Components of a Neanderthal gut microbiome recovered from fecal sediments from El Salt

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A comprehensive view of our evolutionary history cannot ignore the ancestral features of our gut microbiota. To provide some glimpse into the past, we searched for human gut microbiome components in ancient DNA from 14 archeological sediments spanning four stratigraphic units of El Salt Middle Paleolithic site (Spain), including layers of unit X, which has yielded well-preserved Neanderthal occupation deposits dating around 50 kya. According to our findings, bacterial genera belonging to families known to be part of the modern human gut microbiome are abundantly represented only across unit X samples, showing that well-known beneficial gut commensals, such as \textit{Blautia}, \textit{Dorea}, \textit{Roseburia}, \textit{Ruminococcus}, \textit{Faecalibacterium} and \textit{Bifidobacterium} already populated the intestinal microbiome of \textit{Homo} since as far back as the last common ancestor between humans and Neanderthals.
Over the past decade, microbiome research has highlighted the crucial role that the gut microbiome plays in human biology through its pleiotropic influence on many physiological functions, such as human development, immunity, metabolism and neurogenerative processes. This body of knowledge has catalyzed interest in incorporating the gut microbiome into our evolutionary history, as an adaptive partner providing the necessary phenotypic plasticity to buffer dietary and environmental changes. Studies aimed at exploring the ancestral traits of the human gut microbiome are therefore encouraged, as a unique evolutionary perspective to improve our knowledge of gut microbiome assembly and interactions with the human host.

The ancestral configuration of the human gut microbiome has generally been inferred by microbiome data stemming from contemporary populations found across all six human occupied continents who adhere to traditional lifestyles, such as the Hadza hunter-gatherers from Tanzania, the rural Bassa from Nigeria and rural Papuans from Papua New Guinea, among others. However, since this research involved modern populations, no direct information on the ancient human gut microbiome structure can actually be provided. Alternatively, ancient DNA (aDNA) analysis based on shotgun metagenomic sequencing is emerging as an attractive and reliable opportunity to directly investigate the microbial ecology of our ancestors. Paleomicrobiological aDNA studies have traditionally been conducted on dental calculus and bones, providing unique insights into their relevance to human mtDNA and ancient components of the modern human lineage. Nevertheless, to the best of our knowledge, paleofecal samples older than 8,000 years have never been analyzed, leaving an important gap on the pre-historical human gut microbiome configuration.

In this scenario, we attempted to identify ancient human gut microbiome components by shotgun metagenomic analysis of aDNA extracted from archeological sedimentary samples (ES1 to ES7) from the stratigraphic unit (SU) X (subunit Xb-H44) of the Middle Paleolithic open-air site, El Salt (Alicante, Spain). (Fig. 1). The archeological setting of El Salt yielded evidence of recurrent occupation by Neanderthals, our closest evolutionary partner providing the necessary phenotypic plasticity to buffer dietary and environmental changes. In particular, the sedimentary samples ES1-7 have been previously shown to include several millimetric phosphatic coprolites and fecal lipid biomarkers, namely coprostanol and 5β-stigmastanol, with proportions suggesting a human origin. These samples therefore represent, to our knowledge, the oldest known positive identification of human fecal matter. The present work also includes an additional seven new archeological sediments collected in 2018 as a control. Two were from SU X (subunit Xa and Xb, respectively) and the others from surrounding SUs, i.e., upper V (three samples), IX and XI (one sample each) (Fig. 2a). While SUs IX to XI are associated with rich archaeological assemblages, upper SU V yielded very few archaeological remains. We found that samples positive for the presence of fecal biomarkers showed traces of both ancient human mtDNA and ancient components of the modern human gut microbiome. These components included so-called “old friends” and beneficial commensal inhabitants of modern human guts, providing unique insights into their relevance to the biology of the Homo lineage.

**Results and discussion**

**Ancient DNA sequencing and damage assessment.** DNA was extracted from 14 archeological sedimentary samples and prepared for shotgun metagenomics in a dedicated aDNA facility at the Laboratories of Molecular Anthropology and Microbiome Research in Norman (OK, USA) (see Methods). A total of 124,592,506 high-quality paired-end sequences were obtained by Illumina NextSeq sequencing and analyzed for bacterial aDNA. To remove contamination by modern DNA, which is one of the major complications in studies of ancient samples, we evaluated the DNA damage pattern as compared with present-day DNA references. In particular, Skoglund and colleagues translated the pattern of cytosine deamination into a postmortem degradation score (PMDS), which provides information on whether a given sequence is likely to derive from a degraded aDNA molecule. Reads were aligned against all bacterial genomes of the NCBI database, and ancient bacterial reads were recovered by setting PMDS > 5, to minimize the probability of a sequence being from a present-day contaminating source. An average of 2,342 sequences per sample (range, 279–17,901) were obtained, corresponding to a small but consistent fraction of DNA being ancient and derived from bacteria (mean ± SD, 0.069% ± 0.029%) (Supplementary Table 1 and Supplementary Fig. 1). The same procedure was applied to extraction, library and PCR blanks, resulting in the retrieval of a minimal number of 144, 1, and 42 ancient bacterial sequences, respectively. Ancient reads from blanks were assigned to 116 bacterial species that showed no overlap with the sample dataset (Supplementary Data 1). When comparing the fraction of reads with PMDS > 5 per million reads between samples IX, Xa, ES1-7, Xb and XI (i.e., those positive for the presence of fecal biomarkers and/or associated with rich archaeological assemblages), and samples from SU V (i.e., with no or very few archeological remains), the first showed a greater abundance of PMDS > 5 reads (p-value = 0.01, Wilcoxon test) (Supplementary Fig. 2), possibly as a result of the presence of human fecal sediment.

**Detection of ancient human mitochondrial DNA.** In order to detect human aDNA traces in our sample set, we searched for human mitochondrial DNA (mtDNA) sequences in PMDS-filtered metagenomes obtained from the 14 archeological sedimentary samples. Ancient human mtDNA was detected in almost all ES1 to ES7 samples from SU X (Fig. 2b). No traces of mtDNA from other animals were detected. To strengthen these findings, all samples were subjected to target capture of mtDNA with a Neanderthal bait panel (Arbor Biosciences; see Methods), and subsequent sequencing on Illumina NextSeq platform. Based on this analysis, ES1, ES2, ES5 and Xb samples tested positive for the presence of ancient human mtDNA, showing >1000 human mtDNA reads with PMDS > 1, breadth of coverage >10%, Δ ∆% ≥ 0.9 and modern contamination less than 2% (Fig. 2b and Supplementary Table 2). Taken together, this evidence strongly supports human origin for the El Salt samples, particularly those from SU X.

**Profiling of ancient prokaryotic DNA.** As for prokaryotic aDNA, seventeen bacterial and one archaeal phyla were identified in the aDNA sedimentary record of El Salt, with different representation across SUs (Fig. 3). As expected for its wide distribution in nature, Actinobacteria is the most represented phylum, with environmental species from Streptomycetaceae, Pseudonocardiaeces, Micromonosporaceae, Nocardiaceae, Mycobacteriaceae, Microbacteriaceae and Nocardioidaceae families being detected in almost all the SUs. Similarly, the vast majority of sediment samples share a number of ancient sequences assigned to Bacillaceae members, which are known to play...
fundamental roles in soil ecology, where they can persist up to thousands of years, if not longer, due to their ability to form resistant endospores\textsuperscript{33,34}. Another large fraction of aDNA shared by most SUs includes Proteobacteria constituents, especially from Alphaproteobacteria (mainly \textit{Rhodobacteraceae}, \textit{Rhodospirillaceae} and \textit{Sphingomonadaceae} families), Betaproteobacteria (mainly \textit{Comamonadaceae} and \textit{Burkholderiaceae}) and Gammaproteobacteria (with \textit{Xanthomonadaceae} classes). Again, these are...
cosmopolitan bacteria commonly found in both terrestrial and aquatic environments, as free-living organisms or symbionts in different hosts.\textsuperscript{35,36} In light of their DNA damage pattern, it is reasonable to assume that these are truly ancient environmental bacteria that populated archeological sediments. The contamination of archeological remains by environmental bacteria is indeed well expected, as already documented in previous paleomicrobiological aDNA studies.\textsuperscript{15,18,37} For the relative abundances of bacterial families detected across the samples, please see Supplementary Data 2.

Putative components of the Neanderthal gut microbiome. Next, following an approach similar to Weyrich et al.\textsuperscript{18}, who first characterized the oral microbiome from Neanderthal dental calculus, we focused our analysis on intestinal microorganisms. Specifically, in order to identify potential ancient human gut microbiome components, we searched for bacterial genera belonging to the 24 families that have recently been indicated as being common to the gut microbiome of hominids (i.e., \textit{Methanobacteriaceae}, \textit{Bifidobacteriaceae}, \textit{Coriobacteriaceae}, \textit{Bacteroidaceae}, \textit{Porphyromonadaceae}, \textit{Prevotellaceae}, \textit{Rikenellaceae}, \textit{Tannerellaceae}, \textit{Enterococcaceae}, \textit{Lactobacillaceae}, \textit{Streptococcaceae}, \textit{Christensenellaceae}, \textit{Clostridiaceae}, \textit{Eubacteriaceae}, \textit{Lachnospiraceae}, \textit{Oscillospiraceae}, \textit{Peptostreptococcaceae}, \textit{Ruminococcaceae}, \textit{Erysipelotrichaceae}, \textit{Veillonellaceae}, \textit{Desulfovibrionaceae}, \textit{Succinivibrionaceae}, \textit{Enterobacteriaceae}, and \textit{Spirochaetaceae})\textsuperscript{38–45}. Accordingly, while harboring similar family-level gut microbiome profiles, humans and non-human hominids, including our closest living relatives—chimpanzees, can be differentiated on the basis of the particular pattern of associated gut microbiome genera (as well as species and strains) represented within these families.\textsuperscript{45} This strong association between gut microbiome composition and host physiology—known as phyllosymbiosis—is believed to be universal in mammals, essentially as a result of all the physical, chemical and immunological factors that differentiate the intestine of the host species (e.g., type of digestive organs, pH, oxygen level,
host-derived molecules and immune system)\textsuperscript{46}. According to our findings, 210 bacterial species belonging to hominid-associated gut microbiome families, as listed above, are represented in the aDNA from El Salt SU IX, X and XI, with the highest detection rate in samples from SU X and, particularly, in ES1 to ES7 (Fig. 4), for which a human-like host origin had been previously suggested\textsuperscript{25}. In Supplementary Fig. 3, we provide the overall compositional profile of the El Salt samples from SU IX, X and XI restricted to the hominid-associated gut microbiome families, while in Supplementary Fig. 4, the proportions of these families are compared with those of the samples with no or very few archeological remains (i.e., from SU V). The compositional profile of samples from SU IX, X and XI was next compared to publicly available gut microbiomes from contemporary human populations as representative of different subsistence practices, such as Hadza and Matses hunter-gatherers, Tunapuco rural agriculturalists and western urban from Italy and the US\textsuperscript{7,47}.

As shown by the Principal Coordinates Analysis of Bray-Curtis distances between the family-level profiles (Supplementary Fig. 5), the El Salt samples from SU IX, X and XI tend to cluster closer to Tunapuco and Matses, resembling more the “ancestral” human gut microbiome of rural agriculturalists and hunter-gatherers than the urban western gut microbiome\textsuperscript{7}. However, as the degree of degradation of microbial DNA in ancient samples might be different for various gut microbiome components, any conclusions from these compositional data must be taken with due caution.

Further supporting a human-host origin of the bacterial species belonging to the hominid-associated gut microbiome families detected in the El Salt samples from SU IX, X and XI, feces or gastrointestinal tract are the first documented isolation source for 91 species out of 210 (43.3\%), with 60 of these being classifiable as closely related to the human gut (Supplementary Data 3). In the latter subgroup, we can count several species from Lachnospiraceae (including well-known (beneficial) commensal inhabitants of modern human guts, such as Blautia, Coprococcus, Dorea, Fusicatenibacter and Roseburia spp.) and Ruminococcaceae families. Particularly, within Ruminococcaceae, we detected members of Anaerotruncus, Ruminococcus and Subdoligranulum genera, and the butyrate producer Faecalibacterium, one of the human commensal bacteria of greatest current interest, due to its very promising potential as a biomarker of a healthy gut microbiome\textsuperscript{18}. Most of the aforementioned bacterial genera have been reported to account for the phylotypic diversity between human and non-human hominids, showing strong bias towards a human-host\textsuperscript{45}. It is also worth remembering that most of these bacteria are able to produce short-chain fatty acids (mainly acetate and butyrate) from the fermentation of indigestible carbohydrates, through the establishment of complex syntrophic networks. Short-chain fatty acids are today considered metabolic and immunological gut microbiome players with a leading role in human physiology\textsuperscript{49}. In addition, the Xb-H44 samples showed a high number of hits for Bacteroides, Parabacteroides, Alistipes and Bifidobacterium spp., other genera known to prevail in the human gut microbiome of rural agriculturalists and western urban from Italy and the US\textsuperscript{7}. For Bifidobacterium, this is particularly consistent with the propensity of this genus to be maternally inherited across generations. Being capable of metabolizing milk oligosaccharides and acting as a potent immunomodulator, the presence of vertically transmitted Bifidobacterium spp. in the infant gut could have provided important growth benefits to infant Hominidae\textsuperscript{50–52}.

To further characterize the ancient microbial taxa detected in the El Salt samples, we applied the HOPS\textsuperscript{53}–based approach recently used by Jensen et al.\textsuperscript{30}. In short, all the reads were first annotated and, subsequently, the ancient origin of each taxon was authenticated by computing three indicators: (i) the fraction of reads with PMDS > 1, (ii) the negative difference proportion (\(\Delta\%)\) of PMDS > 1 reads, and (iii) their deamination rate at \(5'\). Taxa showing more than 200 assigned reads, more than 50 reads with PMDS > 1, \(\Delta\%) = 1\) and C-T transition at \(5'\) >10\% were
considered to be of ancient origin (see Table 1 and Supplementary Figs. 6–8 for MapDamage plots, coverage plots and edit distance distribution)\(^30\). This in-depth characterization of the microbial metagenomic reads from the El Salt samples allowed us to confirm the presence of several species belonging to the gut microbiome families of hominids (including, among others, Alistipes, Bifidobacterium, Desulfovibrio and Prevotella spp., and Faecalibacterium prausnitzii), showing a read profile consistent with their ancient origin.

As mentioned above, high amounts of coprostanol, a metabolite formed through hydrogenation of cholesterol by specific bacteria in the intestine of higher mammals, were found in some of the El Salt sediments from SU X, with proportions consistent with the presence of human fecal matter\(^25\). We therefore specifically looked for microorganisms capable of this metabolism in the aDNA from El Salt samples. To date, cholesterol-reducing capabilities associated with coprostanol conversion in feces have been suggested for Bifidobacterium, Collinsella, Bacteroidales, Prevotella, Alistipes, Parabacteroides, Enterococcus, Lactobacillus, Streptococcus, Eubacterium, Lachnospiraceae (e.g., Coprococcus and Roseburia) and Ruminococcaceae (e.g., Anaerotruncus, Faecalibacterium, Ruminococcus and Subdoligranulum)\(^54\)–\(^58\), which were all detected, at variable but substantial abundances, within the species belonging to the 24 gut microbiome families (as defined above) in Xa and Xb-H44 subunits. While lending support to the presence of coprostanol in the same layer as reported by Sistiaga et al.\( ^{25} \), our findings on the representation of potential cholesterol-reducing bacteria in Neanderthal feces point to the microbial metabolism of cholesterol as an important function of the human gut microbiome for both modern and ancient humans, and suggest that relatively higher cholesterol intake has been a feature of the human diet at least since the Middle Pleistocene.

Finally, the remaining bacterial species belonging to the hominid gut microbiome families identified in El Salt sediments from SUs IX, X and XI could be sorted into two major source categories: human (or animal) oral and/or pathobiont, and environmental (see Supplementary Fig. 9). In particular, possibly consistent with evidence of dental caries and periodontal disease in Neanderthals\(^18\), we found traces of potential opportunistic pathogens (e.g., Methanobrevibacter oralis, Staphylococcus aureus, S. sanguinis, Propionibacterium acnes, Serratia marcescens, Enterococcus faecalis, Veillonella parvula, Eubacterium lentum, and Prevotella melanogaster)\(^19\)–\(^22\), which were all detected, at variable but substantial abundances, within the species belonging to the 24 gut microbiome families (as defined above) in Xa and Xb-H44 subunits. While lending support to the presence of coprostanol in the same layer as reported by Sistiaga et al.\(^25\), our findings on the representation of potential cholesterol-reducing bacteria in Neanderthal feces point to the microbial metabolism of cholesterol as an important function of the human gut microbiome for both modern and ancient humans, and suggest that relatively higher cholesterol intake has been a feature of the human diet at least since the Middle Pleistocene.

### Table 1 List of the 36 most abundant microbial taxa identified in the El Salt sediments, belonging to the hominid gut microbiome families.

| Species                                 | Reads | Reads with PMDS > 1 | DoC  | >1x (%) | C-T S’ (%) | –Δ % |
|----------------------------------------|-------|---------------------|------|---------|------------|------|
| Alistipes finegoldii                    | 340   | 80                  | 0.003| 0.3     | 11.3       | 1    |
| Alistipes shahii                       | 338   | 68                  | 0.003| 0.3     | 11.9       | 1    |
| Alistipes indistinctus                 | 245   | 62                  | 0.003| 0.2     | 11.4       | 1    |
| Alistipes timonensis                   | 303   | 55                  | 0.003| 0.3     | 11.1       | 1    |
| Alistipes senegalensis                 | 376   | 73                  | 0.003| 0.3     | 13.4       | 1    |
| Alistipes ihumii                       | 406   | 93                  | 0.005| 0.4     | 10.9       | 1    |
| Anaeromassilibacillus senegalensis     | 233   | 54                  | 0.002| 0.2     | 10.2       | 1    |
| Bifidobacterium calitrichos            | 322   | 65                  | 0.003| 0.3     | 10.9       | 1    |
| Bifidobacterium subtilis               | 288   | 54                  | 0.003| 0.3     | 11.7       | 1    |
| Bittarella massiliensis                 | 474   | 110                 | 0.006| 0.5     | 10.5       | 1    |
| Collinsella morbi                      | 543   | 80                  | 0.008| 0.8     | 10.5       | 1    |
| Clostridium perfringens                | 1392  | 194                 | 0.03 | 0.7     | 12.6       | 1    |
| Collinsella ihae                       | 513   | 106                 | 0.006| 0.5     | 10.2       | 1    |
| Collinsella phocaeensis                | 386   | 83                  | 0.003| 0.3     | 11.6       | 1    |
| Desulfovibrio alaskensis               | 286   | 51                  | 0.002| 0.2     | 10.2       | 1    |
| Desulfovibrio alkalitolerans           | 652   | 124                 | 0.007| 0.6     | 10.3       | 1    |
| Desulfovibrio dechloracetivorans       | 608   | 122                 | 0.005| 0.4     | 10.6       | 1    |
| Desulfovibrio fairfieldensis           | 383   | 74                  | 0.003| 0.3     | 12.1       | 1    |
| Desulfovibrio gracilis                 | 289   | 56                  | 0.003| 0.3     | 11.1       | 1    |
| Desulfovibrio legallii                 | 374   | 78                  | 0.004| 0.4     | 10.2       | 1    |
| Desulfovibrio oxyclineae               | 356   | 84                  | 0.003| 0.3     | 10.3       | 1    |
| Desulfovibrio vulgaris                 | 568   | 133                 | 0.005| 0.5     | 10.3       | 1    |
| Faecalibacterium prausnitzii           | 287   | 61                  | 0.003| 0.3     | 12.7       | 1    |
| Fournierella massiliensis              | 313   | 68                  | 0.003| 0.2     | 11.1       | 1    |
| Hungatella (Clostridium) hattheway      | 276   | 53                  | 0.001| 0.1     | 10.4       | 1    |
| Klebsiella pneumoniae                  | 390   | 82                  | 0.002| 0.2     | 10.9       | 1    |
| Paeniclostridium sordelli              | 780   | 86                  | 0.01 | 0.5     | 10.5       | 1    |
| Papillibacter cinnamivorans            | 284   | 53                  | 0.003| 0.3     | 11.3       | 1    |
| Prevotella timonensis                  | 220   | 96                  | 0.004| 0.1     | 15.9       | 1    |
| Prevotella saccharolytica              | 494   | 61                  | 0.008| 0.8     | 12.1       | 1    |
| Pseudobacterchial vulneris             | 278   | 56                  | 0.002| 0.2     | 12.2       | 1    |
| Pseudosulfovibrio indicus              | 605   | 123                 | 0.005| 0.5     | 10.3       | 1    |
| Rikenella microfusus                   | 276   | 54                  | 0.003| 0.3     | 12.0       | 1    |
| Ruminobacteriact lactiformans         | 315   | 57                  | 0.002| 0.2     | 10.1       | 1    |
| Siccibacter turicensis                 | 311   | 58                  | 0.002| 0.2     | 11.2       | 1    |
| Yokenella regensburgei                 | 258   | 56                  | 0.002| 0.1     | 11.8       | 1    |

Depth (DoC) and breadth of coverage (>1x) were calculated using BEDTools. Deamination rates at the 5′ ends of DNA fragments were calculated for the first 10 bases using mapDamage. –Δ % refers to the negative difference proportion introduced by Hübner et al.\(^53\). C-T (%) and –Δ % are computed on PMDS>1 reads.
Treponema vincentii), which have been associated with modern oral and dental diseases in humans\textsuperscript{59–68}. Expectedly, the samples from the upper part of SU V (which are poor in archaeological remains) showed scarce and inconsistent presence of aDNA related to hominin-associated gut microbiome bacterial families. The highest hit counts were found for Clostridium perfringens, Paenibacillus curtulibacter sanguinis, with the first two being environmental opportunistic microorganisms historically associated with human gangrene and the last with acute appendicitis\textsuperscript{69–71}. These findings further support the presence of potential human-like gut microbiome components as being unique to the samples from Xa and Xb, the only sedimentary layers that to date have shown traces of microscopic coprolites and fecal lipid biomarkers of presumed archaic human origin.

In conclusion, by reconstructing ancient bacterial profiles from El Salt Neanderthal feces-containing sediments, we propose the existence of a core human gut microbiome with recognizable coherence between Neanderthals and modern humans, whose existence would pre-date the split between the two lineages i.e., in the early Middle Pleistocene\textsuperscript{72}. Although the risk of fractional contamination by modern DNA can never be ruled out and our data must be taken with some caution, the identification of this ancient human gut microbiome core supports the existence of evolutionary symbioses with strong potential to have a major impact on our health. In particular, the presence of known short-chain fatty acid producers, such as Blautia, Dorea, Roseburia, Ruminococcus, Subdoligranulum, Faecalibacterium and Bifidobacterium, among the gut microbiome of Neanderthals, provides a unique perspective on their relevance as keystone taxa to the biology and health of the Homo lineage. While the former are known to allow extra energy to be extracted from dietary fiber\textsuperscript{73}, strengthening the relevance of plant foods in human evolution, Bifidobacterium could have provided benefits to archaic human mothers and infants as a protective and immunomodulatory microorganism. Furthermore, the detection of so-called “old friend” microorganisms\textsuperscript{74} as putative components of Neanderthal gut microbiome (e.g., Siprochaetaceae, Prevotella and Desulfovibrio) further supports the hypothesized ancestral nature of these human gut microbiome members, which are now disappearing in westernized populations\textsuperscript{11–11}. In the current scenario where we are witnessing a wholesale loss of bacterial diversity in the gut microbiome of the cultural “west”, with the parallel rise in dysbiosis-related autoimmune and inflammatory disorders\textsuperscript{75}, the identification of evolutionally integral taxa of the human holobiont may benefit practical applications favoring their retention among populations living in or transitioning to increasingly microbially deplete contexts. Such therapeutic applications may in the near future include next-generation probiotics, prebiotics or other gut microbiome-tailored dietary interventions.

**Methods**

**Site and sampling.** All samples used for this study were collected from the archaeological site of El Salt, Alicante, Spain. The archaeological team led by B. Galván conducted the excavations under a government permit and following the Spanish heritage law (No. 16/1985, 25 June). All excavated material including the sedimentary material is interpreted as archaeological material so no further permits are required for the presented study. Loose sediment samples (5–10 g) were collected in plastic vials using sterilized spoons (one per sample) after thoroughly cleaning the excavation surface with a vacuum cleaner in order to guarantee removal of any recent dust or sediment blown in from a different location. Lab safety masks and nitrile gloves were used at all times. The samples were collected from two different zones of the current El Salt excavation area (see Fig. 1):

1. **Zone 1.** This is the upper excavation zone. Samples were collected from SU V, Facies 23 (one sample, V1) and Facies 24 (two samples, V2 and V3). This unit has been dated by OSL to 44.7 ± 3.2 ky BP\textsuperscript{76}. Lithologically, it is composed of massive, loose yellowish-brown calcareous silt with coarse sand and isolated larger limestone and travertine clasts. Facies 23 is fine-grained, while Facies 24 (overlying Facies 23) also contains gravel. Unit V has yielded very few archaeological remains (bone fragments and technologically diagnostic flint flakes).

2. **Zone 2.** This is the lower excavation zone. Samples were collected from SUs IX, Xa, Xb and XI, which are a stratified succession of sedimentary layers rich in Middle Paleolithic archaeological remains (charcoal, combustion features and burnt and unburnt bone and flint artifacts). From top to base:

   - **Unit IX (one sample):** is the uppermost layer in this succession. It is discontinuous across the excavation area, comprising a series of dark brown-black sandy silt lenses.
   - **Unit Xa (one sample):** dated by TL to 52.3 ± 4.6 ky BP\textsuperscript{77}, this is a microstratified brownish-yellow deposit of loose calcareous silt sands with few larger clasts.
   - **Unit Xb (eight samples):** similar to Xa, also microstratified but darker (brown) and finer-grained (sandy silts). Seven samples from this unit (ES1-7) were collected from a microstratified combustion structure (H.444) at the top of this layer that yielded human fecal biomarkers\textsuperscript{78}. The other sample was collected from underlying sediment.
   - **Unit XI (one sample):** this is a layer of loose brown silty sand.

**Ancient DNA extraction.** All work was conducted at University of Oklahoma LMAMR ancient DNA laboratory according to the following protocols for coprolite-derived materials.

For DNA extraction, approximately 200 mg were subsampled from each sample material and incubated on a rotator with 400 μl of 0.5 M EDTA and 100 μl of proteinase K (QIAGEN) for 4 h. After that, the samples were subjected to bead-beating with 750 μl of PowerBead solution (QIAGEN) and then extracted using the MinElute PCR Purification kit (QIAGEN) with a modified protocol (method B) described in Hagan et al.\textsuperscript{79} and based on Dubny et al.\textsuperscript{78}, including two cleaning steps before final elution into two 30 μl of EB buffer (QIAGEN).

**Library preparation and sequencing.** Shotgun sequencing indexing libraries were constructed using the NEBNext DNA Library Prep Master Mix set for 454 (New England Biolabs), following the “BEST” (Blunt-End-Single-Tabe) method\textsuperscript{80}, with the hybridization of adapter oligos as per Meyer and Kircher\textsuperscript{81}. Briefly, deaminated (C to U) bases were first partially removed (UDG-half) by uracil DNA glycosylase treatment using USER enzyme\textsuperscript{81}. End overhangs were repaired, creating blunt-end phosphorylated regions for adapter ligation. Oligo adapters were ligated directly to blunt ends and filled in to create priming sites for index primers. After purification with a MinElute column (QIAGEN), indexed libraries were generated in triplicate for each sample using unique forward and reverse barcoded primers. See Supplementary Table 3 for adapter and oligo sequences. The triplicates were pooled, cleaned using Agencourt AmpPure XP magnetic beads (Beckman Coulter), and then run on a Fragment Analyzer (Advanced Analytical) using the high sensitivity NGS standard protocol. Samples containing adapter dimers below the main peak for putative authentic endogenous DNA (i.e., 200–250 bp\textsuperscript{82}), were further cleaned using AMPure XP magnetic beads in a PAGE/NalCi buffer\textsuperscript{83}. Cleaned samples were sequenced on Illumina NextSeq 500 platform (Illumina) at University of Bologna (Bologna, Italy), using paired-end 2 × 73 bp chemistry in order to generate sequences per sample. Quality score exceeded Q30 for more than 95% of the sequenced bases. Sequencing data was pre-processed by retaining only merged reads matching the forward and reverse barcodes with no mismatches using AdapterRemoval\textsuperscript{84}.

**Bioinformatics analysis.** Sequences were analyzed using Burrows-Wheeler Aligner (BWA) aln algorithm and the entire set of bacterial and archaeal genomes available through NCBI ReSeq (downloaded on November 15\textsuperscript{th}, 2017). In particular, we reduced the maximum accepted edit distance (i.e., the threshold of the maximum number of deletions, insertions, and substitutions needed to transform the reference sequence into the read sequence) to 1% (n = 0.01) and set the maximum number of gap openings, the threshold of the maximum number of gaps that can be initiatated to match a given read to the reference to 2, with long gap and seed length disabled (≥1 -16500). These parameters are optimized for the specific types of errors generated by postmortem DNA damage during the alignment of ancient DNA to modern references, as indicated by Schubert and colleagues\textsuperscript{85}. The aligned reads were further filtered for mapping quality ≥20, and only the hits with the best unique match (XO = 1) were considered for analysis in order to minimize the number of false positives. In order to retrieve the entire phylogeny of the assignment, database sequences were previously annotated with the “Tax” tags of the NCBI database using the reference-annotator tool of the MiEGAN utils package\textsuperscript{86}. We then used the calmd program of the samtools suite to recompute the MD tags (containing alignment information, such as mismatches) for all sequences.

To discriminate ancient DNA from modern-day contamination, we calculated the postmortem degradation score (PMDs) distributions\textsuperscript{22}. Sequences with PMDs > 5 were considered ancient (over 5,000 years ago), as reported by Skoglund et al.\textsuperscript{15}, and used for further analysis. The top strains were 1%
abundance as number of reads for each specific taxon. This was then collapsed at family, genus and species level using the command “summarize_taxa.py”. The family-level relative abundances of samples IX, Xa, ES1-7, Xb and XI were compared with publicly available data of the gut microbiota of human populations adhering to different subsistence strategies: urban Italians and Hadza hunter-gatherers from Tanzania (NCBI SRA, Bioproject ID PRJNA278393)14, urban US residents, Matses hunter-gatherers and Tungapoco rural agriculturalists from Peru (NCBI SRA, Bioproject ID PRJNA626906)14. Shotgun sequence datasets were downloaded and processed as El Salt samples, without applying the PMDS filter. 16S rRNA gene representative sequences of bacterial genera for which at least one species was present with more than 4 hits in one El Salt sample, were downloaded from the SILVA repository and used to build a phylogenetic tree using MUSCLE27 and FastTree48. The tree was visualized using the GraPhAn software.89 Finally, bacterial species belonging to families that have recently been indicated as being common to the gut microbiome of hominids,58-45 were specifically sought and visualized for their abundance across El Salt samples by a heat map using the R software. Species membership in other source categories (i.e., human (or animal) oral and/or pathobiont, and environmental) was inferred by searching for PubMed the original article in which the taxonomy was first assigned to that organism, as well as more recent articles reporting its habitat description.

**Independent validation of taxonomic assignments.** To validate the taxonomic assignments of the metagenomic reads recovered from the El Salt samples, we used the same procedure adopted by Jensen and colleagues.90 Specifically, we combined results from samples IX, Xa, ES1-7, Xb and XI (i.e., those positive for the presence of fecal biomarkers and/or associated with rich archaeological assemblages), then aligned the assigned reads to their respective reference genomes and examined edit distances, coverage distributions, and postmortem DNA damage patterns14,53. For the 24 families identified as common to hominid gut microbiome, we chose to further investigate bacterial species with ≥200 assigned reads (including strain-specific reads), for which at least 50 reads showed PMDS > 1 and at least one mismatch in the first 10 bases with respect to the reference genome. We then aligned the taxon-specific reads to the respective reference genome from the NCBI RefSeq database, using bwa aln. MapDamage was used to estimate deamination rates (Supplementary Fig. 6).90 The breadth and depth of coverage were calculated with bedtools91 and visualized with Circos92 (Supplementary Fig. 7). Edit distances for all reads and filtered for PMDS > 1 were extracted from the bam files with the samtools view93 and plotted in R (Supplementary Fig. 8). The negative difference proportion (Δ%) was calculated considering the first 10 bases of reads with PMDS > 1. This metric was proposed by Hübler et al.53 as a measure of decline in abundance as number of reads for each specific taxon. This metric was proposed by Hübler et al.53 as a measure of decline in relative abundance as number of reads for each specific taxon. This metric was proposed by Hübler et al.53 as a measure of decline in relative abundance as number of reads for each specific taxon.

**mtDNA analysis and contamination estimation.** In order to detect human mtDNA, a similar procedure combining BWA (same parameters as above) and the reference annotation tool of the MEGAN Utah package, was applied to the entire set of mitochondrial sequences listed at the MitoSeqs website (https://www.mitomap.org/soz/wiki/bin/view/MITOMAP/MitoSeqs), including all the eukaryotic mitochondrial sequences available at the NCBI database. Only taxa detected in ancient sequences (i.e., with PMDS > 5) with more than 2 hits and not present in the control samples were retained. This procedure allowed us to detect ancient human traces beyond any reasonable doubt, eventually discarding more sequences than necessary. In parallel, capture-enrichment for mtDNA sequencing was performed on the indexed libraries with a Neandertal bait panel, as per the manufacturer’s protocol (version 2019.6, Arbor VitaPrep). In short, libraries were fragmented, blocked and incubated with baits for 48 h. After purification with streptavidin-coated magnetic beads, enriched libraries were amplified and concentrated, before being subjected to a second round of capture. Final libraries were sequenced on an Illumina NextSeq 500 platform (Illumina) at University of Bologna (Bologna, Italy), as described above. As for read processing, we used Schmutzi24 to determine the endogenous consensus mtDNA sequence and to estimate present-day human contamination. Reads were mapped to the mt-Neanderthal reference sequence (NC_011137) and filtered for MAPQ ≥ 30. Haplod variant calls were used to call single nucleotide polymorphism (SNP) and short indels. At the species level, the mtDNA depth of coverage ≥10% of the mitochondrial length was retained for further analysis. The PMDS profile of the reads was computed by PMDTools12. The negative difference proportion (Δ%) was calculated using only reads with PMDS > 1.92 Contamination estimates were obtained using Schmutzi’s mtCont program and a database of putative modern contaminant mtDNA sequences. Samples with ≥1,000 PMDS ≥ 1 reads, breadth of coverage ≥10%, Δ% ≥ 0.9 and mtCont contamination less than 2% were considered to contain ancient human mtDNA.

**Statistics and reproducibility.** No replicates are included, all samples herein analyzed are unique. Filtration test was used to assess differences between samples IX, Xa, ES1-7, Xb and XI (i.e., those positive for the presence of fecal biomarkers and/or associated with rich archaeological assemblages), and samples from SU V (i.e., with no or very few archeological remains) in the number of PMDS > 5 reads, as well as in the relative abundances of the 24 families common to the gut microbiome of hominids.58-45

The significance of data separation in the Bray-Curtis-based Principal Coordinates Analysis between the family-level relative abundance profiles of samples IX, Xa, ES1-7, Xb and XI, and the gut microbiota of urban Italians and Hadza hunter-gatherers from Tanzania14, urban US residents, Matses hunter-gatherers and Tungapoco rural agriculturalists from Peru14 was tested using a permutation test with pseudo-F ratio.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Sequencing data are accessible at the European Nucleotide Archive (ENA; project ID PRJEB41665). Source data are available as Supplementary Data. All sediment samples are readily available from the authors, subject to exhaustion.

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**References**

1. Lynch, S. V. & Pedersen, O. The human intestinal microbiome in health and disease. *N. Engl. J. Med.* 375, 2359–2379 (2016).
2. Davenport, E. R. et al. The human microbiome in evolution. *BMC Biol.* 15, 127 (2017).
3. De Filippo, C. et al. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc. Natl Acad. Sci. USA* 107, 14691–14696 (2010).
4. Yakob, L. V. et al. Human gut microbiota community structures in urban and rural populations in Russia. *Nat. Commun.* 4, 2469 (2013).
5. Schnorr, S. L. et al. Gut microbiome of the Hadza hunter-gatherers. *Nat. Commun.* 5, 3654 (2014).
6. Martinez, I. et al. The gut microbiota of rural Papua New Guineans: composition, diversity patterns, and ecological processes. *Cell Rep.* 11, 527–538 (2015).
7. Obregon-Tito, A. J. et al. Subsistence strategies in traditional societies distinguish gut microbiomes. *Nat. Commun.* 6, 6505 (2015).
8. Sankaranarayanan, K. et al. Gut microbiome diversity among Cheyenne and Arapaho individuals from Western Oklahoma. *Curr. Biol.* 25, 3161–3169 (2015).
9. Girard, C., Tomas, N., Aymot, M. & Shapiro, B. J. Gut microbiome of the Canadian Arctic Inuit. *mSphere* 2, e00297–e00316 (2017).
10. Ayeni, F. A. et al. Infant and adult gut microbiome and metabolome in rural Basa and urban settlers from Nigeria. *Cell Rep.* 23, 3056–3067 (2018).
11. Iba, A. R. et al. Gut microbiome transition across a lifestyle gradient in Himalaya. *PLoS Biol.* 16, e2003396 (2018).
12. Skoglund, P. et al. Separating endogenous ancient DNA from modern day contamination in a Siberian Neandertal. *Proc. Natl Acad. Sci. USA* 111, 2229–2234 (2015).
13. Moeller, A. H. et al. Cosepation of gut microbiota with hominids. *Science* 353, 380–382 (2016).
14. Key, K. M., Poth, C., Krause, J., Herbig, A. & Bos, K. J. Mining metagenomic data sets for ancient DNA: recommended protocols for authentication. *Trends Genet.* 33, 508–520 (2017).
15. Philips, A. et al. Comprehensive analysis of microorganisms accompanying human archaeological remains. *Gigascience* 6, 1–13 (2017).
16. Warinner, C. et al. Pathogens and host immunity in the ancient human oral cavity. *Nat. Genet.* 46, 336–344 (2014).
17. Rasmussen, S. et al. Early divergent strains of *Yersinia pestis* in Eurasia 5,000 years ago. *Cell* 163, 571–582 (2015).
18. Weyrich, L. S. et al. Neandertal behaviour, diet, and disease inferred from ancient DNA in dental calculus. *Nature* 544, 357–361 (2017).
19. Eckburg, P. B. et al. Diversity of the human intestinal microbial flora. *Science* 308, 1635–1638 (2005).
20. Tito, R. Y. et al. Phyloptying and functional analysis of two ancient human microbiomes. *PLoS ONE* 3, e3703 (2008).
21. Tito, R. Y. et al. Insights from characterizing extinct human gut microbiomes. *PLoS ONE* 7, e51146 (2012).
80. Meyer, M. & Kircher, M. Illumina sequencing library preparation for highly multiplexed target capture and sequencing. Cold Spring Harb. Protoc. 2010, pdb.prot5448 (2010).
81. Rohland, N., Harney, E., Mallick, S., Nordenfelt, S. & Reich, D. Partial uracil-DNA-glycosylase treatment for screening of ancient DNA. Philos. Trans. R. Soc. Lond. B Biol. Sci. 370, 20130624 (2015).
82. Ziesemer, K. A. et al. Intrinsic challenges in ancient microbiome reconstruction using 16S rRNA gene amplification. Sci. Rep. 5, 16498 (2015).
83. Rohland, N. & Reich, D. Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. Genome Res. 22, 939–946 (2012).
84. Lindgreen, S. AdapterRemoval: easy cleaning of next-generation sequencing reads. BMC Res. Notes 5, 337 (2012).
85. Schubert, M. et al. Improving ancient DNA read mapping against modern reference genomes. BMC Genomics 13, 178 (2012).
86. Huson, D. H. et al. MEGAN community edition – interactive exploration and analysis of large-scale microbiome sequencing data. PLoS Comput. Biol. 12, e1004957 (2016).
87. Edgar, R. C. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics 5, 113 (2004).
88. Price, M. N., Dehal, P. S. & Arkin, A. P. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol. Biol. Evol. 26, 1641–1650 (2009).
89. Asnicar, F., Weingart, G., Tickle, T. L., Huttenhower, C. & Segata, N. Compact graphical representation of phylogenetic data and metadata with GraPhlAn. PeerJ 3, e1029 (2015).
90. Jónsson, H., Ginolhac, A., Schubert, M., Johnson, P. L. & Orlando, L. mapDamage2.0: fast approximate Bayesian estimates of ancient DNA damage parameters. Bioinformatics 29, 1682–1684 (2013).
91. Quinlan, A. R. & Hall, I. M. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics 26, 841–842 (2010).
92. Kryszewinski, M. et al. Circos: an information aesthetic for comparative genomics. Genome Res. 19, 1639–1645 (2009).
93. Li, H. et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25, 2078–2079 (2009).
94. Renaud, G., Slon, V., Duggan, A. T. & Kelso, J. Schmutzi: estimation of contamination and endogenous mitochondrial consensus calling for ancient DNA. Genome Biol. 16, 224 (2015).

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Competing interests
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