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Wet Lab Protocols Matter: Choice of DNA Extraction and Library Preparation Protocols Bias Ancient Oral Microbiome Recovery

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ABSTRACT

Ancient DNA (aDNA) analysis of archaeological dental calculus has provided a wealth of insights into ancient health, demography and lifestyles. However, the workflow for ancient metagenomics is still evolving, raising concerns about reproducibility. Few systematic investigations have examined how DNA extraction methods and library preparation protocols influence ancient oral microbiome recovery, despite evidence from modern populations suggesting that they do. This leaves a gap in our understanding of how wet-lab protocols impact aDNA recovery from dental calculus. In this study, we apply two DNA extraction and two library preparation methods in the aDNA field on dental calculus samples from Hungary and Niger. Samples from each context have similar chronological ages, but differences in their levels of aDNA preservation are notable, providing additional insights into how the efficacy of wet-lab protocols is impacted by sample preservation. Several metrics were employed to assess intra- and inter-sample variability, such as DNA fragment length recovery, GC content, clonality, endogenous content, DNA deamination and microbial composition. Our findings indicate that both DNA extraction and library preparation protocols can considerably impact ancient DNA recovery from archaeological dental calculus. Furthermore, no single protocol consistently outperformed the others across all assessments, and the effectiveness of specific protocol combinations depended on the preservation of the sample. These findings highlight the challenges of meta-analyses and underscore the need to account for technical variability. Lastly, our study raises the question of whether the field should strive to standardise methods for comparability or optimise protocols based on sample preservation and specific research objectives.

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1 | Introduction

The cyclic mineralization of dental plaque—a biofilm formed when microorganisms adhere to the tooth surface—leads to the formation of dental calculus (Lieverse 1999; White 1997). Dental calculus accumulates over an individual's lifetime, creating a long-term record of their oral microbiome—the diverse community of bacteria, archaea, viruses, fungi and parasites inhabiting the human mouth (Baker et al. 2024; Wade 2013; Dewhirst et al. 2010). The oral microbiome plays a pivotal role in systemic health, with established links to cardiovascular disease, inflammatory bowel disease, rheumatoid arthritis and colorectal cancer (Baker et al. 2017; Herrero et al. 2018; Hajishengallis and Chavakis 2021). These associations have made understanding the relationship between the evolutionary history of the oral microbiome and these diseases a key area of interest. The study of ancient DNA (aDNA) preserved in archaeological dental calculus has substantially contributed to these ongoing efforts (Wright et al. 2021; Weyrich et al. 2015; Warinner et al. 2015; Adler et al. 2013; Warinner et al. 2014; Mann et al. 2018). By applying next generation sequencing to dental calculus, these approaches have gleaned novel insights into ancient health (Weyrich et al. 2017; Warinner et al. 2014; Wright et al. 2024; Gancz et al. 2023), diets (Adler et al. 2013; Ottoni et al. 2021; Quagliariello et al. 2022; Fellows Yates, Velsko, et al. 2021), demography (Ottoni et al. 2021; Velsko et al. 2024) and lifestyles (Eisenhofer et al. 2020; Ottoni et al. 2021).

While significant strides have been made in ancient oral microbiome research over the past decade (Der Sarkissian et al. 2021; Ziesemer et al. 2015; Fagernäs et al. 2020; Tromp et al. 2017; Eisenhofer et al. 2024), variability in ancient metagenomic workflows remains a challenge for reproducibility (Gancz et al. 2023; Farrer et al. 2021; Fellows Yates et al. 2020; Borry et al. 2024; Fellows Yates, Andrades Valtueña, et al. 2021; Fellows Yates, Velsko, et al. 2021; Wright 2025). For instance, various DNA extraction and library preparation protocols are used in the field (Moore and Weyrich 2021; Fellows Yates et al. 2020; Ottoni et al. 2021; Quagliariello et al. 2022). Addressing this variability is critical, as research on modern populations demonstrate that the choice of DNA extraction (Lim et al. 2018; Teng et al. 2018; Yang et al. 2020) and library preparation methods (Tourlousse et al. 2021; Jones et al. 2015; Poulsen et al. 2022) can significantly influence both DNA and microbiome composition recovery. To bridge this gap, our study systematically compares two DNA extraction methods and two library preparation methods that have been designed to recover aDNA from archaeological biomaterials.

Currently, two commonly used aDNA extraction methods are available in the field because of their effectiveness in recovering short, fragmented DNA. One of the first DNA extraction methods developed was by Rohland and Hofreiter (2007) (hereafter referred to as the QG method). The QG method involves the digestion of a biological sample using ethylenediaminetetraacetic acid (EDTA) and proteinase K, followed by a DNA purification step with a silica-based binding buffer containing a high concentration of guanidinium thiocyanate. This combination facilitates efficient DNA release while minimising the presence of PCR inhibitors. Dabney et al. (2013) modified the original QG extraction protocol to improve the recovery of highly degraded DNA fragments, particularly those shorter than 50 bp. This modified protocol, hereafter referred to as the PB method, uses a binding buffer composed of sodium acetate, isopropanol and guanidinium hydrochloride to enhance the binding efficiency of short DNA fragments in a silica matrix. While ongoing improvements to both methods have been made since their inception (Weyrich et al. 2017; Hagan et al. 2020; Rohland et al. 2010; Gamba et al. 2016; Brotherton et al. 2013), no systematic comparison of their effectiveness in recovering aDNA from dental calculus has been done.

Much like DNA extraction techniques, the effectiveness of commonly employed library preparation methods in the aDNA field has been benchmarked with teeth and bone (Carøe et al. 2018; Gansauge and Meyer 2013; Meyer and Kircher 2010; Wales et al. 2015). Most library construction protocols for aDNA research were designed for Illumina sequencing-based platforms (i.e., sequencing-by-synthesis) and can be classified into two broad categories: the double-stranded library (DSL) and the single-stranded library (SSL) construction methods. The DSL library method developed by Meyer and Kircher (2010) is widely used in both paleomicrobiology and paleogenomic fields (Orlando et al. 2021). For this method, the ends of DNA molecules are first repaired and then ligated to double-stranded adapters (Briggs et al. 2007; Meyer and Kircher 2010; Kircher et al. 2012). While most of the steps for this protocol have remained the same (Psonis et al. 2021), it has undergone a few revisions since its original development (Allentoft et al. 2015; Carøe et al. 2018). The SSL method, in contrast, was initially introduced by Gansauge and Meyer (2013). The protocol for the SSL method involves denaturing all DNA molecules in a DNA extract into single-stranded form, which, in theory, allows for higher conversion of DNA fragments into adapter-ligated molecules compared to DSL protocols (Bennett et al. 2014; Wales et al. 2015; Gansauge et al. 2017). Although the SSL has shown to be more effective in recovering aDNA from teeth and bone (Bennett et al. 2014; Wales et al. 2015), few research teams utilize this method, primarily due to its increased cost and longer protocol duration compared to their double-stranded approach counterparts. These barriers of the SSL protocols have been addressed by the development of the Santa Cruz Reaction (SCR) method, a SSL approach that substantially reduces both cost and laboratory processing time compared to earlier SSL methods (Kapp et al. 2021). However, despite its advantages, the SCR method has yet to see widespread adoption in the ancient oral microbiome field.

Examining the effectiveness of combining different DNA extraction and library preparation methods for recovering aDNA from archaeological dental calculus is essential, as these protocols have been shown to significantly influence aDNA recovery. For instance, the PB extraction method paired with SSL preparation was particularly effective in recovering short fragments (< 100 bp) (Dabney et al. 2013). Similarly, Gamba et al. (2016) found that the QG method paired with the DSL preparation increases clonality compared to other methods. However, much of the benchmarking work to date has focused on dentin or bone (e.g., Dabney et al. 2013; Gamba et al. 2016; Barlow et al. 2016), and often on single-organism systems (e.g., Sproul and Maddison 2017; Hahn et al. 2022). Since DNA extraction protocols have been shown to impact microbial community

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TABLE 1 | Metadata for samples included in this project.

SampleID	Country	Site	Museum/Grave number	Sample date
18393_Niger_QG_DSL	Niger	Gobero	G3B63A	5200-2200BCE
18393_Niger_PB_DSL	Niger	Gobero	G3B63A	5200-2200BCE
18393_Niger_PB_SSL	Niger	Gobero	G3B63A	5200-2200BCE
18393_Niger_QG_SSL	Niger	Gobero	G3B63A	5200-2200BCE
18398_Niger_QG_DSL	Niger	Gobero	G5B1A	5200-2200BCE
18398_Niger_PB_DSL	Niger	Gobero	G5B1A	5200-2200BCE
18398_Niger_PB_SSL	Niger	Gobero	G5B1A	5200-2200BCE
18398_Niger_QG_SSL	Niger	Gobero	G5B1A	5200-2200BCE
18400_Niger_QG_DSL	Niger	Gobero	G3B3B	5200-2200BCE
18400_Niger_PB_DSL	Niger	Gobero	G3B3B	5200-2200BCE
18400_Niger_PB_SSL	Niger	Gobero	G3B3B	5200-2200BCE
18400_Niger_QG_SSL	Niger	Gobero	G3B3B	5200-2200BCE
18416_Hungary_QG_DSL	Hungary	Balatonszarszo Kis-Erdei Dulo	789	5500-5000BCE
18416_Hungary_PB_DSL	Hungary	Balatonszarszo Kis-Erdei Dulo	789	5500-5000BCE
18416_Hungary_PB_SSL	Hungary	Balatonszarszo Kis-Erdei Dulo	789	5500-5000BCE
18416_Hungary_QG_SSL	Hungary	Balatonszarszo Kis-Erdei Dulo	789	5500-5000BCE
18421_Hungary_QG_DSL	Hungary	Alsonyek	1791	5000-4500BCE
18421_Hungary_PB_DSL	Hungary	Alsonyek	1791	5000-4500BCE
18421_Hungary_PB_SSL	Hungary	Alsonyek	1791	5000-4500BCE
18421_Hungary_QG_SSL	Hungary	Alsonyek	1791	5000-4500BCE
18427_Hungary_QG_DSL	Hungary	Alsonyek	1318	5000-4500BCE
18427_Hungary_PB_DSL	Hungary	Alsonyek	1318	5000-4500BCE
18427_Hungary_PB_SSL	Hungary	Alsonyek	1318	5000-4500BCE
18427_Hungary_QG_SSL	Hungary	Alsonyek	1318	5000-4500BCE

recovery in coprolites (Hagan et al. 2020), the broader effectiveness of these protocols for dental calculus is relatively unexplored. Because dental calculus has unique chemical and biological properties—particularly its dense calcium phosphate matrix and its potential for co-extracted inhibitors (Mann et al. 2018; White 1997)—it remains unclear whether the findings from bone and dentin studies translate directly. Therefore, it is crucial to assess whether and how DNA extraction and library preparation protocols influence aDNA recovery from dental calculus, particularly in studies aiming to characterize ancient microbial communities.

To explore whether DNA extraction and library preparation protocols influence the sequencing data generated from archaeological dental calculus, we investigate the impact of laboratory methods on calculus samples from two geographically distinct locations: Niger and Hungary. Despite the samples sharing similar chronological ages, there were notable differences in their preservation states. This design, therefore, provides a unique opportunity to evaluate how wet lab protocols perform across varying preservation contexts. In addition, we analysed the two datasets separately to avoid confounding biological variability with geography, given its strong association with ancient oral microbiome composition (Ottoni et al. 2021). Specifically, we assess whether wet-lab protocols affect the recovery of DNA fragment lengths, GC content, clonality, endogenous content, DNA deamination and microbial composition.

2 | Materials and Methods

2.1 | Export Permissions and Sample Provenance

This research was evaluated and approved by the University of Adelaide Human Research Ethics Committee (H-2012-108). The ancient dental calculus samples analysed in this study were derived from Hungary ($n = 3$) and Niger ($n = 3$) (Table 1; Data S1). All three samples from Niger were recovered from Gobero, an archaeological site situated in the Sahara that was intensively occupied 9500 cal years BP and 4500 BP (Garcea et al. 2013). These individuals are estimated to have lived between approximately 5200 and 2200 BCE, spanning the middle

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to late Holocene occupation of the region (Sereno et al. 2008). One sample from Hungary was collected from an individual from Balatonszárszó–Kis-erdei-dűlő, dated to the Early Neolithic period (approximately 5500–5000 BCE) (Oross and Marton 2012), while two calculus samples were collected from individuals from Alsonyek, dating between 5000 and 4500 BCE (Osztás et al. 2012).

2.2 | Sample Collection Procedures

Supragingival dental calculus deposits were dislodged from the surface of tooth samples using a sterile dental pick (Figure 1). Gentle pressure was applied in parallel to the tooth surface in order to avoid enamel damage as previously described (Weyrich et al. 2015). Collected fragments were then stored in sterile, sealed zip bags for transportation to the ancient DNA facility at the Australian Centre for Ancient DNA (ACAD), University of Adelaide, Australia. These samples were selected for comparison because, while both groups were of similar chronological age (~7000 years BP), their aDNA preservation was expected to be different. This expectation stemmed from previous findings, which indicated that authentic ancient human DNA was recovered from the respective teeth of the Hungarian samples (Haak et al. 2015; Lipson et al. 2020) but not the samples from Niger.

Extraction blanks (EBCs) were included at the beginning of each sample series to monitor potential contamination introduced during sample processing. Prior to DNA extraction, an empty tube was moved around within the still-air hood (the one that would be used in which the dental calculus samples were handled) for approximately 20 s during each decontamination batch. These EBCs were processed in parallel with the calculus samples using the same DNA extraction protocols.

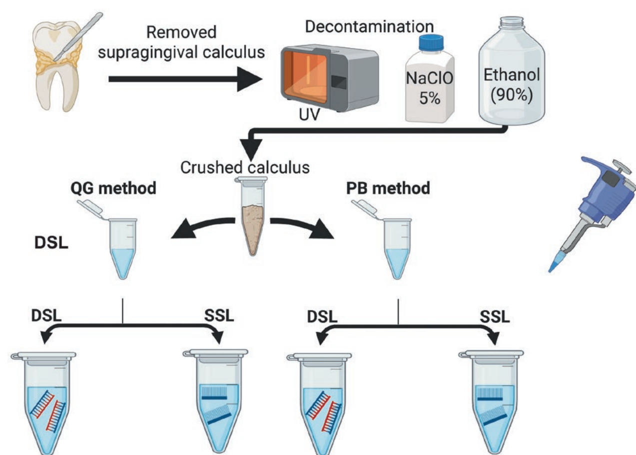

FIGURE 1 | Schematic overview of the experimental design. Dental calculus samples from three individuals in Niger and three individuals in Hungary were each divided into two roughly equal pieces and placed into separate sampling tubes for DNA extraction. Two silica-based DNA extraction methods were employed: the QG method, based on the protocol described Rohland and Hofreiter (2007), and the PB method, based on the protocol described by Dabney et al. (2013). Extracted DNA was then used to construct sequencing libraries with one of the following two approaches: a double-stranded library (DSL) method following Meyer and Kircher (2010), or a single-stranded library (SSL) method following a modified protocol originally described by Gansauge and Meyer (2013). This design enabled a systematic comparison of extraction and library preparation protocols across different geographic and preservation contexts.

2.3 | Facility Protocols

All sample processing and laboratory procedures were performed in the specialised aDNA facility at the University of Adelaide (ACAD). The facility is equipped with positive air pressure, undergoes daily sanitation with a 3% sodium hypochlorite (NaClO), and is exposed to ultraviolet light each night for disinfection. All experiments in this study were performed within ultraviolet light-treated, still-air hoods located in isolated, still-air rooms. All personnel accessed the facility using a dedicated single access room and wore disposable full body suits, gloves and face masks and followed strict facility workflows.

2.4 | Sample Decontamination and Digestion

For decontamination, we divided each sample into two visually equal portions and subjected them to the following protocol: samples were placed in a sterile plastic dish and exposed to UV radiation for 15 min on each side. Next, each sample was submerged in 5% bleach and then purified water for 5 min, followed by immersion in 90% ethanol for 3 min to remove any residual bleach. The samples were air dried for 5 min within a sterile container and then transferred to a sterile plastic bag. While inside the bag, the calculus was crushed with a steel hammer. The corner of the plastic bag was then removed, and the sample was poured into a sterile 2 mL screw cap tube.

2.5 | QG Method

Crushed calculus samples prepared using the QG method followed protocols previously described in Brotherton et al. (2013), which were adapted from Rohland and Hofreiter (2007). Briefly, samples were decalcified by adding the powderized sample to a sterile tube containing 1.8 mL of 0.5 M ethylenediaminetetraacetic acid (EDTA), 100 μL of 10% sodium dodecyl sulphate (SDS) and 20 μL of 20 mg/mL proteinase K and left to rotate at 55°C for 20 h. Following a day of digestion, released DNA was bound to a silica suspension with 3 mL of modified QG buffer (Qiagen), pelleted, and washed twice in 80% ethanol. Next, the cleaned and dried silica was resuspended in 100 μL of Tris–HCl buffer twice to elute the DNA. Eluted DNA was aliquoted and stored at −20°C until amplification. Notably, all the DNA and RNA-free certified water (Invitrogen Ultrapure distilled water) used to create the reagents was opened fresh or was frozen upon aliquoting to prevent microbial growth and contamination.

2.6 | PB Method

Samples prepared with the PB method were decontaminated and digested with the same protocols described for the QG method. Following digestion, the steps for the PB method followed those described in Moore and Weyrich (2021),

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which were adapted from Dabney et al. (2013). Briefly, the PB binding buffer consisted of Qiagen PB buffer 12.2 mL, 7 μL Tween-20 and 378 μL NaOAc (3 M). Additional reagents needed for this protocol included 100 μL silica solution, 200 μL TLE buffer and 1.8 mL 80% ethanol in molecular grade water. Each crushed calculus sample was added to a 15 mL conical tube in a still air hood. Next, 12.6 mL of the PB binding buffer was added and 100 μL silica. All samples were centrifuged for 3 min at 19,500 *g* in a microcentrifuge. Next, the supernatant of samples was transferred into a sterile 15 mL tube. Tubes were then placed on a rotary mixer for 1 h at room temperature. Following this step, samples were centrifuged for 5 min at 4400 *g*. The supernatants were discarded, and 900 μL of 80% ethanol was added to each pellet to resuspend it using a long reach pipette tip by mixing up and down. The resuspended solution was then transferred to DNA LoBind tubes. All samples were then centrifuged for 1 min at 14,000 rpm in a microcentrifuge. A total of 100 μL TLE was added to each dried pellet and resuspended using a vortex. Thirty microlitres DNA extract can be placed into a tube.

2.7 | DSL Method

DNA libraries constructed using the DSL method were prepared following the protocols described in Meyer and Kircher (2010) with minor modifications. Approximately 20 μL of DNA extract from the PB and QG method was treated with T4 polynucleotide kinase and T4 DNA polymerase (New England Biolabs) for 15 min at 25°C. Reactions were cleaned using a MinElute Reaction Cleanup kit (Qiagen). Truncated Illumina adapter sequences with 5 bp unique barcodes (Meyer and Kircher 2010) were ligated onto double-stranded DNA molecules using T4 DNA ligase (Fermentas) for 60 min at 22°C. A Qiagen MiniElute Clean-up kit was utilized to remove excess ligase; adapter sequences were filled using a Bst DNA polymerase (New England Biolabs) for 30 min at 37°C, followed by denaturation of the polymerase at 80°C for 10 min. The resulting reaction was then used as a template in five independent PCR reactions (12 μL DNA-free dH_2O, 2.5 μL 10× buffer, 2.5 μL 25 mM $MgCl_2$, 0.25 μL HiFi DNA polymerase and 5 μL of the Bst reaction mixture) under the following conditions: 12 min at 94°C; 13 cycles of 30 s at 94°C, 30 s at 60°C, 45 s at 72°C and 10 min at 72°C. PCR reactions were pooled and cleaned using Ampure PCR purification (Agencourt). Libraries were then reamplified under the same conditions using GAII indexing primers (Meyer and Kircher 2010) to include a single P7 (3′) index sequence unique to each sample, re-pooled, and cleaned again using Ampure.

2.8 | SSL Method

Single-stranded DNA libraries were prepared following the protocol of Gansauge et al. (2017), with minor modifications. First, 20 μL DNA extract from the PB and QG method of each sample was dephosphorylated using FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher) to remove 5′ and 3′ phosphate groups. Each reaction was incubated at 37°C for 30 min and then heat-inactivated at 75°C for 10 min. Following dephosphorylation, the DNA was denatured by heating to 95°C for 2 min and immediately snap-cooled on ice to preserve single-stranded fragments. A biotinylated adapter was ligated to the 3′ end of single-stranded DNA using T4 DNA Ligase (ThermoFisher). The ligated products were immobilised on Dynabeads C1 streptavidin-coated magnetic beads (Invitrogen) and washed thoroughly to remove unbound DNA. A primer complementary to the biotinylated adapter was then annealed, and the second strand was synthesised using Klenow Fragment (3′ → 5′) (ThermoFisher). Next, a blunt-end double-stranded adapter was ligated to the newly synthesised strand using T4 DNA Ligase, followed by a second bead immobilisation and wash step. After adapter ligation, libraries were heat-denatured to release the library molecules from the beads, and the supernatant was retained for amplification.

Pre-amplified libraries were subjected to indexing PCR using P5 and P7 primers containing sample-specific barcodes. PCR was performed using 13 cycles, following quantification to determine the appropriate cycle number for balanced amplification across samples.

2.9 | Library Pooling and Sequencing

All DSL and SSL indexed libraries were purified with AMPure XP beads (Beckman Coulter), quantified with an Agilent TapeStation, and pooled at equimolar concentrations. Final pools were quantified using qPCR (Applied Biosystems). Libraries were sequenced on the Illumina NextSeq platform (Illumina, USA) using the 2 × 150 bp configuration.

2.10 | Bioinformatic Processing and Analyses

Sequencing data was converted into FASTQ file format using the Illumina bcl2fastq (v1.8.4) software. The raw FASTQ files were then demultiplexed, trimmed and collapsed using AdapterRemoval v2 (Schubert et al. 2016) based on the unique P5/P7 barcoded adapters (–minlength 25, –minquality 25, –trimns, –trimqualities, –collapse), resulting in analysis-ready reads. Seqkit (v2.6.1) was used to calculate the number of reads, average read lengths and GC content for each sample. Clonality (i.e., percentage of unique reads) was calculated using an in-house script. We tested statistical significance for these assessments using a two-way ANOVA with the *aov()* function in R (v.4.1.1). Scripts for these analyses can be found on this GitHub page (https://github.com/microARCHlab/AncientCalculusLabMethods_2024).

The analysis-ready reads were taxonomically binned with the nucleotide alignment option in the MEGAN Alignment Tool (MALTn; v.0.3.8) (Herbig et al. 2016). The reads were aligned against an in-house RefSeq ('RefSeq') database that was previously published (Eisenhofer and Weyrich 2019) and includes 47,696 archaeal and bacterial genome assemblies at scaffold, chromosome and complete levels. The resulting alignment-based blast-text files were then converted into RMA files using the blast2rma script included with the program MEGAN6 (v 6.11.1) (Huson et al. 2016) with the following last common ancestor (LCA) parameters: Weighted-LCA = 80%, minimum bitscore = 42, minimum *E*-value = 0.01, minimum support

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percent = 0.1. The resulting RMA6 files were imported into MEGAN6. We used MEGAN6 CE to also export species-level BIOM tables using the taxonNameToCount summarized option and excluded the non-assigned reads.

2.11 | Preservation Assessment

To assess the preservation of the endogenous content in the samples, we used SourceTracker2 (v2.0.1) (Knights et al. 2011). The comparative data for this analysis came from studies analysing modern dental calculus, modern dental plaque, soil and skin, as well as the EBCs from this study (Table S2), as described in Gancz et al. (2023). Reads for the source samples were processed using the same bioinformatics pipeline as the samples in this study. The comparative sources were selected for the following reasons: they were generated using Illumina shotgun sequencing strategies and yielded at least 1000 mapped reads to our NBCI-RefSeq database. The species-level table for the sources was combined with the samples in this study. We employed SourceTracker2 with the following parameters: alpha2 = 1.0, sink_rarefaction_depth = 5573, source_rarefaction_depth = 1489. The rarefaction depth for the sinks was set to 5573 reads, based on the sequencing depth of sample 18,393, which had the lowest read count among the calculus samples that had at least 1000 reads. The rarefaction depth for the source samples was set to 1489 reads, based on sample 19,028, which represented the lowest sequencing depth among the reference source datasets with at least 1000 reads (Table S3). Samples with less than 1000 counts were excluded from this analysis. The results for this analysis were visualised using R (v.4.1.1).

Post-mortem damage of individual microbial genomes was assessed with MapDamage (v2.0) (Jónsson et al. 2013) by mapping analysis-ready reads to Anaerolineaceae bacterium oral taxon 439 (ASM171754v1), *Methanobrevibacter oralis* DSM 7256 (ASM163927v1) and *Olsenella* sp. *taxon 807* (strain F0089) (ASM118951v2). These microbes were selected because they were abundant across many samples in the Niger (Figure S1) and Hungary (Figure S2) datasets, and have been well documented in other ancient dental calculus studies (Weyrich et al. 2017; Ottoni et al. 2021; Quagliariello et al. 2022; Eisenhofer et al. 2020; Granehäll et al. 2021). For the single-stranded libraries, we incorporated the single-stranded option in mapDamage to ensure the correct detection of C → T substitutions at both ends of the reads.

In addition to mapDamage, we utilised ChangePoint (Gancz et al. 2023; Liu 2019). Unlike MapDamage, ChangePoint does not rely on mapping reads to a reference genome but rather it uses a likelihood ratio test to assess whether the ends of DNA fragments exhibit an enrichment of thymines (T) at the 5′ ends and adenines (A) at the 3′ ends. Because it operates reference-free, ChangePoint assumes that such nucleotide patterns, when observed across a metagenomic dataset, reflect a community dominated by authentic aDNA, with deamination signals dispersed across multiple taxa. Samples are considered to represent authentic ancient metagenomes if their adjusted *p*-values are less than 0.05. To optimise the performance of ChangePoint, we subsampled each sample to 100,000 reads.

2.12 | Microbial Compositional Analysis

Sample 18393_Niger_PB_SSL was removed from all compositional analyses because of its low assigned read count (400) (Figure S3). We then proceeded with downstream analyses on the remaining 23 samples. While pooling the Nigerien and Hungarian datasets would have increased statistical power, this approach would likely obscure the intra-sample variability we aim to assess, as region-level differences in microbial composition would dominate the signal (Ottoni et al. 2021). By stratifying analyses by country, we are better able to isolate and interpret the effects of DNA extraction and library preparation protocols on key variables such as preservation state, DNA fragment length recovery, GC content, clonality, endogenous content, DNA deamination and microbial diversity and composition, all of which have been shown to be key metrics for assessing aDNA and ancient microbiome recovery (Gamba et al. 2016; Mann et al. 2018; Wales et al. 2015; Hagan et al. 2020).

2.13 | Taxonomic Analyses and Contaminant Filtering

To evaluate the influence of wet-lab protocols on microbial composition, we conducted taxonomic analyses using an unfiltered dataset. The unfiltered dataset was used to assess whether certain DNA extraction or library preparation protocols were more prone to recovering known contaminant taxa, which could indicate protocol-specific biases in contamination susceptibility. In parallel, we used the *decontam* package (v.1.26.0) (Davis et al. 2018) to identify and remove contaminant species based on frequency patterns. We tested three contamination score thresholds with *decontam*—0.10, 0.25 and 0.50. The 0.50 threshold, which has been validated in previous studies (Fellows Yates, Velsko, et al. 2021), identified two oral taxa—*Ottowia* sp. oral taxon 894 and *Actinomyces israelii*—as contaminants, although most species in this dataset were non-oral species (Table S9). Based on this and prior literature, we proceeded with the dataset filtered at the 0.50 threshold. Furthermore, we removed features that were not found in at least two samples and had less than 10 counts in the entire dataset, as these are likely spurious alignments (Tables S10 and S11). Following this filtering, samples 18398_Niger_PB_DSL, 18398_Niger_QG_DSL and 18400_Niger_PB_DS had less than 1000 counts. Consequently, they were excluded from all the analyses on the filtered dataset. This refined dataset enabled us to determine whether the observed patterns in microbial diversity and composition held after excluding probable contaminants. By comparing results from both unfiltered and filtered datasets, we were able to assess the robustness of protocol-related trends and understand how taxonomic profiles shift in response to contaminant filtering, as well as how the entire metagenome (endogenous and exogenous signatures) is influenced by these methods.

Differences in alpha diversity (observed species) were assessed using a Kruskal–Wallis test. This test was applied for both overall group and pairwise comparisons. For beta diversity, the DEICODE plugin in QIIME2 (v.2024.10) (Martino et al. 2019) was used to centered-log transform the species-level

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tables and convert them into Aitchison distance matrices. We performed permutational multivariate analysis of variance (PERMANOVA) using the adonis function in QIIME2 with 999 permutations, applying the following model: Aitchison distance matrix ~ "ExtractionType" × "LibraryMethod". We further explored beta diversity using principal coordinates analysis (PCoA) based on Aitchison distances at the species level. Statistical significance for all tests was set at < 0.05.

2.14 | Associations Between Microbial Abundance and Laboratory Methods

We employed Microbiome Multivariable Association with Linear Models (MaAsLin2) (v.1.20.0) to carry out differential abundance analyses on species-level tables exported from MEGAN (Mallick et al. 2021). The default MaAsLin2 parameters were utilized (taxonomic feature prevalent in a minimum of 10% of all samples and minimum percentage relative abundance 0.01) with the exception that the corrected max_significance threshold was set to 0.05. The "ExtractionType" and "LibraryMethod" columns in the metadata were inputted as "fixed_effects" in the model.

3 | Results

3.1 | Fragment Length Recovery

Ancient DNA datasets are characterised by highly fragmented sequences, resulting in short read lengths (Sawyer et al. 2012; Pääbo 1989). To evaluate how laboratory protocols influence fragment length recovery, we first assessed the effects of DNA extraction and library preparation methods (Table S4; Figures S3–S5). In the Niger dataset, neither DNA extraction nor library preparation methods had a significant effect on fragment length recovery. For the DNA extraction comparison, samples prepared with the QG method recovered longer fragments (67.60 bp, standard deviation [SD] = 25.471) than the PB method (61.42 bp, SD = 18.060), but this difference was insignificant ($F = 0.199$, $p = 0.667$). For the comparison of library preparations, the DSL method recovered longer fragments (69.25 bp, SD = 30.211) than the SSL method (59.77 bp, SD = 5.587), but the difference was not significant ($F = 0.469$, $p = 0.513$). When comparing the combination of the DNA extraction and library preparation methods, the interactions between the two did not affect fragment length recovery ($F = 0.008$, $p = 0.930$).

For the samples from Hungary, the QG method recovered longer fragments (82.72 bp, SD = 30.625) than its PB counterpart (56.13 bp, SD = 8.871), and these differences were significant ($F = 53.92$, $p = 0.0000805$). Whereas for the library preparation analysis, the DSL method recovered significantly longer fragments (86.57 bp, SD = 26.303) than the SSL method (52.28 bp, SD = 6.597) ($F = 89.68$, $p = 0.0000127$). The interaction between DNA extraction and library preparation methods was also significant ($F = 31.6$, $p = 0.000498$). Overall, DNA extraction and library methods did not significantly impact fragment recovery of these poorly preserved samples, but they did for these well-preserved samples.

3.2 | GC Content

We explored whether DNA extraction and library protocols impact the GC content of samples (Table S4; Figure S6). For the Niger dataset, the QG method (mean = 48.23%, SD = 7.110) and PB method (mean = 47.90%, SD = 6.483) yielded similar GC content ($F = 0.045$, $p = 0.838$). However, the GC content between the DSL method (mean = 53.85%, SD = 1.466) and SSL method (mean = 42.28%, SD = 3.194) was significantly different ($F = 52.303$, $p = 0.0000896$). The interaction between the DNA extraction and library preparation methods was insignificant ($F = 0.003$, $p = 0.959$).

For the Hungarian dataset, the mean GC content for samples extracted with the QG method was 48.14% (SD = 5.363) and for the PB method was 47.95% (SD = 6.503), showing no significant difference (ANOVA: $F = 0.009$, $p = 0.92$). In contrast, a significant difference was observed between library preparation methods: samples prepared with the DSL protocol had a higher GC content (mean = 52.70%, SD = 2.796) than SSL (43.39%, SD = 3.351) (ANOVA: $F = 23.187$, $p = 0.001$). There was no difference in GC content between the interaction of the two methods ($F = 0.479$, $p = 0.51$). These findings suggest that, irrespective of preservation status, the choice of library preparation protocol can influence GC content recovery, while the DNA extraction method did not have a discernible impact.

3.3 | Clonality

We explore whether laboratory protocols impacted the clonality of the recovery of DNA from dental calculus samples (Table S4; Figure S7). For samples from Niger, neither DNA extraction method nor library preparation method significantly impacted the recovery of clonal sequences, while mild differences were observed. The QG method recovered a lower percentage of unique reads (mean = 12.71%, SD = 6.247) than the PB method (mean = 27.61%, SD = 31.22), but these differences were not significant ($F = 1.121$, $p = 0.303$). While samples prepared with the DSL method yielded a lower percentage of unique reads (mean = 17.00%, SD = 5.229) than the SSL method (mean = 23.31%, SD = 33.103), these differences were also not significant ($F = 0.218$, $p = 0.653$). The interaction between the two protocols did not have a significant interaction ($F = 1.015$, $p = 0.343$).

A similar trend was observed for the samples from Hungary. There were minor differences in the percentage of unique reads between the QG (mean = 78.12%, SD = 32.936) and PB methods (mean = 94.22%, SD = 2.315) ($F = 1.331$, $p = 0.282$), as well as between DSL (mean = 92.34%, SD = 5.662) and SSL (mean = 80.00%, SD = 33.500) methods ($F = 0.782$, $p = 0.402$). The combination of methods also had a minimal impact on the percentage of unique reads recovered ($F = 0.540$, $p = 0.483$). This suggests that DNA extraction and library preparation methods can have little impact on the clonality observed in ancient metagenomic libraries.

3.4 | Microbial Source Tracking

To authenticate the microbial content in the dental calculus samples, we used SourceTracker2 (Knights et al. 2011).

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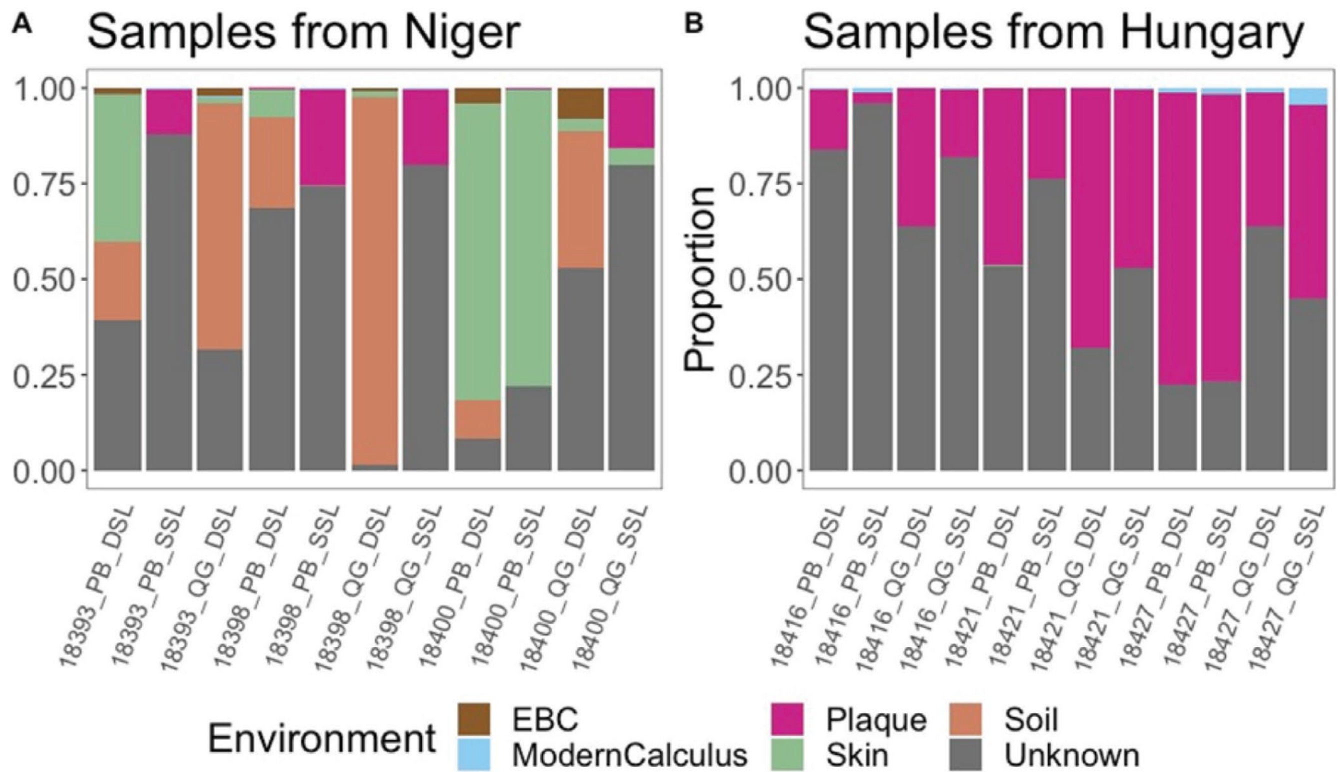

FIGURE 2 | SourceTracker2 results. SourceTracker2 was applied using extraction blank controls (EBCs) processed alongside the dental calculus samples, modern calculus, plaque, skin, and soil samples as sources and dental calculus samples as sinks. Sink rarefaction depth was 5573 and source rarefaction depth was 1489. The SourceTracker2 results for the A) Nigerien and B) Hungarian dataset were visualised using *ggplot* in R.

SourceTracker2 predicts the potential origins of the microbial DNA found in the dental calculus samples from Niger and Hungary (Table S5; Figure 2). For samples from Niger, the DNA extraction method had minimal influence on the recovery of oral DNA (i.e., DNA sequences matching with modern dental calculus or plaque sources) as the QG (mean = 7.2%, SD = 0.002) and the PB (mean = 6.4%, SD = 0.002) methods had similar yields (two-way ANOVA, F = 0.039, p = 0.850). This is not the case with library preparation methods. The SSL method was much more effective in recovering oral DNA (mean = 14.8%, SD = 0.002) than the DSL method (mean = 0.05%, SD = 0.0002) (two-way ANOVA, F = 12.847, p = 0.009). Notably, the PB + SSL approach yielded the most oral DNA for samples 18,393 (12.0%) and 18,398 (25.20%), while the QG + SSL method yielded the most amount of oral DNA for sample 18,400 (15.50%). However, there was not signfiicant interaction between DNA extraction and library preparation (tw-oway ANOVA, F = 0.373, p = 0.561). Furthermore, the DSL method tended to recover more DNA attributed to contaminant sources, such as EBC, skin, and soil, across the samples (mean = 66.20%, SD = 25.70%) than their SSL counterparts (mean = 16.50%, SD = 34.20%).

Similar to the Niger dataset, the choice of DNA extraction method did not have a significant effect on the recovery of endogenous content (two-way ANOVA, F = 0.027, p = 0.873). However, unlike the Niger dataset, library preparation was not significant (two-way ANOVA, F = 0.334, p = 0.579), although the DSL method had yielded a higher percentage of endogenous content for the Hungarian samples (mean = 46.70%, SD = 0.005) than the SSL method (mean = 37.4%, SD = 0.015). Notably, for the Hungarian group, all samples had less than 1% attributed to contaminant source (DSL mean = 0.04%, SD = 0.0002; SSL mean = 0.05%, SD = 0.0003). Together, these results suggest that library preparation plays a significant part in recovering endogenous content from poorly preserved samples, but not for well-preserved samples and can influence recovery of contaminant DNA.

3.5 | Laboratory Protocols Impact Recovery of Damage Profiles of Samples

We explored whether the methodologies for DNA extraction and library preparation methodologies had an impact on the ability to analyse deamination rates with MapDamage2. We mapped analysis-ready reads from each sample to three microbes: Anaerolineaceae bacterium oral taxon 439 (ASM171754v1), *Methanobrevibacter oralis* (strain DSM 7256) (ASM163927v1) and *Olsenella* sp. taxon 807 (strain F0089) (ASM118951v2). We selected these microbes because of the relatively high abundance found across samples (Figures S1 and S2). For the Niger dataset, samples prepared with the DSL method did not exhibit deamination patterns expected for ancient samples (Table S6; Figures S8–S10). This result is likely due to the insufficient number of uniquely mapped reads. None of the samples prepared with the DSL method yielded more than two unique reads to the three microbes. Samples prepared with the SSL method yielded more uniquely mapped reads, albeit there was a substantial difference between the groups based on extraction methods. Samples prepared with the QG extraction and SSL methods yielded more unique reads

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mapped (mean = 1415.44; SD = 1702.23) than samples prepared with the PB extraction and SSL method (mean = 116.56; SD = 113.86) (ANOVA, $F = 5.217$; $p = 0.036$). These results suggest that the QG + SSL method was the only protocol that consistently recovered a sufficient number of reads—defined here as at least 100 uniquely mapped reads—to allow for preliminary assessment of deamination patterns in the poorly preserved samples. While this threshold provides some indication of aDNA authenticity, it still falls short of the commonly accepted benchmark of 1000 uniquely mapped reads to confidently determine whether a microbe is genuinely ancient (Mann et al. 2023).

DNA extraction and library preparation methods seem to have less influence on the well-preserved samples from Hungary. Most samples from Hungary prepared with the DSL method exhibit expected A → T and G → C misincorporations at the 5′ and 3′ ends, respectively (Table S6; Figures S11–S13). Only sample 18,427 prepared with the PB + DSL methods yielded less than 100 unique mapped reads to the *M. oralis* genome. Unlike the results for the Niger dataset, neither DNA extraction nor library preparation method played a significant role in yielding more uniquely mapped reads. However, the SSL method did yield a significantly larger number of uniquely mapped reads to the *M. oralis* genome (mean = 6034.83) than the DSL method (mean = 678.67) (ANOVA: $F = 6.284$; $p = 0.03$).

To assess the overall damage profiles for the samples from Hungary and Niger, we employed ChangePoint, a reference-free damage profiler (Liu 2019; Gancz et al. 2023). Our ChangePoint results indicate that the samples from Niger supported the MapDamage results in that an insignificant amount of DNA fragments in the samples were enriched with T's on their 5′ ends and C's on their 3′ ends (Table S7; Figure S14). All samples from Hungary, on the other hand, exhibited expected aDNA-associated damage signatures, irrespective of DNA extraction and library preparation methods employed (Table S7; Figure S14). These findings suggest that laboratory methods can significantly impact the recovery of authenticated aDNA sequences from ancient microbes.

3.6 | Alpha Diversity

We explored the impact that both DNA extraction and library preparation methods have on alpha diversity using the observed species index. For the unfiltered Niger dataset, neither DNA extraction ($H = 1.256$, $q = 0.749$) nor library method had a significant effect ($H = 1.256$, $q = 0.262$) on alpha diversity. This pattern remained consistent for the filtered dataset, where DNA extraction method ($H = 0.000$, $q = 1.000$) and library preparation ($H = 0.000$, $q = 1.000$) were not statistically significant (Figure 3A,B; Table S12). For the unfiltered Hungarian dataset, DNA extraction method was not significantly associated with alpha diversity ($H = 0.926$, $q = 0.336$), but library preparation protocol was significant ($H = 4.689$, $q = 0.03$). This pattern also held for the filtered Hungarian dataset when analysing DNA extraction ($H = 0.930$, $q = 0.335$) and library preparation ($H = 4.364$, $q = 0.037$). In both Hungarian datasets, the DSL protocol recovered a greater number of species compared to the SSL method (Figure 3C,D; Table S12). In summary, these results suggest that library preparation method can have a notable impact on the recovery of alpha diversity of well-preserved samples, whereas it may not for samples with poor aDNA preservation.

3.7 | Compositional Analysis

We evaluated whether DNA extraction and library preparation methods influenced beta diversity (i.e., compositional differences) via adonis and PCoA. For the unfiltered dataset including samples from Niger, both DNA extraction ($R^2 = 0.180$, $p = 0.039$) and library preparation ($R^2 = 0.224$, $p = 0.022$), as well as their interaction ($R^2 = 0.260$, $p = 0.005$), were associated with beta diversity (Table S13; Figure S15A). A similar pattern was observed for the filtered dataset: DNA extraction was not significantly associated with microbial composition ($R^2 = 0.088$, $p = 0.667$), whereas library preparation remained significant and explained a substantial portion of the variation ($R^2 = 0.588$, $p = 0.017$) (Figure 4A; Table 2).

A different pattern emerged for the samples from Hungary in the unfiltered dataset, where both DNA extraction ($R^2 = 0.099$, $p = 0.374$) and library preparations ($R^2 = 0.111$, $p = 0.321$) were not significantly associated with overall composition. This analysis also indicated that the two protocols were not significantly interacting ($R^2 = 0.052$, $p = 0.669$) (Table S13). These results were also supported by our PCoA (Figure S15B), where no clear segregation between samples based on their DNA extraction or library method was observed. This pattern persisted in the filtered dataset, where neither DNA extraction method ($R^2 = 0.088$, $p = 0.667$), library preparation protocol ($R^2 = 0.070$, $p = 0.640$), nor their interaction ($R^2 = 0.027$, $p = 0.986$) showed a significant association with community composition (Table 2). Overall, this suggests that DNA extraction and library preparation methods can significantly impact the microbial composition recovered from poorly preserved samples but not in these well-preserved samples.

3.8 | Differential Abundance Analysis

To explore whether differentially abundant species were associated with DNA extraction or library preparation method, we utilised MaAsLin2 (Mallick et al. 2021), treating both variables as fixed effects. In the unfiltered Niger dataset, 22 species were found to be significantly associated with library preparation method (Figure 5; Table S8). Interestingly, the analysis of the filtered dataset indicated that library preparation was significantly associated with the abundance of 22 of the 25 differentially abundant species (Table S14). All 22 species were enriched in samples prepared with the DSL method and are likely contaminants that were not removed during filtering, as they are not typical oral taxa (e.g., *Anaplasma phagocytophilum*, *Rhodoplanes* sp. *Z2 YC6860*, and *Xenophilus azovorans*). These results further support the interpretation that the DSL method may recover more contaminant DNA in poorly preserved samples, which is supported by the SourceTracker analysis.

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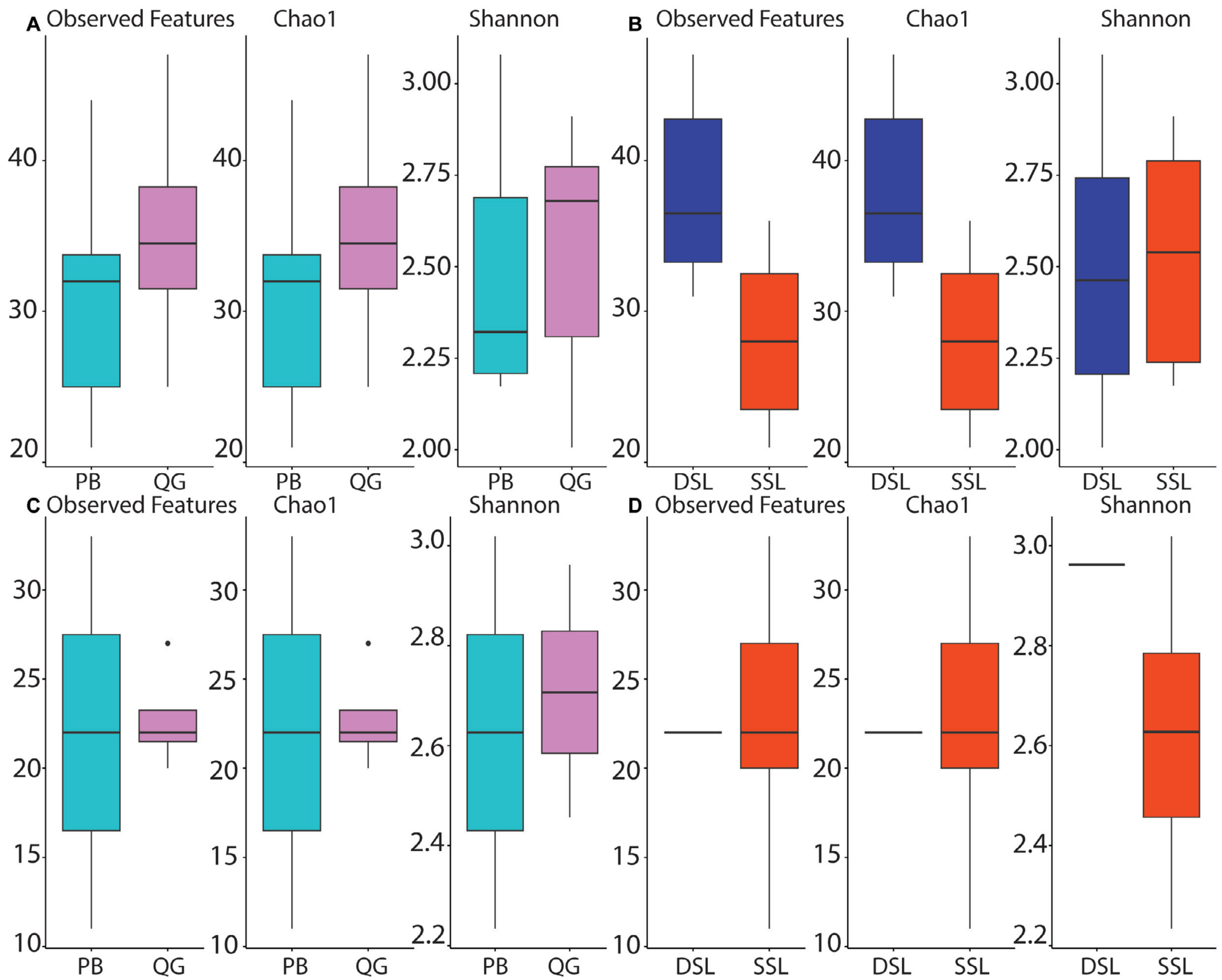

FIGURE 3 | Box plots comparing alpha-diversity within each cohort based on observed species, Chao1, and Shannon. (A, B) The alpha diversity results for the dataset including Niger samples and (C, D) are for the dataset from Hungary. The plots were generated using the plot_richness() function in the phyloseq package. (A) Alpha diversity for Niger dataset by DNA extraction method. (B) Alpha diversity for Niger dataset by library preparation method. (C) Alpha diversity for Hungary dataset by DNA extraction method. (D) Alpha diversity for Hungary dataset by library preparation method. Only the Hungary samples processed with the single stranded method showed a significant decrease in alpha diversity compared to samples processed with double stranded libraries ($H=4.689$, $q=0.03$).

No species were differentially abundant in the Hungarian unfiltered dataset (Table S8) or the filtered dataset (Table S14). This finding, in contrast to the Niger dataset, may suggest that library preparation has little influence on the recovery of endogenous oral species in well preserved samples.

4 | Discussion

4.1 | Wet-Lab Protocols and Preservation State Shape Ancient Metagenome Signatures

Our study highlights the importance of accounting for the variation attributed to DNA extraction and library preparation methods when recovering aDNA from archaeological dental calculus, while also emphasising that their effects are shaped by the preservation state of the sample. Previous work, largely focused on bone or dentin (e.g., Gamba et al. 2016; Dabney et al. 2013), established that extraction and library protocols can influence endogenous DNA yield and fragment length recovery. However, our findings demonstrate that those insights cannot be directly translated to dental calculus, likely due to its distinct biological and chemical properties compared to dentin (Mann et al. 2018; White 1997). Critically, we show that for well-preserved dental calculus, laboratory methods can affect the recovery of aDNA across several key metrics, such as fragment length, GC content, and diversity. In contrast, within poorly preserved samples, methodological effects were more limited or inconsistent, suggesting that these biases may be less prevalent in well-preserved material compared to that of poor preservation. This nuance adds an important dimension to ongoing conversations about protocol optimization in ancient microbiome research and potential meta-analyses of ancient oral microbiomes compared across laboratories or research studies (Velsko et al. 2024; Wright 2025).

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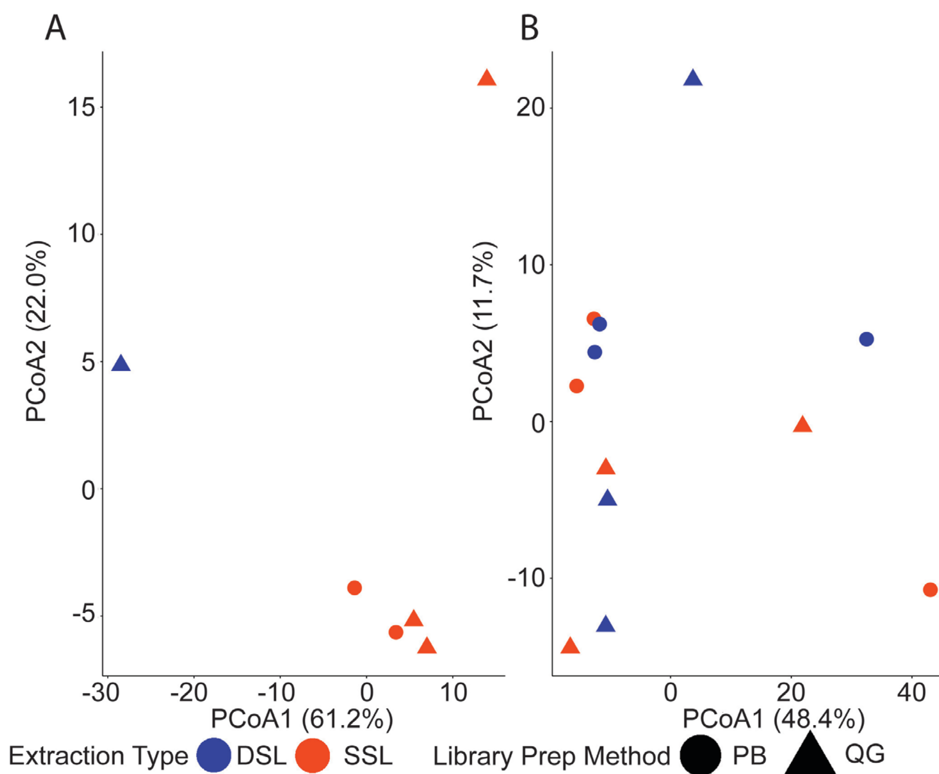

FIGURE 4 | PCoAs of filtered datasets on the filtered Nigerien and Hungarian datasets based on Aitchison distance at the species level. (A) PCoA of the Niger dataset shows a clear separation in microbial composition between samples processed with SSL method and those with the DSL method. (B) In contrast, the PCoA of the Hungarian dataset does not show a consistent pattern of separation between SSL and DSL methods.

TABLE 2 | Adonis results for the filtered dataset at the species level. *Indicates signicance at q (FDR corrected) equal to or less than 0.05.

	df	R^2	Pr(>F)
Results for Niger			
Extraction type	1	0.088	0.667
Library method	1	0.588	0.017*
Residuals	3	0.324	NA
Total	5	1	NA
Results for Hungary			
Extraction type	1	0.091	0.458
Library method	1	0.070	0.640
Extraction type:library method	1	0.027	0.986
Residuals	8	0.812	NA
Total	11	1	NA

4.2 | Methodological Biases and the Recovery of Oral Microbiome Signal

Our SourceTracker2 results and diversity analyses suggest that the library preparation method, more than the DNA extraction protocol, influences the recovery of the taxonomic composition from calculus samples. While previous studies suggested that SSL methods may better preserve short or damaged fragments (Bennett et al. 2014; Wales et al. 2015), we observe that their impact can be context-specific. For instance, in poorly preserved Niger samples, the SSL method appeared more effective in recovering oral taxa despite low overall preservation. Conversely, in better-preserved Hungarian samples, the DSL libraries recovered more total oral DNA than the SSL method. These results support the idea that SSL methods may offer better sensitivity for highly degraded samples.

4.3 | Wet Laboratory Methods Can Impact the Ability to Authenticate aDNA

Our use of both mapDamage and ChangePoint suggests that, while aDNA authentication primarily reflects preservation state, the choice of DNA extraction method and library preparation protocol can influence the recovery of DNA with damage patterns. For example, among the poorly preserved Niger samples, only those processed with the QG + SSL method yielded sufficient reads for authentication with mapDamage, a pattern consistent with previous observations (Bennett et al. 2014; Wales et al. 2015). In contrast, no clear differences were observed across different laboratory protocols in the better-preserved Hungarian samples, also aligning with prior studies suggesting that a DSL strategy is likely sufficient to recover authentic ancient metagenomic profiles when preservation is good (Wales et al. 2015).

Taken together, these findings suggest that while preservation remains the key determinant of authentic aDNA signal, researchers working with highly degraded calculus samples

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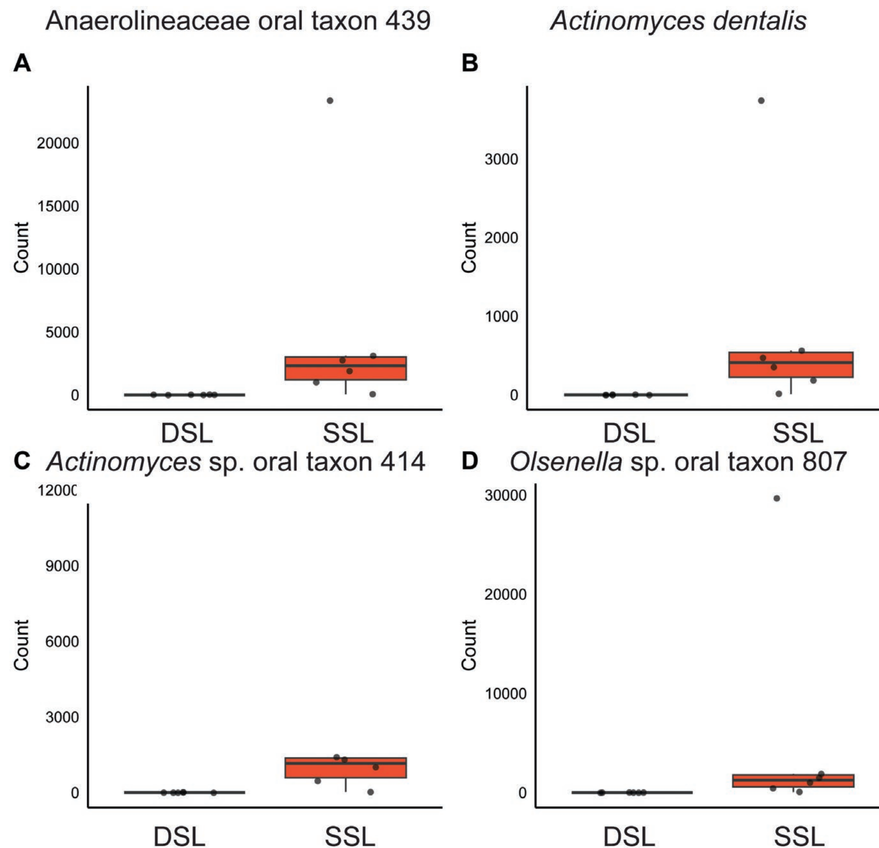

FIGURE 5 | Abundances of oral species identified in the unfiltered Niger dataset as differentially abundant using MaAsLin2. This figure highlights the relative abundances of (A) Anaerolineaceae oral taxon 439, (B) *Actinomyces dentalis*, (C) *Actinomyces* sp. oral taxon 414, and (D) *Olsenella* sp. oral taxon 807. These species are known to be associated with the oral microbiome and have frequently been recovered in archaeological studies, serving as indicators of authentic ancient oral communities. The results suggest that samples processed using the SSL preparation method recovered higher abundances of these ancient oral taxa compared to the double-stranded library (DSL) method. This supports the interpretation that the SSL method is more effective in capturing fragmented and damaged DNA characteristics of authentic ancient oral microbes.

may benefit from using the QG+SSL protocol or a mixture of approaches to maximise authentication potential. However, the SSL step may not be necessary for well-preserved samples. Nevertheless, method choice should be decided upon the research question and expected preservation status.

4.4 | DNA Extraction and Library Protocols Impact the Recovery of Oral Microbiome Diversity

While several studies have shown that DNA extraction and library preparation methods influence the microbial diversity obtained from modern oral samples (Teng et al. 2018; Vesty et al. 2017; Rosenbaum et al. 2019), others indicated that wet lab protocols play a minimal role (Lazarevic et al. 2013). Nonetheless, it is difficult to discern whether the findings in these previous studies are applicable to ancient oral microbiome research since dental calculus has unique characteristics. Whether either procedure impacts the recovery of ancient oral microbiomes has not been examined in detail. Both DNA extraction and library preparation protocols impacted the alpha diversity observed in the Niger samples. For the Hungarian samples, only the choice of laboratory preparation method was associated with alpha diversity. A similar trend was observed when evaluating whether laboratory procedures influenced the recovery of beta diversity from samples. For the Niger samples, the DNA extraction method and library preparation, independently, as well as their interaction, were significantly associated with beta diversity. This was not the case with the Hungarian samples, as neither DNA extraction nor library preparation was associated with microbial composition. Taken together, these data suggest that wet lab protocols influence the alpha and beta diversity for poorly preserved samples to a greater extent than they do for well-preserved samples.

Our study also represents the first attempt to investigate whether aDNA extraction or library preparation protocols selectively enrich or deplete certain species within a dataset. In the unfiltered Niger dataset, we found that samples prepared using the SSL method exhibited a higher abundance of 22 species, some of which are oral species (e.g., Anaerolineaceae bacterium oral taxon 439, *Actinomyces dentalis*, *Actinomyces* sp. oral taxon 414 and *Olsenella* sp. oral taxon 807). However, no differentially abundant species were detected in either the unfiltered or filtered Hungarian datasets. These findings suggest that, for samples with adequate aDNA preservation, library protocol may have minimal impact on the recovery of specific taxa relative to the DSL method. In conclusion, the choice of library preparation may be critical when working with poorly preserved samples.

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4.5 | Limitations to Our Study

There are some limitations to this study. First, our study has a small sample size regarding the number of samples collected from each context, which limits the scope of drawing broad conclusions. A larger dataset could offer a more comprehensive understanding of these methods and how they affect aDNA recovery. However, it is important to acknowledge that obtaining and processing such a dataset will come with higher costs and decisions around the irreversible destruction of valuable ancient calculus samples for technical analysis.

Another limitation is that the comparison in our study includes samples from only two specific archaeological contexts. The efficiency of the laboratory methods may vary in burial contexts with different environmental processes. For example, burial environments in more acidic environments (pH 3.5–4.5) are known to be more destructive environments for the biomolecular integrity than alkaline soils (7.5–8.0) (Kendall et al. 2018). Moving forward, accounting for whether certain protocols improve the recovery of DNA based on their burial context could prove to be noteworthy.

We also did not account for other confounding variables such as sample weight, post-excavation treatment, or collection and storage procedures. These factors, although rarely documented in studies, have been shown to influence DNA preservation in studies of modern microbial communities (Armstrong et al. 2021; Luo et al. 2016; Jenkins et al. 2018; Franceschetti et al. 2024; Singh and Okpeku 2024). This underscores the need for further research into pre-sequencing factors that may influence aDNA recovery from archaeological dental calculus samples.

Finally, while the SSL method was useful in recovering authentic aDNA from poorly preserved samples in our study, updates to this method (e.g., SCR protocol) may offer comparable or even improved recovery at a significantly lower cost. Exploring whether the SCR protocol is susceptible to the same biases here could help evaluate its potential as an alternative to the methods employed in our study, especially in large-scale projects or in settings where resources are limited.

5 | Conclusion

Although both DNA extraction and library preparation methods have shown to influence the recovery of DNA from metagenomic samples (Teng et al. 2018; Vesty et al. 2017; Rosenbaum et al. 2019), little has been done to investigate how wet lab protocols impact the DNA recovery from archaeological dental calculus. Overall, this study indicates that DNA extraction and library preparation methods are critical factors in the recovery of ancient oral microbiomes, especially in samples of varying states of preservation. These findings carry important implications for data comparability, which is essential for conducting meta-analyses and to assessing the robustness of individual study findings. However, the lack of standardisation in ancient oral microbiome research may be limiting the field from achieving data comparability and reproducibility. In some cases, methodological differences could explain why research teams reach different conclusions on important topics, such as whether diet and cultural transitions led to shifts in ancient oral microbiomes (Adler et al. 2013; Weyrich et al. 2017; Ottoni et al. 2021; Quagliariello et al. 2022; Fellows Yates, Andrades Valtueña, et al. 2021). Differing wet lab protocols among research teams could play a part in these conflicting findings and interpretations. Systematic meta-analyses incorporating data across studies could explain the inconsistency in the field.

The evidence presented here indicates no singular approach yields the most optimal results in all aspects of data quality, irrespective of preservation state, implying that no single "optimal standard" currently exists. This raises a fundamental question about whether the field should prioritize standardisation or optimization of methods. Standardising protocols could mitigate technical variation across datasets, promoting more reproducible and replicable results. Conversely, optimising methods for each dataset may enhance accuracy for individual samples but would complicate data integration. As such, our study highlights the delicate balance between standardisation and optimization, emphasising the importance of well-documented methodologies, transparent reporting, and thoughtful consideration of the unique attributes of each sample. As aDNA analysis of dental calculus continues to shed light on the past, it is essential to address how wet lab protocols influence the recovery of ancient oral microbiomes.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The sequencing data for this project can be found on the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under the accession number: PRJNA1031139. All scripts and analytical code used in the project are available on GitHub: https://github.com/microARCHlab/AncientCalculusLabMethods_2024.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** men70054-sup-0001-DataS1.docx. **Tables S1–S14:** men70054-sup-0002-TableS1-S14.xlsx.

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