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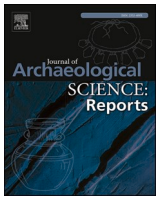

Ancient DNA and paleoproteomic analysis on Roman Imperial-era individuals from Histria, Romania

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ABSTRACT

Ancient biomolecules have become an increasingly important part of archaeological investigations interested in understanding population movements and health. Despite their ability to elucidate historically-attested contexts of human mobility and interaction between different cultural groups, biomolecular techniques are still underutilized in certain historical and archaeological contexts. One such context is the Roman Imperial *limes*, or border zone, along the lower reaches of the Danube, which saw more than five hundred years of migration, conflict, and accommodation among a wide range of populations, from Mediterranean settlers to steppe pastoralists. In this region, more than a century of archaeological investigation has unearthed the remains of tens of thousands of Roman-era individuals. However, only a limited number of contexts have undergone biomolecular analyses. While these deceased humans may offer an untapped reservoir of biomolecular information, many were collected during a period when the standard precautions and protocols for ancient biomolecular research were not yet established. Because contamination is a major barrier for successfully recovering ancient DNA and proteins, conducting a pilot study to assess bimolecular preservation of a small representative dataset of human remains before embarking on a more extensive research program may prevent unnecessary sampling. This study applies ancient DNA and paleoproteomic techniques to human remains from a Roman-period cemetery at Histria, a site located just south of the Danube at the edge of the Roman province of Moesia Inferior. The individuals from whom we sampled dentin and dental calculus were excavated between the 1940s and 1980s and were housed at the Francisc J. Rainer Institute since. Our results suggest that both microbial and human ancient DNA is preserved in the dental calculus and dentin samples. We also successfully recovered sex-specific amelogenin peptides in tooth enamel from three individuals, including a juvenile. In conclusion, our results are encouraging, signifying the feasibility of future aDNA and paleoproteomic research for this skeletal collection. Our analyses also showcase how sex estimation with genomic and proteomic methods may contradict traditional osteological approaches. These findings not only offer deeper insights into the lives of these individuals but also show promise for the investigation of broader anthropological questions, such as the impact of Roman annexation in this region.

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1. Introduction

The degree to which Roman conquest of territories beyond Italy changed the culture, demography, health, and lifeways of the populations of those territories has been debated for centuries. In recent years, the idea that local groups were assimilated into a homogenous "Roman" culture—the process of "Romanization"—has been replaced by an acknowledgement of the diversity and contingency of identities in the Roman provinces (Mattingly, 2013; Oltean, 2007; Woolf, 1994). Both archaeological and textual sources have been used to demonstrate that many local populations maintained their cultural practices or, at least, developed creolized identities when experiencing Roman annexation *de jure* (Alcock, 1997, 1996; Dalaison, 2014; Madsen, 2014; Webster, 2001). Since the 1980s, bioarchaeological research has investigated the differential effects of Roman rule on the health and diet of individual members of these groups, as well as trends correlated with "Roman" or "local" collective identities. These studies have identified significant differences in nutrition, general health, and prevalence of disease, according to the status, age, sex, and geographic origins of individuals in Roman cemeteries (Bonsall, 2014; Marklein, 2020; Pitts and Griffin, 2012; Redfern et al., 2018; Rohnbogner and Lewis, 2016). For instance, isotopic analyses show that diet varied across populations in Italy itself during the Imperial period (1st-3rd centuries CE) (Killgrove and Montgomery, 2016; Killgrove and Tykot, 2018, 2013). Dietary isotopic analysis for people residing in proximity to the city of Rome suggests that they consumed a substantial quantity of marine resources, whereas those residing at a greater distance from the urban hub had consumed large quantities of C4 plants, probably millet, a grain often described unfavorably in literary sources (Killgrove and Tykot, 2013). Similar investigations using isotopic analysis have been employed to understand the dietary practices of populations living in the Roman provinces of Britannia (Britain), Africa Proconsularis (North Africa), Achaea (Greece), and Asia (Anatolia) (Borstad et al., 2018; Chenery et al., 2010; Dotsika and Michael, 2018; Keenleyside et al., 2009; Lösch et al., 2014). Just as isotopic analyses have expanded our understanding of Roman-period diets, the incorporation of ancient DNA (aDNA) and paleoproteomic methodologies into the bioarchaeological toolkit offer new perspectives into the life histories of individuals who lived during the height of the Roman Empire.

Both aDNA and paleoproteomic approaches can aid in the reconstruction of osteobiographies of ancient individuals because they are intertwined with the social and biological processes that take place during the development of the human body (Fig. 1). These analyses not only provide additional avenues for examining skeletal collections, but also yield novel evidence to address a broad spectrum of anthropological and archaeological inquiries (Gancz et al., 2023; Hendy et al., 2018; Orlando et al., 2021; Weyrich et al., 2015; Wright et al., 2021). For example, aDNA techniques have yielded significant insights into ancient migrations (Antonio et al., 2019; Eisenhofer et al., 2020; Hervella et al., 2015), demography (Ozga et al., 2016; Ziesemer et al., 2019), health (Warinner et al., 2014; Weyrich et al., 2017), diet (Mann et al., 2020; Weyrich et al., 2017), and behaviors (Weyrich et al., 2017). An investigation of the oral microbiome composition of a Japanese population from the Edo period (400–150 years Bp) sheds light on how gender roles within this society might account for the observed discrepancy in the frequency of periodontal disease between males and females (Eisenhofer et al., 2020). In an ancient Mediterranean context, aDNA evidence also revealed that individuals inhabiting the ancient city of Rome were quite diverse, with ancestries that can be linked to regions across Europe, the Near East, and North Africa (Antonio et al., 2019). A recent study indicates that the genetic landscapes for several central Mediterranean sites dating to the Iron Age were also heterogeneous (Moots et al., 2023). Furthermore, paleoproteomic approaches have been used to test hypotheses related to diet (Wilkin et al., 2020), health (Fotakis et al., 2020), social stratification (Jersie-Christensen et al., 2018), and demography, particularly with respect to biological sex (Lugli et al., 2019; Parker et al., 2019; Stewart et al., 2017). In the last case, ancient peptides have already had profound implications on previous interpretations of Roman mortuary practices, as exemplified by the case of the "Lovers of Modena". Contrary to the initial estimate of one male and one female, the identification of sex-specific peptides from two individuals interred together, holding hands, revealed that both were biological males (Lugli et al., 2019). This method holds significant promise for accurately estimating biological sex, defined here as the biological state of being male or female as indicated by the presence of sex chromosomes (Fausto-Sterling et al., 2012). Because traditional osteological methods are unable to estimate sex of infants and juveniles (Parker et al., 2019), the capacity of paleoproteomic approaches to accurately make such assessments can provide crucial evidence regarding ancient population dynamics and health.

This study assesses aDNA and paleoproteomic preservation from a group of individuals interred in a Roman-era cemetery at the site of Histria, on the western shore of the Black Sea near the Danube *limes* (Roman border region). Our central research question concerns the viability of ancient DNA and proteins from a group of individuals who were previously excavated at the site without following widely-used precautions typical for these biomolecular techniques. For instance, the archaeologists at the excavation did not wear any sort of protective gear (*e.g.*, surgical mask, hair net, and sleeves to cover arms) when handling the remains. Taking such precautions has been shown to

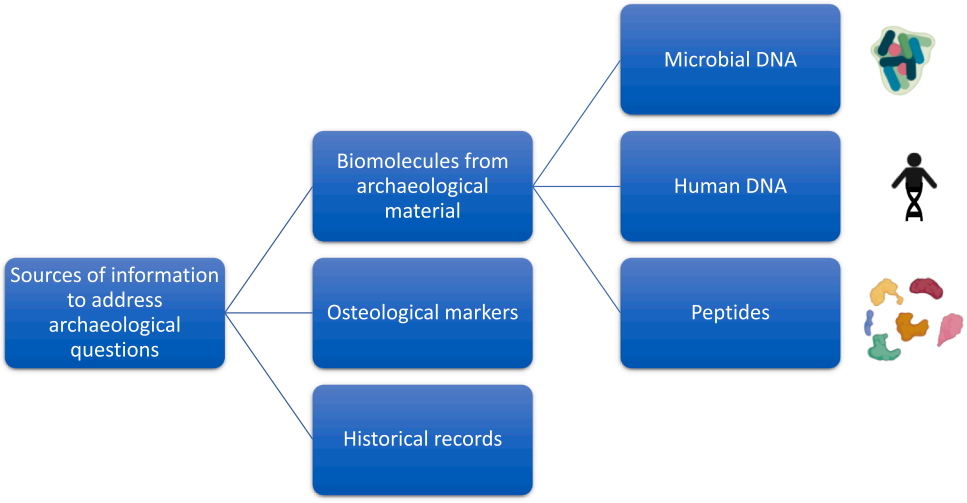

Fig 1. Biomolecules from dental calculus and dentin can provide additional evidence to address archaeological questions.

minimize contamination (Llamas et al., 2017; Orlando et al., 2021). Thus, while the application of aDNA and paleoproteomic analyses in tandem to these individuals could help in uncovering novel insights, it is possible that the lack of precautions taken during the time of excavating these human remains, and their storage conditions since then could have significantly impacted their biomolecular preservation. Several factors impact the integrity of aDNA (Farrer et al., 2021, 2018; Kistler et al., 2017; Mann et al., 2020) and paleoproteins (Hendy, 2021; Hendy et al., 2018). Therefore, assessing preservation with a small number of samples that were subject to similar taphonomic circumstances and post-excavation treatment before initiating an extensive program may help minimize unnecessary destructive analyses (Austin et al., 2019).

With these considerations in mind, our study aims to 1) evaluate the preservation of the microbial and human DNA in both dentin and dental calculus samples; 2) reconstruct the taxonomic profile of microbes within the dental calculus samples; 3) estimate mitochondrial haplotypes for human individuals; and 4) estimate the biological sex of three individuals using a combination of genomic and proteomic strategies, in order to compare the outcomes of these analyses with estimations based on osteological data. We successfully retrieved aDNA from two dentin samples and two calculus samples from individuals who were at least 20 years of age and were buried between the 2nd and 6th centuries CE. In addition, we recovered sex-specific peptides from the dental enamel of two adults and one juvenile (3–5 years of age) using an acid etching approach. Our analyses of aDNA and paleoproteomic data yields promising insights into the biomolecular preservation for the human remains housed at the Francisc J. Rainer Institute of Anthropology.

2. Materials and methods

2.1. The site of Histria

Histria was established along the western Black Sea coast and near the mouth of the Danube (Fig. 2) by Greek settlers from Miletus in Asia Minor during the 7th century BCE (Avram et al., 2004). For a time, it was one of the most important Greek trading centers in the Black Sea region. However, it suffered a series of destructions as a result of conflicts between different groups in the region, and by the 1st century BCE the community was struggling to survive. Roman influence in the region seems to have stabilized the situation in the early 1st century CE (Jones, 2016). Trajan's conquest of Dacia in the early 2nd century CE led to the construction of new walls and multiple civic buildings in Histria. When the city was reestablished as part of the Roman province of Moesia Inferior, a former residential area referred to by modern scholars as the "Plateau" was used as a necropolis between the 2nd and 6th centuries CE. Many of the burials in this area were excavated between the late 1940s and 1980s (Coja, 1979). While the presence of human remains were recorded by the excavators (Condurachi et al., 1962, 1957), osteological analyses, such as age and sex estimations, were not performed on these remains until rather recently (Crețu, 2018; Crețu et al., 2020; Soficaru and Mihăilescu-Bîrliba, 2011).

2.2. Archaeological context for the samples

All individuals in this study were interred in the Plateau necropolis, and all but one were excavated by members of the Vasile Pârvan Institute of Archaeology from the 1940s to the 1980s. A juvenile from whom we collected a tooth was recovered from a different area of the "Plateau" in the course of the first field season of the Histria Multiscalar Archaeological Project in 2018 and was included for paleoproteomic analysis. Individuals recovered during 20th century excavations and the 2018 campaign were inhumed in simple earth-cut graves, delimited on one or more sides with a few tiles or small slabs of green schist.

Because all but one of these individuals were excavated in the 20th century, information regarding their context is partial or uncertain. The scarcity of grave goods and the simplicity of burial practices imply a non-elite status for these individuals. The archaeological report regarding the individuals from whom samples ROM_3D and ROM_10D were collected indicated that they were buried without any grave goods in the same tomb (Condurachi et al., 1962). The individual who provided samples ROM_5C and ROM_5D was found in an area of graves without goods, is also likely from the Roman period. For the individual from whom sample ROM_9C was taken, we have more extensive documentation. This individual was found in a supine position along with another adult and child. This individual was reported to be a female buried next to an adult individual identified as male, who was placed in a contracted position. Sex estimation for both of these individuals was based on a previous osteological analysis (Soficaru et al., 2014). This burial was furnished with glass beads of a type that can be assigned to the 3rd or 4th century CE. In contrast with samples from burials

Fig 2. Map showing the location of Histria.

excavated in the 20th century campaigns, ROM_11 was collected immediately after being unearthed from a juvenile burial excavated in 2018 by the Histria Multiscalar Archaeological Project (HMAP) team in the course of stratigraphic test-trenching on the Plateau. The context for this individual is well documented: the grave was bounded to the south by small slabs of green schist and friable white limestone and contained the remains of a single individual with head to the west, severely disturbed by ground-squirrel burrows. There were no grave goods, but radiocarbon analysis of one of the individual's ribs provided a calibrated date of 433–566 CE at the 95.4% confidence interval (SI Appendix).

In line with typical aDNA protocols (Llamas et al., 2017), personnel handling the human remains from the grave unearthed in 2018 wore nitrile gloves, long sleeves, and face masks in both the field and the storeroom on site, where samples were taken and stored in sterile sample tubes. The remains of individuals recovered in the 20th century, on the other hand, had been transferred decades ago to the Francisc J. Rainer Institute of Anthropology in Bucharest, Romania, for further analysis and curation after excavation. For a previous study, these human remains were washed with tap water and subsequent examination to derive estimations regarding sex, age, and pathologies (Soficaru et al., 2014). They were then housed in wooden or cardboard boxes in the storerooms at the Institute until the present. No specific measures were taken either during or after excavation to prevent DNA contamination.

2.3. Sample collection

We collected dentin and dental calculus samples from 11 individuals. A total of seven dental calculus and six dentin samples were collected from these individuals for aDNA analysis, while the dental enamel of two adult individuals from that group and one juvenile from the 2018 excavation was used for the paleoproteomic analysis (Table S1). During the collection process, nitrile gloves, a disposable face mask, goggles, and a hair net were worn. The calculus was removed from the surfaces of the teeth using a sterile dental scaler and deposited in polypropylene tubes. Gloves were changed after each sample collection and the blade was sanitized after each use with bleach. A single tooth from each individual was removed with gloved hands and was then directly placed into a polypropylene tube. With the exception of the 2018 burials, only individuals with multiple teeth and multiple deposits of calculus were selected. When possible, calculus deposits on molars were selected because they were often the largest size. We also selected teeth with intact roots. Samples were stored at room temperature and were transported in a sealed package to the ancient DNA laboratory facilities at the Laboratories of Molecular Anthropology and Microbiome Research (LMAMR) at the University of Oklahoma in Norman, Oklahoma USA.

2.4. Laboratory work

The LMAMR aDNA laboratory maintains positive air pressure, HEPA filtered air, daily ultraviolet (UV)-treatment, and regular bleach cleanings, all of which minimize the amount of modern DNA contaminating the samples. DNA extractions were performed in a PCR hood. All researchers entered the facility via a dedicated entry room and wore full-body Tyvek suits, layers of gloves, hairnets, dedicated footwear, and facemasks.

2.4.1. Decontamination, DNA extraction, and amplification

For both the aDNA and the paleoproteomic analysis, all work was conducted in a room dedicated to ancient biomolecular research at LMAMR. For the aDNA analysis, we followed the decontamination and authentication methods outline in a previously published protocol (Jacobson et al., 2020). Both calculus and dentin samples were wiped down with a 2 % bleach (NaOCl) solution to remove loose dirt and debris. Before the dental calculus was removed from the tooth, a second dampened wipe soaked with molecular grade water was used to remove residual bleach. Both sample types were then UV irradiated for 1 min per side. A Dremel rotary tool was used to separate the root of the tooth from the crown. Between 120.8 and 220.8 mg of the tooth were crushed to a coarse powder. After subsampling for both calculus and teeth, both sample types were washed for 15 min in a 0.5 M EDTA solution wash to remove contaminants and minerals. Additional 0.5 M EDTA was added, and the sample was decalcified for three days with proteinase K (Qiagen). DNA concentrations for these samples were measured with a Qubit following an initial round of library prep (Table S2). The libraries for these initial samples failed quality control assessments.

A second round of DNA extraction was performed but was limited to only two calculus and two dentin samples (Table S2). The weight for the calculus samples ranged from 3.1 to 4.0 mg, while that of the dentin samples ranged from 103.3 to 105.5 mg (Table S2). As with the first round of extractions, the second round included an extraction blank control (ExtBlnk) and a library blank control (LibNeg) to monitor contamination. The remainder of the discussion of aDNA methods applies only to those four samples extracted in the second round.

The DNA was extracted from the samples using a previously-described protocol (Dabney et al., 2013). Each sample was processed with a silica column/guanidine HCl purification method performed using a Qiagen MinElute™ PCR purification column. All DNA was then eluted in 60 μl of 10.0 mM Tris-Cl/0.5 mM EDTA Buffer. These DNA extracts were converted into Illumina DNA sequencing libraries.

2.4.2. Library preparation

Metagenomic libraries were constructed using a previously described protocol (Carøe et al., 2018). We performed the end repair step by utilizing 16 μl reactions which consisted of T4 DNA ligase reaction buffer, dNTPs, T4 PNK, and T4 DNA polymerase and 14 μl of DNA extract. These cocktails were incubated for 30 min at 20° C and 30 min at 65° C. After the end-repair step, we ligated the Illumina adapters in 20 μl reactions with 1 μl of BEDC3 adapter mix (0.60 μM). We incubated the reactions for 30 min at 20° C and for 10 min at 65° C which were then followed by 20 min at 80° C. For the adapter fill-in step, we added isothermal amplification buffer, dNTPs, water, and Bst 2.0 Warmstart Polymerase which was then incubated for 20 min at 65° C and for 20 min at 80° C in a final volume of 30 μl. Each reactions was then purified utilizing modified Speedbeads (Rohland and Reich, 2012). To assess the amount of DNA present in each sample, we performed a quantitative PCR (qPCR, Lightcycler 480 Roche) analysis. Each library was amplified in triplicate 25 μl PCR reactions which included 4 μl template, 12.5 μl of a 2x KAPA HiFi HotStart Ready master mix, 6 μl H20, 1 μl BSA (2.5 mg/ml), and 0.75 μl each of barcoded forward and reverse indices (0.3 μM). The thermocycler conditions were set for 5 min at 95° C which was then followed by 12–25 cycles of 20 seconds at 98° C, 15 s at 60° C, and 30 s at 72° C. All of these steps were then followed by an elongation step which was set for 1 min at 72° C. We then purified the pooled triplicate amplified libraries by utilizing Speedbeads and then made an elution in 30 μl EB. We quantified the libraries with another round of qPCR. We estimated the fragment size for each library with a Fragment Analyzer. Following this step, we pooled the libraries in equimolar ratios. Finally, we used a Pippin Prep to select DNA molecules between 150 and 500 bp.

2.4.3. Shotgun sequencing

The pool was sequenced on an Illumina NextSeq 500 using a paired-end, 2 x 150 bp, rapid-run chemistry at the Oklahoma Medical Research Foundation. The raw read sequencing files can be found on the NCBI website with the project number PRJNA70418.

2.4.4. Enamel protein extraction and purification

We also applied a modified acid etching protocol described in a previous study (Stewart et al., 2017) to three enamel samples (ROM_3D, ROM_10D, and ROM_11D). In brief, we washed the enamel with 3 % H_2O_2 for 30 s and then rinsed with ultra-pure water. We performed an initial etch by lowering the tooth onto 60 μl of 5 % (vol/vol) HCL. This initial etch was discarded and was then followed by a second 2-min etch,

which was retained. Next, a C18 resin-loaded ZipTip (ZTC18S096; EMD Millipore) was conditioned with 100 % acetonitrile three times, and then conditioned with 0.1 % (vol/vol) formic acid, with each draw discarded. Afterwards, the etch solution was adhered to the ZipTip by pipetting the solution up and down 10 times, with the last draw discarded. The ZipTip was washed six times with 0.1 % (vol/vol) formic acid, with each wash discarded. The extracted peptides were then lyophilized, and then resuspended with 20 μl 0.1 % FA in HPLC grade water. 8 μl of the reconstructed sample was loaded onto the analytical column (Acclaim PepMap 75 75um x 250um C18 5um, 100 Å) for each sample.

2.4.5. Tandem mass spectrometry

LC-MS/MS experiments were carried out on a Thermo Accela HPLC system, which was coupled with a Thermo Orbitrap Elite mass spectrometer (ThermoFisher Scientific, Bremen, Germany). The RPLC conditions are listed here. The mobile phase A (MPA) was 0.1 % FA, and the mobile phase B (MPB) was 0.1 % FA in ACN. A 100-min gradient from 3 % MPB to 35 % MPB at a flow rate of about 400 nL/min was applied for the separation. After that, the column was regenerated by 90 % MPB for 5 min, and equilibrated by 97 % MPA for 15 min. The electrospray voltage was set to 2.6 kV throughout the experiments. The temperature of the inlet capillary was set to 275 °C. MS data acquisition was performed by setting the resolving power of 240,000 at $m/z = 400$ with maximum ion injection time equal to 1000 (2 microscans). We performed 2 microscans because this increases signal-to-noise ratio. MS/MS was performed by collision induced dissociation (CID) with 35 % normalized energy. The maximum ion injection was set as 500 ms. The auto gain control (AGC) value was set as 1 x 10^6 for Full MS scans, and 5 x 10^5 for MS/MS scan. Data dependent MS/MS acquisition was used as the top five most abundant precursor ions in the MS full scan were selected for MS/MS analysis with an isolation window of 3.0.

The proteomic data are publicly available on MassIVE (accession no. MSV000093976) and Zenodo (https://doi.org/10.5281/zenodo.10551784).

2.5. Bioinformatic processing, data handling, and analysis

Downstream processing included data quality assessment and filtering, damage patterns assessment, microbial taxonomic profiles, mtDNA haplotypes, and sex estimation. The raw sequencing data was processed with AdapterRemoval2 (v.2.3.2) to remove low quality and adapter sequences with the following flags: –trimns –trimqualities –minqualities 20 –collapse –minlength 30. The resulting analysis-ready merged reads were used for downstream analyses (Table S3).

2.5.1. Ancient DNA authentication

To assess whether the sequencing reads were ancient, we used MapDamage 2.0 to evaluate the cytosine deamination rates of the microbial and human DNA (Jónsson et al., 2013). For this analysis, the analysis-ready reads were mapped with bwa aln (v0.7.17) against five references: hg19 genome, human mitogenome (rCRS), *Methanobrevibacter oralis* (JMRO1 strain), *Streptococcus anginosus* (C238 strain), and the GreenGenes database with the following options: -l 1000 -n 0.01. The following BAM file of mapped-only reads was then processed with Samtools (v1.10) (Li et al., 2009) (Table S3). Damage profiles were visualized in R (v.3.6.3) (Team, 2018) with the tidyverse set of packages (Wickham et al., 2019).

To assess the preservation of the overall microbial community in the samples, we used Sourcetracker (v1.0) (Knights et al., 2011). Sourcetracker applies a Bayesian approach to make predictions about the origin of a community in a sample by using the taxonomic inventories from well-characterized communities (*e.g.* skin, gut, oral, extraction blank controls, and soil) in a sample. This analysis included sequencing reads that can be found in dental calculus (Mann et al., 2018; Weyrich et al., 2017), human gut (Costea et al., 2017; Gopalakrishnan et al., 2018; Ormerod et al., 2016; Rampelli et al., 2015), plaque (Lloyd-Price et al., 2017; Schmidt et al., 2014), saliva (Aleti et al., 2019; Lassalle et al., 2018), skin (Schmedes et al., 2017; Tirosh et al., 2018), and soil studies (DOE Joint Genome Institute, 2017; Lin et al., 2014) (Table S6). The taxonomic profiling of the analysis-ready reads was performed with the closed-reference OTU-picking approach against the GreenGenes database (version 13.7) using the same parameters on the samples in the present study. The resulting OTU table was processed with the summarize_taxa_through_plots script to collate OTUs at the genus level. The OTU table was rarefied to 1900 counts (sample ExtBlnk had the lowest number of counts) using the single_rarefaction script (Table S7). Sourcetracker was then applied using the following options: -r 0, –train_rarefaction 0.

2.5.2. Metagenomic taxonomic characterization

Because each taxonomic alignment program has inherent biases when profiling microbial communities (Velsko et al., 2018), we used two: QIIME1 (v. 1.9.1) (Caporaso et al., 2010) and MetaPhlAn2 (Truong et al., 2015). For the QIIME analysis, we used bowtie2 (Langmead and Salzberg, 2012) to map the analysis-ready to the GreenGenes (v13.8) database (DeSantis et al., 2006). Samtools (v1.10) (Li et al., 2009) was then used to filter and sort the mapped reads, as well as remove PCR duplicates. Next, taxonomic binning was performed against the GreenGenes 16S rRNA gene database (version 13.8) by preclustering at 97 % sequence similarity with the pick_closed_reference_otus.py script and the following options: –max_accepts 500, max_rejects 500, –word_length 12, –stepwords 20, –enable_rev_strand_match True. Results are found in Table S4.

Additionally, Metataxonomic Phylogenetic Analysis (MetaPhlAn) (v.2.7.7) was also used to estimate phylum, genus, and species abundances using a marker gene database. Analysis-ready reads were taxonomically binned using the metaphlan2.py script. Results are found in Table S5.

To visualize the taxonomic profiles from each program, a species-level heatmap was generated using the R heatmap() function (Dixon, 2003).

2.5.3. Mitochondrial DNA haplogroup assignment

HaploGrep was used on the two dentin samples to determine their mitochondrial haplotypes. The HaploGrep pipeline (https://haplogrep.uibk.ac.at/) can classify the mtDNA haplotypes present within a sample. Following AdapterRemoval, analysis-ready reads were then aligned with bwa mem (minimum seed length 15, mismatch penalty of 1) with the rCRS as a reference which was the proceeded with the following steps: 1) duplicate marking with picard MarkDuplicates with samtools; 2) variant calling using samtools mpileup (-C 50 to adjust mapQ); 3) varscan mpileup2cns (java -Xmx16g -d64 -jar VarScan.v2.4.4.jar mpileup2cns –output-vcf 1); and, 4) haplogroup identification using haplogrep.

2.5.4. Mitochondrial DNA contamination estimates

We used the program Schmutzi on the mitochondrial alignments from ROM_5D and ROM_10D samples to examine the amount of mtDNA contamination. The schmutzi.pl script was used on the bam files that were mapped to the rCRS reference along with the following options: –lengthDeam 5, –library double.

2.5.5. Sex identification with genetic methods

We used to the rx_compute.r script (Mittnik et al., 2016) to estimate the biological sex of sample ROM_5C, ROM_5D, ROM_9C, and ROM_10D using the default settings.

2.5.6. Database search for proteomic data

The collected raw files were converted to mzXML format through MsConvert (Adusumilli and Mallick, 2017) and searched against the human proteome (UniprotKB, 10/15) with MS-GF+ (Kim and Pevzner,

2014), as well as specifically against the human amelogenin protein sequences AMELX (Q99217) and AMELY (Q99218) to estimate sex (UniProt Consortium, 2015).

3. Results and discussion

3.1. Sequencing data

We generated 79,961,021 DNA sequences across two dental calculus and two dentin samples (range 17,373,684–21,305,627) and two extraction blank controls (EBCs) (range 858,532–2,373,466) (Table 1). The average fragment lengths for each biological sample were similar (62.58–89.72) and fall within the expected range for aDNA samples (Knapp and Hofreiter, 2010).

3.2. aDNA authentication

To determine whether the microbial and human DNA sequences are ancient, we used MapDamage2 (Jónsson et al., 2013). The quality-filtered reads for each sample and EBC were mapped to five references using bwa aln: GreenGenes (v13.8), two oral microbes (*Methanobrevibacter oralis, Streptococcus anginosus*), the human genome (hg19), and the human mitochondrial genome (rCRS) (S1 Appendix). The calculus samples exhibit expected damage patterns for the GreenGenes, *S. anginous,* and hg19 mappings. Sample ROM_5C showed damage for *M. oralis* but ROM_9C did not, because it had only 22 reads mapped to this reference, too low to provide reliable estimates. Both dentin samples exhibited patterns of damage for the human nuclear (hg19) and mitochondrial (rCRS) genomes. In summary, the dental calculus samples contain authentic ancient microbial and human DNA, while the dentin samples contain authentic ancient human DNA.

To assess the preservation of the overall microbial community in the samples, we used Sourcetracker (v1.0). Sourcetracker predicts that 92.15 % of the microbial DNA in ROM_5C and 87.70 % in ROM_9C could be attributed to human dental calculus (Fig. 3). The low proportion of oral microbiome DNA sequences in ROM_10D was expected (<1.0 %), since this was a dentin sample. The high relative proportion of dental calculus microbes in ROM_5D was surprising (72.9 %) as dentin samples generally only exhibit slight signals of human oral microbiome (Mann et al., 2018). Trace amounts of calculus retained during the DNA extraction process could explain these results; this source is likely, as the taxonomic profile for this sample is similar to its calculus counterpart. These results suggest that both calculus samples, as well as one dentin sample, retained strong dental calculus signatures that are distinct from the EBCs and potential contaminant sources, such as soil and skin.

3.3. Oral-specific species identified in the dental calculus samples

The QIIME1 pipeline identified a total of 498 genera among the samples. Among the genera identified in the calculus and dentin samples were *Streptococcus, Actinomyces*, and *Fusobacterium* which are all associated with the human oral microbiome (Dewhirst et al., 2010). MetaPhlAn2 identified 424 taxa, including several key oral species in the calculus samples, such as *Tannerella forsythia, Treponema denticola,* and *Corynebacterium matruchotii* (Fig. 4) (Table S5). The identification of these species is noteworthy because they play a critical role in oral health (Dewhirst et al., 2010; Wade, 2013) and have a deep relationship with human evolution (Fellows Yates et al., 2021). While the first two are part of the red complex—a group of bacteria associated with chronic periodontitis (Mohanty et al., 2019)—the third is associated with non-diseased subgingival plaque (Paster et al., 2001). Although the implications from the presence of these microbes together remain unclear, investigating these relationships may facilitate a better understanding of oral health in the area.

3.4. Mitochondrial DNA analysis for the dentin samples

We utilized HaploGrep to infer mitogenome haplotype (Kloss-Brandstätter et al., 2011). HaploGrep was only applied to the dentin samples (ROM_5D and ROM_10D) as the calculus samples had an insufficient number of mapped reads to the rCRS reference to generate reliable results (<1,000) (Mann et al., 2023). The analysis estimated that the ROM_5D sample belonged to the U5b2a5 haplotype, while ROM_10D belonged to I3a1 (Table S8).

To authenticate the haplotype assignments, we utilized Schmutzi, which iteratively estimates the amount of endogenous mitochondrial genome while also estimating the amount of contamination by utilizing a database of potential contaminant mitochondrial genomes (Renaud et al., 2015). As with the HaploGrep analysis, the mtDNA reads mapping to the rCRS reference were used for the Schmutzi analysis. Overall, results indicate that there were low levels of mtDNA contamination in both ROM_5D and ROM_10D (<5%) (Table S8). These results are only preliminary, but they are encouraging, especially considering both haplogroups have been identified in other populations inhabiting areas

Table 1
An overview of the archaeological and laboratory samples included in this study.

Sample ID	Type of Sample	Type of Analysis	Locality	Excavation date and reference	Date of burial	Estimated sex based on osteological analysis	Estimated sex based on molecular analysis
ROM_3D	Dentin	Osteological & Proteomic	Sector X, NV Camp, burial 3	1959: MCA 8, 1962, 401–402	Undated, but probably 2nd-6th c. CE	F	M
ROM_5C	Calculus	Osteological & Genomic	Section E, burial 7	1972: SCIVA 1974, 36	Undated, but probably 2nd-6th c. CE	F	NA
ROM_5D	Dentin	Osteological & Genomic	Section E, burial 7	1972: SCIVA 1974, 36	Undated, but probably 2nd-6th c. CE	F	NA
ROM_9C	Calculus	Osteological & Genomic	Section Z2, burial 5	1955: MCA 4, 1957, 48	3rd-4th century CE from grave goods	F	NA
ROM_10D	Dentin	Osteological, Genomic, & Proteomic	Section X, NV Camp, Skeleton 2, Burial 3	1959: MCA 8, 1962, 401–402	Undated, but probably 2nd-6th c. CE	M	F
ROM_11D	Dentin	Osteological & Proteomic	Test Trench 3 burial 1	2018: CCA 2020, 205	433–566 CE (14C, 95.4 % confidence)	NA	F
Extract negative	EBC	Genomic	Norman, OK	NA	NA	NA	NA
Library negative	EBC	Genomic	Norman, OK	NA	NA	NA	NA

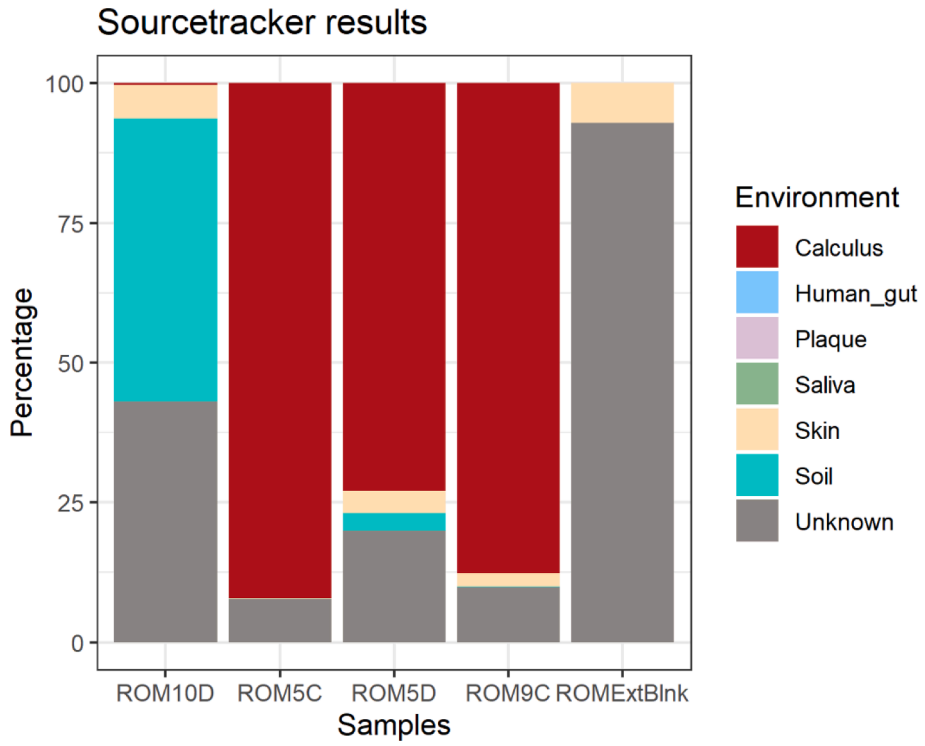

Fig 3. Genus-level Sourcetracker results for dental calculus (ROM_5C & ROM_9C), dentin (ROM_5D & ROM_10D), and extractiona blank (ROMExtBlank). Results indicate that most of the DNA (>50%) in the calculus samples can be attributed to DNA found in other dental calculus studies.

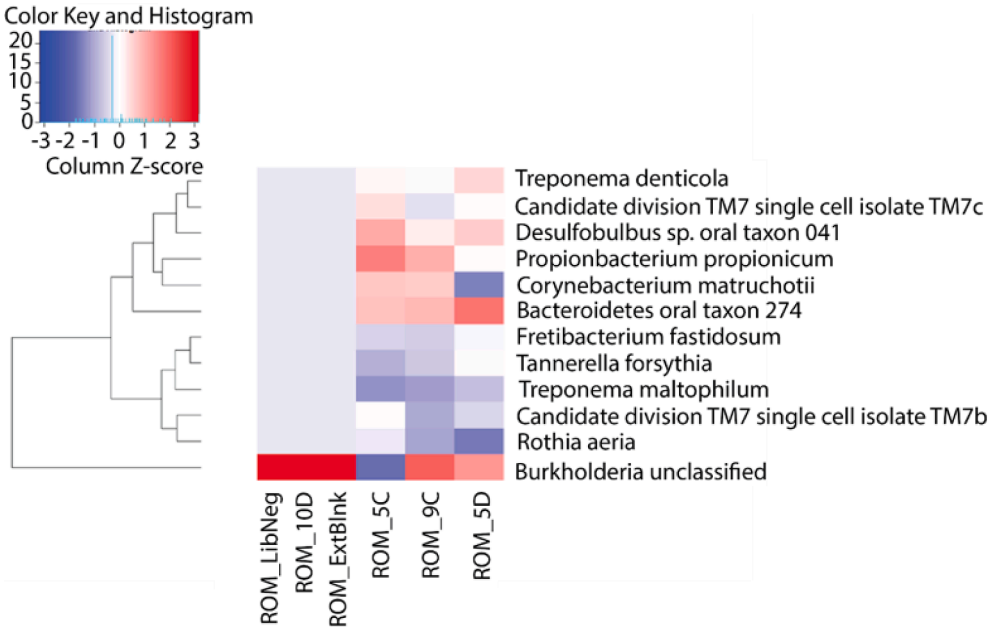

Fig 4. MetaPhlAn2 identifies several oral species across samples.
A species-level heatmap generated using the heatmap() function in R. The 12 species represented had a relative abundance of at least 0.01 and were present in at least 2 samples. The scale ranges from −3 to 3 on a $\log_2$ scale. As shown by the Color Key and Histogram, red represents highly abundant species, whereas blue represents rarer types. The results indicate that MetaPhlAn2 identified *Burkholderia* in not only the extraction blanks but also the biological samples, suggesting that it is a likely laboratory contaminant, which has been documented in previous studies (Eisenhofer et al., 2019; Salter et al., 2014). However, none of the oral species were identified in the controls, indicating that there was no cross contamination.

near the Black Sea (Juras et al., 2017; Revesz, 2016). Characterizing the prevalence of these haplogroups in Histria, as well as others, and whether their frequencies change during and after Roman annexation could provide deeper insights into the population dynamics in this region.

3.5. Sex estimation using osteological, genomic, and proteomic analyses

Estimating the biological sex of archaeological skeletons has been a standard practice to test questions related to division of labor and social stratification (Meindl et al., 1985; Milner et al., 2008). While pelvic and

cranial measurements can accurately estimate the sex of well-preserved adults, their effectiveness is dependent on skeletal preservation and population variation (Cox and Mays, 2000). Moreover, the accuracy of estimating sex based on visually assessing the sciatic notch and skull features are susceptible to biases and can be confounded by factors such as age, particularly for juvenile populations (Lewis, 2007), population variation, and cultural practices, including cranial modification (Walker, 2008, 2005). Genomic and proteomic approaches offer a valuable advantage as they mitigate some of the biases inherent to osteological methods.

Contrary to a previous study that reported high congruency in sex estimates between osteological and biomolecular approaches (Buonasera et al., 2020), our findings revealed a mixed consistency (Table S1 & S9). In comparing osteological with genetic estimations, only in the case of sample ROM_9C did the osteological and genomic methods reach the same sex estimate, both suggesting the sample was from a biological female. For ROM_5D, osteological analysis estimated sex as female, while genomic analysis suggests a male. Conversely, for ROM_10D, osteological analysis estimated sex as male, contradicting the genomic and proteomic analyses, which suggest a female.

It is important to interpret the sex estimates with the genomic analyses cautiously, particularly samples ROM_5D and ROM_9C, as each had less than 100,000 reads mapped to the sex chromosomes. Previous analyses indicate that this threshold is crucial for obtaining reliable sex estimates (Buonasera et al., 2020). As such, the sex estimate for sample ROM_10D based on the genomic analysis is likely the only reliable estimate, given the number of mapped reads to the X chromosome (n = 139,146) and the small number to the Y chromosome (n = 65).

The addition of proteomic analysis can provide further validation of the sex estimation, especially when aDNA preservation is poor. We

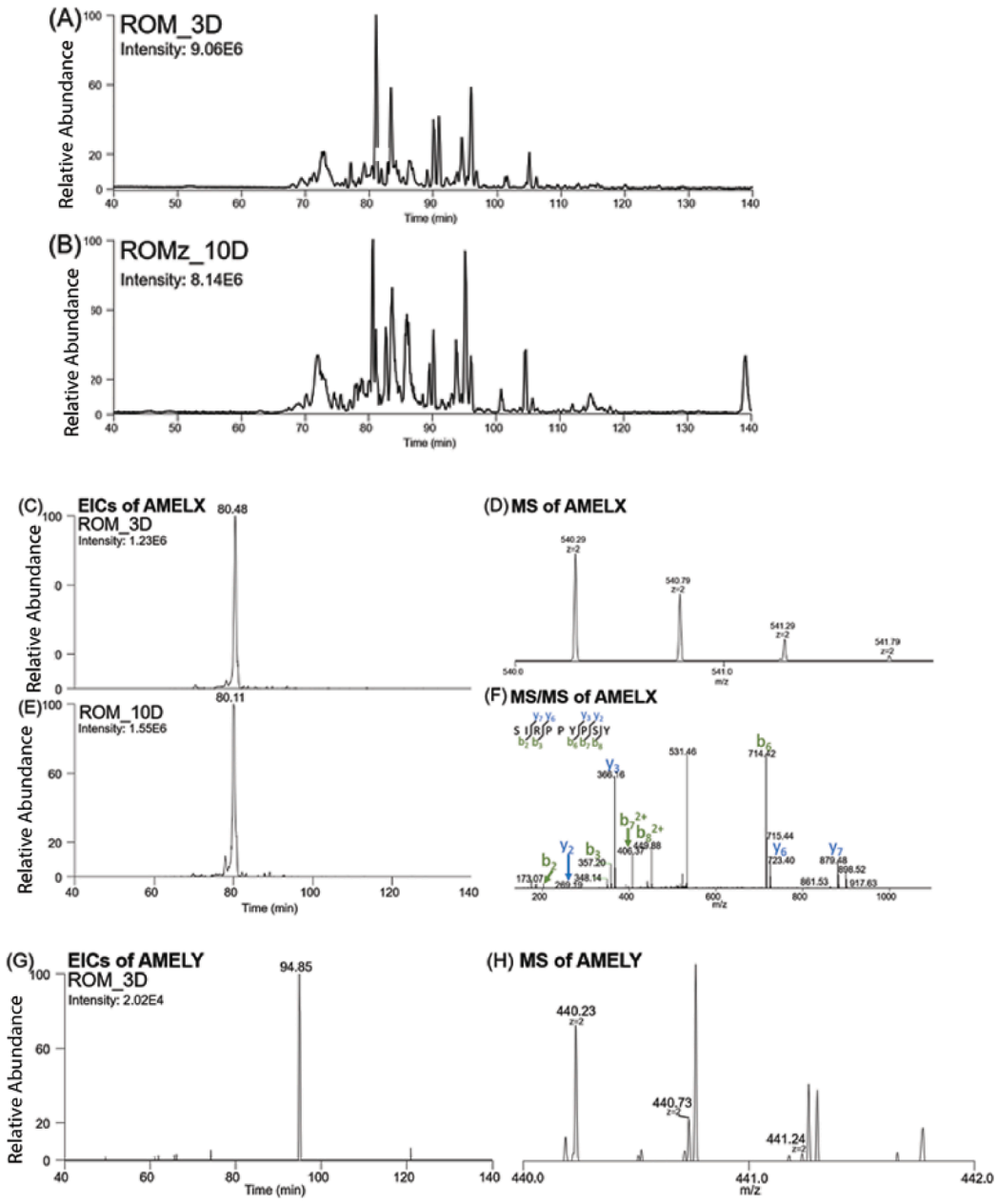

Fig 5. Sex-specific peptides recovered from ROM_3D and ROM_10D enamels. Both (A) and (B) show a representative base peak chromatogram (BPC) (300–1,600 *m/z*) for samples ROM_3D and ROM_10D, respectively. (C) Extracted ion chromatogram (EIC) of AMELX in ROM_3D. (D) MS full scan of AMELX in ROM_3D. (E) EIC of AMELX in ROM_10D. (F) MS/MS spectrum of AMELX in ROM_10D. (G) EIC of AMELY for ROM_3D. (H) EIC of AMELY in ROM_3D. AMELY was not detected in ROM_10D or ROM_11D.

performed a proteomic approach to estimate the biological sex of three samples (ROM_3D, ROM_10D, and ROM_11D), two of which (ROM_3D and ROM_10D) had previously been assigned sex using osteological analysis (Soficaru et al., 2014) and one of which (ROM_10D) also had sex estimation from genomic analysis. These previous osteological analyses concluded that the individual represented by ROM_3D was biologically a female, while the individual represented by ROM_10D was biologically a male. Sex estimation based on osteological analysis was not performed for the juvenile because the individual was between the ages of 3–5 years old at the time of death.

The proteomic results successfully identified the presence of the AMELX_HUMAN peptide in all three samples, but the AMELY_HUMAN peptide was only identified in ROM_3D (Fig. 5). These results indicate that ROM_3 was a biological male and suggest that samples ROM_10D and ROM_11D came from biological females. While the results for the adult individuals contradict the osteological sex estimation, the result for ROM_10D is consistent with the genomic analysis, which also indicated that the tooth sample came from a biological female (Rx = 1.071921, 95 % CI 1.013845–1.129997) (Table S9). While these results are promising, it is important to note that we cannot rule out a false negative result (*e.g.,* the AMELY peptide may not be detected because of its low frequency) (Mikšík et al., 2023; Parker et al., 2021).

These results highlight the utility of biomolecular approaches to estimate the chromosomal sex of archaeological adult and juvenile populations. While sex estimation using osteological methods is generally accurate (Soficaru et al., 2014) and in many cases is corroborated by proteomic and genomic results for the same individuals (Buonasera et al., 2020; Stone et al., 1996), in the case of sample ROM_10D, the genetic and proteomic results are congruent with each other but contradict the osteological sex estimation. This preliminary study also suggests that proteomic approaches can be useful in estimating the sex of juvenile populations at Histria. As traditional osteological techniques cannot estimate the sex of children, this minimally-destructive proteomic method offers an important tool for gaining new insights into the demography of past juvenile populations.

4. Conclusions

This is the first study to apply aDNA and paleoproteomic methods to dental calculus, dentin, and enamel from individuals who were interred in the necropolis at Histria. Our results indicate that despite the skeletal collection being maintained in less-than-ideal conditions and the lack of precautions by previous researchers to minimize contamination, the biomolecular preservation in this collection is generally robust. The aDNA in the dental calculus samples consists mainly of oral microbes (>80 %) and exhibits authentic damage profiles. Moreover, the human aDNA in the dentin samples exhibits expected patterns of deamination. Because this data was generated with shotgun sequencing, a more targeted approach, such as hybridization enrichment capture, would likely yield higher-resolution data to perform population genomic structure analyses. Our combined paleoproteomic and genomic analyses for estimating biological sex also provide noteworthy results, because sex estimates with conventional osteological methods conflicted with biomolecular results for the same individuals. While the proteomic approach has been documented to be a sensitive method with the highest confidence assignments (Buonasera et al., 2020), it too has limitations. Potentially, poor amelogenin preservation can result in false negative results. Nonetheless, it has the advantage of being minimally destructive and suitable for any remains with minimal preservation of dental enamel.

Our sample size is small, but our findings show promise for future ancient oral microbiome, host genomic, and proteomic research for human remains from Histria. Because the aDNA and paleoproteomic preservation for the skeletal collection are viable, our results may also provide some insights for research on anthropological collections at other institutions that were similarly collected and stored under less-than-ideal conditions. Moreover, applying these biomolecular methods to a larger dataset can help explore how the Roman Empire impacted the lives of individuals at Histria. The integration of biomolecular data with isotopic and radiocarbon data will also improve our understanding of how the Roman Empire transformed the lives of individuals living along the Danube, and of the long-term consequences of its shifting frontiers.

Ethics declarations

The authors declare no competing interests.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The sequencing data is available on NCBI with this project number: PRJNA704183.

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Appendix A. Supplementary data

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