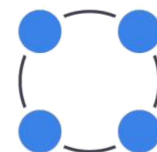

Ancient Oral Chronicles: Unveiling Microbial Secrets Through Dental Calculus

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Abstract

Metagenomic analysis of diverse microbial communities provides a powerful lens through which we can explore ecosystem dynamics and human health[1]. Leveraging next-generation sequencing (NGS) techniques, this study delves into the microbial archives preserved within dental calculus samples extracted from human remains dating back over a millennium[2]. The metagenome of the discovered and analyzed bacterial community reveals a wealth of information – ranging from insights into historical diseases and dietary patterns to the evolutionary trajectories of typical oral bacteria.
By scrutinizing 16S rRNA genes and employing shotgun sequencing, the meticulous dissection of the taxonomy composition of ancient dental calculus unveils remarkable findings. Specifically, two out of the four individuals examined likely suffered from severe forms of periodontal disease. These revelations not only illuminate the past but also underscore the enduring relevance of microbial studies in shaping our understanding of human health.

Keywords: Metagenomics, Paleogenomics, Human microbiome

Introduction

The study of ancient human oral microbiomes provides a fascinating glimpse into the microbial communities that coexisted with our ancestors[3]. By analyzing dental calculus samples from individuals dating back over a millennium, we can unravel the mysteries of historical health, dietary habits, and ecological interactions[2,4]. In this research, we delve into the metagenomes of ancient dental calculus, aiming to understand the composition, evolution, and functional properties of these microbial communities.

Dental calculus, or mineralized dental plaque, serves as a unique reservoir of ancient biomolecules. It perfectly preserves microbial and host DNA, proteins, and metabolites, making it an invaluable source for studying the oral microbiome of the past. Through the analysis of dental calculus, we gain insights into the microbial ecosystems that shaped human health and disease over time[5,6].

Among the microbial players in ancient dental calculus, the «red complex» stands out. This group of bacteria is consistently associated with severe forms of periodontal disease. Notably, the red complex includes three key species:

- *Porphyromonas gingivalis*: known for its virulence factors, *P. gingivalis* plays a central role in periodontitis progression.
- *Tannerella forsythia*: contributes to tissue destruction and inflammation in periodontal disease.
- *Treponema denticola*: associated with subgingival plaque and periodontitis pathogenesis.

Understanding the prevalence and impact of the red complex in ancient oral microbiomes informs our knowledge of historical health challenges[7,8].

Shotgun sequencing analysis reveals regions of zero coverage in the modern *T. forsythia* 92A2 strain. These regions likely represent genetic material acquired during evolution which highlights the dynamic nature of the oral microbiome over millennia.

Root samples were also explored, which exhibit different bacterial phyla proportions and compositions compared to calculus samples. These variations reflect ecological niches and host interactions, providing insights into ancient oral ecosystems.

Studying ancient oral microbiomes not only enriches our understanding of the past but also has implications for contemporary health. By bridging history and modern science, we gain valuable insights into the human condition.

Materials and methods

16S rRNA Gene Sequencing

DNA samples were sequenced, yielding single-ended reads of the V5 region of the 16S ribosomal RNA using a Roche GS Junior instrument (454). The original research data are publicly available in the NCBI Sequence Read Archive (SRA) under accession number SRP029257 (BioProject PRJNA216965). QIIME2 v2023.2.0[9,10] ("Quantitative Insights Into Microbial Ecology"), an open-source bioinformatics pipeline, was employed to analyze raw DNA sequencing data for microbiome characterization.

The QIIME2 pipeline encompassed the following steps:

1. Raw reads underwent quality control and trimming.
2. Denoising and Dereplication processes were applied.
3. Chimeric sequences were filtered out.
4. DADA2 v1.30.0[11], integrated within QIIME2, was utilized for ASV clustering.
5. Trimming and filtering parameters were set as follows: --p-trim-left 32 and --p-trunc-len 150, tailored to barcode and primer lengths and quality control distribution.
6. The QIIME2 Naive Bayes classifier, trained on 16S rRNA data using SILVA reference sequences and taxonomy files, was employed to analyze the taxonomic composition of the samples.
7. Each step of the analysis was visualized using QIIME2.

Shotgun Sequencing of Dental Calculus Microbiome

Shotgun sequencing was conducted on a DNA sample extracted from the dental calculus microbiome of individual G12 (skeleton dating from AD 950-1200) who suffered from

periodontal disease. The resulting reads were assembled into contigs. To facilitate comparison, we obtained a precomputed assembly of the ancient *Tannerella forsythia* genome specific to the G12 individual.

Metagenome Assembly:

- The shotgun sequencing data from the G12 sample were assembled into contigs.
- A precomputed assembly for the *Tannerella forsythia* 92A2 genome specific to the G12 individual was obtained.

Alignment and Annotation:

- The G12 metagenome assembly was aligned to the *Tannerella forsythia* 92A2 reference genome using BWA-mem v0.7.17[12].
- Samtools v1.19.2[13] was used to compress, sort, and index the aligned reads against the reference genome.
- Basic statistics of the resulting alignment were obtained using the samtools flagstat command.

Conversion and Annotation Visualization:

- The alignment results were converted to .bed format using BEDtools v2.31.1[14] (bedtools bamtobed).
- Genome regions absent in the ancient strain were subtracted from the GFF3 annotation using bedtools intersect (with the -v option).
- The resulting annotation file was manually inspected using bash scripting.
- Visualization of the annotated regions was performed using the Integrative Genomics Viewer (IGV) desktop application.

Results

Amplicon Sequencing

Initially, our sequencing results yielded approximately 5000 reads per analyzed sample, with a median read length of 179 nucleotides. However, upon quality assessment, we observed a decline in read quality scores starting at base 180. Considering the lengths of barcodes and primer sequences used during library preparation and the decrease in read quality, we performed trimming.

Using the DADA2 v1.30.0 pipeline, chimeric sequences within the V5 16S regions were filtered out. These regions are particularly informative for profiling oral metagenomes, as they are consistently present across metadata samples. Remarkably, over 90% of reads in each sample successfully passed all filters and were confirmed as non-chimeric.

Feature Distribution

Analysis resulted in the clustering of amplicon sequences into amplicon sequence variants (ASVs), which serve as a higher-resolution analogue of traditional operational taxonomic units (OTUs). Notably, we observed that only a few dominant features were consistently present in the majority of samples. This outcome aligns with our focus on conservative sequences.

Microbiome Composition

Figure 1 illustrates the relative abundance of bacterial phyla in the calculus samples. Seven dominant phyla were identified: *Firmicutes, Proteobacteria, Bacteroidetes, Synergistetes, Actinobacteria, Fusobacteria,* and *Spirochaetes*. Interestingly, the root samples also contained bacteria from *Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria*, albeit in different proportions and compositions.

These findings provide valuable insights into the diversity and taxonomic composition of ancient oral microbiomes, shedding light on the historical health and ecological context of the studied individuals.

Shotgun Sequencing Insights

During shotgun sequencing, only 1.83% of the metagenomic contigs from dental calculus aligned to the *Tannerella forsythia* 92A2 genome. Analysis of the alignment results revealed intriguing regions of zero coverage in the modern strain – likely acquired during evolution. Specifically, 194 coding sequences associated with these regions were identified.

Acquired Genes and Their Functions: These acquired genes fall into distinct functional groups, shedding light on the strain’s adaptation mechanisms:

1. Restriction-Modification System: These genes are involved in defense against foreign DNA.
2. Mobile Genetic Elements: Elements that facilitate horizontal gene transfer.
3. DNA Repair and Post-Translational Modification: Crucial for maintaining genome stability.
4. Antibiotic Biosynthesis and Drug Resistance: Reflecting the strain’s interactions with its environment.
5. Metabolism: Genes related to nutrient utilization and energy production.

Discussion

Metagenomic analysis of dental calculus revealed a fascinating glimpse into the oral microbiome of individuals from the past millennium[15,16]. By combining 16S rRNA gene sequencing and shotgun sequencing, we were able to characterize the overall bacterial composition and gain deeper insights into specific pathogens associated with periodontal disease[17].

The 16S rRNA gene sequencing identified a diverse range of bacterial phyla within the dental calculus samples, including *Firmicutes, Proteobacteria, Bacteroidetes, Synergistetes, Actinobacteria, Fusobacteria,* and *Spirochaetes*. This finding aligns with previous studies on the oral microbiome, highlighting the presence of a complex and dynamic bacterial community within the human mouth.

Interestingly, our analysis identified two individuals with a high abundance of bacteria associated with the «red complex». This complex, consisting of *Tannerella forsythia, Treponema denticola,* and *Porphyromonas gingivalis*, is a well-established group of pathogens known to be a major contributor to severe forms of periodontal disease. The presence of the red complex in these ancient samples suggests that periodontal disease has been a prevalent health concern throughout human history[18]. This aligns with recent paleomicrobiological studies that have identified evidence of the red complex in dental calculus from prehistoric populations.

The shotgun metagenomic sequencing of the *T. forsythia* genome from one individual (G12) provided even more granular details. 194 coding sequences potentially acquired through horizontal gene transfer were identified, suggesting the strain's ability to adapt and evolve over time. Notably, these

acquired genes were associated with functions like restriction-modification systems (defense against foreign DNA), mobile genetic elements (facilitating horizontal gene transfer), and antibiotic biosynthesis (resistance mechanisms). These findings highlight the dynamic nature of bacterial genomes and their capacity to adapt to changing environments, including the development of resistance to antibiotics – a concern that continues to be highly relevant in modern medicine[19].

In conclusion, this study demonstrates the power of metagenomic analysis in unraveling the mysteries of the ancient human microbiome. By analyzing dental calculus, we were able to identify a diverse bacterial community and even pinpoint evidence of the red complex, a prevalent cause of periodontal disease. Additionally, shotgun sequencing provided valuable insights into the evolutionary trajectory of specific pathogens, highlighting their ability to adapt and acquire new functionalities.

Supplementary materials

Lab journal can be found in GitHub repository.

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Table 1 Basic statistics of initial sequencing reads and their statistics after their trimming and filtering.

Sample-id	Initial reads	Filtered reads	Passed filter, %	Denoised	Non-chimeric	Input non-chimeric, %
S10-V5-Q-B61-calc	5957	5695	95.60	5575	5559	93.32
S14-V5-P-B17-calc	4491	4282	95.35	4218	4218	93.92
S15-V5-R-B78-calc	4212	4037	95.85	3950	3911	92.85
S16S17-V5-K1-G12-root	5788	5599	96.73	5427	5361	92.62
S16S17-V5-K2-G12-root	5272	5066	96.09	4884	4862	92.22
S18S19-V5-L-B17-root	4955	4790	96.67	4680	4680	94.45
S20S21-V5-M-B61-root	5516	5396	97.82	5240	5182	93.94
S22S23-V5-N-B78-root	4695	4534	96.57	4382	4261	90.76
S8-V5-O-G12-calc	5362	5199	96.96	5108	5092	94.96

Table 2 Examples of several protein coding genes acquired by the *Tannerella forsythia* 92A2 strain.

Product	Protein_id	Protein_group
AIPR family protein	WP_014224073.1	restriction modification system
DDE-type integrase/transposase/recombinase	WP_014223573.1	mobile genetic element
IS1 family transposase	WP_099046116.1	mobile genetic element
IS110 family transposase	WP_014225157.1	mobile genetic element
IS1380 family transposase	WP_041590984.1	mobile genetic element
DNA mismatch endonuclease Vsr	WP_041590610.1	DNA repair
GNAT family N-acetyltransferase	WP_007366527.1	post-translational modification
SPASM domain-containing protein	WP_157755359.1	post-translational modification
radical SAM peptide maturase	WP_014224286.1	post-translational modification
class I lanthipeptide	WP_041590503.1	antibiotic biosynthesis
lanthionine synthetase C family protein antibiotic dehydratase family protein thiopeptide-type bacteriocin biosynthesis protein ParA family protein	WP_014223583.1	antibiotic biosynthesis
conjugative transposon protein TraJ	WP_041590506.1	antibiotic biosynthesis
conjugative transposon protein TraM	WP_041590507.1	antibiotic biosynthesis
conjugative transposon protein TraN	WP_007366517.1	HGT of plasmids
tetracycline resistance ribosomal protection protein	WP_007366507.1	HGT of plasmids
RteC domain-containing protein	WP_007366504.1	HGT of plasmids
TetR/AcrR family transcriptional regulator	WP_007366503.1	HGT of plasmids
dihydrofolate reductase family protein	WP_007366526.1	antibiotic resistance
beta-ketoacyl-ACP synthase III	WP_007366522.1	antibiotic resistance

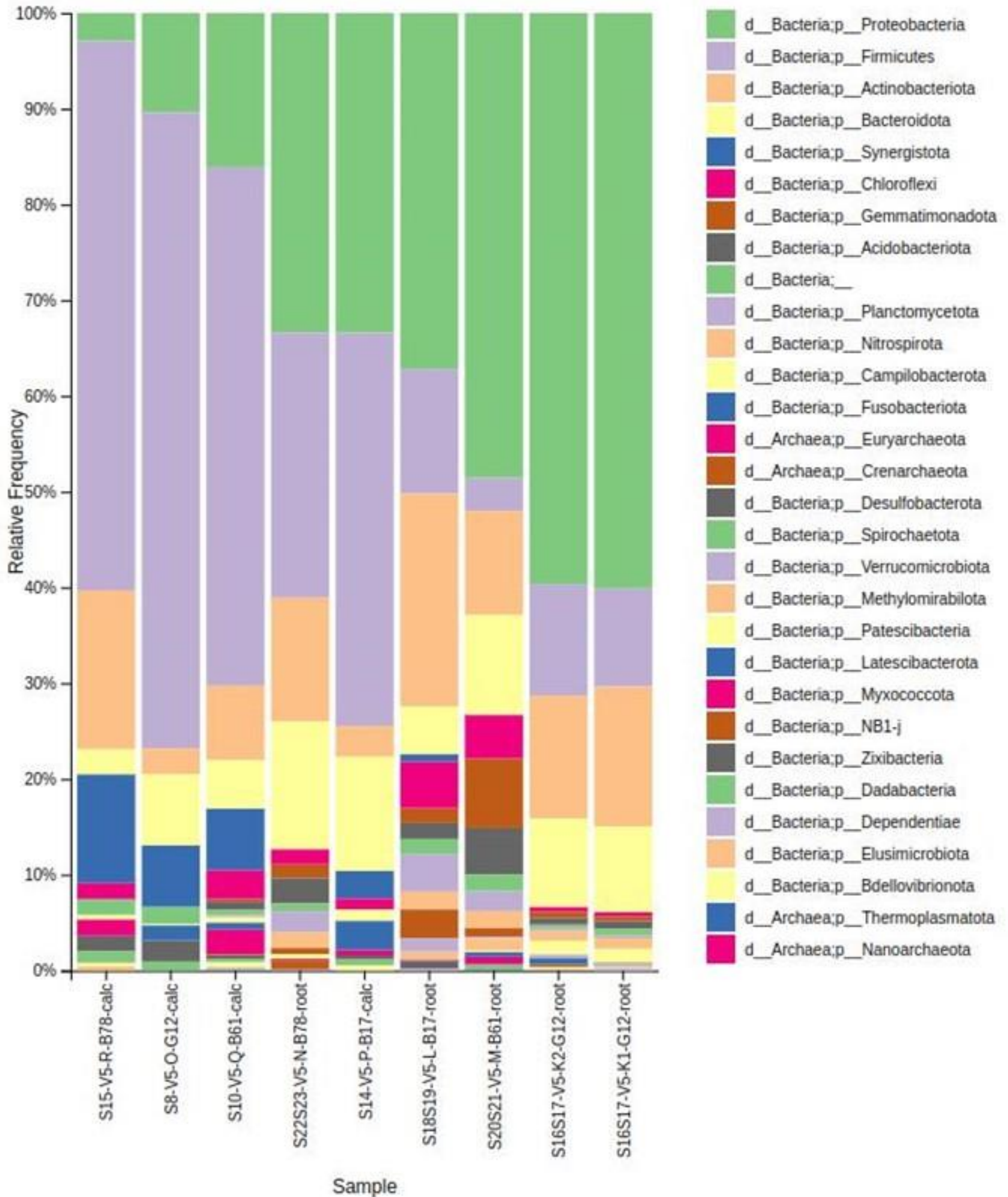

Figure 1 Taxonomic composition of analyzed ancient samples.