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Tracing back ancient oral microbiomes and oral pathogens using dental pulps from ancient teeth

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The dental pulp is the internal portion of teeth that contains highly vascularized soft tissues, which are protected by hard and mineralized structures (cementum, enamel, and dentin). Thanks to this protection, dental pulps are exceptional sources of ancient DNA, not only human but also from pathogens that produce systemic infections.1,2 In the past few years, several works have used high-throughput sequencing on ancient dental pulp samples to reconstruct the genomes of ancient pathogenic bacteria such as *Yersinia pestis* (the etiological agent of plague, including the Black Death), which dates back to centuries and millennia before the present.3,4 However, the microbial communities found in living dental pulps have been scarcely studied and it is not known whether ancient dental pulps also keep a record of oral bacteria and periodontal diseases from the past.

Here, we analyzed 16S rRNA amplicon sequencing data sets from ancient and modern dental pulps and provide an unprecedented exploration of their associated microbiomes. We combined these samples with other data sets generated by different laboratories and grouped them in eight categories: (1) Ancient dental pulps, (2) Modern dental pulps, (3) Modern root canals (which are equivalent to dental pulps, but without teeth extraction), (4) Ancient complete teeth that were ground and homogenized, (5) Modern oral cavity surfaces, (6) Saliva, (7) Ancient dental calculus, and (8) Soils (see Table 1 and the methods section from supplementary text for detailed sample description).

A beta-diversity analysis based on the unweighted Unifrac (UU) distance showed an overlap between modern and some of the ancient dental pulp samples, which were markedly different from ancient dental calculus, modern saliva, and oral cavity samples (Fig. 1a). A second group of ancient dental pulp samples clustered together with ancient complete ground teeth and closer to soil than to other samples from the oral cavity. To further explore the differences among microbial communities of the eight groups of samples, we performed a pairwise ANalysis Of SIMilarity (ANOSIM) statistical test5 and we obtained low *R*-values (the lower the *R*, the less significant are the differences between communities) when comparing modern root canal samples and modern dental pulps were compared to ancient dental pulp samples (Fig. 1a, upper panel). Similarly, ancient ground teeth also showed low *R*-values when compared to ancient dental pulps, probably indicating a significant contribution of the dental pulp fraction to the microbial profile found in the complete teeth preparation. Overall, these results suggested that dental pulps samples contained distinct microbial communities that differed from other oral cavity samples, and which could be detected even centuries after death.

When only dental pulps and complete ground teeth were analyzed, we found three well-defined clusters, one containing modern dental pulp and modern root canal samples, one containing ancient dental pulps and complete ground teeth from European samples of different periods of time (Table 1 for details), and one containing ancient Nigerian dental pulp samples (Fig. 1b). Although the perspective of the principal coordinate analysis (PCoA) in Fig. 1a seemingly shows an overlap between blanks of extraction and polymerase chain reaction (PCR) (the only 5 that amplified from the 12 blanks tested) with some dental pulp samples, Fig. 1b shows that these blanks were clearly different from samples. Our results indicate that although the microbial profiles of ancient and modern dental pulps are close when contrasted to samples of different nature, significant differences can be found between dental pulp samples from different sources. The variations observed between different populations and time
periods also point out the relevance of extending these characterizations to individuals from different geographical origins.

To explore in detail the taxonomic composition in the different groups of samples, we analyzed the complete data sets classified at genus level (Fig. 1c). All genera detected in the five blanks mentioned above were excluded from the analysis. We found two main and distinct clusters, one containing soil samples (in yellow) and one containing samples retrieved from the oral cavity (saliva and oral sites, in green), with several genera that were mutually exclusive between both groups. In agreement with results from Fig. 1a, ancient dental pulps and complete ground teeth from European samples showed a higher degree of environmental contamination, with several genera that are typically found in soil but not in the oral cavity and modern dental pulps, as well as intermediate richness values between both groups. However, despite the presence of these contaminants, we could detect a clear signal of genera that are typically found in oral samples and are totally absent in soil. On the other hand, ancient dental pulps from Nigeria, which also showed a clear record of oral bacteria, showed a much lower sign of contamination and also presented several genera that were exclusively found in these samples. Since ancient African teeth have never been analyzed before, we cannot rule out the possibility that the latter is due to particularities in the oral microbiome of these populations.

Using a Kruskal–Wallis statistical test, we could first identify which genera presented significant differences between modern oral microbiomes and soils and then estimate the proportion of genera in ancient samples (dental pulps, complete ground teeth, and dental calculus) presenting oral-like profiles (see Supplementary results and Supplementary Table 1 in the Supplementary information file for further detail). We found that 30–50 % of the oral-associated genera were also enriched in ancient dental pulp, a proportion that was much higher than in complete ground teeth and relatively similar to that found in dental calculus (Supplementary Table 1). The statistical analyses also revealed a number of genera that were not associated with modern oral samples, but that showed significantly higher proportion in ancient samples compared to soil. These genera could potentially correspond to either bacteria-enriched post-mortem that may participate in decomposition or, alternatively, particular residents of the ancient oral microbiomes analyzed in this study (Supplementary Tables 2 and 3). All together, our results indicate that although ancient dental pulps are invaded by environmental bacteria after death, they also conserve a good record of the microbial communities that are naturally present in the oral cavity and particularly in dental pulps.

We finally explored the presence of pathogenic bacteria in all groups of samples (i.e., unambiguous hits against known pathogens). We found almost exclusively hits against oral pathogenic bacteria (Fig. 1d) from 18 different species, a group that is not overrepresented in the database used. From these, 12 were found in at least one ancient sample. The analysis of individual samples showed a high variability, with positive hits in only a limited number of ancient samples (Supplementary Figure 1). This is probably determined by the natural variability in the prevalence of oral pathogens among individuals and even among teeth of the same oral cavity, although it could also be influenced by the conservation of bacterial DNA in these samples.

We did not detect any systemic pathogen that could have likely caused the death of any of the analyzed individuals. We therefore demonstrate that ancient dental pulps can also conserve a good record of ancient oral pathogens that were likely producing localized bacteremia. These results are in agreement with previous
studies showing that modern oral pathogens were already present in ancient populations.\textsuperscript{8,9} Here, we showed the first evidence that DNA from oral pathogens can be also recovered from ancient dental pulp samples.

In this work we showed that dental pulps are not only highly precious sources of ancient human and blood-borne pathogens DNA, but also from the oral microbes that prevailed in association with our ancestors. This work brings new perspectives for studying the evolution of human oral microbiomes and the spread of periodontal diseases in ancient populations, through dental pulp metagenomics.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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