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Sequencing ancient calcified dental plaque shows changes in oral microbiota with dietary shifts of the Neolithic and Industrial revolutions

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The importance of commensal microbes for human health is increasingly recognized[1–5], yet the impacts of evolutionary changes in human diet and culture on commensal microbiota remain almost unknown. Two of the greatest dietary shifts in human evolution involved the adoption of carbohydrate-rich Neolithic (farming) diets[6,7] (beginning ~10,000 years before the present[6,8]) and the more recent advent of industrially processed flour and sugar (in ~1850)[9]. Here, we show that calcified dental plaque (dental calculus) on ancient teeth preserves a detailed genetic record throughout this period. Data from 34 early European skeletons indicate that the transition from hunter-gatherer to farming shifted the oral microbial community to a disease-associated configuration. The composition of oral microbiota remained unexpectedly constant between Neolithic and medieval times, after which (the now ubiquitous) cariogenic bacteria became dominant, apparently during the Industrial Revolution. Modern oral microbiotic ecosystems are markedly less diverse than historic populations, which might be contributing to chronic oral (and other) disease in postindustrial lifestyles.

Commensal microbiota comprise the majority of cells in the body and have a key role in human health[1–5,10]. However, their evolution remains poorly understood, and detailed genetic records from commensal bacteria have yet to be recovered from the archaeological record. Dental calculus is ubiquitous in both present-day and ancient human populations[11], and microscopic analysis has shown that it accurately preserves bacterial morphology over millennia[12–14]. Dental calculus develops when dental plaque, an extremely dense bacterial biofilm[15], becomes mineralized with calcium phosphate[16]. Bacteria in calculus become locked in a crystalline matrix similar to bone[16] (**Supplementary Fig. 1**), with deposits occurring both above and below the gum or gingiva (supra- and subgingivally)[17]. Calculus represents one of the few sources of preserved human and hominid microbiota, and genetic analysis has the potential to create a powerful new record of dietary impacts, health changes and oral pathogen genomic evolution deep into the past. In addition, oral bacteria are transferred vertically from the primary caregiver(s) in early childhood[18] and horizontally between family members later in life[18,19], making archaeological dental calculus a potentially unique means of tracing population structure, movement and admixture in ancient cultures, as well as the spread of diseases.

The increased consumption of domesticated cereals (wheat and barley in the Near East) beginning with the Neolithic period was associated with a marked increase in the prevalence of dental calculus and oral pathology[20]. These oral diseases include dental caries (tooth decay)[1] and periodontal disease (an infection causing damage to the supporting connective tissues of the tooth and resorption of bone)[21], both of which were rare in pre-Neolithic hunter-gatherer societies[20] and early hominins[22]. Caries and periodontal disease are both polymicrobial, plaque-mediated infections, thought to result from perturbation of a healthy, ecologically balanced oral biofilm[23,24] that can occur because of dietary changes, such as the increased consumption of fermentable carbohydrates[25,26]. Caries has become a major endemic disease, affecting 60–90% of school-aged children in industrialized countries, whereas periodontal disease occurs in 5–20% of the adult population worldwide[27]. Notably, oral bacteria are also associated with many systemic diseases, including arthritis[28], cardiovascular disease[3] and diabetes[4], in addition to other diseases of the oral cavity[1,2].

We collected a mixture of supra- and subgingival calculus samples (determined morphologically; **Supplementary Note**) from the teeth of 34 prehistoric European human skeletons (11 males, 11 females and 12 of unknown sex, ranging in age from <20 to >60 years at death; **Supplementary Table 1**), dating from before the Mesolithic period

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Table 1 Archaeological and anthropological samples used in the study

Sample ID	Museum	Group or culture	Period (years BP)	Location
12011	VI-1 B	Hunter-gatherer	Mesolithic/Paraneolithic (7,550–5,450)	Dudka, Poland
12012	VI-7 A	Hunter-gatherer	Mesolithic/Paraneolithic (7,550–5,450)	Dudka, Poland
12013	VI-14 A	Hunter-gatherer	Mesolithic/Paraneolithic (7,550–5,450)	Dudka, Poland
12015	VI-2 D	Hunter-gatherer	Mesolithic/Paraneolithic (7,550–5,450)	Dudka, Poland
12016	VI-1 A	Hunter-gatherer	Mesolithic/Paraneolithic (7,550–5,450)	Dudka, Poland
12017	VI-7 A	Hunter-gatherer	Mesolithic/Paraneolithic (7,550–5,450)	Dudka, Poland
8215	HK2000:4083a, 613.1	LBK	Neolithic (7,400–6,725)	Halberstadt-Sonntagsfeld, Germany
8240	HK2000:4228a, 861	LBK	Neolithic (7,400–6,725)	Halberstadt-Sonntagsfeld, Germany
8247	HK2000:4233a, 870	LBK	Neolithic (7,400–6,725)	Halberstadt-Sonntagsfeld, Germany
8275	HK2000:7374a, 1324	LBK	Neolithic (7,400–6,725)	Halberstadt-Sonntagsfeld, Germany
8277	HK2000:4014b, 413.1	LBK	Neolithic (7,400–6,725)	Halberstadt-Sonntagsfeld, Germany
4331	HK2004:9463a, 6255.1	Bell Beaker	Neolithic (4,450–4,000)	Quedlinburg XII, Germany
9436	HK, 43	LN/BA	Late Neolithic/Bronze Age (4,150–3,600)	Benzingerode-Heimburg, Germany
8890	T82GF	Bronze Age	Bronze Age (4,100–2,800)	Yorkshire, England
8891	14Barrow 163	Bronze Age	Bronze Age (4,100–2,800)	Yorkshire, England
8894	T98	Bronze Age	Bronze Age (4,100–2,800)	Yorkshire, England
8326	2095	Jewbury	Late Medieval (750–650)	York, England
8330	2106	Jewbury	Late Medieval (750–650)	York, England
8332	4440	Jewbury	Late Medieval (750–650)	York, England
8477	2357	Jewbury	Late Medieval (750–650)	York, England
8482	2654	Jewbury	Late Medieval (750–650)	York, England
8814	4161	Jewbury	Late Medieval (750–650)	York, England
8863	4485	Jewbury	Late Medieval (750–650)	York, England
8333	R5287	Raunds Furnells	Early Medieval (1,100–850)	Northamptonshire, England
8335	R5252	Raunds Furnells	Early Medieval (1,100–850)	Northamptonshire, England
8337	R5136	Raunds Furnells	Early Medieval (1,100–850)	Northamptonshire, England
8341	R5206	Raunds Furnells	Early Medieval (1,100–850)	Northamptonshire, England
8868	R5157	Raunds Furnells	Early Medieval (1,100–850)	Northamptonshire, England
8869	R5229	Raunds Furnells	Early Medieval (1,100–850)	Northamptonshire, England
8873	5228	St. Helen-on-the-Walls	Late Medieval (1,000–400)	York, England
8874	5241	St. Helen-on-the-Walls	Late Medieval (1,000–400)	York, England
8877	5113	St. Helen-on-the-Walls	Late Medieval (1,000–400)	York, England
8878	5203	St. Helen-on-the-Walls	Late Medieval (1,000–400)	York, England
8883	5244	St. Helen-on-the-Walls	Late Medieval (1,000–400)	York, England
1	NA	European descent	0	Adelaide, Australia
2	NA	European descent	0	Adelaide, Australia
3	NA	European descent	0	Adelaide, Australia
4	NA	European descent	0	Adelaide, Australia
5	NA	European descent	0	Adelaide, Australia
6	NA	European descent	0	Adelaide, Australia
7	NA	European descent	0	Adelaide, Australia
8	NA	European descent	0	Adelaide, Australia
9	NA	European descent	0	Adelaide, Australia
10	NA	European descent	0	Adelaide, Australia

Mesolithic/Paraneolithic is the terminology used for the transitional cultures of the forest zone of eastern Europe. Further information about the ancient calculus and modern plaque and calculus samples is provided in **Supplementary Table 1**.

(before farming) to the medieval period. The samples were collected from the remains of the last hunter-gatherers in Poland and the earliest farming culture in Europe (the Linear Pottery Culture, LBK), as well as late Neolithic (Bell Beaker Culture), early and later Bronze Age, and medieval rural and urban populations (**Table 1**; description provided in the **Supplementary Note**). All work on ancient DNA was conducted in a physically isolated, specialist laboratory dedicated to ancient environmental and bacterial DNA research at the Australian Centre for Ancient DNA, using strict decontamination and authentication protocols (**Supplementary Note**). We extracted bacterial DNA from sterilized ancient calculus samples ($n = 34$) and generated PCR amplicon libraries of the 16S rRNA gene, targeting three hypervariable regions (V1, V3 and V6) with barcoded primers (**Supplementary Tables 2** and **3**). In addition, primers specific to *Streptococcus mutans* and *Porphyromonas gingivalis* were used to detect oral pathogens in ancient dental calculus (**Supplementary Table 2**). We compared the ancient samples to modern calculus ($n = 6$) and plaque ($n = 13$) samples that were extracted and sequenced in an analogous manner (Online Methods and **Supplementary Tables 3** and **4**). We also extracted and sequenced bacterial DNA from within the teeth that provided the ancient calculus samples to determine the background bacterial contribution of the postmortem depositional environment ($n = 6$; **Supplementary Note**). Amplicons generated from extracted samples and multiple extraction blanks were sequenced using both conventional and pyrosequencing technology. Of the 998,575 sequences generated, we discarded ~50% after quality filtering and

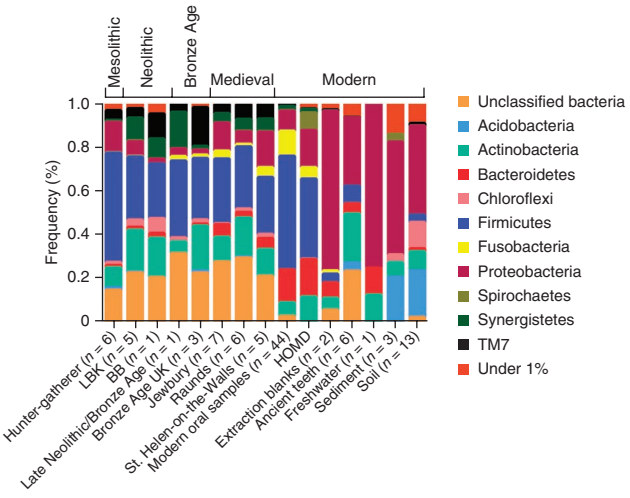

Figure 1 Phylum-level microbial composition of ancient dental calculus deposits. The distribution is similar to that of modern oral samples and distinct from those of non-template controls, ancient human teeth and environmental samples. The phylum frequencies for the V3 region are presented for the ancient calculus samples (BB, Bell Beaker), modern oral samples, which included pyrosequenced (calculus, plaque and saliva[31]) and cloned (plaque[1,2,21]) data, non-template controls (or extraction blanks), ancient human teeth and environmental samples (freshwater, sediments and soils[34–40]) (**Supplementary Table 1**). Phylum frequencies from HOMD were generated from partial and full-length sequences of the 16S rRNA gene. The phyla with a frequency of <1% include ABY1_OD1, AD3, Armatimonadetes, BRC1, CCM11b, Chlamydiae, Chlorobi, Cyanobacteria, Elusimicrobia, Euryarchaeota, Fibrobacteres, GAL15, Gemmatimonadetes, GN02, GN04, GOUTA4, KSB1, Lentisphaerae, NC10, Nitrospirae, NKB19, OP11, OP3, OP9, PAUC34f, Planctomycetes, SBR1093, SC3, SC4, SM2F11, SPAM, Spirochaetes, SR1, Tenericutes, Thermi, TM6, Verrucomicrobia, WPS-2, WS3 and ZB2.

denoising to remove sequences containing PCR and sequencing errors[29], leaving 451,241 sequences (**Supplementary Table 4**).

At the phylum level, the bacterial composition of ancient calculus was similar to that of modern oral samples and sequences from the Human Oral Microbiome Database (HOMD)[30] but markedly distinct from the compositions identified for laboratory reagents (extraction blanks) and environmental samples (soils, sediments and water) and within the ancient teeth themselves (**Fig. 1**, **Supplementary Fig. 2** and **Supplementary Note**). The archaeological calculus was dominated by Firmicutes (33% for the V3 region, which was the most phylogenetically informative fragment; **Supplementary Note**), which was found at a frequency comparable to those in both the HOMD (37%), and modern oral samples (average 50%; **Supplementary Fig. 3**)[1,2,21,31]. Again, the distribution was clearly distinguishable from those generated with the bacterial sequences obtained from extraction blanks (6% Firmicutes, $P = 0.003$), environmental samples (1.6% Firmicutes, $P < 0.001$) and within the ancient teeth (8% Firmicutes, $P < 0.001$), which were all dominated by Proteobacteria (73%, $P = 0.2$; 56%, $P < 0.001$; 31%, $P = 0.005$, respectively). The sequences from the extraction blanks were typical of bacterial communities found in clean-room environments[32] and non-template controls[33]. In addition to Firmicutes, the ancient dental calculus samples contained all 15 phyla commonly found in the modern human oral cavity[30], with high percentages of Actinobacteria (19%), as is observed in modern calculus deposits (7%). We have shown that dental calculus from samples that are thousands of years old preserve representative and informative microbial signatures of past human-associated microbiota.

Phylogenetic analyses of the β diversity, which measures the number of operational taxonomic units (OTUs) that are unique between the groups (**Supplementary Note**), confirmed that the V3 sequences from ancient calculus were clearly more similar to those from modern dental calculus, plaque and saliva samples[1,2,21,31] than to those from environmental samples[34–40] (**Fig. 2a**,**b**, **Supplementary Fig. 4** and **Supplementary Table 5**). Similar patterns were observed for sequences from V1 and V6 (**Supplementary Fig. 5** and **Supplementary Note**). Furthermore, the bacterial sequences of the ancient calculus samples clustered separately from the sequences present within the ancient teeth ($P = 0.002$; **Supplementary Fig. 6** and **Supplementary Table 6**). Overall, these results strongly suggest that DNA sequences from ancient calculus samples are not derived as a result of contamination from the postmortem environment.

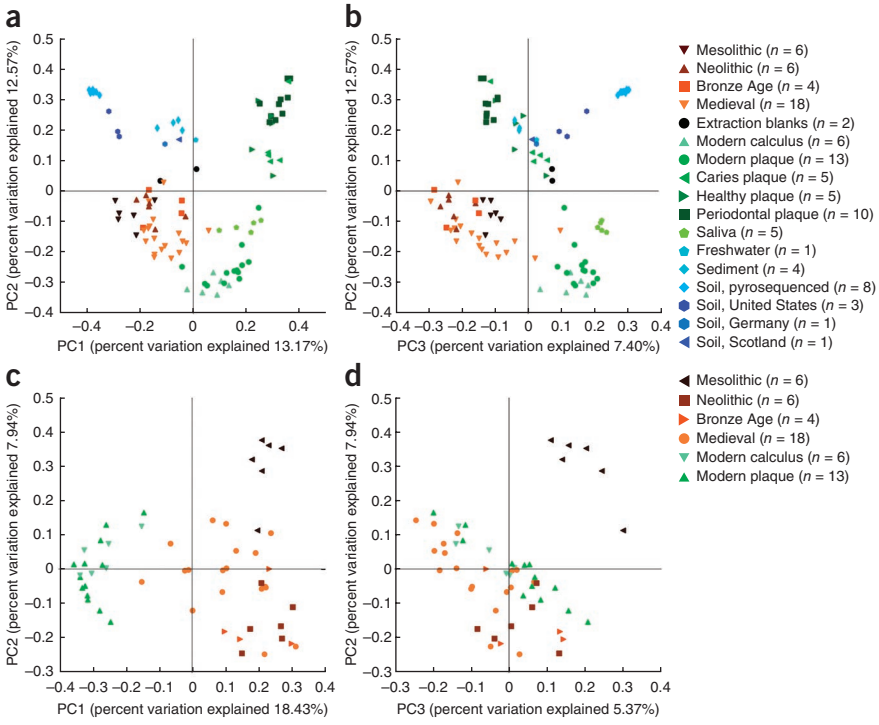

Figure 2 Principal-components plot of β diversity. Principal-components analysis (PCA) shows a close phylogenetic relationship between ancient dental calculus and modern oral samples, both of which are distinct from the non-template controls and environmental samples. β diversity was calculated for all samples (**Supplementary Note**) using the UniFrac metric for the V3 region, and PCA was applied to the unweighted UniFrac distances. (**a**,**b**) Plots of the first and second components (PC1 and PC2) (**a**) and the second and third components (PC2 and PC3) (**b**) from PCA clustered the ancient dental calculus samples with the modern oral pyrosequenced data (calculus, plaque and saliva), which were separated from the environmental samples and extraction blanks. (**c**,**d**) Restricted PCA plots of PC1 and PC2 (**c**) and PC2 and PC3 (**d**) that only include ancient and modern oral pyrosequencing samples separated the hunter-gatherer (Mesolithic) samples from modern, medieval and Neolithic samples.

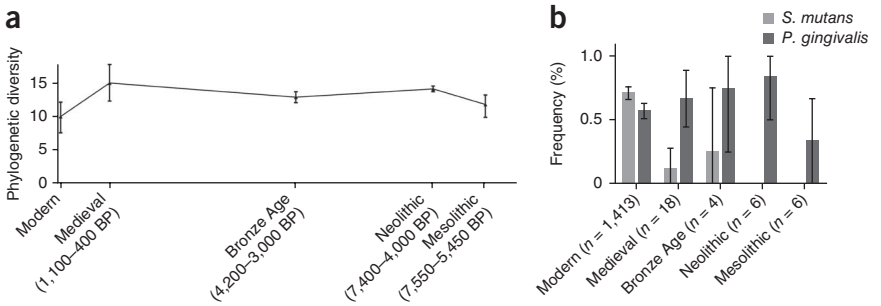

Figure 3 Changes in the diversity and composition of oral microbiota. (**a**) For the V3 region sequences, we estimated the phylogenetic diversity[50] (**Supplementary Note**) of the archaeological dental calculus samples ($n = 34$) and compared them to modern calculus ($n = 6$) and plaque ($n = 13$). We estimated phylogenetic diversity from only classified, Gram-positive bacterial sequences to minimize the influence of taphonomic bias (**Supplementary Note**). Diversity was calculated at a depth of 34 sequences and bootstrapped to assess the robustness of the pattern. Error bars represent bootstrapped diversity values generated by sampling 255 replicates without replacement. BP, years before the present. (**b**) Specific primers were used to amplify sequences unique to the oral pathogens *S. mutans* and *P. gingivalis*. Error bars represent bootstrapped frequencies generated by sampling 255 replicates without replacement.

The temporal transect of ancient dental calculus samples provides the first idea of the timing and nature of change in human oral bacterial composition and diversity over the last 7,500 years. The composition of oral microbiota underwent a distinct shift with the introduction of farming in the early Neolithic period (**Fig. 2c**,**d**), with the earlier hunter-gatherer groups having fewer caries- and periodontal disease–associated taxa (**Fig. 3**). This is consistent with skeletal evidence showing marked increases in periodontal disease[41] following the transition to an agricultural diet, suggesting a major impact on the human oral ecosystem around this time. This is thought to be caused by increased amounts of soft carbohydrate foods compared with hunter-gatherer diets[26]. After the transition to agriculture in the early Neolithic period, there was a notable consistency in the composition of bacteria through the medieval period (~400 years before the present) (**Fig. 3**), in parallel with the broad similarity of food-processing technologies during these times[9]. In contrast, today's oral environment is much less biodiverse and is dominated by potentially cariogenic bacteria (for example, *S. mutans*; **Fig. 3a**,**b**, **Supplementary Figs. 7–9** and **Supplementary Table 7**).

Random forest analysis was used to identify the taxa that discriminate the different time periods (**Supplementary Note**). This analysis showed that Clostridia taxa, such as Clostridiales (importance score = 0.014 ± 0.002) and non-pathogenic oral microbial family Ruminococcaceae (importance score = 0.0035 ± 0.0009), were predictive of hunter-gatherer microbial communities compared to early agriculturists (ratio of baseline error to observed error = 3.5; **Supplementary Table 8**). Farming groups from the Neolithic and medieval periods were discriminated by both non-pathogenic taxa, such as Clostridiales Incertae Sedis (importance score = 0.014 ± 0.003), and decay-associated Veillonellaceae (importance score = 0.012 ± 0.0038). Farming populations also had more periodontal disease–associated taxa, including *P. gingivalis* and members of the *Tannerella* and *Treponema* genera than did hunter-gatherers. Although there is also a strong association between periodontal disease and individual age at death[42], we found periodontal disease–associated taxa across a range of ages, including the youngest individual in the study (3–4 years old, ID 8247).

Random forest analysis showed that only a limited number of taxa distinguished modern oral environments from farming groups in the medieval and Neolithic periods (ratio of baseline error to observed error = 4.0; **Supplementary Table 9**). These taxa include decay-associated Veillonellaceae (importance score = 0.021 ± 0.004), in addition to Lachnospiraceae (importance score = 0.019 ± 0.007) and Actinomycetales (importance score = 0.0013 ± 0.0005). Notably, the frequency of *S. mutans* is significantly higher in modern samples than in preindustrial agricultural samples ($P < 0.0001$; **Fig. 3b** and **Supplementary Note**), indicating that caries-associated bacteria have only become dominant after medieval times. This change is most likely associated with the onset of the Industrial Revolution, which began some 200 years ago and represents the largest change in food production and processing technology since the shift to farming[9]. The Industrial Revolution saw the production of refined grain and concentrated sugar from processed sugar beet and cane[9], generating mono- and disaccharides, which are the main substrates for the microbial fermentation that lowers plaque pH and causes enamel demineralization[26].

Overall, it is clear that modern Europeans have much lower oral microbial diversity than either Mesolithic or preindustrial Neolithic groups ($P < 0.001$; **Supplementary Table 7**), including fewer bacteria associated with good health (Ruminococcaceae), periodontal disease–associated taxa (for example, *P. gingivalis* and members of the *Tannerella* and *Treponema* genera), similar to early agriculturists, and a markedly higher abundance of (now ubiquitous) pathogens

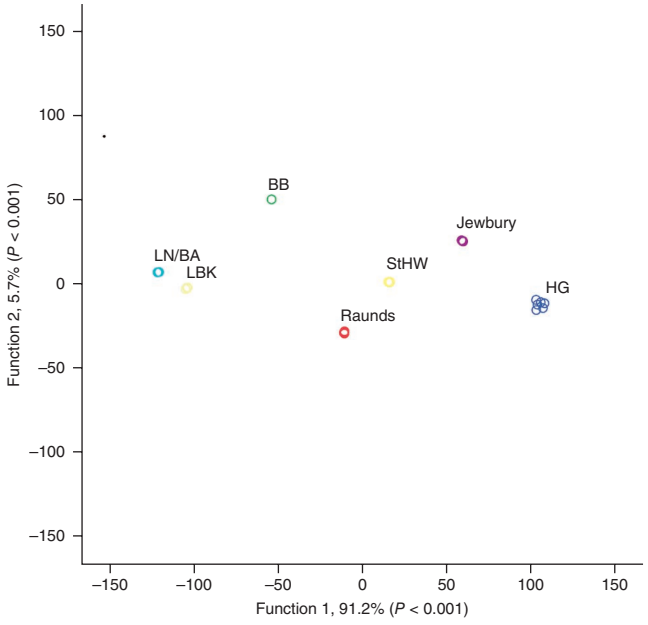

Figure 4 Discriminant analysis of β diversity. Discriminant analysis was applied to the principal coordinates generated from the unweighted UniFrac distances calculated from the V3 region sequences. Each individual is represented by a circle and colored according to archaeological grouping (HG, hunter-gatherer; LN/BA, late Neolithic/Bronze Age; StHW, St. Helen-on-the-Walls). The majority of phylogenetic variation (91.2%) was described by the first discriminant function, showing that individuals from the same archaeological groups cluster according to microbial composition.

such as *S. mutans* (**Fig. 2** and **Supplementary Note**). Perhaps more notably, the decline in overall oral microbial diversity indicates that, over the past few hundred years, the human mouth has become a substantially less biodiverse ecosystem. In both human-associated microbiota[43–45] and macroecological[46,47] contexts, higher phylogenetic diversity is associated with greater ecosystem resilience and productivity. Therefore, the modern oral environment is likely to be less resilient to perturbations[48] in the form of dietary imbalances or invasion[49] by pathogenic bacterial species.

Major changes in carbohydrate intake in human history seem to have affected the ecosystem of the mouth, opening up pathological niches for periodontal disease in the early Neolithic period and caries in the recent past. These data are potentially important for assessing current associations with systemic diseases: for example, it has been proposed that periodontal disease might contribute to the development of diabetes and heart disease[25] through the production of a prolonged inflammatory state[3]. However, although the frequency of these systemic diseases has risen over the last few decades[25], our data show that the abundance of periodontal disease–associated bacteria has been relatively stable since the introduction of farming (for example, *P. gingivalis*; **Fig. 3**). This indicates that, although periodontal disease might contribute to pathogenesis, it is probably not a factor in the rising incidence of these systemic diseases.

Our research has identified a powerful new avenue for bioanthropological research, which promises to provide the first detailed genetic records of the evolution of human microbiota. This provides the potential to directly examine the effects of nutritional and cultural transitions (**Fig. 4**, **Supplementary Figs. 10** and **11,** and **Supplementary Note**) on human health through time and to record the genomic evolution of human commensals and pathogens.

URLs. Human Microbiome Project, http://www.hmpdacc.org/tools_protocols/tools_protocols.php.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Sequence data have been deposited in GenBank under accession ERP002107.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

C.J.A., A.C., K.D., A.W.W., J.P., K.W.A., G.T., J.K. and W.H. designed the study. C.J.A., K.D., K.W.A., A.S., W.H., A.C. and J.K. collected samples. C.J.A. and L.S.W. extracted and amplified DNA from dental calculus. C.J.A. and L.S.W. analyzed sequence data. A.W.W. performed 454 sequencing. C.J.A.B. performed α diversity bootstrapping analyses. C.J.A., A.C. and K.D. wrote the manuscript. All authors discussed the results and contributed to writing the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Further details for the ancient dental calculus samples, including archaeological information, preparation methodology and authentication criteria, are provided in the **Supplementary Note**.

DNA extraction, PCR, cloning and sequencing. We extracted DNA from 0.05–0.2 g of sterilized and powdered ancient dental calculus and included a non-template control for every three extractions. Ancient dental calculus deposits, modern calculus and plaque samples, and non-template controls were lysed in 1 ml of lysis buffer containing 0.5 M EDTA (pH 8), SDS (10%) and Proteinase K (20 mg/ml). Samples in lysis buffer were rotated for 24 h at 55 °C. After sample lysis, DNA was isolated using the QIAamp DNA Investigator kit (Qiagen). DNA was eluted in a final volume of 100 µl and extracts were stored at 4 °C. Tooth samples were extracted using protocols described previously[51]. Independent extractions were not possible owing to the small size of samples; commonly, only one calculus deposit per individual was available for DNA analysis.

PCR was used to amplify microbial DNA in the ancient dental calculus samples, modern oral samples, tooth samples and extraction controls using both primers for the universal microbial 16S rRNA gene and primers specific for the oral pathogens *S. mutans* (*GtfB* gene) and *P. gingivalis* (16S rRNA gene) (**Supplementary Table 2**). We also attempted (unsuccessfully) to amplify human mitochondrial DNA from the ancient dental calculus samples. For all primer sets, the PCR conditions included 2 U of AmpliTaq Gold (Applied Biosystems) in a 25-µl volume using 1× Buffer Gold, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP (Fermentas), 400 µM of each primer, 1 mg/ml rabbit serum albumin (RSA, Sigma-Aldrich), ShrimpDNase (Affymetrix) at 0.002 U/µl and 2 µl of DNA extract. ShrimpDNase was used to remove microbial contamination from PCR reagents before the amplification reaction and was added to the PCR mixture (minus the extract), samples were incubated at 37 °C for 15 min and the enzyme was then inactivated by heating the mixture to 65 °C for 15 min. For the specific primers, the thermocycling conditions consisted of an initial enzyme activation step at 95 °C for 6 min, followed by 45 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s and elongation at 72 °C for 30 s, with a single final extension step at 60 °C for 10 min. We used 40 cycles to amplify 16S rRNA universal sequence, and the annealing temperature was 50 °C. Each set of PCRs included multiple extraction and PCR blanks. All PCR products were visually examined by electrophoresis on 3.5% agarose TBE gels. Specific PCR products were purified using 5 µl of amplified product, exonuclease I (0.8 U/µl) and shrimp alkaline phosphatase (1 U/µl). Mixtures were heated to 37 °C for 40 min and then heat inactivated at 80 °C for 10 min. Purified amplicons were sequenced bidirectionally using PCR primers and the BigDye Terminator 3.1 kit (Applied Biosystems) according to the manufacturer's instructions. Sequencing products were purified using a Multiscreen$_{HTS}$ Vacuum Manifold (Millipore) according to the manufacturer's protocol. Sequencing products were separated on the 3130xl Genetic Analyzer (Applied Biosystems), and the resulting sequences were edited using Sequencher (version 4.7).

We cloned the 16S rRNA gene universal amplicons to monitor contamination within the ancient samples and non-template controls and to assess the suitability of calculus samples for 454 sequencing. PCR products were purified using Agencourt AMPure (Beckman Coulter) according to the manufacturer's instructions and cloned using a StrataClone PCR cloning kit (Stratagene). Clones were added directly to the colony PCR mix, which contained 10× HotMaster Buffer (Eppendorf), 0.5 U/µl HotMaster Taq (5Prime) and 10 µM of forward and reverse M13 primers (**Supplementary Table 2**) in a 25-µl reaction. The thermocycling conditions consisted of an initial step at 94 °C for 10 min and 35 cycles of 94 °C for 20 s, 55 °C for 10 s and 65 °C for 45 s, with a single extension of 65 °C for 10 min. Colony PCR products were visually inspected on 2% agarose TBE gels, and products were purified and sequenced using the same protocols as described for the products of the specific primers.

454 GS FLX Titanium sequencing. Pyrosequencing (GS-FLX Titanium) was used to examine 34 ancient dental calculus samples, 19 modern oral samples (6 calculus and 13 plaque), 6 tooth samples and 2 extraction blanks. For the ancient calculus samples, modern oral samples and non-template controls, three hypervariable regions of the 16S rRNA gene (V1, V3 and V6) were amplified using the described conditions. For the tooth samples, only the V3 region was amplified, using the same conditions. The forward and reverse primers contained 454 Lib-L kit A and B adaptors, respectively, at the 5′ end. The forward primer also contained sample-specific barcodes (**Supplementary Table 3**) that were developed by the Human Microbiome Project. The barcode sequences had not previously been used in either the Australian Centre for Ancient DNA or the Wellcome Trust Sanger Institute, where the 454 sequencing was performed. Hence, all sequences retrieved that did not contain a barcode were assumed to be contaminants and were discarded. Each region of the 16S rRNA gene was amplified twice (on different days), and duplicates were pooled for 454 sequencing to minimize the potential impact of preferential sequence amplification.

Filtering, OTU selection, alignment and taxonomic assignment of 454 sequences. Sequences from the GS FLX Titanium platform were processed using the QIIME (version 1.5.0) software package[52]. Quality filtering was performed to remove sequences that were either under 60 bp in length (potential primer dimers), contained ambiguous bases, had primer or barcode mismatches, contained homopolymers that exceeded 6 bases or had an average quality score below 25. The remaining sequences ranged between 60 and 210 bp in length. The quality-filtered sequences were denoised[53] and chimera checked to remove sequences containing errors produced during pyrosequencing and PCR, respectively, which resulted in the removal of ~50% of the sequences that were identified as having ambiguous flow data (**Supplementary Table 4**). However, we found that sequence classifications and β diversity analyses were comparable between the data set on which only quality filtering had been performed and the denoised data set, as has previously been shown[53]. Similar sequences were binned into OTUs using optimal UCLUST[54] at a 95% likeness. Clustering is more commonly performed at 97%; however, a 95% cutoff has been found to classify OTUs more accurately for closely related, short sequences[55]. Representative sequences from each OTU were aligned using PyNAST[52] against the GreenGenes core set, with a minimum length of 60 bp and identity of 75%. PyNAST aligns the short GS FLX–generated sequences (60–210 bp) against the full 16S rRNA gene. Columns that solely contained gaps were removed from the alignment before building phylogenetic trees. To overcome the difficulty in aligning highly variable 16S rRNA gene sequences, it is common to hide or lane mask regions where at least 50% of the base composition is not conserved[56]. We did not hide variable regions because lane-masked alignments can mute the phylogenetic diversity observed[55]. The gap-filtered sequences were taxonomically assigned using the RDP classifier and nomenclature[57].

Detailed descriptions of the analyses performed on the ancient dental calculus, modern oral and extraction blank sequences are described in the **Supplementary Note**, including information on β diversity, α diversity, random forest and discriminant analyses.

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