found to be associated with caries, such as *Actinomyces* and *Stenotrophomonas* [19••]. It is important to note that the modern hunter-gatherer populations examined, while indicative of the impact of this diet type, are not strictly living a traditional lifestyle. For example, the Amerindian population assessed had a strong history of tobacco usage, which may have influenced the oral microbiome composition [19••].

The association between the adoption of a cereal-based diet and the development of a caries-causing oral microbiome has been suggested by the increase in abundance [15•] and population expansion [16•] of the caries-initiating bacteria, *S. mutans* with the development of agriculture. This bacterium was observed in greater abundance among ancient agriculturists compared to that among hunter-gatherers [15•]. Furthermore, maximum likelihood analysis of complete genomes from 57 *S. mutans* clinical strains indicated that this bacteria started expanding exponentially approximately 10,000 years ago, which coincides with the development of agriculture in the Near East [16•]. The impact of a more carbohydrate-based diet on the expansion of *S. mutans* is also reflected in the genes found to be under positive selection, which were involved in sugar metabolism and acid tolerance [16•]. These findings indicate that the adoption of a cereal-based diet placed selective pressure on the oral microbiome, making it more cariogenic.

The above-discussed studies primarily focused on compositional changes in the oral microbiome, as opposed to functional changes, which as previously detailed may be more clinically relevant to the health-caries transition. A landmark study examining functional changes in the oral metagenome of ancient hunter-gatherers compared oral bacterial sequences obtained from the dental calculus of five Neanderthals to ancient agriculturists and modern humans [40••]. This study revealed that the 16S–NGS approach did not capture the complete microbial diversity when compared to the metagenomic sequence data. However, akin to the previous 16S results showing an overall change in the oral microbiome composition with adoption of a cereal-based diet, there was an overall change in the function of the oral microbiome. Hierarchical clustering of the oral metagenomic sequence data revealed a split between hunter-gatherers and agriculturists, indicating that diet change was associated with a shift in the functional potential of the oral microbiome.

Other Lifestyle Factors

The impact of lifestyle changes during human evolution on the oral microbiome is not limited to diet. In particular,

the development of oral hygiene practices and introduction of medications have most likely also contributed to the increasingly cariogenic state of the oral microbiome. However, the direct impact of these changes is more difficult to assess, given their more gradual introduction compared to the hunter-gatherer to agricultural dietary transition. Metagenomic analysis of the dental calculus from Neanderthals has indicated that oral hygiene and medications have impacted the makeup and function of the oral microbiome [40••]. This study was able to recover a draft genome of a new archaeal species, *Methanobrevibacter oralis* subsp. *neandertalensis* from the Neanderthal dental calculus. Genomic comparison between the modern human-associated *Methanobrevibacter oralis* and ancient *Methanobrevibacter oralis* subsp. *neandertalensis* revealed the ancient sequence lacked genes encoding antiseptic resistance (*qacE*) and bacterial immunity (CRISPR Cas2 and Cas6) that potentially reflect hygiene differences between modern humans and Neanderthals.

Conclusion

Genetic analysis of the oral microbiome from ancient and modern humans has revealed that the development of an agricultural diet has been associated with a change in the composition and function of this microbial community. Some of these changes, such as the increased abundance and population expansion of caries-associated bacteria, have meant our oral microbiome is now more prone to developing caries. However, it is difficult to assess how past lifestyle changes have influenced current oral health, as the microbiome transition between health and caries is still unresolved. In terms of compositional change in the oral microbiome between health and caries, due to the large degree of inter-individual variation seen between individuals' oral microbiome profiles, population-level studies are required to resolve this question. In terms of functional change in the oral microbiome between health and caries, again, there is a need for studies on a greater number of individuals, and from varied populations with different lifestyles. At present, genetic studies have revealed that lifestyle changes, such as diet, have impacted our oral microbiome and oral health during human history.

Compliance with Ethical Standards

Conflict of Interest All authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

References

Papers of particular interest, published recently, have been highlighted as:

- Of importance
- Of major importance

1. Petersen PE, Bourgeois D, Ogawa H, Estupinan-Day S, Ndiaye C. The global burden of oral diseases and risks to oral health. *Bull World Health Organ.* 2005;83(9):661–9.
2. Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, Dewhirst FE, et al. Bacteria of dental caries in primary and permanent teeth in children and young adults. *J Clin Microbiol.* 2008;46(4):1407–17. doi:10.1128/JCM.01410-07.
3. Dewhirst FE, Chen T, Izard J, Paster BJ, Tanner AC, Yu WH, et al. The human oral microbiome. *J Bacteriol.* 2010;192(19):5002–17. doi:10.1128/JB.00542-10.
4. Lederberg J, Mc Cray A. ‘Ome Sweet ‘Omics—a genealogical treasury of words. Philadelphia: Scientist Inc; 2001.
5. D’Aiuto F, Orlandi M, Gunsolley JC. Evidence that periodontal treatment improves biomarkers and CVD outcomes. *J Clin Periodontol.* 2013;40(Suppl 14):S85–105. doi:10.1111/jcpe.12061.
6. Chavarry NG, Vettore MV, Sansone C, Sheiham A. The relationship between diabetes mellitus and destructive periodontal disease: a meta-analysis. *Oral Health Prev Dent.* 2009;7(2):107–27.
7. Lopez NJ, Smith PC, Gutierrez J. Higher risk of preterm birth and low birth weight in women with periodontal disease. *J Dent Res.* 2002;81(1):58–63.
8. Braidwood RJ, Howe B, Reed CA. The Iranian Prehistoric Project: new problems arise as more is learned of the first attempts at food production and settled village life. *Science.* 1961;133(3469):2008–10. doi:10.1126/science.133.3469.2008.
9. Richards MP, Schulting RJ, Hedges RE. Archaeology: sharp shift in diet at onset of Neolithic. *Nature.* 2003;425(6956):366. doi:10.1038/425366a.
10. Cordain L, Eaton SB, Sebastian A, Mann N, Lindeberg S, Watkins BA, et al. Origins and evolution of the Western diet: health implications for the 21st century. *Am J Clin Nutr.* 2005;81(2):341–54.
11. Larsen CS. Biological changes in human-populations with agriculture. *Annu Rev Anthropol.* 1995;24:185–213.
12. Wolfe ND, Dunavan CP, Diamond J. Origins of major human infectious diseases. *Nature.* 2007;447(7142):279–83. doi:10.1038/nature05775.
13. Smith BH. Patterns of molar wear in hunter-gatherers and agriculturalists. *Am J Phys Anthropol.* 1984;63(1):39–56. doi:10.1002/ajpa.1330630107.
14. Grine FE, Gwinnett AJ, Oaks JH. Early hominid dental pathology: interproximal caries in 1.5 million-year-old *Paranthropus robustus* from Swartkrans. *Arch Oral Biol.* 1990;35(5):381–6.
15. Adler CJ, Dobney K, Weyrich LS, Kaidonis J, Walker AW, Haak W, et al. Sequencing ancient calcified dental plaque shows changes in oral microbiota with dietary shifts of the Neolithic and Industrial revolutions. *Nat Genet.* 2013;45(4):450–5. doi:10.1038/ng.2536. **This manuscript represents the first study to genetically analyse the composition of the oral microbiome from ancient dental calculus.**
16. Comejo OE, Lefebure T, Bitar PD, Lang P, Richards VP, Eilertson K, et al. Evolutionary and population genomics of the cavity causing bacteria *Streptococcus mutans*. *Mol Biol Evol.* 2013;30(4):881–93. doi:10.1093/molbev/mss278. **This article presents a comprehensive picture of the population history of *Streptococcus mutans*.**
17. Nasidze I, Li J, Schroeder R, Creasey JL, Li M, Stoneking M. High diversity of the saliva microbiome in Batwa Pygmies. *PLoS One.* 2011;6(8):e23352. doi:10.1371/journal.pone.0023352.
18. Contreras M, Costello EK, Hidalgo G, Magris M, Knight R, Dominguez-Bello MG. The bacterial microbiota in the oral mucosa of rural Amerindians. *Microbiology.* 2010;156(Pt 11):3282–7. doi:10.1099/mic.0.043174-0.
19. Clemente JC, Pehrsson EC, Blaser MJ, Sandhu K, Gao Z, Wang B, et al. The microbiome of uncontacted Amerindians. *Sci Adv.* 2015;1(3) doi:10.1126/sciadv.1500183. **This paper presented the first large genetic study of the human microbiome in hunter-gatherer populations.**
20. Kilian M, Chapple IL, Hannig M, Marsh PD, Meuric V, Pedersen AM, et al. The oral microbiome—an update for oral healthcare professionals. *Br Dent J.* 2016;221(10):657–66. doi:10.1038/sj.bdj.2016.865.
21. Walters W, Hyde ER, Berg-Lyons D, Ackermann G, Humphrey G, Parada A, et al. Improved bacterial 16S rRNA gene (V4 and V4-5) and fungal internal transcribed spacer marker gene primers for microbial community surveys. *mSystems.* 2016;1(1) doi:10.1128/mSystems.00009-15.
22. Ghannoum MA, Jurevic RJ, Mukherjee PK, Cui F, Sikaroodi M, Naqvi A, et al. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS Pathog.* 2010;6(1):e1000713. doi:10.1371/journal.ppat.1000713.
23. Pride DT, Salzman J, Haynes M, Rohwer F, Davis-Long C, White RA 3rd, et al. Evidence of a robust resident bacteriophage population revealed through analysis of the human salivary virome. *ISME J.* 2012;6(5):915–26. doi:10.1038/ismej.2011.169.
24. Matarazzo F, Ribeiro AC, Feres M, Faveri M, Mayer MP. Diversity and quantitative analysis of Archaea in aggressive periodontitis and periodontally healthy subjects. *J Clin Periodontol.* 2011;38(7):621–7. doi:10.1111/j.1600-051X.2011.01734.x.
25. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev.* 1986;50(4):353–80.
26. Loesche WJ, Grenier E. Detection of *Streptococcus mutans* in plaque samples by the direct fluorescent antibody test. *J Dent Res.* 1976;55:87–93.
27. van Houte J. Role of micro-organisms in caries etiology. *J Dent Res.* 1994;73(3):672–81.
28. Munson MA, Banerjee A, Watson TF, Wade WG. Molecular analysis of the microflora associated with dental caries. *J Clin Microbiol.* 2004;42(7):3023–9. doi:10.1128/JCM.42.7.3023-3029.2004.
29. Becker MR, Paster BJ, Leys EJ, Moeschberger ML, Kenyon SG, Galvin JL, et al. Molecular analysis of bacterial species associated with childhood caries. *J Clin Microbiol.* 2002;40(3):1001–9.
30. Yang F, Zeng X, Ning K, Liu KL, Lo CC, Wang W, et al. Saliva microbiomes distinguish caries-active from healthy human populations. *ISME J.* 2012;6(1):1–10. doi:10.1038/ismej.2011.71. **This manuscript presented the first large-scale, next-generation sequencing analysis of the oral microbiome composition in health and caries.**
31. Kianoush N, Adler CJ, Nguyen KA, Browne GV, Simonian M, Hunter N. Bacterial profile of dentine caries and the impact of pH on bacterial population diversity. *PLoS One.* 2014;9(3):e92940. doi:10.1371/journal.pone.0092940.
32. Rocas IN, Alves FR, Rachid CT, Lima KC, Assuncao IV, Gomes PN, et al. Microbiome of deep dentinal caries lesions in teeth with symptomatic irreversible pulpitis. *PLoS One.* 2016;11(5):e0154653. doi:10.1371/journal.pone.0154653.
33. Jiang W, Ling Z, Lin X, Chen Y, Zhang J, Yu J, et al. Pyrosequencing analysis of oral microbiota shifting in various caries states in childhood. *Microb Ecol.* 2014;67(4):962–9. doi:10.1007/s00248-014-0372-y.

34. Belda-Ferre P, Alcaraz LD, Cabrera-Rubio R, Romero H, Simon-Soro A, Pignatelli M, et al. The oral metagenome in health and disease. *ISME J*. 2012;6(1):46–56. doi:10.1038/ismej.2011.85. **This manuscript presented the first next-generation sequencing analysis of the oral metagenome in health and caries.**
35. Yang F, Ning K, Zeng X, Zhou Q, Su X, Yuan X. Characterization of saliva microbiota's functional feature based on metagenomic sequencing. *Spring*. 2016;5(1):2098. doi:10.1186/s40064-016-3728-6.
36. Boraas JC, Messer LB, Till MJ. A genetic contribution to dental caries, occlusion, and morphology as demonstrated by twins reared apart. *J Dent Res*. 1988;67(9):1150–5. doi:10.1177/00220345880670090201.
37. Wang X, Shaffer JR, Weyant RJ, Cuenco KT, DeSensi RS, Crout R, et al. Genes and their effects on dental caries may differ between primary and permanent dentitions. *Caries Res*. 2010;44(3):277–84. doi:10.1159/000314676.
38. Holbrook WP, Amadottir IB, Takazoe I, Birkhed D, Frostell G. Longitudinal study of caries, cariogenic bacteria and diet in children just before and after starting school. *Eur J Oral Sci*. 1995;103(1):42–5.
39. Seow WK, Clifford H, Battistutta D, Morawska A, Holcombe T. Case-control study of early childhood caries in Australia. *Caries Res*. 2009;43(1):25–35. doi:10.1159/000189704.
40. Weyrich LS, Duchene S, Soubrier J, Arriola L, Llamas B, Breen J, et al. Neanderthal behaviour, diet, and disease inferred from ancient DNA in dental calculus. *Nature*. 2017; doi:10.1038/nature21674. **This manuscript represents the first study to genetically analyse the oral metagenome of Neanderthals from ancient dental calculus.**