Received: 13 August 2019 | Revised: 31 October 2019 | Accepted: 9 November 2019

DOI: 10.1002/ajpa.23978

RESEARCH ARTICLE

American Journal of PHYSICAL ANTHROPOLOGY | WILEY

Comparison of extraction methods for recovering ancient microbial DNA from paleofeces

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Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: EXC 2051 #390713860; Max-Planck-Gesellschaft; National Institute of General Medical Sciences, Grant/Award Number: R01GM089886

Abstract

Objectives: Paleofeces are valuable to archeologists and evolutionary biologists for their potential to yield health, dietary, and host information. As a rich source of preserved biomolecules from host-associated microorganisms, they can also provide insights into the recent evolution and changing ecology of the gut microbiome. However, there is currently no standard method for DNA extraction from paleofeces, which combine the dual challenges of complex biological composition and degraded DNA. Due to the scarcity and relatively poor preservation of paleofeces when compared with other archeological remains, it is important to use efficient methods that maximize ancient DNA (aDNA) recovery while also minimizing downstream taxonomic biases.

Methods: In this study, we use shotgun metagenomics to systematically compare the performance of five DNA extraction methods on a set of well-preserved human and dog paleofeces from Mexico (~1,300 BP).

Results: Our results show that all tested DNA extraction methods yield a consistent microbial taxonomic profile, but that methods optimized for ancient samples recover significantly more DNA.

Conclusions: These results show promise for future studies that seek to explore the evolution of the human gut microbiome by comparing aDNA data with those generated in modern studies.

KEYWORDS

ancient DNA, coprolite, gut microbiome, paleogenomics

1 | INTRODUCTION

The gut microbiome is a core component of human biology, contributing to a range of physiological functions from digestion to host immunity. To date, most studies of the human gut have focused on industrialized societies, but recent research on hunter-gatherer, horticulturalist, and pastoralist cultures has revealed previously unknown microbial diversity in populations living more traditional lifeways (Obregon-Tito et al., 2015; Schnorr et al., 2014; Yatsunenko et al., 2012). Such studies suggest that industrialized societies may have

undergone recent changes in gut microbiome structure and function related to changing dietary and sanitation practices; however, testing this hypothesis requires the recovery of well-preserved gut microbiota from the archeological record. Paleofeces, alternatively known as coprolites, are the preserved remnants of feces from humans or other animals. Preserving only under extraordinary conditions, such as rapid desiccation or freezing, paleofeces are relatively uncommon in the archeological record; however, those specimens that do survive have the potential to shed light on the evolution of the gut microbiome (Schnorr, Sankaranarayanan, Lewis Jr., & Warinner, 2016; Warinner, Speller, Collins, & Lewis Jr., 2015).

The genetic comparison of paleofeces and fresh feces, however, presents two major challenges. The first is the degraded nature of ancient DNA (aDNA) itself, and the second is that reconstruction of a complex microbial community, such as the gut microbiome, can be influenced by DNA extraction methods (Wesolowska-Andersen et al., 2014). The aDNA is both low in abundance and highly fragmented, and as a result, aDNA extraction methods necessitate a minimum of handling steps and chemical reagents, as well as a specialized silica binding protocol, in order to mitigate DNA loss and efficiently recover short aDNA fragments (Dabney et al., 2013). On the other hand, modern microbiome reconstruction depends on the efficiency with which bacterial lysis occurs during DNA extraction, which is influenced by differences in cell wall structure, spore formation, and other factors. In order to reduce data variability in modern microbiome studies, many laboratories and large-scale projects have attempted to standardize DNA extraction methods. For example, both the Earth Microbiome Project (Marotz et al., 2017; Thompson et al., 2017) and the Human Microbiome Project (HMP) (Aagaard et al., 2013) have recommended using the Qiagen PowerSoil (formerly MoBio PowerSoil) DNA extraction kit, and consequently, this kit has become relatively standard in modern microbiome research. To ensure efficient cell lysis, this kit uses extensive mechanical, chemical, and enzymatic lysis steps, followed by inhibitor removal using proprietary chemicals, and finally a DNA purification and concentration step using a silica spin column. In addition, some variants of this protocol have also advocated using an initial heat lysis step (Obregon-Tito et al., 2015).

The fact that the standard methods for ancient and modern DNA extraction differ so greatly is a potential problem that could introduce bias when generating and comparing DNA sequence data obtained from paleofeces and fresh feces using two different protocols. However, on the other hand, using the same extraction method for both sample types is problematic because it would very likely yield suboptimal results for one sample type. For example, using an optimized aDNA protocol for modern microbiome DNA extraction is expected to result in inefficient cellular lysis and biased taxonomic recovery, and using an unmodified commercial DNA extraction kit for paleofeces would likely result in unacceptable aDNA losses.

To address this problem and determine the impact of extraction protocol on DNA recovery and reconstructed microbial profiles obtained from paleofeces, we systematically tested a panel of five DNA extraction protocols on well-preserved human and dog paleofeces from the Cueva de los Muertos Chiquitos archeological site. Located near the Rio Zape river in Durango, Mexico and dating to ~1,300 BP, paleofeces from this site have been shown to contain well-preserved aDNA deriving from gut-associated bacteria (Tito et al., 2008; Tito et al., 2012). To assess protocol performance, we compare both total DNA yield and reconstructed microbial community structure. All genetic sequence data was generated using a shotgun metagenomics approach in order to avoid known aDNA amplification biases due to length polymorphisms in the 16S rRNA gene (Ziesemer et al., 2015). Overall, our findings show that protocols developed specifically for the recovery of aDNA result in significantly higher DNA yields compared with commercial DNA extraction kits. Importantly, however, all extraction protocols resulted in consistent taxonomic profiles from paleofeces, indicating that aggressive cell lysis and inhibitor removal steps are not necessary to efficiently recover DNA from paleofeces. Consequently, genetic sequences obtained from paleofeces using optimized aDNA extraction protocols can be compared with previously published gut microbiome data obtained using commercial kits without systematic taxonomic bias due to extraction protocol differences.

2 | MATERIALS AND METHODS

2.1 | Samples and study design

Three paleofeces samples were selected for analysis from the Cueva de los Muertos Chiquitos archeological site, a cave situated approximately 15 m above the Rio Zape in Durango, Mexico. This site contains evidence of storage, agave feasting, waste disposal, and burial and is associated with the Loma San Gabriel Culture, a group of rural agriculturalists who occupied the area from approximately 1,200 to 1,400 years ago (Jimenez et al., 2012). Analysis of dental casts in quids from the site shows that at least 49 people were involved in feasting activity at the site (Hammerl, Baier, & Reinhard, 2015). Wooden materials associated with the paleofeces have been dated to 1,300 ± 100 BP (Brooks, Kaplan, Cutler, & Whitaker, 1962). The paleofeces were excavated from a midden above an adobe floor. The adobe floor sealed the remains of several partially mummified child burials, as well as offerings. This site was remarkably deep and the refuse deposits were separated from the entry of the cave by approximately 10 m. This distance was composed of rock fall. Inside the rock fall was the midden overlying the adobe floor and extending a further 4–5 m deeper into the cave. This unusually isolated archeological context deep within a cave has contributed to their exceptional preservation, and paleofeces from this site have been previously shown to preserve DNA from gut microbiome-associated bacterial taxa (Tito et al., 2008; Tito et al., 2012). Paleofeces found within the midden are consistent with human and dog feces. The samples in this study are a subsample of 36 specimens analyzed for parasite remains (Jimenez et al., 2012). One putative dog sample (Zape 2) and two human samples (Zape 5 and Zape 28) of paleofeces were selected for analysis. A canine

origin for Zape 2 was suspected based on the presence of previously identified dog-associated parasites identified within the fecal material (Cleeland, Reichard, Tito, Reinhard, & Lewis Jr, 2013; Jimenez et al., 2012), and later confirmed in this study (see in the following). Because human and dog paleofeces make up the majority of paleofeces reported at archeological sites, we include paleofeces from both host species in this study.

2.2 | DNA extraction

DNA was extracted from paleofeces following five protocols, here designated A–E: the Human Microbiome Project standard protocol using the PowerSoil kit (A); an aDNA-optimized modified MinElute protocol for bone extraction following Dabney et al. (2013) (B); and three variants of these protocols (C–E) (Figure 1). Extractions were

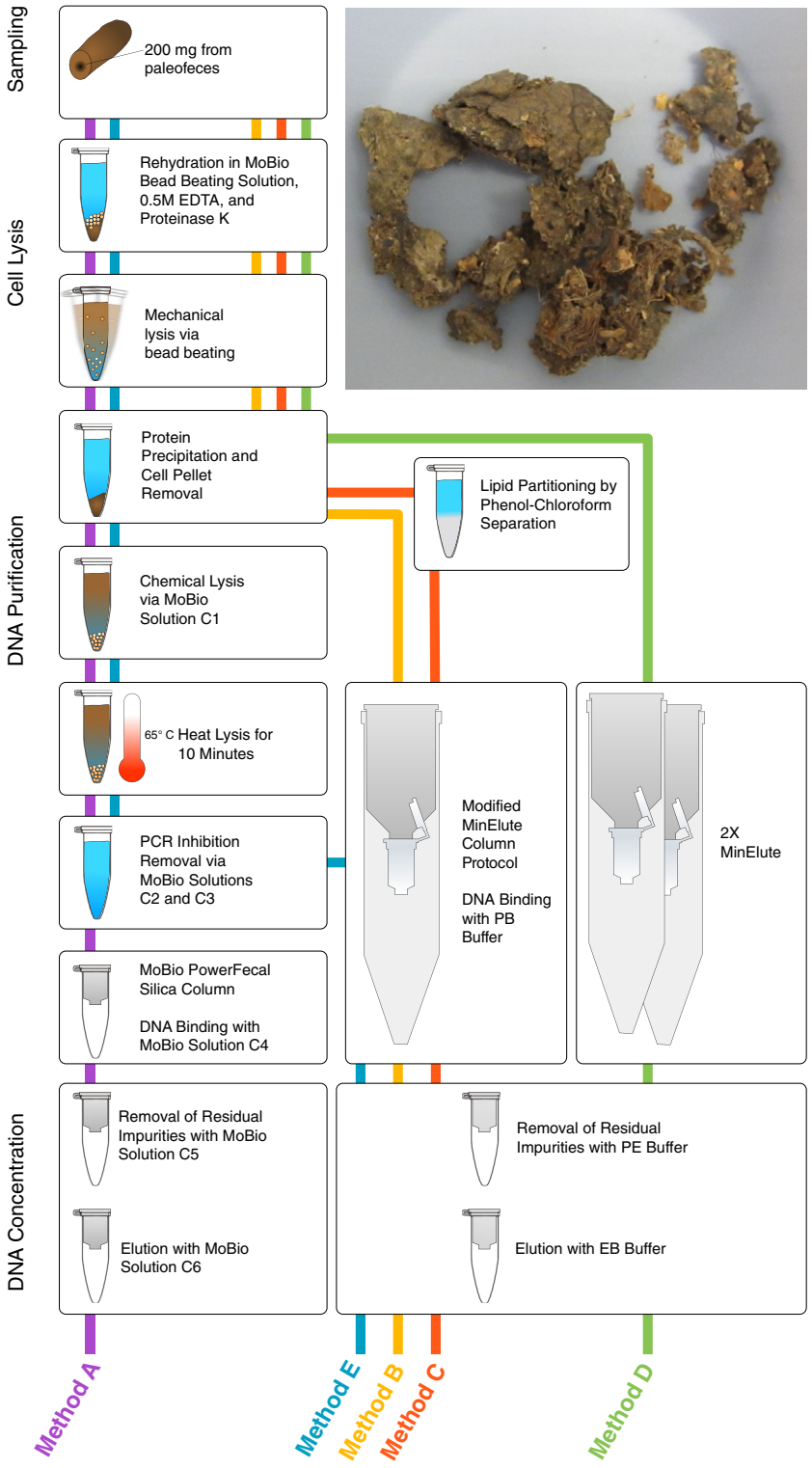

FIGURE 1 Overview of study design. This study was designed to test the influence of DNA extraction method on DNA recovery and reconstructed microbial community structure from paleofeces. Five different DNA extraction methods (A–E) were compared, and the protocol steps included in each method are indicated by colored lines: Method A: Human Microbiome Project (HMP) Protocol with PowerSoil Kit; Method B: Modified MinElute Protocol; Method C: Phenol-Chloroform + Modified MinElute Protocol; Method D: Split Modified MinElute Protocol; Method E: HMP Protocol + Modified MinElute Protocol. For each protocol, three paleofeces specimens were analyzed in duplicate, resulting in a total of 30 DNA extractions. Inset shows the paleofeces of Zape 2 prior to sampling

performed in an ISO-6 cleanroom dedicated to ancient biomolecule research at the University of Oklahoma's Laboratories of Molecular Anthropology and Microbiome Research following established guidelines for aDNA research. Each extraction protocol was performed in duplicate, and non-template (negative) extraction controls were processed alongside each protocol to monitor for contamination. Prior to extraction, ~2.2 g of material was homogenized from each of the paleofeces; because of the fragmentary nature of the paleofeces, this sampling was performed on whole fecal pieces, which were then homogenized by milling to eliminate potential spatial biases. For each extraction, a subsample of approximately 200 mg was resuspended in a solution of 400 μL of 0.5 M EDTA and 100 μL Proteinase K (Qiagen, >600 mAU/ml) to form a slurry for DNA extraction. Following extraction and purification, DNA was quantified using a Qubit 3.0 fluorometer with a double-stranded DNA High Sensitivity assay (Table S1).

2.2.1 | Extraction Method A: Human Microbiome Project protocol with PowerSoil kit

This method follows the HMP protocol for the MoBio PowerSoil DNA extraction kit and was used as a test of the efficacy of a standard microbial extraction protocol on DNA recovery from paleofeces. In brief, the paleofeces suspension was added to a PowerBead tube containing 750 μL guanidine thiocyanate and garnet bead solution, and samples were rotated for 2 hr at RT. The sample was then incubated at 65°C for 10 min with 60 μL of solution C1, followed by bead beating for 10 min. Following centrifugation for 1 min at 17900 rcf, the supernatant was then processed according to the PowerSoil kit manufacturer's instructions. In total, this protocol involves mechanical lysis, chemical lysis, heat lysis, chemical removal of inhibitors, and silica purification steps. Final DNA elution was performed with 60 μL of Solution C6 after a 5 min RT incubation of the elution buffer on the column.

2.2.2 | Extraction Method B: Modified MinElute protocol

This method was selected as a test of a highly efficient aDNA extraction protocol developed for bone (Dabney et al., 2013) on paleofeces as a source material. While this protocol has been shown to be highly effective for the recovery of aDNA from mineralized tissues, including bone, dentine, and dental calculus (Mann et al., 2018), it has yet to be empirically tested on paleofeces. In brief, the paleofeces suspension was added to a PowerBead tube containing 750 μL guanidine thiocyanate and garnet bead solution, and samples were rotated for 4 hr at RT, followed by bead beating for 10 min. Samples were then spun down at 3400 rcf for 5 min, and the supernatant was then added to 14 mL of Qiagen PB buffer. This was then centrifuged in a MinElute column (Qiagen) attached to a Zymo-Spin V column (Zymo Research) for 4 min at 1500 rcf, rotated 90°, and then centrifuged for an additional 2 min. The column was then dry spun for 1 min at 3400 rcf and washed twice with 700 μL Qiagen PE buffer at 9400 rcf. DNA was eluted from the column after a 5 min RT incubation in two rounds of 30 μL of Qiagen EB buffer at 17900 rcf for a total volume of 60 μL.

2.2.3 | Extraction Method C: Phenol-chloroform + modified MinElute protocol

Previous experiments in our laboratory with paleofeces have resulted in the occasional clogging of silica columns with an unidentified gel-like substance. This protocol aims to circumvent this issue by chemically isolating nucleic acid through phenol-chloroform separation prior to silica binding. In this protocol, the paleofeces suspension was added to a PowerBead tube containing 750 μL guanidine thiocyanate and garnet bead solution, and samples were rotated for 4 hr at RT, followed by bead beating for 10 min and centrifugation at 3400 rcf for 5 min. DNA was then separated from the supernatant via phenol-chloroform extraction. In brief, the supernatant was added to a new microcentrifuge tube containing 750 μL of 25:24:1 mixture of phenol: chloroform:isoamyl alcohol, vortexed, then centrifuged at 17900 rcf for 1 min. The aqueous phase was transferred to a new microcentrifuge tube with an additional 750 μL of the phenol:chloroform: isoamyl alcohol mixture, vortexed, and again centrifuged at 17900 rcf for 1 min. The resulting aqueous phase was then added to 750 μL of 24:1 chloroform:isoamyl alcohol mixture, vortexed, and centrifuged at 17900 rcf for 1 min before being processed according to Method B, and DNA was eluted after a 5 min RT incubation in two rounds of 30 μL of Qiagen EB buffer for a total volume of 60 μL.

2.2.4 | Extraction Method D: Split modified MinElute protocol

Nearly identical to Method B, this protocol differs only in splitting the silica binding step across two MinElute columns in order to mitigate clogging problems encountered when loading extraction lysates onto a single column. For this, the supernatant was divided into two equal aliquots, each of which was added to a MinElute column with 7.5 mL of Qiagen PB buffer and processed according to the protocol described in Method B. For each of the two columns, DNA was eluted after a 5 min RT incubation in two rounds of 15 μL of Qiagen EB buffer, and the four elutions were then pooled for a total volume of 60 μL.

2.2.5 | Extraction Method E: HMP protocol + modified MinElute protocol

Methods A and B involve starkly different cell lysis and inhibitor removal procedures, but share a similar DNA purification and concentration step using a silica column. However, the manufacture and reported binding capacity of the columns used in Methods A and B differ. Specifically, Method B uses a Qiagen MinElute column, which

is marketed as efficiently retaining DNA fragments as short as 70 bp, while the retention properties of the silica column spin filter used in Method A are not specified and may not retain short fragments. In order to distinguish the effects of the lysis and inhibitor removal procedures from the size selection of the silica column, we devised a hybrid method that combines the two protocols. The paleofeces suspension was added to a PowerBead tube containing 750 μL guanidine thiocyanate and garnet bead solution, and DNA was then extracted according to Method A up to the centrifugation step following the addition of 200 μL of Solution C3. The resulting supernatant was then purified and concentrated on a Qiagen MinElute column following Method B, and DNA was eluted after a 5 min RT incubation in two rounds of 30 μL of Qiagen EB buffer for a total volume of 60 μL.

2.3 | Library construction and sequencing

Double-stranded shotgun Illumina libraries were constructed for each extract following the protocol developed by Meyer and Kircher (2010), with modifications. Briefly, 100 ng of DNA (or 30 μL of extract for negative controls and low yield samples) as measured by the Qubit fluorometer high-sensitivity assay was used to construct indexed libraries with the NEBNext DNA Library Prep Master Set (E6070) according manufacturer instructions, but replacing SPRI bead purification with a silica column-based purification (Qiagen MinElute PCR Purification kit). In brief, the ends of template molecules were repaired with T4 polymerase and polynucleotide kinase to create blunt end templates with phosphorylated 5′ ends. Blunt end IS1/IS3 and IS2/IS3 adapters were then ligated to template molecules at a concentration of 0.5 μM. Adapter overhangs were filled in by Bst DNA polymerase at 37°C for 30 min, followed by an inactivation step at 80°C for 20 min and −20°C overnight. Shotgun libraries were constructed for each sample and negative control by PCR amplification with Kapa HiFi Uracil+. The libraries were then purified by silica column (Qiagen MinElute PCR Purification kit) and quantified with a BioAnalyzer 2,100 using the High Sensitivity DNA reagents. MinElute centrifugation speeds were modified to 3,400 rcf, 9,400 rcf, and 17,900 rcf for binding, washing, and elution steps, respectively. All libraries were pooled in equimolar amounts, size selected to 150–600 bp using a PippinPrep 2% agarose gel, and sequenced by Illumina HiSeq 2×100 paired-end sequencing.

2.4 | Data analysis

2.4.1 | Data filtering and quality control

Sequenced reads were initially assessed for quality with FastQC. Library pooling and sequencing for sample Zape 2 Method B replicate 2 resulted in fewer than 10 K sequences and was excluded from subsequent analysis. The remaining paleofeces samples were each sequenced to an average depth of approximately 11 million reads (median, 11.1 M reads; range, 3.4–59.9 M reads). Sequencing adapters were removed and paired-end reads were merged with AdapterRemoval 2.0 using the following parameters: —maxns 0, —trimqualities, —minquality 30, —collapse, —minlength 25, and —minalignmentlength 10. Residual adapter contamination was removed by mapping all reads to full adapter constructs with Bowtie 2.3.0 (Langmead & Salzberg, 2012) in local alignment mode. The resulting collapsed, analysis-ready reads were then used for downstream analysis. A summary of sequencing and preprocessing statistics is provided in Table S2.

2.4.2 | Assessment of method impact on DNA yield

The quantity of DNA recovered by each extraction as measured by the Qubit fluorometer double-stranded high-sensitivity assay was normalized to the amount of starting material used. Next that the mean quantity of DNA recovered by Method A in each sample was calculated, and the fold change in each sample for each additional method was calculated by dividing its yield by the mean of Method A. Significant differences in fold change were tested between all methods using a pairwise Wilcoxon rank sum test corrected for multiple testing by the Benjamini–Hochberg method (Benjamini & Hochberg, 1995) in R. To determine if there was any effect by method on the length of DNA molecules recovered, all analysis-ready reads were mapped to the reference genomes for 21 bacterial taxa present within the dataset (Table S3), and the insert lengths of all mapped reads were computed. The median insert length was calculated for each species in each sample, and their distributions are shown in Figure S1.

2.4.3 | Microbial community profiling

Analysis-ready reads were mapped to the Greengenes 16S rRNA gene database (v.13.8, pre-clustered at 97% similarity) (DeSantis et al., 2006) using bowtie2 (v.2.3.0; Langmead & Salzberg, 2012). Successfully mapped reads (Table S2) were selected and combined into an input FASTA file for use with the QIIME package (Caporaso et al., 2010). Closed-reference operational taxonomic unit (OTU) clustering was performed in QIIME v1.9.1 using the same pre-clustered database and the following parameters: —max_accepts 500, —max_rejects 500, —word_length 12, —stepwords 20, and —enable_rev_strand_match TRUE. In order to determine the effect of extraction method on the observed microbial community of the paleofeces, the resulting BIOM tables were merged into a single BIOM table using the script *merge_otu_tables.py* in QIIME (Data S1), and beta diversity was calculated using the weighted UniFrac metric (Lozupone, Lladser, Knights, Stombaugh, & Knight, 2011) via the script *beta_diversity_through_plots.py*. Principal coordinates were extracted from the output file and visualized using R.

2.4.4 | Assessment of paleofeces microbial preservation

To determine whether the reconstructed microbial communities observed were endogenous to the paleofeces, Bayesian source

tracking was performed using SourceTracker v2.1.0 (Knights et al., 2011). Reference fecal metagenomes obtained from industrialized human populations (Sankaranarayanan et al., 2015), non-industrialized human populations (Obregon-Tito et al., 2015), and domesticated dogs (Li, Lauber, Czarnecki-Maulden, Pan, & Hannah, 2017), as well as soil metagenomes (Johnston et al., 2016) were prepared in QIIME following the same procedures used for paleofeces (see above), and the BIOM files were merged with those of the current study for use with SourceTracker2. Then, to determine whether the paleofeces DNA exhibits appropriate molecular behavior for aDNA, such as DNA damage, the merged reads from each sample were mapped to the genome of *Prevotella copri* (DSM 18205), and mapDamage 2.0 (Jonsson, Ginolhac, Schubert, Johnson, & Orlando, 2013) was used to measure the frequency of nucleotide deaminations that are characteristic of aDNA. *P. copri* was selected because it has been reported in both human and dog gut microbiota (Li et al., 2017; Tett et al., 2019).

2.4.5 | Host determination of paleofeces

Finally, two strategies were employed to confirm the previous identification of Zape 2 as dog paleofeces rather than representing other possible scenarios, such as human paleofeces with a dietary signal from dog consumption. First, all merged reads from each sample were competitively mapped to both the human (hg19) and dog (CanFam 3.1) reference genomes using bowtie2 (v.2.3.0; Langmead & Salzberg, 2012) in local mode, and the number of reads mapping exactly once to each was log10 transformed and compared under the assumption that host DNA would be higher than other eukaryotic, non-host DNA for a given paleofeces sample. Next, the combined BIOM file containing OTUs from the paleofeces and reference fecal metagenomes was processed in QIIME. Beta diversity was again computed using the weighted UniFrac metric and visualized using principal coordinates analysis under the assumption that the microbial communities present in Zape 5 and Zape 28 would cluster with modern human feces, while Zape 2 would cluster with dog feces.

3 | RESULTS AND DISCUSSION

3.1 | DNA recovery by extraction method

Overall DNA recovery differed by paleofeces sample, with Zape 2 yielding the highest average amount of DNA (median, 6.7 ng/mg; range, 0.8–11.7 ng/mg), followed by Zape 5 (median, 2.9 ng/mg; range, 0.02–6.7 ng/mg), and Zape 28 (median, 2.5 ng/mg; range, 0.03–7.2 ng/mg) (Table S1). DNA recovery also differed significantly by extraction method (Figure 2), and the lowest DNA yields were observed for extraction methods using the PowerSoil kit (Methods A and E). DNA recovery was lowest using the HMP protocol (Method A) and only slightly improved with the substitution of a MinElute silica column in place of the kit's silica spin filters (Method E). This suggests that a large proportion of the DNA lost during extraction using the Powersoil kit occurs during the cell lysis and/or inhibitor removal stage prior to the silica column purification step.

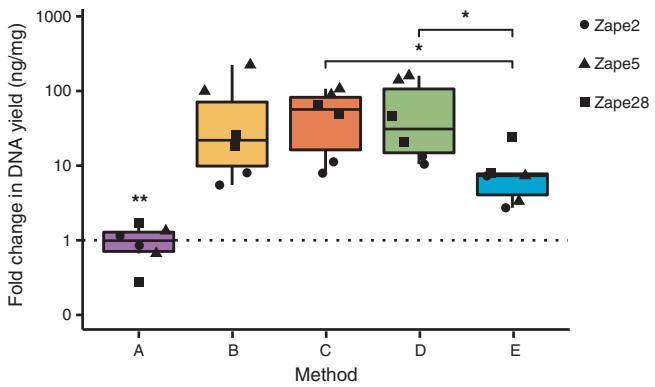

FIGURE 2 Choice of DNA extraction method affects DNA recovery from paleofeces. Log-fold change in the normalized yield of DNA (ng DNA per mg paleofeces) is shown compared with the median DNA yield using method A for each sample. Statistical comparisons were made using a pairwise Wilcoxon test with Benjamini–Hochberg correction for multiple tests. Methods b–e all resulted in significantly higher median DNA yield than method A (**, $p < .01$); other significant pairwise differences are indicated by *, $p < .05$

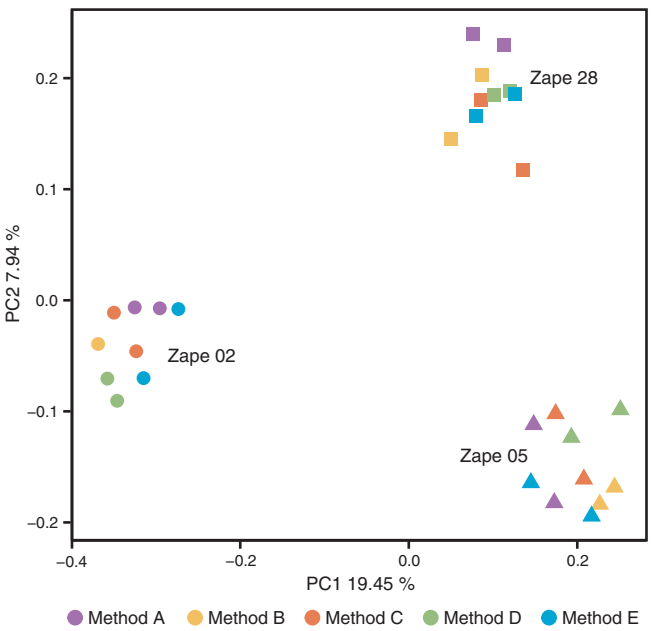

FIGURE 3 Sample origin, not DNA extraction method, is primary determinant of microbial community structure in paleofeces. Principal Coordinates Analysis (PCoA) analysis of weighted UniFrac beta-diversity in paleofeces indicates that DNA extraction method has little influence on reconstructed microbial structure. This contrasts with studies of modern feces, where extraction method has been shown to introduce systematic taxonomic biases

To further investigate the high degree of DNA loss observed in Method A, we compared the DNA fragment lengths recovered using the five protocols (Figure S1). Although protocols B-E were expected to retain shorter DNA fragments due the MinElute silica column, we did not observe significant differences between any of the methods, but rather high inter-sample variation. This supports our conclusion that the cell lysis and/or inhibitor removal steps of the PowerSoil kit protocol likely had the greatest impact on DNA recovery.

Methods B, C, and D all performed well, and differences between these methods were non-significant. However, using two silica columns to expand the total DNA binding capacity and reduce clogging (Method D) resulted in the highest median DNA yields, and we found that this method was more straightforward to implement than Method B, which frequently required cleaning steps to dislodge clogged material. Method C incorporated a phenol: chloroform purification step that was intended to remove unwanted material prior to silica column filtration, but we found that it did not reduce clogging of the columns. Because Method C uses hazardous chemicals and did not outperform the other methods, we do not recommend its use. Overall, we recommend Method D on the basis of DNA recovery and ease of use.

3.2 | Effect of DNA extraction protocol on microbial community reconstruction

The microbial community structure reconstructed from modern feces has been shown to be highly influenced by choice of DNA extraction method (Wesolowska-Andersen et al., 2014). By contrast, here we find that reconstructed microbial profiles from paleofeces are highly similar regardless of DNA extraction protocol and that differences in sample beta diversity are primarily driven by the paleofeces from which the sample derives (Figure 3). This finding suggests that cellular degradation over time has sufficiently weakened the cellular structure of the ancient microbial cells such that even highly simplified DNA extraction protocols, such as those used in Methods B and D, recover a microbial community similar to complex and highly aggressive protocols, such as Method A.

3.3 | Gut microbiome preservation

To assess gut microbiome preservation in the paleofeces, Bayesian source tracking of the microbial taxa (OTUs) present in each sample was performed using published reference metagenomes of human feces from industrialized and non-industrialized populations, dog feces, and soil (Figure 4a). We found that the majority of the OTUs present are consistent with microbes found within human or dog feces rather than soil, indicating well-preserved paleofeces. Additionally, all three paleofeces exhibited DNA damage patterns consistent with authentic aDNA (Figures 4b and S2). The high degree of endogenous gut microbiome preservation we observe is consistent with previous studies that have reported good molecular preservation of paleofeces at this site (Tito et al., 2008; Tito et al., 2012).

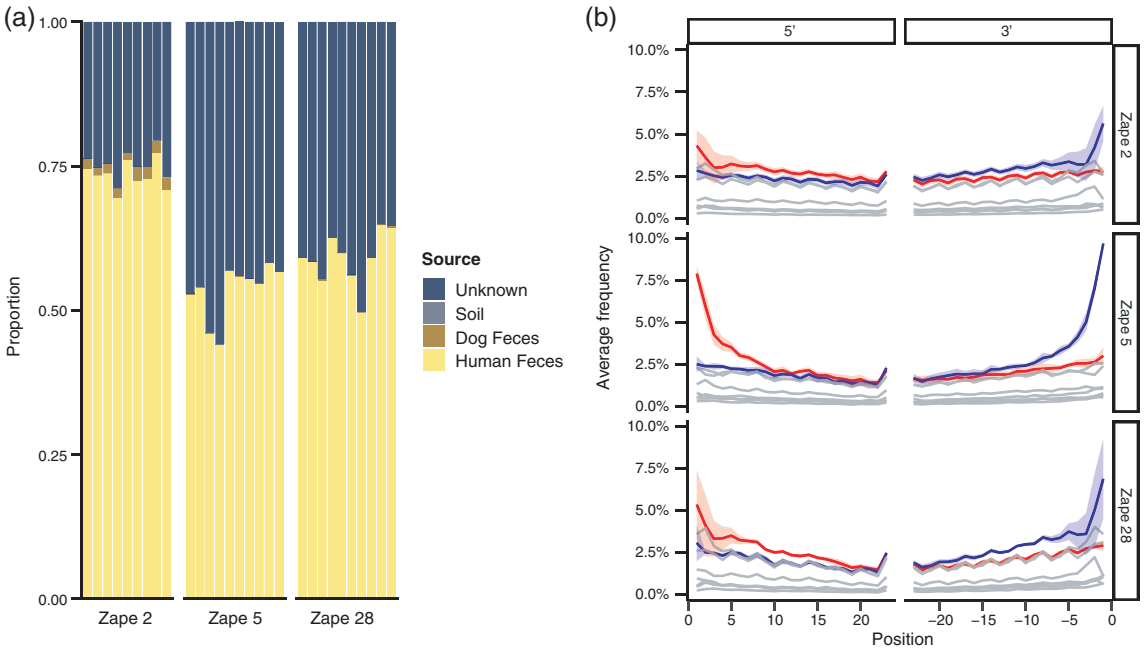

FIGURE 4 Zape paleofeces exhibit characteristics of an authentic ancient gut microbiome. (a) Bayesian source tracking of microbial taxa present in paleofeces suggests a high degree of gut microbiome preservation and low soil contamination; only Zape 2 shows evidence for dog-associated taxa. (b) Terminal per sample mean nucleotide deamination rates across all methods calculated using mapDamage 2 confirm the presence of typical aDNA damage patterns in sequences mapping to *P. copri* (DSM 18025), a bacterial species present in the feces of both humans and dogs. Red lines indicate C > T; blue lines indicate G > A; all other substitutions are marked in gray

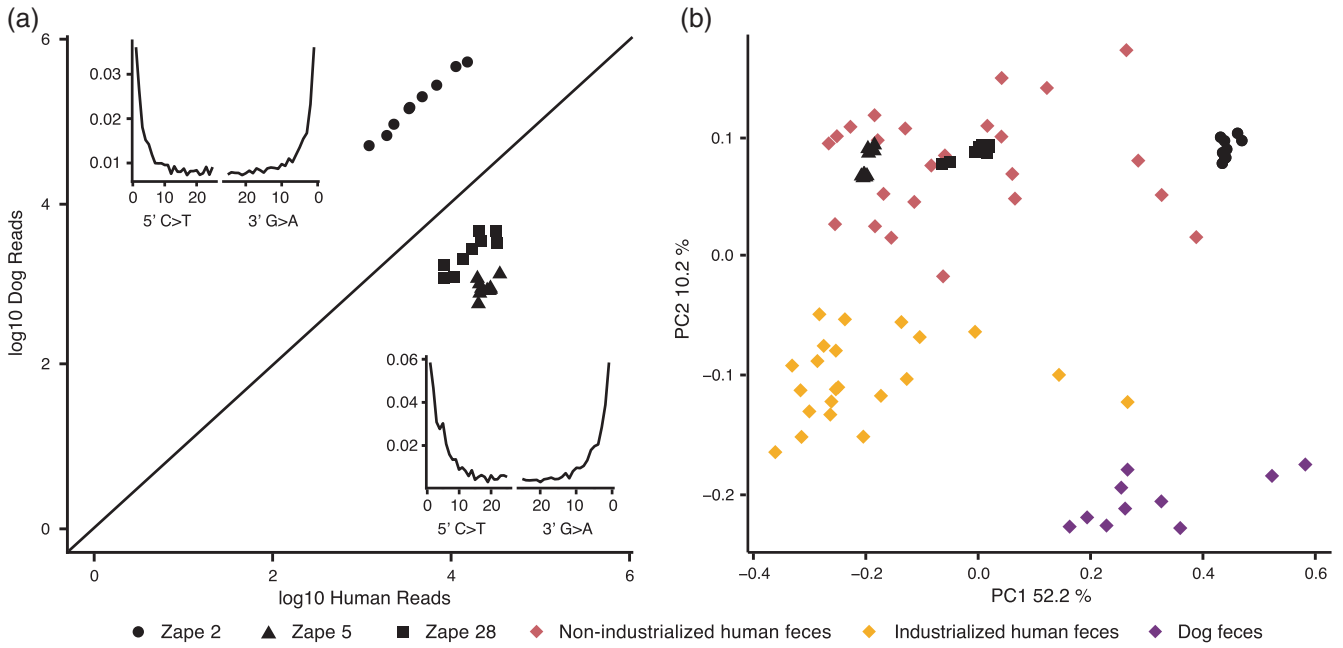

FIGURE 5 Host assessment of paleofeces. (a) Comparison of the log10-transformed number of DNA sequences mapping uniquely to the dog and human genomes from paleofeces. The solid diagonal line indicates an equal number of reads aligning to both genomes; points falling above the line contain more dog DNA (Zape 2), while those falling below the line contain more human DNA (Zape 5, Zape 28). Insets show representative DNA damage profiles for human and dog genomic DNA obtained using method D (Upper: dog, Zape 2; Lower: human, Zape 28). (b) Microbial beta diversity analysis of paleofeces and reference metagenomes. Zape 5 and Zape 28 fall within the distribution of feces from modern non-industrialized human populations, while Zape 2 falls outside reference distributions, but within the range of dog feces on the first principal coordinate. Analysis was performed using Principal Coordinates Analysis (PCoA) of weighted UniFrac distances

3.4 | Host confirmation of paleofeces

The host origin of each paleofeces sample was next investigated by comparing the relative proportion of human and dog DNA sequences in each sample. Paleofeces samples Zape 5 and Zape 28 exhibited a 7–26-fold excess of human DNA sequences, suggesting a human origin. By contrast, Zape 2 contained a 40-fold excess of dog DNA sequences, suggesting a canine origin (Figure 5a). Further analysis of both the dog and human DNA sequences revealed that they exhibit damage patterns typical of aDNA, suggesting that they are endogenous to the sample and not contaminants (Figure 5a).

Microbial community profiling further supported these findings (Figure 5b). Beta diversity analysis of weighted UniFrac distances revealed that the microbial communities of the Zape 5 and Zape 28 paleofeces fall within the distribution of reference fecal metagenomes from non-industrial human populations, supporting a human origin. By contrast, Zape 2 falls outside this distribution, and, although displaced in principle coordinate two, Zape 2 clusters along the first principal coordinate within the range of modern dog feces. Bayesian source tracking further identified a dog fecal contribution in Zape 2, whereas the fecal components of Zape 5 and Zape 28 were both estimated to be exclusively of human origin (Figure 4a). The additional human fecal contribution estimated by SourceTracker2 for Zape 2, as well as its displacement in principle coordinate two outside the range of reference dog feces may result from the fact that no reference fecal metagenomes are currently available for dogs from non-industrialized contexts.

4 | CONCLUSIONS

Paleofeces are a valuable resource for investigating the evolution of the gut microbiome and the diet and health of past peoples, but until now no systematic studies had been conducted to assess the performance and potential biases of existing DNA extraction protocols on the recovery and reconstruction of microbiome profiles. In this study, we compared five DNA extraction methods on a panel of well-preserved human and dog paleofeces and evaluated methodological performance on the basis of DNA recovery and taxonomic composition. We found that DNA extraction methods that have become field-standard in modern microbiome studies, such as the HMP PowerSoil protocol, recover significantly less DNA from paleofeces than methods that have been developed and optimized for ancient skeletal material. Additionally, we found that aDNA optimized-methods do not negatively impact the structure of the reconstructed microbial communities when compared with the HMP PowerSoil protocol, and in fact, all DNA extractions tested in this study yielded highly similar microbial communities. This finding supports future research that seeks to compare metagenomic data generated using optimized protocols for both ancient and modern fecal samples. For paleofeces, we

recommend the use of Method B or D for this purpose, with Method D favored if clogging occurs during spin filtration.

ACKNOWLEDGMENTS

This work was supported by the US National Institutes of Health R01GM089886 (to C.M.L., C.W., and K.S.), the Deutsche Forschungsgemeinschaft EXC 2051 #390713860 (to C.W.), and the Max Planck Society.

AUTHOR CONTRIBUTIONS

C.W., C.A.H., and K.S. designed the research. R.H., C.A.H., and S.S. performed the experiments. R.H., A.H., and K.S. analyzed the data. K.S. supervised microbiome informatics and statistical analysis. K.R., C.M.L., K.S., and C.W. provided materials and resources. R.W. and C.W. wrote the article, with input from the other co-authors.

DATA AVAILABILITY STATEMENT

Genetic data are available in the European Nucleotide Archive (ERA) under the accession PRJEB33577.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Hagan RW, Hofman CA, Hübner A, et al. Comparison of extraction methods for recovering ancient microbial DNA from paleofeces. *Am J Phys Anthropol*. 2020;171:275–284. https://doi.org/10.1002/ajpa.23978