1 **Wet-lab methods matter: Extraction and library preparation protocols impact oral microbiome**
2 **recovery from archaeological dental calculus**

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17 Keywords: ancient DNA, oral microbiome, metagenomics, archaeology, dental calculus

18 **Abstract**

19 Applying next-generation sequencing and metagenomic strategies to archaeological dental calculus has
20 facilitated the reconstruction of oral microbiomes of individuals who lived in the past, greatly enhancing
21 our understanding of their health, demography, and lifestyles. Nevertheless, the workflow for ancient
22 metagenomics is continually evolving, which raises concerns regarding reproducibility. While research
23 shows that the selection of DNA extraction methods and library preparation protocols influences observed
24 microbial profiles of present-day populations, no similar systematic evaluation has been conducted on
25 ancient oral microbiomes. This leaves a gap in our understanding about the degree to which variations in
26 wet-lab methodology introduce variability in results. Here, we apply two of the most widely used DNA
27 extraction and library preparation methods on dental calculus samples from Hungary and Niger. The
28 samples from each context date to similar temporal periods but have varying preservation states.
29 Specifically, we explore DNA fragment length recovery, GC content, clonality, endogenous content, DNA
30 deamination, and microbial composition. Our results indicate that both DNA extraction and library
31 preparation protocols considerably impact DNA recovery from archaeological dental calculus. They also
32 suggest that no single protocol consistently outperformed others in all assessments. This underscores the
33 necessity for further research to include laboratory methodologies as factors when carrying out meta-
34 analyses. Accounting for such technical variation is essential for enhancing the reproducibility of results
35 and advancing our knowledge of how ancient oral microbiomes respond to environmental and cultural
36 shifts.

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38 Keywords: ancient DNA, dental calculus, oral microbiome, metagenomics

39 ## 1. Introduction

40 Dental calculus develops during the cyclic mineralization of dental plaque, a biofilm created when
41 microorganisms adhere to the tooth surface[1,2]. During this process, biomolecules from dietary and
42 environmental sources are also entrapped in the dental calculus matrix[3,4]. If not removed by dental
43 hygienic practices, dental calculus accumulates throughout the life of an individual, providing a long-term
44 record of their oral microbiome—the collection of bacteria, archaea, viruses, fungi, and parasites
45 occupying the human mouth[5]. Oral microbiome research has provided new insights into how oral
46 microbes play a role in cardiovascular disease, inflammatory bowel disease, rheumatoid arthritis, and
47 colorectal cancer[6–8]. Applying ancient DNA (aDNA) and metagenomic strategies to archaeological
48 dental calculus has also afforded the ability to reconstruct oral microbiomes from past populations[4,9–
49 13], which has gleaned novel insights about their health[12,14], diets[11,15–17], demography[18], and
50 lifestyles[15,19].

51 Despite significant strides in ancient oral microbiome research over the past decade[20–23], standardizing
52 protocols in the field has been slow. Although a few studies examine the efficiency of laboratory
53 methods, these have primarily focused on the sample collection and decontamination steps[24–27]. As
54 such, there remains a significant gap in understanding how other steps in ancient metagenomic
55 workflows impact downstream results and reproducibility. In particular, the field currently lacks
56 standardized protocols for DNA extraction and library preparation. This presents a challenge as
57 previous studies on modern microbiomes demonstrate that the choice of DNA extraction[28–30] and
58 library preparation[31–33] methods influence DNA recovery, and subsequently, the observed microbial
59 community of samples. We address this lacuna of knowledge in our study by comparing two widely
60 used DNA extraction methods and two library preparation methods in ancient DNA research. Our
61 analyses offer us insights into the relationship between laboratory methods employed and intra-sample
62 variability.

63 Currently, there are two widely-used DNA extraction methods in the field, due to their recovery rates of
64 short, fragmented DNA. One method was developed by Rohland and Hofreiter (2007)[40] (hereafter
65 referred to as the QG method). The QG method involves digestion of bone or tooth sample with
66 ethylenediaminetetraacetic acid (EDTA) and proteinase K and is followed by a purification step that
67 includes a binding buffer consisting of silica and a high concentration of guanidinium thiocyanate to
68 ensure efficient DNA release and reduce inhibition of DNA amplification. This method was modified by
69 Dabney et al. (2013)[41] (hereafter referred to as the PB method). The PB method improves the recovery of
70 shorter DNA fragment sizes (<50 bp) by adding a binding buffer consisting of sodium acetate and
71 isopropanol and guanidinium thiocyanate which replaces guanidine hydrochloride. While ongoing
72 improvements to both methods have been made since their inception[14,42–44], there has not been a
73 systematic assessment of their effectiveness in recovering aDNA from dental calculus.

74 Much like DNA extraction techniques, the effectiveness of the majority of library preparation methods in
75 the field have been validated with teeth and bone[35,36,45,46]. Most library construction protocols for aDNA
76 research were designed for Illumina sequencing-based platforms (*i.e.,* sequencing-by-synthesis) and can
77 be classified into two broad categories: the double-stranded library (DSL) and the single-stranded library
78 (SSL) construction methods. The DSL library method developed by Meyer and Kircher (2010)[45] is widely
79 used in both in paleomicrobiology and paleogenomic fields. For this method, the ends of DNA molecules
80 are first repaired and are then ligated to double-stranded adapters[45,47,48]. While most of the steps for this
81 protocol have remained the same[34], it has undergone a few revisions since its original development[35,49].
82 The SSL method, initially introduced by Gansauge and Meyer (2013)[36], involves the denaturing of all
83 DNA molecules within an extract into single-stranded form which often leads to a higher conversion of

84 DNA fragments into an adapter-ligated form, when compared to DSL libraries[37,46,50]. Although this
85 approach can be more effective when working with highly degraded archaeological samples, few research
86 teams utilize this method, primarily due to its increased cost and longer protocol duration when compared
87 to their double-stranded approach counterparts. The Santa Cruz Rection (SCR) is a more recently
88 developed SSL-based method that has substantially reduced both cost and laboratory processing time
89 compared to earlier SSL methods[39]. However, the question remains whether the benefits justify the
90 expenses associated with updating existing procedures for research teams, especially teams applying
91 shotgun sequencing. For instance, a study comparing DSL and SSL methods on whole beetle specimens
92 found no difference in endogenous DNA content[51]. Similarly, another study reported no difference in
93 endogenous DNA content or insert length between DSL and SSL when applied to specimens from a
94 vertebrae museum collection[52].

95 Research has evaluated the effectiveness of various extraction and library preparation methods in
96 combination for the retrieval of DNA from archaeological samples. For instance, Dabney et al. (2013)
97 compared the QG extraction method against the PB method[40], coupled with the SSL method[36]. Using
98 bones from ancient cave bears as their DNA source, their results showed that shorter DNA fragments
99 were obtained with the PB extraction method coupled with the SSL method. Using ancient equine bone
100 samples, Gamba et al. (2016)[44] examined the efficiency of the QG and PB extraction methods followed
101 by the DSL library. They found that the PB method coupled with the DSL method were more efficient in
102 recovering short aDNA fragments and that the QG method coupled with the DSL library method increases
103 the clonality (duplication rate/rate of non-unique fragments) of aDNA fragments. Barlow et al. (2016)[53]
104 compared the QG and PB methods on ancient bear bones, coupled with the DSL and SSL methods. Their
105 results indicate that the PB method coupled with the SSL resulted were the most efficient in recovering
106 shorter DNA fragments. They also showed that while extraction methods had no impact on GC content,
107 the library preparation method did have a significant impact, with samples prepared via the DSL method
108 having a higher GC content than the samples prepared with the SSL method. Together, these studies
109 demonstrate that the type of extraction and library preparation methods employed have an impact on
110 DNA recovery from archaeological biomaterials.

111 To explore whether DNA extraction and library preparation protocols influence the data generated from
112 archaeological dental calculus, we investigate the impact of such methods have on calculus samples from
113 two geographically distinct locations: Niger and Hungary. Despite the samples sharing similar
114 chronological ages, notable differences in thermal ages were observed based on their respective human
115 DNA data. This study design allows us to explore the potential impact of these wet lab procedures on
116 intra- and inter-sample variability, accounting for preservation state. Specifically, we assess whether wet-
117 lab protocols affect variables such as DNA fragment length recovery, GC content, clonality, endogenous
118 content, DNA deamination, and microbial composition.

119 ## 2. Material and Methods

120 ### *2.1 Ancient dental calculus sample collection*

121 While not required by the institution, this research was evaluated and approved by the University of
122 Adelaide Human Research Ethics Committee (H-2012-108). Ancient dental calculus was sampled from
123 six individuals who were buried in Hungary (n = 3) and Niger (n = 3) (Table 1). While both groups were
124 of similar age (~7,000 yBP), the preservation state for each were expected to be significantly different.
125 The samples from Hungary were expected to have a greater amount of endogenous DNA content because
126 their respective tooth samples yielded a high amount of endogenous human nuclear DNA[54,55]. Samples

127 from Niger, on the other hand, were expected to have poor DNA preservation because the respective tooth
128 samples yielded no detectable amounts of endogenous DNA (data not yet published).

129 Supragingival dental calculus deposits were dislodged from the surface of tooth samples using a sterile
130 dental pick. Gentle pressure was applied in parallel to the tooth surface in order to avoid enamel damage
131 as previously described[10]. Collected fragments were then stored in sterile, sealed zip bags for
132 transportation to the ancient DNA facility at the Australian Centre for Ancient DNA (ACAD), University
133 of Adelaide, Australia.

134 *2.2 Facility protocols*

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

141 *2.3 Sample decontamination, DNA extraction, library preparation and sequencing*

142 To decontaminate the dental calculus samples, each one was irradiated with Ultraviolet (UV) for 15
143 minutes on each side, soaked in two mL of 5% sodium hypochlorite for three minutes, rinsed in 90%
144 ethanol for a minute, and dried at room temperature for two minutes with a delicate task wipe[25]. All
145 chemical solutions were diluted with DNA-free water that was aliquoted into sterile 50 mL tubes and
146 stored in the freezer until the day of use. Samples were then placed into sterile, plastic tubes and crushed
147 into powder on the side of the tube with tweezers that were previously cleaned with 5% hypochlorite,
148 rinsed with 90% ethanol, and UV-treated for 30 minutes.

149 We divided each sample into two visually equal portions and conducted two distinct extractions (QG and
150 PB) on each of the subsampled portions (Figure 1; Table S1). The QG method was done using an in-
151 house adapted strategy, as previously described in Brotherton et al. (2013), but with decreased buffer
152 volumes (1.72 ml lysis extraction buffer; 1.6 ml EDTA; 100 µl SDS; and 20 µl l 20 mg ml^{-1} proteinase K)
153 and 3 ml of guanidine DNA-binding buffer (QG buffer from Qiagen (Germany))[57], as described in
154 Weyrich et al. (2017). The PB method was based on the method developed by Dabney et al. (2013),
155 which involved adding sodium acetate (420 µl), isopropanol (20 µ) guanidine hydrochloride to the
156 binding buffer (Qiagen). The method includes an increase in the ratio of the volume of binding buffer (14
157 ml of guanidine DNA-binding buffer) to that of the lysis buffer (1 ml lysis buffer which consisted of 900
158 µl EDTA, 80 µ l ddH_20, and 20 µl 20 mg ml^{-1} proteinase K) and further modified with the use of silica
159 suspension instead of silica columns, as described in Weyrich et al. (2017)[14]. We produced two
160 independent DNA extracts for each sample, reflecting both the QG and PB extraction methods. For each
161 extraction method, two negative controls were processed alongside the samples after the decontamination
162 step and were then sequenced.

163 Extracts from both the PB and QG methods were then used to create DSL and SSL libraries. The DSL
164 were created using the protocols described originally in Meyer and Kircher (2010) and implemented for
165 dental calculus in Weyrich et al. (2017)[14]. The first amplification step involved 13 cycles of PCR
166 amplification with P5/P7 barcoded adapters, followed by an additional 13 cycles with the addition of a
167 GAII-index and sequencing primers.

168 The SSL method was adapted from the protocol developed by Gansauge et al. (2017)[37] with minor
169 modifications. First, the 5' and 3' termini were dephosphorylated using FastAP (ThermoFisher).
170 Subsequently, the DNA was denatured at 95°C for 2 minute and rapidly cooled by immersion in an ice
171 block. Following the cooling step, a biotinylated adapter was ligated to the 3' terminus using T4 DNA
172 Ligase (Thermo Scientific). The biotinylated adapters were then immobilized on C1 beads (Invitrogen),
173 pulled down, and washed. An extension primer was annealed to the ligated adapter, and a second strand
174 was synthesized using the Klenow Fragment (Thermo Scientific), followed by a second C1 bead pull-
175 down and wash step. Next, a double-stranded blunt-end adapter was ligated to the 3' terminus of the
176 synthesized strand with T4 DNA Ligase (Thermo Scientific). After this step, a third C1 bead pull-down
177 and wash step was performed. Lastly, the reactions were heat denatured, and the pre-amplified library was
178 collected with the supernatant.

179 All libraries were then purified using Ampure XP (Beckman Coulter, USA), quantified using an Agilent
180 TapeStation (Agilent Technologies, USA), and pooled at equimolar concentrations. Final pools were
181 quantified using an Applied Biosystems Real Time qPCR machine. Libraries were sequenced on the
182 Illumina NextSeq platform (Illumina, USA) using the 2 x 150 bp configuration.

183 *2.4 Bioinformatic processing and analyses*

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

193 The analysis-ready reads were taxonomically binned with the nucleotide alignment option in the MEGAN
194 Alignment Tool (MALTn; v. 0.3.8)[58]. The reads were aligned against an in-house RefSeq ('RefSeq')
195 database that was previously published[59] and includes 47,696 archaeal and bacterial genome assemblies at
196 scaffold, chromosome, and complete levels. The resulting alignment-based blast-text files were then
197 converted into RMA files using the blast2rma script included with the program MEGAN6 (v 6.11.1)[60]
198 with the following last common ancestor (LCA) parameters: Weighted-LCA=80%, minimum bitscore =
199 42, minimum E-value=0.01, minimum support percent=0.1. The resulting RMA6 files were imported into
200 MEGAN6. We used MEGAN6 CE to also export phylum-, genus-, and species-level BIOM tables using
201 the taxonNameToCount summarized option and the non-assigned reads were excluded.

202 *2.5 Preservation assessment*

203 To assess the preservation of the endogenous content in the samples, we used SourceTracker2 (v2.0.1)[61].
204 The comparative data for this analysis came from studies analyzing modern dental calculus, modern
205 dental plaque, soil, and skin, as well as the EBCs from this study (Table S2), as described in Gancz,
206 Farrer et al., (2023)[18]. Reads for the source samples were processed using the same bioinformatics
207 pipeline as the samples in this study. The comparative sources for this analysis were included in a
208 previous study[18] and were selected for the following reasons: they were generated using Illumina shotgun
209 sequencing and had enough data to yield at least 1,000 reads mapped to the NBCI-RefSeq database. The
210 species-level table for the sources was combined with the samples in this study. We employed
211 SourceTracker2 with the following parameters: alpha2=1.0, sink_rarefaction_depth=5573,

source_rarefaction_depth=1489). The rarefaction depth for the sinks was determined based on the sequencing depth of sample 18393, whereas for the sources, it was chosen based on the sequencing depth of sample 19028 (Table S3). Samples with less than 1000 counts were excluded from this analysis. The results for this analysis were visualized using R.

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[14–16,19,62].

To understand the overall damage preservation of the aDNA in our samples, we performed a ChangePoint analysis[18]. ChangePoint infers deamination by assessing the proportions of 3' adenines and 5' thymines at the ends of DNA reads within a sample. It then models the relationship between the distance from the end of the reads and the amount of deamination in a sample with a likelihood ratio test. Samples are considered to represent authentic ancient metagenomes if their adjusted p-values are less than 0.05. To optimize the performance of ChangePoint, we subsampled each sample to 100,000 reads.

2.6 Microbial compositional analysis

Sample 18393_Niger_PB_SSL was removed from all compositional analyses because of its low assigned read count (400). We then proceeded with downstream analyses on the remaining 23 samples. While most aDNA analyses would include utilizing a bioinformatic step to remove contaminant taxa, such as the use of *decontam*[63], we chose to skip this step in order to identify whether contaminant taxa could be driving some of the microbial signatures and whether certain methods enriched them.

We calculated differences in alpha diversity (observed species) using the Kruskal-Wallis test with Chao1 and Shannon's Diversity metrics. For beta-diversity, the DEICODE plugin in QIIME2[64] was used to centered-log transform the species-level tables and convert them into Aitchison distance matrices. Using the adonis function in QIIME2 with 9,999 permutations, we employed the following model: Aitchison distance matrix ~ "ExtractionType" * "LibraryMethod". We furthered explored the beta diversity of samples using principal coordinates analysis (PCoA) based on Aitchison distances at the species level. Statistical significance for all tests was set at 0.05.

2.7 Associations between microbial abundance and laboratory methods

We employed Microbiome Multivariable Association with Linear Models (MaAsLin2) to carry out differential abundance analyses on species-level tables exported from MEGAN[65]. 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 and the normalization parameter set to "CLR". The "ExtractionType" and "LibraryMethod" columns in the metadata were used as covariates were inputted in the "fixed_effects" option.

Scripts for this project can be found on GitHub (https://github.com/microARCHlab/AncientCalculusLabMethods_2024).

3. Results

3.1 Fragment length recovery

We first assessed the influence of DNA extraction and library preparation methods on fragment length recovery (Table S4; Figure S3, S4, and S5). For the samples from Niger, neither DNA extraction nor library preparation methods had a significant effect on fragment length recovery. For the DNA extraction analysis, samples prepared with the QG method recovered longer fragments (67.60 bp, 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 also 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 impact the recovery fragment length recovery ($F=0.008$, $p=0.93$).

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, these findings suggest that DNA extraction and library methods in this study did not impact fragment length recovery in poorly preserved samples, but they did for 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 (48.23%, SD=7.110) and PB method (47.90%, SD=6.483) yielded similar GC content ($F=0.045$, $p=0.838$). However, the GC content between the DSL method (53.85%, SD=1.466) and SSL method (42.28%, SD=3.194) was significantly different ($F=52.303$, $p=0.0000896$). The interaction between the DNA extraction and library preparation methods were insignificant ($F=0.003$, $p=0.959$).

For the Hungary dataset, the GC content for the QG (48.14%, SD=5.363) and PB (47.95%, SD=6.503) methods was similar ($F=0.009$, $p=0.92$). In contrast, the difference in GC content between the DSL (52.70%, 2.796) and SSL (43.39%, SD=3.351) was significant ($F=23.187$, $p=0.001$). There was no difference in GC content between the interaction between the two methods ($F=0.479$, $p=0.51$). These findings suggest that, irrespective of preservation status, library preparation protocols play a role in influencing the recovery of GC content, whereas DNA extraction methods did not have a discernable impact in this study.

3.3 Clonality

We explored 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, as only mild differences were observed. The QG method recovered a lower percentage of unique reads (12.71%, SD=6.247) than the PB method (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 (17.00%, SD=5.229) than the SSL method (23.31%, SD=33.103), these differences were also not significant ($F=0.218$, $p=0.653$). The interaction between the two protocols was also not meaningful ($F=1.015$, $p=0.343$).

A similar trend was observed for the samples from Hungary. There were minor differences in percentage of unique reads between the QG (78.12%, SD=32.936) and PB methods (94.22%, SD=2.315) ($F=1.331$,

p=0.282), as well as between DSL (92.34%, SD=5.662) and SSL (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 have little impact on the overall clonality observed in these ancient metagenomic libraries.

3.4 Microbial source tracking

To authenticate the microbial content in the dental calculus samples, we used SourceTracker2[61]. SourceTracker2 predicts the potential origins of the microbial DNA found in the dental calculus samples from Niger and Hungary (Table S6; Figure 2). For samples from Niger, 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 (Average=7.2%, SD=0.002) and the PB (Average=6.4%, SD=0.002) methods had similar yields. This is not the case with library preparation methods. Only samples prepared with the SSL method yielded any discernible oral DNA (Average=14.8%, SD=0.002). The DSL method, on the other hand, did not yield any indiscernible amount of oral DNA (Average=<0.04%, SD=0.0002). Interestingly, the PB+SSL approach yielded the most oral DNA for samples 18393 (12.0%) and 18398 (25.2%), while the QG+SSL method yielded the most amount of oral DNA for sample 18400 (15.5%). Furthermore, the DSL method consistently recovered more DNA attributed to contaminant sources, such as EBC, skin, and soil, across the samples (Average=66.2%, SD=25.7) than their SSL counterparts (Average=16.5%, SD=34.2).

In stark contrast, the DSL method recovered more endogenous content across Hungarian samples (46.7%, SD=0.005) than the SSL method (37.4%, SD=0.015). Moreover, unlike the case with the samples from Niger, the recovery of contaminant DNA between the SSL (Average=0.05%, SD=0.0003) and DSL (Average=0.04%, SD=0.0002) methods were minimal. Interestingly, samples 18416 and 18421 prepared via the DSL method yielded a higher percentage of oral DNA than the SSL method, whereas QG+SSL method yielded more oral DNA (50.7%) than the QG+DSL method (34.8%) in 18427. For the Hungarian samples, all samples had less than 1% attributed to contaminant sources, indicating neither DNA extraction nor library preparation methods significantly impacted the recovery of endogenous content in this data set.

3.5 Authenticating the damage profiles of samples

We explored whether the methodologies for DNA extraction and library preparation methodologies had an impact on the ability generate reads exhibiting deamination with MapDamage2. We mapped analysis-ready reads from each sample to three microbes: *Anaerolineaceae* bacterium oral taxon 439 (ASM171754v1), *Methanobrevibacter oralis* DSM 7256 (ASM163927v1), and *Olsenella sp taxon 807* (strain F0089) (ASM118951v2). We selected these microbes because of the relative high abundance found across samples (Figure S1 & S2). For the Niger dataset, the MapDamage2 results corroborate with the SourceTracker2 results, as library preparation protocol seemed to play a larger role in recovering oral microbial DNA. Most samples did not exhibit deamination patterns that is expected for ancient samples (Figure S7, S8, and S9). For instance, all samples prepared with either the PB+DSL or QG+DSL methods did not have enough unique mapped reads to the microbial genomes to provide reliable results (<100)[66] (Table S5). While the single-stranded libraries yielded more unique mapped reads, there was a substantial difference between the groups based on extraction methods. All but one sample (18400 prepared with the QG+SSL) had at least 100 unique mapped reads to the three genomes, whereas samples prepared with PB+SSL method, only three samples with at least 100 unique mapped reads. These findings indicate that the QG+SSL method was the most effective in recovering enough DNA for these oral microbes from poorly preserved samples.

339 DNA extraction and library preparation methods seem to have less an influence on the well-preserved
340 samples from Hungary. Most samples from Hungary exhibit expected A→T and G→C misincorporations
341 at the 5' and 3' ends, respectively (Figure S10, S11, and S12). Only sample 18427 prepared with the
342 PB+DSL methods yielded less than 100 unique mapped reads to the *M. oralis* genome. In summary, the
343 method had little effect on the ability to recover enough endogenous DNA to assess deamination patterns
344 for well-preserved samples.

345 To assess the overall damage profiles for the samples from Hungary and Niger, we employed
346 ChangePoint (Table S5; Figure S11). Our ChangePoint results indicate that the samples from Niger
347 supported the MapDamage results in that an insignificant amount of DNA fragments in the samples were
348 enriched with T's on their 5' ends and C's on their 3' ends. All samples from Hungary, on the other hand,
349 exhibited expected aDNA-associated damage signatures, irrespective of DNA extraction and library
350 preparation method employed. These findings suggest that laboratory methods did not differentially
351 impact the recovery of authentic aDNA from samples of varying preservation states.

352 *3.6 Alpha diversity*

353 We explored the impact that both DNA extraction and library preparation methods have on alpha
354 diversity using the observed species index. For the Niger samples, neither DNA extraction (H=1.256,
355 q=0.749) nor library method (H=1.256, q=0.262) had a significant effect on alpha diversity across this
356 dataset, likely due to the low recovery of oral sequences. However, the opposite was true for the better-
357 preserved calculus samples from Hungary, as the library preparation protocol significantly impacted alpha
358 diversity (H=4.689, q=0.03). The double-stranded library protocol recovered a significantly greater
359 number of species (44.67 observed species) compared to the single-stranded method (29.33 observed
360 species (*t-test*: p=0.007). DNA extraction method, in contrast, did not show a significant association
361 (H=0.926, q=0.336). These overall patterns in alpha diversity remained consistent when using other alpha
362 diversity indices (Figure 3), suggesting that rare species and relative abundance did not have a meaningful
363 influence. In summary, these results suggest that choice of library preparation method has a notable
364 impact on the recovery of alpha diversity of well-preserved samples, but not poorly preserved samples
365 where the signal is minimal. This also indicates that different library preparation methods should be
366 included in downstream metadata to assess this effect in metanalyses.

367 *3.7 Beta diversity*

368 We evaluated whether DNA extraction and library preparation methods influenced beta diversity (*i.e.,*
369 compositional differences) via adonis and PCoA. The dataset for Niger indicates both DNA extraction
370 (p=0.039, R^2=0.180, adonis) and library preparation (p=0.022, R^2=0.224), as well as their interactions
371 (p=0.005, R^2=0.260), were associated with beta diversity (Aitchison distance of CLR-transformed species
372 counts; Table 2). These results were further supported by PCoA (Figure 4A), where distinct clustering
373 based on the chosen DNA extraction and library preparation method is observed.

374 A different pattern emerged for the samples from Hungary, where both DNA extraction (p=0.374,
375 R^2=0.099, adonis) and library preparations (p=0.321, R^2=0.111, adonis) had no significant effect, as well
376 as no significant interaction between the two (p=0.669, R^2=0.052, adonis). These results were further
377 supported by our PCoA (Figure 4B), where no clear segregation was observed between samples based on
378 their DNA extraction and library method. Overall, this suggests that DNA extraction and library
379 preparation methods impact the microbial composition recovered from poorly preserved samples but not
380 well-preserved samples.

381 *3.8 Differential abundance*

382 To explore whether differentially abundant genera or species were associated with DNA extraction or
383 library preparation method, we utilized MaAsLin2[65] (Table S7). We treated both DNA extraction and
384 library preparation as fixed effects. No species were enriched in the Niger dataset according to DNA
385 extraction or library preparation method. For Hungarian dataset, only *Eggerthia catenaformis*
386 (coef=0.827, q=0.008,) and *Bacteroidetes oral taxon 274* (coef=0.738, q=0.020,) were significantly
387 enriched in the SSL group for the Hungarian dataset (Figure 5). This finding indicates that some
388 microbial species may be differentially identified based on the library method employed.

389 4. **Discussion**

390 4.1 *Both DNA extraction and library preparation methods impact aDNA recovery from dental calculus*

391 In this study, we observe a strong association between DNA extraction and library preparation methods
392 and their influence on aDNA recovery and downstream analyses. Our findings also show that the
393 effectiveness of these wet-lab methods is tied to the preservation state of dental calculus samples, adding
394 nuance to our understanding. For example, previous research concluded that the PB method recovered
395 more DNA molecules, endogenous DNA content, and greater library complexity than the QG method[44].
396 Our findings indicate that this is not straightforward for dental calculus research. While the average
397 fragment length recovery for the PB method was significantly shorter than the QG method for well-
398 preserved samples, the choice of DNA extraction method minimally influenced the average fragment
399 length recovery for poorly preserved samples. Likewise, library preparation method had no significant
400 effect on the average fragment length recovery for poorly preserved samples. However, it did demonstrate
401 an impact on well-preserved samples.

402 Unlike a previous study in which the QG method yielded a higher percentage of GC content than the PB
403 method[44], we found no significant difference between the two. On the other hand, we observed an
404 association between library preparation method and GC content, which remains consistent across
405 preservation states. This finding is consistent with a previous investigation[46]. Our analysis also concurs
406 with previous studies that found the SSL method exhibits less bias toward high GC content compared to
407 the DSL method[46,69,70]. Overall, these results underscore the importance of considering library preparation
408 when analyzing GC content of samples.

409 Our analysis indicates that the recovery of unique reads (*i.e.,* clonality) showed no association with DNA
410 extraction method, irrespective of preservation state. This contradicts findings from a previous study
411 examining bones[44], hinting at a potential differences in the properties of dental calculus. Despite the
412 expectation that the SSL method should yield a higher percentage of unique reads compared to the DSL
413 method[46], our results indicate otherwise. We did not observe a significant difference in library preparation
414 method when analyzing the poorly preserved and better-preserved samples.

415 *4.2 Laboratory methods influence the recovery of types of microbial taxa in dental calculus*

416 Our SourceTracker2 analysis indicates that the library preparation procedure, rather than DNA extraction
417 method, significantly influences the recovery of endogenous content from calculus samples. While most
418 of the DNA across the Niger samples were attributed to either contaminant or unknown sources, the SSL
419 method recovered marginal amounts of oral DNA. This finding is consistent with previous studies
420 indicating the SSL method recovers more endogenous DNA than the DSL method, especially for samples
421 with poor DNA preservation[46,50]. Conversely, library preparation protocol did not impact the recovery of
422 contaminant DNA for the Hungarian samples. However, the DSL method consistently yielded a higher
423 proportion of oral DNA than the SSL method for the Hungarian samples. In summary, our findings

424 underscore the importance of considering the library preparation procedure in DNA recovery from dental
425 calculus samples.

426 *4.3 Deamination is associated with the general preservation state of a sample and unaffected by the*
427 *selection of laboratory methods employed*

428 Detection of aDNA damage in archaeological dental calculus samples is crucial for their validation and
429 authentication. In this study, we utilized MapDamage[71,72] to examine the deamination patterns of specific
430 microbes and ChangePoint[18] to assess deamination of the overall community. MapDamage indicated that
431 the SSL method recovered enough endogenous DNA to perform authentication, which is in accordance
432 with the SourceTracker2 results. We did not find any observable differences in being able to detect
433 deamination across the Hungarian samples prepared with different laboratory methods.

434 ChangePoint showed that neither DNA extraction nor library preparation protocols had a discernible
435 effect on the recovery of DNA with deamination in this study. While MapDamage results suggested that
436 the SSL method could recover some oral microbial DNA from a few Niger samples, our ChangePoint
437 analysis revealed that most of the DNA did not exhibit deamination, indicating it was not authentically
438 ancient. Moreover, ChangePoint indicated that significant proportions of the DNA for all the samples
439 from Hungary were ancient, suggesting that no specific laboratory method hinders the recovery of
440 endogenous DNA from well-preserved samples. Together, these findings suggest that while certain
441 methods may affect DNA recovery in specific contexts, overall, well-preserved samples appear to such
442 influences, with ancient DNA being successfully recovered regardless of the laboratory method utilized.

443

444 *4.4 DNA extraction and library protocols impact the recovery of oral microbiome diversity*

445 While some studies have shown that DNA extraction and library preparation methods influence the
446 microbial diversity obtained from modern oral samples[29,73,74], others suggested that wet lab protocols play
447 a minimal role[75]. Nonetheless, it is difficult to discern whether the findings in these previous studies on
448 modern DNA are always applicable to ancient oral microbiome research, as aDNA has unique
449 characteristics. Whether either procedure impacts the recovery of ancient oral microbiomes has not been
450 examined in detail. We observed that both DNA extraction and library preparation protocols were
451 associated with the alpha diversity of the Niger samples. For the Hungarian samples, only the choice of
452 laboratory preparation method was associated with alpha diversity. A similar trend was observed when
453 evaluating whether laboratory procedures influenced the recovery of beta diversity from samples. For the
454 Niger samples, DNA extraction method and library preparation, independently, as well as their
455 interaction, were significantly associated with beta diversity. This was not the case with the Hungarian
456 samples as neither DNA extraction nor library preparation were associated with microbial composition.
457 Taken together, these data suggest that wet lab protocols influence the alpha and beta diversity for poorly
458 preserved samples to a greater extent than they do for well-preserved samples. Regardless, information on
459 the DNA extraction type and library preparation procedures should be included in downstream
460 applications, such that these effects can be tested in metanalyses moving forward.

461 Our study represents the first attempt to investigate whether aDNA extraction or library preparation
462 protocols selectively enrich or deplete certain species within dataset. We found no species in the Niger
463 dataset that were enriched based on DNA extraction or library preparation method. However, in the
464 Hungarian dataset, two species were enriched when using the SSL method: *E. catenaformis* and
465 Bacteroidetes oral taxon 274. *E. catenaformis,* initially isolated from human faeces in 1935[76], is a Gram-
466 positive bacterium[77]. Limited information is available regarding the properties of *Bacteroidetes oral taxon*

467 *274*. Nevertheless, a previous study noted that this taxon was enriched in several ancient populations[78].
468 Interestingly, this study employed a silica-column based method similar to our PB method, coupled with
469 the DSL method. These findings suggest that the SSL method may yield greater DNA recovery for
470 specific taxa compared to the DSL method. Notably, SourceTracker2 results indicated that the SSL
471 method yielded less oral DNA than the DSL method for the Hungarian samples. These combined results
472 may suggest that while the DSL method may recover a higher yield of oral DNA, the SSL method may be
473 more effective in recovering the DNA from specific oral taxa.

474 ### 4.5 *Limitations to our study*

475 There are a few limitations in our study. First, our study has a small sample size. This limited number
476 may not provide a fully representative assessment of the performance of different DNA extraction and
477 library preparation methods, limiting the scope of drawing broad conclusions. A larger dataset with a
478 more extensive sample size could offer a more comprehensive understanding of these methods and how
479 they affect aDNA recovery. Another limitation is that the comparison in our study includes samples from
480 only two specific archaeological contexts that were already shown to have vastly different recovery rates
481 of human DNA. The efficiency of the laboratory methods may vary in burial contexts with different
482 environmental processes. For example, burial environments in more acidic environments (pH 3.5-4.5) are
483 known to be more destructive environments for the biomolecular integrity than alkaline soils (7.5-8.0)[79].
484 Moving forward, accounting for whether certain protocols improve the recovery of DNA based on their
485 burial context could prove to be noteworthy, although we appreciate the difficulties in assessing DNA
486 preservation in individuals without prior human DNA assessment. It is also important to acknowledge
487 that we did not account for how post-excavation treatments could impact the aDNA recovery of calculus
488 samples. Such information is rarely documented in many cases, yet they introduce variability and could
489 confound the interpretation of results.

490

491 ## 5. **Conclusion**

492 Although both DNA extraction and library preparation methods have shown to influence the recovery of
493 DNA from samples[29,73,74], little has been done to investigate how wet lab protocols impact the DNA
494 recovery from archaeological dental calculus. Overall, our findings here indicate that DNA extraction and
495 library preparation methods are critical factors in the recovery of ancient oral microbiomes, especially in
496 samples of varying states of preservation. These findings carry important implications in data
497 comparability, which is essential for conducting meta-analyses to assess the robustness of individual
498 study findings. However, the lack of standardization in ancient oral microbiome research has limited the
499 field from achieving data comparability. In some cases, laboratory methodological differences could
500 contribute to why research teams reach different conclusions on important topics, such as whether diet
501 and cultural transitions led to shifts in ancient oral microbiomes[11,14–17], in addition to a multitude of other
502 confounding factors, such as oral geography and archaeological site differences.

503 The evidence presented here indicates no singular approach yields the most optimal results in all aspects
504 of data quality, irrespective of preservation state. The findings imply that no single “optimal standard”
505 currently exists. This raises a fundamental question about whether the field should prioritize
506 standardization or optimization of methods. Standardizing protocols could mitigate technical variation
507 across datasets, promoting more reproducible and replicable results. Conversely, optimizing methods for
508 each dataset may enhance accuracy for individual samples but would complicate data integration. As
509 such, our study highlights the delicate balance between standardization and optimization, emphasizing the
510 importance of well-documented methodologies, transparent reporting, and thoughtful consideration of the

511 unique attributes of each sample. As we continue to uncover the mysteries of the past through aDNA
512 analysis, it is paramount to address these questions and develop a more nuanced understanding that
513 benefits individual studies and the broader field.

514

515 **Data availability**

516 The sequencing data for this project can be found on the Sequence Read Archive
517 (https://www.ncbi.nlm.nih.gov/sra) under the accession number: PRJNA1031139.

518

519 **Acknowledgements**

520 We thank Professor Alan Cooper for his efforts in this study.

521 **Tables**

SampleID	Continent	Present-day Country of origin	DNA extraction method	Library preparation method
18393_Niger_QG_DSL	Africa	Niger	QG	DSL
18393_Niger_PB_DSL	Africa	Niger	PB	DSL
18393_Niger_PB_SSL	Africa	Niger	PB	SSL
18393_Niger_QG_SSL	Africa	Niger	QG	SSL
18398_Niger_QG_DSL	Africa	Niger	QG	DSL
18398_Niger_PB_DSL	Africa	Niger	PB	DSL
18398_Niger_PB_SSL	Africa	Niger	PB	SSL
18398_Niger_QG_SSL	Africa	Niger	QG	SSL
18400_Niger_QG_DSL	Africa	Niger	QG	DSL
18400_Niger_PB_DSL	Africa	Niger	PB	DSL
18400_Niger_PB_SSL	Africa	Niger	PB	SSL
18400_Niger_QG_SSL	Africa	Niger	QG	SSL
18416_Hungary_QG_DSL	Europe	Hungary	QG	DSL
18416_Hungary_PB_DSL	Europe	Hungary	PB	DSL
18416_Hungary_PB_SSL	Europe	Hungary	PB	SSL
18416_Hungary_QG_SSL	Europe	Hungary	QG	SSL
18421_Hungary_QG_DSL	Europe	Hungary	QG	DSL
18421_Hungary_PB_DSL	Europe	Hungary	PB	DSL
18421_Hungary_PB_SSL	Europe	Hungary	PB	SSL
18421_Hungary_QG_SSL	Europe	Hungary	QG	SSL
18427_Hungary_QG_DSL	Europe	Hungary	QG	DSL
18427_Hungary_PB_DSL	Europe	Hungary	PB	DSL
18427_Hungary_PB_SSL	Europe	Hungary	PB	SSL
18427_Hungary_QG_SSL	Europe	Hungary	QG	SSL

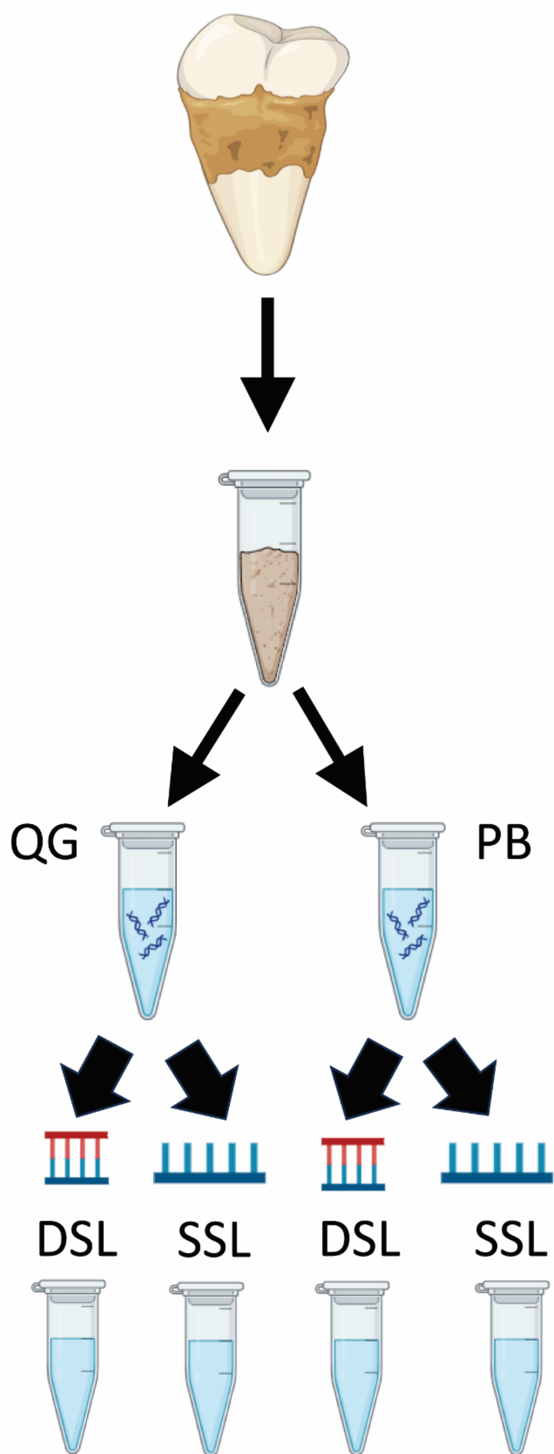
522 Table 1. Metadata for samples included in this project

523

Niger	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
ExtractionType	1	4.326	4.326	3.755	0.180	0.039
LibraryMethod	1	5.386	5.386	4.675	0.224	0.022*
ExtractionType:LibraryMethod	1	6.262	6.262	5.435	0.260	0.005**
Residuals	7	8.065	1.152	NA	0.335	NA
Total	10	24.038	NA	NA	1.000	NA
Hungary	**Df**	**SumsOfSqs**	**MeanSqs**	**F.Model**	**R2**	**Pr(>F)**
ExtractionType	1	2.541	2.541	1.075	0.099	0.374
LibraryMethod	1	2.855	2.855	1.208	0.111	0.321
ExtractionType:LibraryMethod	1	1.337	1.337	0.566	0.052	0.669
Residuals	8	18.911	2.364	NA	0.737	NA
Total	11	25.644	NA	NA	1.000	NA

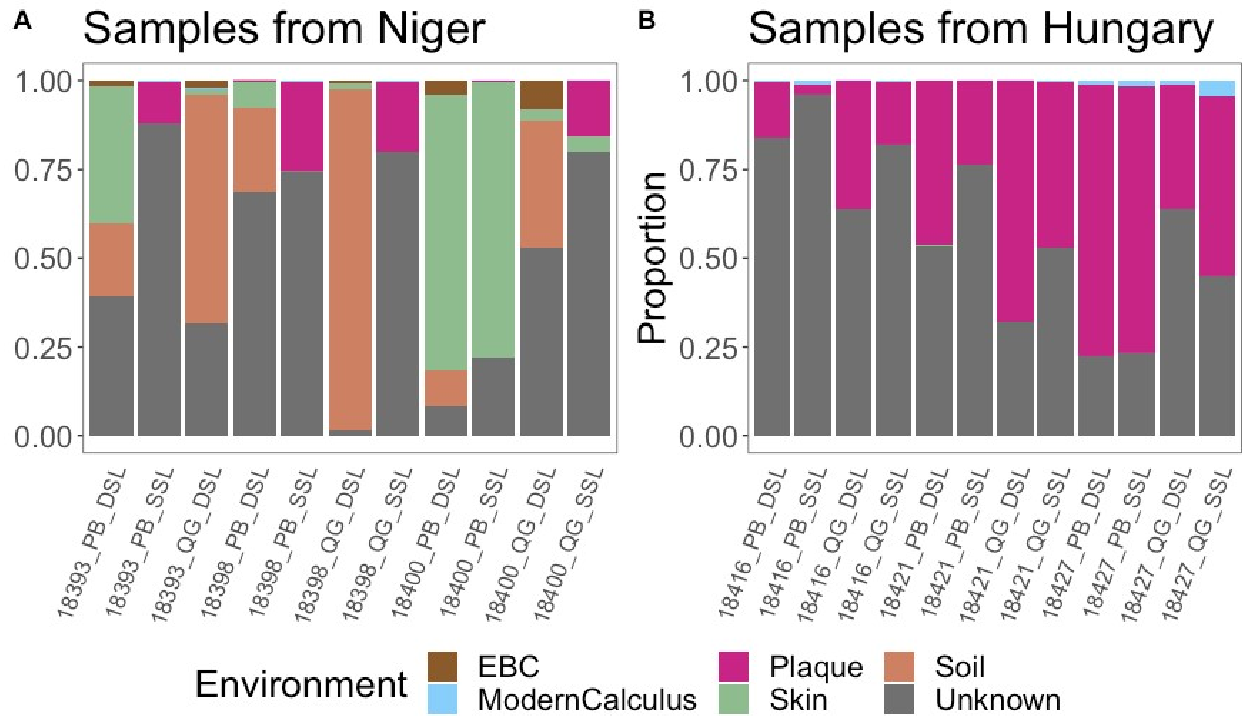
524 Table 2. Adonis results at the species level.

525



526

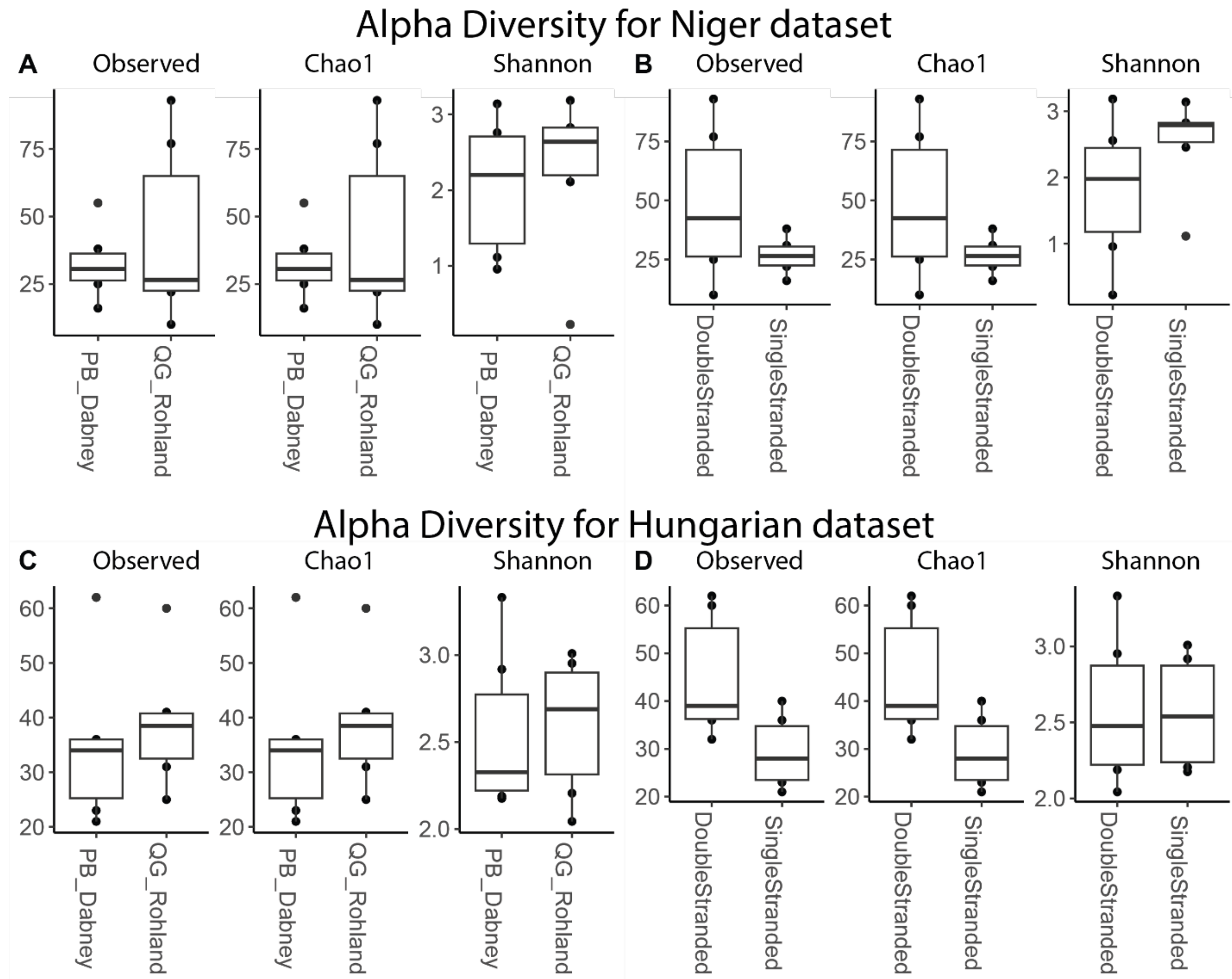
527 Figure 1 Experimental design. Three calculus samples from Niger and three from Hungary were split into
528 roughly equal amounts and places into two separate sampling tubes for DNA extraction. Method QG,
529 silica-based DNA extraction method following Rohland & Hofreiter (2007); method PB, column-based
530 DNA extraction method following Dabney *et al.* (2013); DSL method, double-stranded library
531 preparation method following Meyer and Kircher (2010), as modified by Weyrich *et al.* (2017); method
532 SSL, single-stranded library method following Gansauge *et al.* (2017).



533

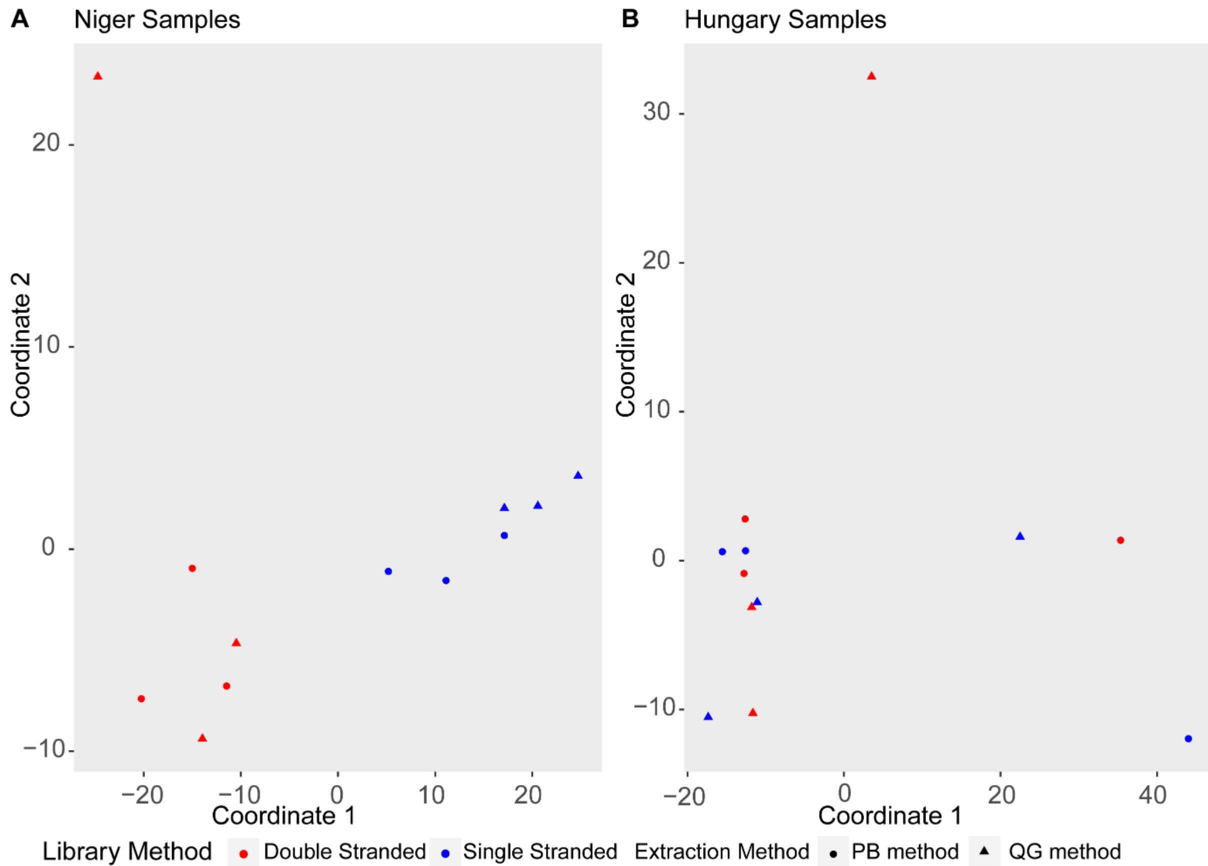
534 Figure 2. SourceTracker2[61] was applied using extraction blank controls (EBCs) processed alongside the
535 dental calculus samples, modern calculus, plaque, skin, and soil samples as sources and dental calculus
536 samples as sinks. The following parameters were used: alpha2=1.0, sink_rarefaction_depth=5573,
537 source_rarefaction_depth=1489). The rarefaction depth for the sinks was determined based on the
538 sequencing depth of sample 18393, whereas for the sources, it was chosen based on the sequencing depth
539 of sample 19028. Sample 18393 prepared with the QG and SSL methods was removed from this analysis
540 because it had less than 1000 counts. The SourceTracker2 results file was visualized using *ggplot* in R.

541



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

549

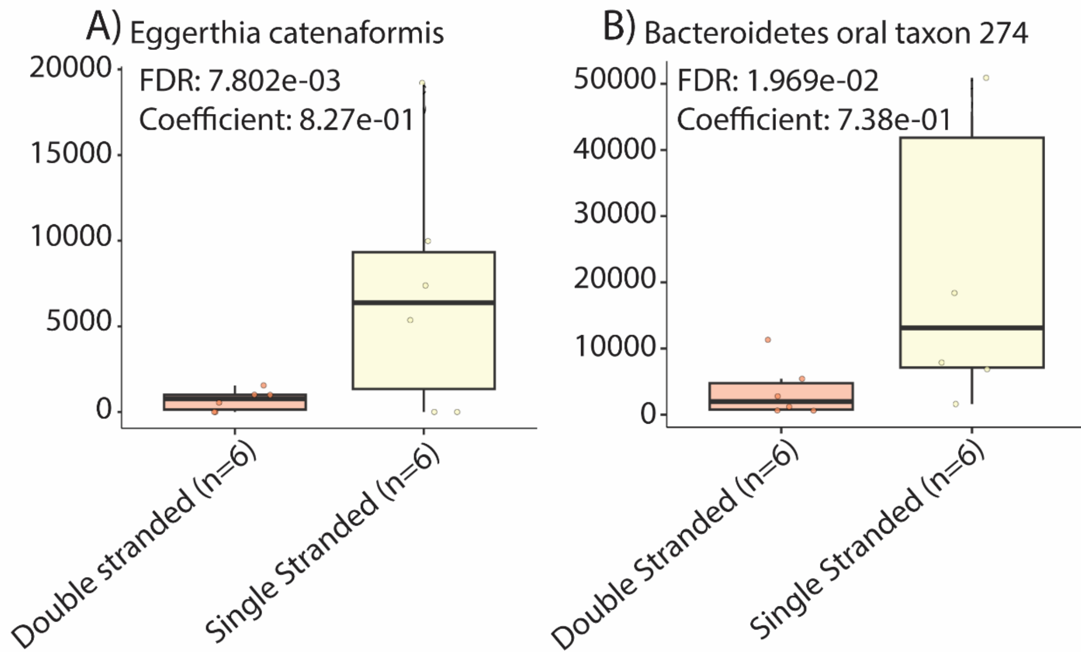


550

551 Figure 4. Beta diversity represented by a principal coordinate analysis (PCoA) for the A) Niger and B)
552 Hungary datasets based on Aitchison distance at the species level. CoAs for Niger (A) and Hungarian (B)
553 samples. A multifactorial permutational analysis of variance of the Aitchison distances indicated that
554 DNA extraction (R^2=0.180, p=0.039), and library preparation (R^2=0.224, p=0.022), as well as their
555 interaction (R^2=0.260, p=0.005), methods had a strong association with the oral microbiome composition
556 of the Niger dataset. The analysis for the Hungary dataset, in contrast, indicates that DNA extraction,
557 library preparation, and their interaction were not significantly associated with the oral microbial
558 communities ($p>0.05$).

559

560



561 Figure 5. In multivariable linear modeling with MaAsLin2, we set DNA extraction and library preparation
562 methods as fixed effects. After adjusting for these covariates, no species were associated DNA extraction
563 nor library preparation for the Niger dataset after FDR correction (<0.05; Table S7). For the Hungary
564 dataset, two species (A) Eggerthia catenaformis and B) *Bacteroidetes* oral taxon 274) were significantly
565 associated with library preparation method. Specifically, both species were enriched in the single stranded
566 samples. Little is known about the properties of these microbial species, leaving open questions about the
567 factors contributing to their enrichment.

568

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