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1 Paleomicrobiology of the human digestive tract: a review.

2

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14

15 **Abstract**

16 The microbiota is a hot topic of research in medical microbiology, boosted by
17 culturomics and metagenomics, with unanticipated knowledge outputs in physiology
18 and pathology. Knowledge of the microbiota in ancient populations may therefore be
19 of prime interest in understanding factors shaping the coevolution of the microbiota
20 and populations. Studies on ancient human microbiomes can help us understand
21 how the community of microorganisms presents in the oral cavity and the gut was
22 shaped during the evolution of our species and what environmental, social or cultural
23 changes may have changed it. This review cumulates and summarizes the
24 discoveries in the field of the ancient human microbiota, focusing on the remains
25 used as samples and techniques used to handle and analyze them.

26

27 **Keywords**

28 Coprolite; dental calculus; microbiome; microbiota; mummified tissue;
29 paleomicrobiology; paleogenomics; paleoproteomics.

30 **1. Introduction**

31 In the last 20 years, there has been an exponential increase in interest in the
32 microbiome and its effect on human health. We currently know that the microbiome
33 plays a fundamental role in the host via the development, maintenance and priming
34 of the immune system [1–3], defense against pathogens [4,5], metabolism, digestion
35 and the production of vitamins [6]. Furthermore, slight modification of the bacterial
36 equilibrium of the microbiome (dysbiosis) due to changes in diet, physical exercise or
37 antibiotic treatments can lead to major consequences for the host [7–11]. Recent
38 studies have focused their attention on the composition of the microbiome of our
39 ancestors and its evolution throughout history. Particular attention was given to the
40 periods of major revolutions in human lifestyles, such as the Neolithization process
41 (debuted 12,000 years BCE [12]) and the industrialization period (1760-1840 CE).
42 With the Neolithization process, we observed the transition from a nomadic hunter-
43 gatherer diet characterized by the consumption of only wild and unprocessed food
44 foraged and hunted from the environment [13] to a sedentary one based on
45 agriculture and livestock. Then the industrialization period arose the advent of
46 modern sanitation and Western diet, characterized by food that has been processed
47 and modified and has a low quantity of vegetables and fibers [14–16].

48 In the literature, there is some confusion about the definition of the terms
49 “microbiota” and “microbiome” and are often used interchangeably. The term
50 “microbiota” refers to all the microbial taxa associated with the host [17], while
51 “microbiome” can refer to “the ecological community of commensal, symbiotic, and
52 pathogenic microorganisms that literally share our body space and have been all but

53 ignored as determinants of health and disease” [18] or to “the collective genome of
54 our indigenous microbes” [19].

55 Diets of past and present human populations are very variable and mostly depend
56 on resource availability and ecological settings [20]. Comparative studies on the
57 microbiota among human populations with different dietary habits have shown how
58 differences in diet are reflected in differences in the microbiota composition [14,16].
59 For instance, De Filippo and colleagues [21] showed how children from urban
60 Europe, with a modern Western diet, and children from rural Africa, with a diet very
61 high in fibers, present differences in microbiota composition that can be attributed to
62 an evolutionary adaptation to the diet composition, allowing each population to
63 maximize the nutritional value of each diet. Other studies on mouse models have
64 shown how changing diet correlated with changes in the microbial composition of the
65 gut microbiota [22]. Moreover, Groussin et al. [23] demonstrated how microbiota
66 composition reconstructed from ancient mammal remains could be used to
67 reconstruct ancient diets of these animals and that information about dietary shift can
68 be recorded by the gut microbiome. For these reasons, ancient microbiota studies
69 are not restricted to the microbial diversity and composition of the ancient remains
70 but also to the dietary information that is preserved inside these specimens. The first
71 study to target the ancient DNA of multiple species of bacteria on human remains
72 was conducted by Ubaldi et al, analyzing the gut microbiome from the colon of a
73 mummy recovered in Cuzco, Peru, that dated back to the 10th-11th century CE [24].
74 Following by many other PCR-based studies on coprolites and mummified samples
75 [25–27]. In 2008, Tito et al. performed the first study on an ancient microbiota using
76 NGS techniques, and from that point, the number of ancient microbiome analyses
77 multiplied [28].

78 Retrieving information on ancient human microbiomes is not easy and is strictly
79 dependent on the availability of suitable samples for the analysis. Dental calculus
80 and coprolite are the only two human remains that persist long enough to be used for
81 these studies, with the exceptions of frozen and mummified tissues [29]. In this
82 review, we will present the remains used as starting samples for ancient human
83 microbiota studies and the methods used to retrieve this information.

84 **2. Literature search strategy and terminology use**

85 All the papers used for this review were obtained from PubMed and Google Scholar.

86 The keywords used alone or in combination for the search were “paleomicrobiology”,

87 “ancient”, “human”, “paleo”, “microbiota”, “microbiome”, “oral”, “dental calculus”,

88 “gut”, “intestinal”, “feces”, “faeces”, “paleofeces”, “paleofaeces”, “coprolite”, “latrines”,

89 “mummy”, “mummified”, “tissue”, “metagenomic”, “shotgun”, and “proteomics”.

90 Only studies written in English and on samples of human origin that targeted multiple

91 species from the digestive tract microbiota of the host were considered. The results

92 of this bibliographical research are shown in Table 1 (last search March 2021).

93 As described in the introduction section, the two terms “microbiota” and “microbiome”

94 are often used interchangeably, and since a discussion on the terminology was not

95 one of the purposes of this review, we used the two terms as synonyms, following

96 the definition given by Lederberg [18].

97 **Contamination prevention and authentication**

98 Paleomicrobiological studies on ancient molecules must follow standard protocols to
99 avoid modern exogenous contamination and ensure the authenticity of the results
100 [30]. Contaminating molecules can come from the burial site, handling, transport,
101 storage, and laboratory environments; from the materials and instruments used
102 during the analysis; and from the operators at each step of the study [31,32].
103 Regardless of the starting sample type, all the steps of the analysis of ancient
104 molecules must be performed in a dedicated clean hood and laboratory, ideally
105 exclusively dedicated to work on ancient remains, excluding any positive controls,
106 and including several negative controls that should be handled strictly in parallel with
107 investigated samples [31]. Authentication of ancient DNA can be performed by
108 checking for fragmentation (ancient DNA rarely exceeds 200-300 bp length) [33,34]
109 and degradation (e.g., assessing the PCR success related to the size of the
110 amplicon of a selected DNA sequence) [33]. Multiple bioinformatics tools can
111 contribute to the authentication of ancient molecules. Software like MapDamage 2.0
112 [35,36] quantify the patterns of damage in NGS datasets generated from ancient
113 DNA and therefore help differentiate endogenous from exogenous/contaminating
114 DNA. Other tools like SourceTracker [33,37] estimate the proportion of DNA
115 sequences that come from the source environments to determine whether the
116 reconstructed microbial communities observed are endogenous to the samples.
117 Moreover, directly comparing the DNA sequences obtained from different tissues
118 with those from soil from the site where samples have been collected through
119 bioinformatic analysis may facilitate the interpretation of the data [34].

120 **3. Ancient Oral Cavity Microbiota.**

121 **3.1. Dental calculus**

122 Dental calculus, or tartar, is a mineralized bacterial biofilm that forms on the teeth as
123 a result of the action of bacteria and saliva in the oral cavity and is very common in
124 adults without active dental hygiene [29]. Dental calculus presents some
125 characteristics that make it the most reliable source for information about the oral
126 microbiota. It is common and relatively abundant in archaeological sites; it has the
127 capability to preserve ancient biomolecules, avoiding environmental contaminants;
128 and it contains a high quantity of DNA originating from the oral microbiome
129 [29,38,39]. In fact, it can be found on teeth in all past and present human populations
130 and can survive for thousands and even millions of years. Accordingly, dental
131 calculus remains were obtained from a 12-8 million-year-old fossil of the ape
132 *Sivapithecus* [40].

133 Geographical distribution of the specimens analyzed for the oral microbiota are
134 represented in Figure 1.

135 **3.1.1. Analysis of ancient DNA from dental calculus.**

136 After the recent confirmation of the presence and abundance of DNA in dental
137 calculus [41], multiple studies have successfully extracted and analyzed DNA
138 sequences from this type of sample. Different protocols have been applied for DNA
139 extraction in the past. In general, it is very similar to apply the extraction methods
140 used for other types of samples, such as bones and teeth, with a “lysis step” followed
141 by a “purification step”. The lysis step usually relies on decalcification through
142 ethylenediaminetetraacetic acid (EDTA) with or without Tris-HCl and protein
143 digestion using proteinase K with or without sodium-dodecyl-sulfate (SDS), with a

144 variable incubation time and temperature (usually 24 h at 56 °C) [38,42–46].
145 Occasionally, in the lysis step, Tris-HCl and SDS are added to the sample to improve
146 decalcification and protein digestion [38,47]. After the lysis step, purification of DNA
147 is performed using the phenol:chloroform:isoamyl alcohol (25:24:1) method [47]
148 preferentially followed by Qiagen MinElute column purification [38,43,45,46] or using
149 commercial kits for DNA purification, such as the EZ1 DNA tissue kit (Qiagen)
150 [44,48] and QIAamp DNA Investigator Kit (Qiagen) [42]. It is also possible to use an
151 in-house silica-based method for the extraction and purification of ancient DNA [20].

152 Polymerase chain reaction (PCR) amplification and subsequent sequencing can be
153 used to analyze DNA sequences specific for microorganisms from the oral microbial
154 community. PCR primers were specifically designed for five species of bacteria,
155 *Actinomyces naeslundii*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*,
156 *Streptococcus gordonii* and *Streptococcus mutans*, by De La Fuente et al. [47],
157 which were detected in dental calculus from 4,000-year-old to modern samples.
158 Specific primers are useful for the identification of single species of microorganisms,
159 such as pathogens or common microbes of the oral microbiota; to determine the
160 conservation of DNA in the sample; and to study specific genes [49].

161 The amplification of a conserved bacterial region of the 16S ribosomal RNA (16S
162 rRNA) gene and subsequent high-throughput sequencing yields information about
163 the diversity of bacteria present in the samples, describing the bacterial community
164 of the ancient oral microbiota [20,38,42,50,51]. Using this method, it is possible to
165 identify hundreds of species with a single PCR amplification [49]. Adler et al. [42]
166 used the results of high-throughput sequencing to compare the bacteria present in
167 the oral cavity of Mesolithic, Neolithic, Bronze Age, medieval and modern individuals,

168 reporting a reduction in the bacterial diversity and a shift toward cariogenic bacterial
169 dominance after medieval times, concomitant with the industrial revolution [42].
170 Following the same principle, regions conserved among any organism of interest can
171 be amplified. PCR sequencing of 16S rRNA and methyl coenzyme M reductase
172 (*mcrA*) genes was used to detect and describe the methanogen community present
173 in the oral human microbiota, allowing the identification of multiple archaea, such as
174 *Candidatus Methanobrevibacter* sp. N13, *Methanobrevibacter oralis* and
175 *Methanoculleus bourgensis* [44]. Unfortunately, this approach has limited
176 applicability when the aDNA is known to be highly degraded and fragmented or
177 when the targeted region contains extensive length polymorphisms that induce a
178 bias toward taxa with the shortest amplicon lengths [43].

179 In contrast to targeted gene amplification, shotgun sequencing allows the
180 sequencing of random DNA fragments present in the sample independently of their
181 origin (eukarya, bacteria, archaea, and viruses), overcoming targeted sequencing
182 problems [38,43]. This approach allows the analysis of all the sequences inside the
183 sample at the same time without any prior knowledge, providing both taxonomic and
184 functional information [49]. Shotgun metagenomics applied to ancient human dental
185 calculus allowed the characterization of the oral microbiota from multiple
186 archaeological sites [20,38,39], providing information about antibiotic resistance and
187 metabolic functions of ancient bacteria [20,38]. Data obtained with this approach
188 showed a possible correlation between behavior, diet, and health and the oral
189 microbiota composition of two populations of European Neanderthals [20]. This
190 finding suggested a central role of meat consumption in early hominid microbiota
191 composition and that the repertoire of microorganisms preserved in dental calculus
192 could be used to derive the dietary behavior of ancient populations by comparing the

193 shotgun data obtained to those obtained for other ancient calculus specimens from
194 populations with a known diet. Moreover, the shotgun metagenomic approach also
195 allowed the reconstruction of draft genomes of organisms present in ancient dental
196 calculus, such as the bacterium *Tannerella forsythia* [38] and the archaeon
197 *Methanobrevibacter oralis* [20].

198 **3.1.2. Analysis of ancient protein from dental calculus.**

199 Similar to shotgun metagenomics for nucleotides, shotgun proteomics allows the
200 detection and study of extracted peptides from ancient human dental calculus [52].
201 Despite the better preservation capability of proteins than DNA molecules, proteomic
202 approaches are relatively new in the field of paleomicrobiology, providing valuable
203 information about the diet, microbiota function and composition, host health and
204 immune response [53,54]. Warriner and colleagues showed how tandem mass
205 spectrometry (MS/MS) used for shotgun proteomics can produce information about
206 the presence of bacterial species-specific proteins, virulence factors, and antigenic
207 proteins, as well as human immune response proteins (antimicrobial gene products)
208 [38,55]. In addition, it has been shown that mass spectrometry for proteomic analysis
209 can be used to describe the oral human metabolome (the collection of all the low-
210 weight metabolites produced by metabolism [46]), providing important information
211 about salivary and microbiome-related proteins [46] and dietary information from
212 ancient populations [56,57].

213 **3.1.3. Microscopical analysis on dental calculus.**

214 Microscopic analysis of dental calculus allowed the detection of aliments and the
215 presence of calcified microorganisms [58,59]. Examination through scanning
216 electron microscopy (SEM) can be performed without treating the sample, allowing

217 complete preservation [60], while optical microscopy and transmission electron
218 microscopy (TEM) need a pretreatment to be performed that partially or completely
219 disrupts the sample [60]. For SEM, a preliminary step for the decalcification of the
220 sample is performed using hydrochloric acid (HCl) at different concentrations [38,61].
221 For TEM, ancient samples follow routine procedure, with treatment with osmium
222 tetroxide fixative and epoxy resin embedding [41]. Optical microscopy can be used to
223 distinguish between gram-positive and gram-negative bacteria [38,60,62]. Optical
224 and electron microscopy usually do not allow the identification of species but can
225 provide important pieces of information about the presence, dimension and shape of
226 microorganisms in the samples [59]. Microscopic analysis can also be used for
227 biomolecular investigations, using gold-labeled antibody TEM to detect the presence
228 of DNA [41]; fluorescence microscopy and polyclonal antibodies for the identification
229 of specific bacteria, such as *Streptococcus mutans* [62]; and fluorescent *in situ*
230 hybridization (FISH) to reveal archaea, such as *Candidatus Methanobrevibacter* sp.
231 [44].

232 **3.2. Other sources of ancient oral microbiota.**

233 Despite the vast majority of studies on ancient oral microbiota have been performed
234 on dental calculus, some authors tried to think outside of the box obtaining
235 interesting results. In 2019, Jensen and colleagues analyzed a chewed birch pitch
236 from Denmark dated to 5,700 years ago and revealed the presence of preserved
237 DNA material. Starting from this specimen, they reconstructed a complete ancient
238 human genome and identified microbial DNA sequences that reflected the oral
239 microbiome of the person who chewed the pitch [63]. This work highlighted the

240 potential utility of chewed birch pitch in the field of paleomicrobiology for the study of
241 ancient microbiota.

242 Moreover, the analysis of buccal swabs from Inca mummies through shotgun
243 proteomics analysis was used to describe active states of infection on an individual
244 at the time of death by providing information on the immune response and potentially
245 on the pathogens present when combined with DNA amplification [64].

246 **4. Ancient Gut Microbiota.**

247 **4.1. Coprolites and palaeofaeces.**

248 The primary sources for information about the ancient gut microbiota are coprolites,
249 which are “any formed fecal mass remains, including mineralized, desiccated, or
250 frozen feces and even the intestinal contents of mummies” [65]. These remains can
251 be preserved for thousands or even millions of years [66], providing information
252 about the gut microbiota components, the presence of pathogens and the diet of
253 past human and animal populations [67]. In fact, traces of aliments consumed by the
254 host, such as bones of small animals, feathers, seafood shells, and plant seeds, are
255 commonly found in coprolites [65,68–71]. Moreover, it is possible to correlate the
256 presence of animal parasites with the diet of an ancient population [70,72,73].

257 **4.1.1. Molecular analysis on coprolites.**

258 DNA analysis can potentially produce additional information about the complete set
259 of species inside the coprolite and the evolution of these organisms. To reduce
260 environmental contamination, extraction of DNA is performed on the core of the
261 coprolites after the removal of a few millimeters (3 mm - 1 cm) of coprolite surface.
262 Multiple protocols and commercial kits are available for DNA extraction from fecal

263 and soil samples and are also used on ancient samples, e.g., UltraClean Fecal DNA
264 Isolation Kit (MOBIO) [33] and PowerSoil® DNA Isolation Kit (MOBIO) [70,72–74].
265 Protocol specific for coprolites that does not require commercial kits have also been
266 developed, such as the one from Kuch *et al.* [75]. In 2019, Hagan and colleagues
267 tested the efficacy of 5 different methods for DNA extraction from ancient fecal
268 material [36], indicating that despite every tested method being valuable, methods
269 used for modern microbiota analysis recovered less DNA than methods developed
270 and optimized for ancient bone material [39,76].

271 Massive sequencing of specific amplified regions of the DNA, such as 16S rDNA,
272 provided information about the prokaryotic community inside the coprolite [34,70,74].
273 The same approach can be used for the eukaryotic community, e.g., by using
274 primers that target the 18S ribosomal DNA (18S rDNA) or Internal transcribed spacer
275 (ITS) region for fungal diversity [70,74]. Whole-genome shotgun sequencing does
276 not require previous amplification of the DNA and gives information on the entirety of
277 DNA sequences present in the sample, allowing both taxonomic and functional
278 characterization [28,34,73,77]. Furthermore, pathogen DNA sequences, including
279 those of *Clostridium botulinum*, *Trypanosoma cruzi* and human papillomaviruses
280 (HPVs) [34], as well as drug resistance genes [34,77], are detected. Moreover, viral
281 metagenomes from coprolites have been described [72,78].

282 **4.1.2. Microscopic and immunoassay analysis on coprolites.**

283 Rehydration of samples is the preliminary step for subsequent microscopic analysis.
284 Different protocols have been developed for coprolite rehydration [79]. These
285 protocols all rely on using 0.5% trisodium phosphate (Na_3PO_4) aqueous solution
286 sometimes complemented with 5% glycerinated water and formalin (Le Bailly 2010)

287 or hydrochloric acid (HCl) to dissolve the calcium carbonates [80]. This step can last
288 from 24 h to one week [81,82] and can be combined with a sedimentation step
289 [68,73,77]. Microscopic analysis allows the identification of multicellular parasites,
290 such as helminth eggs [73,77,83]; protozoan cysts [84–86]; and food traces [87,88].
291 Parasite identification relies on morphometric characterization with light and electron
292 microscopy, which is based on the experience and ability of the operator. Cases
293 have been reported in which pollen grains, which are morphologically similar to
294 pinworm eggs, have been confused for parasitic eggs [89]. The use of confocal laser
295 scanning microscopy (CLSM) has been revealed to be a powerful tool for the
296 identification of parasites in coprolites. This technique allows better morphological
297 identification, detects intrinsic autofluorescent molecules and has the great
298 advantage of being less destructive to the specimen, allowing further analyses to be
299 performed (e.g., molecular analysis) on the same specimen [90]. Moreover,
300 immunofluorescence assays (IFAs) or enzyme-linked immunosorbent assays
301 (ELISAs) have been used for the detection of intestinal protozoa, such as *Giardia*
302 spp. [84,91–95], *Cryptosporidium* spp. [84,95,96], and *Entamoeba histolytica* [93–
303 95,97,98].

304 **4.1.3. Culture-dependent analysis on coprolites.**

305 Finally, a culture-dependent investigation was performed on a 14th-century coprolite
306 found in Belgium in parallel to a metagenomic analysis [77]. A portion from the inner
307 part of the coprolite was inoculated into Schaedler and R2A liquid and solid culture
308 media and maintained for several days at 30 °C under various atmospheres created
309 using gas-generating pouch systems (BD Gas Pak™ EZ). Small colonies were
310 observable after 10 days of incubation, and ten different bacterial species

311 (*Paenibacillus macerans*, *Bacillus jeotgali*, *Staphylococcus pasteurii*, *Staphylococcus*
312 *epidermidis*, *Staphylococcus cohnii*, *Micrococcus luteus*, *Pseudomonas geniculata*,
313 *Stenotrophomonas maltophilia*, *Bacillus horti* and *Clostridium magnum*) were
314 identified by matrix-assisted laser desorption/ionization time-of-flight mass
315 spectrometry (MALDI-TOF-MS) and 16S rRNA gene sequencing. Additionally, a
316 portion from the inner part of the coprolite was cultured in BD BACTEC, Lytic/10
317 Anaerobic and Aerobic bottles enriched with 7 mL of defibrinated sheep blood and
318 incubated at 37 °C. After two days, cultures were diluted and plated onto COS
319 culture plates (bioMérieux) and reincubated at 37 °C under different atmospheres.
320 Eight different bacterial species, *Rhodanobacter sp. organism*, *Paenibacillus sp.*
321 *organism*, *Paenibacillus macerans*, *Paenibacillus thiaminolyticus*, *Paenibacillus*
322 *ehimensis*, *Staphylococcus arlettae*, *Propionibacterium acnes* and *Enterobacter*
323 *cloacae*, were isolated and identified from the blood-enriched cultures using MALDI-
324 TOF-MS and 16S rRNA gene sequencing.

325 **4.2. Mummified tissues.**

326 Other than coprolites, natural and artificial mummified tissues are also valuable
327 sources for ancient human microbiota studies. DNA, proteins, and even entire
328 parasites (eggs, larvae, and adults) have been found in mummified tissues. Well-
329 preserved mummies offer the rare possibility to study microbiota both inside and
330 outside of the digestive tract, allowing the identification of specific pathogens of
331 certain organs, i.e., *Helicobacter pylori* [99], and even compare the microbial
332 communities present in different organs from the same individual [25]. Mummified
333 tissues from the digestive tract are used for the identification of the microbial
334 community of the gut, stomach, and oral cavity. Similar to the methods used for

335 coprolite analysis, mummified tissues can be analyzed for parasite and protozoan
336 detection through microscopy. The rehydration step can be performed following the
337 same methods used for coprolites with trisodium phosphate [79] or with a solution of
338 potassium hydroxide at 4-7% [79,100].

339 Techniques of 16S rRNA gene high-throughput sequencing and metagenomics
340 performed on mummified digestive tract tissues were used for the characterization of
341 ancient microbiota. The presence of DNA sequences homologous to many
342 organisms of medical interest were also detected, such as *Clostridium botulinum*,
343 *Trypanosoma cruzi*, HPVs, *Pseudomonas* spp., and *Leishmania donovani*
344 [24,101,102]. The genomes of 5,300-year-old *Helicobacter pylori* [99], *Clostridium*
345 *perfringens*, *Clostridium* sp. Ade.TY, *Clostridium algidicarnis*, and *Pseudomonas*
346 *veronii* [102] were reconstructed and used for phylogenetic analysis to study the
347 evolution of these organisms. Moreover, metagenomic analysis allowed the
348 identification of sequences homologous to bacteriophages [103], metabolic profiles
349 [101], multiple sequences associated with antibiotic resistance and virulence-related
350 genes in old mummified gut tissues [34,102,104]. Data obtained from mummified gut
351 remains could also potentially be used to infer ancient dietary habits [104].

352 Organs connected to the digestive tract have been studied using microscopy or
353 DNA-related techniques for the presence of pathogens and parasites: studies on
354 gallbladder samples detected *Clonorchis sinensis* [105], *Mycobacterium tuberculosis*
355 [106]; studies on liver samples detected hepatitis B virus [107].

356 Geographical distribution of the specimens analyzed for the gut microbiota are
357 represented in figure 1.

358 **5. Perspectives**

359 We have just begun to understand the relationships between diet, health,
360 pathological conditions, and the composition of our microbiota, but it is clear that the
361 gut microbiota plays a role in the health and disease conditions of humans. This
362 explains the strong interest in medical research for this subject. Combining modern
363 techniques and protocols used in the field of paleomicrobiology for the analysis and
364 the extraction of data from ancient human remains, we can study the past of
365 humankind and its microbiota. An evolutionary framework that leads us to
366 understand how these changes in human lifestyle have influenced changes in the
367 microbiota can be built through the comparison of the microbiota composition among
368 different eras and with data on the microbiota in the modern world. Studies on the
369 microbiota of our ancestors can help us describe the evolutionary path that led
370 humans and their microbiota to where we are now, revealing how changes in diet,
371 innovations in modern medicine, hygiene, and antibiotics have changed the
372 composition and, therefore, how the characteristics of our microbiota have shaped
373 humans as a species.

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817 **Declaration of competing interest**

818 The authors have no competing interests to declare.

819 **Table 1. Scientific studies performed on ancient human digestive tract**
820 **microbiota catalogued per archaeological site, dating, sample type, and**
821 **method used for the analysis.** Only studies written in English, published before
822 March 2021, and on samples of human origin that targeted multiple species from the
823 digestive tract microbiota of the host were considered.

Archaeological site	Present-day country	Dating	Sample type	Method	References
Cuzco	Peru	10th-11th century CE	Mummified colon	PCR-based techniques	[24]
Similaun mountain	Italy	3400-3100 BCE	Mummified stomach and colon	PCR-based techniques	[25]
Cuzco	Peru	980-1170 CE	coprolites	PCR-based techniques	[26]
Forni glacier (Alta Val di Pejo, northern Italy)	Italy	1918 CE	Mummified colon	PCR-based techniques	[27]
Similaun mountain	Italy	3400-3100 BCE	Mummified colon	PCR-based techniques	[27]
La Cueva de los Chiquitos Muertos', Rio Zape, Durango Durango	Mexico	600 - 700 CE	coprolites	Shotgun metagenomic	[28]
Caserones	Chile	400 CE	coprolites	16S rRNA Gene High-Throughput Sequencing	[33]
Hind's Cave	Texas	6000 BCE	coprolites	16S rRNA Gene High-Throughput Sequencing	[33]
La Cueva de los Chiquitos Muertos', Rio Zape, Durango Durango	Mexico	600 - 700 CE	coprolites	16S rRNA Gene High-Throughput Sequencing	[33]
Benzingerode-Heimburg	Germany	2150-1600 BCE	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
Dudka	Poland	5550-3450 BCE	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
Halberstadt-Sonntagsfeld	Germany	5400-4725 BCE	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
Northamptonshire	England	900-1150 CE	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
Quedlinburg XII	Germany	2450-2000 BCE	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
York	England	1250-1350 CE	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
York	England	1000-1600 CE	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
Yorkshire	Engalnd	Bronze age	dental calculus	16S rRNA Gene High-Throughput Sequencing	[42]
Sorcé, island of Vieques	Puerto Rico	180 - 600 CE	coprolites	16s rRNA gene and ITS region PCR-based techniques	[74]
Tecla, Guayanilla	Puerto Rico	180 - 600 CE	coprolites	16s rRNA gene and ITS region PCR-based techniques	[74]
Namur	Belgium	14th-century	coprolites	shotgun metagenomic	[78]
Namur	Belgium	14th-century	coprolites	culture-base method	[77]
Namur	Belgium	14th-century	coprolites	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[77]
Sorcé, island of Vieques	Puerto Rico	5 to 1170 CE	coprolites	16S rRNA Gene High-Throughput Sequencing	[70]
Tecla, Guayanilla	Puerto Rico	5 to 1170 CE	coprolites	18S rRNA Gene High-Throughput Sequencing	[70]
Similaun mountain	Italy	3400-3100 BCE	gingival tissue biopsy	PCR-based techniques	[108]
Similaun mountain	Italy	3400-3100 BCE	Mouth swab	PCR-based techniques	[108]
St. Petri church and convent complex in Dalheim	Germany	950-1200 CE	dental calculus	mass spectrometry (MS)-based proteomics profiling	[38]
St. Petri church and convent complex in Dalheim	Germany	950-1200 CE	dental calculus	Shotgun metagenomic	[38]
Cuzco	Peru	11th century CE	paleofaeces	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[34]
Cuzco	Peru	11th century CE	mummified colon	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[34]
Anse à la Gourde	Guadalupe	975-1395 CE	Dental calculus	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[43]

Camino del Molino, Caravaca de la Cruz, Murcia	Spain	2340–2920 BCE	Dental calculus	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[43]
Lavoutte	St. Lucia	990–1255 CE	Dental calculus	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[43]
Middenbeemster	Netherlands	1611–1866 CE	Dental calculus	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[43]
Rupert's Valley	St. Helena	1840–1872 CE	Dental calculus	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[43]
Samdzong	Nepal	400–650 CE	Dental calculus	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[43]
Tickhill, Yorkshire	UK	1450–1600 CE	Dental calculus	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[43]
Avosnes	France	1300 CE	dental calculus	PCR-based techniques	[44]
Douai	France	1710-1712 CE	dental calculus	PCR-based techniques	[44]
Forbach	France	1813 CE	dental calculus	PCR-based techniques	[44]
Les Fedons	France	1590 CE	dental calculus	PCR-based techniques	[44]
Martigues	France	1720-1721 CE	dental calculus	PCR-based techniques	[44]
Saint-Mitre-les-Remparts	France	1500-1700 CE	dental calculus	PCR-based techniques	[44]
Cuzco	Peru	980-1170 CE	Mummified descending colon	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[103]
Cuzco	Peru	14-15th century CE	Mummified abdominal viscera	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[103]
Cuzco	Peru	1410–1530 CE	Mummified transverse colon	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[103]
Cuzco	Peru	980-1170 CE	Mummified descending colon	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[101]
Cuzco	Peru	14-15th century CE	Mummified abdominal viscera	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[101]
Cuzco	Peru	1410–1530 CE	Mummified transverse colon	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[101]
Similaun mountain	Italy	3400-3100 BCE	Mummified distal gut	shotgun metagenomic	[102]
Leicester, UK (NGR SK 586038), Oxford Street	UK	2nd–5th century CE	dental calculus	mass spectrometry (MS)-based proteomics profiling	[109]
Rome, Isola Sacra	Italy	1st–3rd century CE	dental calculus	mass spectrometry (MS)-based proteomics profiling	[109]
York, UK (NGR: SE 59324510 & SE 59285095), Driffield Terrace	UK	2nd–4th century CE	dental calculus	mass spectrometry (MS)-based proteomics profiling	[109]
Sorcé, island of Vieques	Puerto Rico	10 - 385 CE	Dental calculus	16S rRNA gene high-throughput sequencing	[50]
Cuzco	Peru	980-1170 CE	Mummified descending colon	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[104]
Cuzco	Peru	14-15th century CE	Mummified abdominal viscera	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[104]
Cuzco	Peru	1410–1530 CE	Mummified transverse colon	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[104]
Naples, Basilica of St. Domenico Maggiore	Italy	15-16th century CE	Mummified colon and abdominal viscera	16S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[104]
Radcliffe Infirmary Burial Ground collection	England	1770-1855 CE	Dental calculus	mass spectrometry (MS)-based metabolites characterization	[46]
Tjærby, Jutland, Denmark	Denmark	1100–1450 CE	dental calculus	mass spectrometry (MS)-based proteomics profiling	[55]
Anse à la Gourde	Guadalupe	975-1395 CE	dental calculus	shotgun metagenomic	[39]
Arbulag Soum, Khövsgöl,	Mongolia	930-1650 BCE	dental calculus	shotgun metagenomic	[39]
Camino del Molino, Caravaca de la Cruz, Murcia	Spain	2340-2920 BCE	dental calculus	shotgun metagenomic	[39]
Kilteasheen	Ireland	600-1300 CE	dental calculus	shotgun metagenomic	[39]
Middenbeemster	Netherlands	1611-1866 CE	dental calculus	shotgun metagenomic	[39]
Norris Farms	Illinois (USA)	1300 CE	dental calculus	shotgun metagenomic	[39]
Samdzong	Nepal	400-650 CE	dental calculus	shotgun metagenomic	[39]

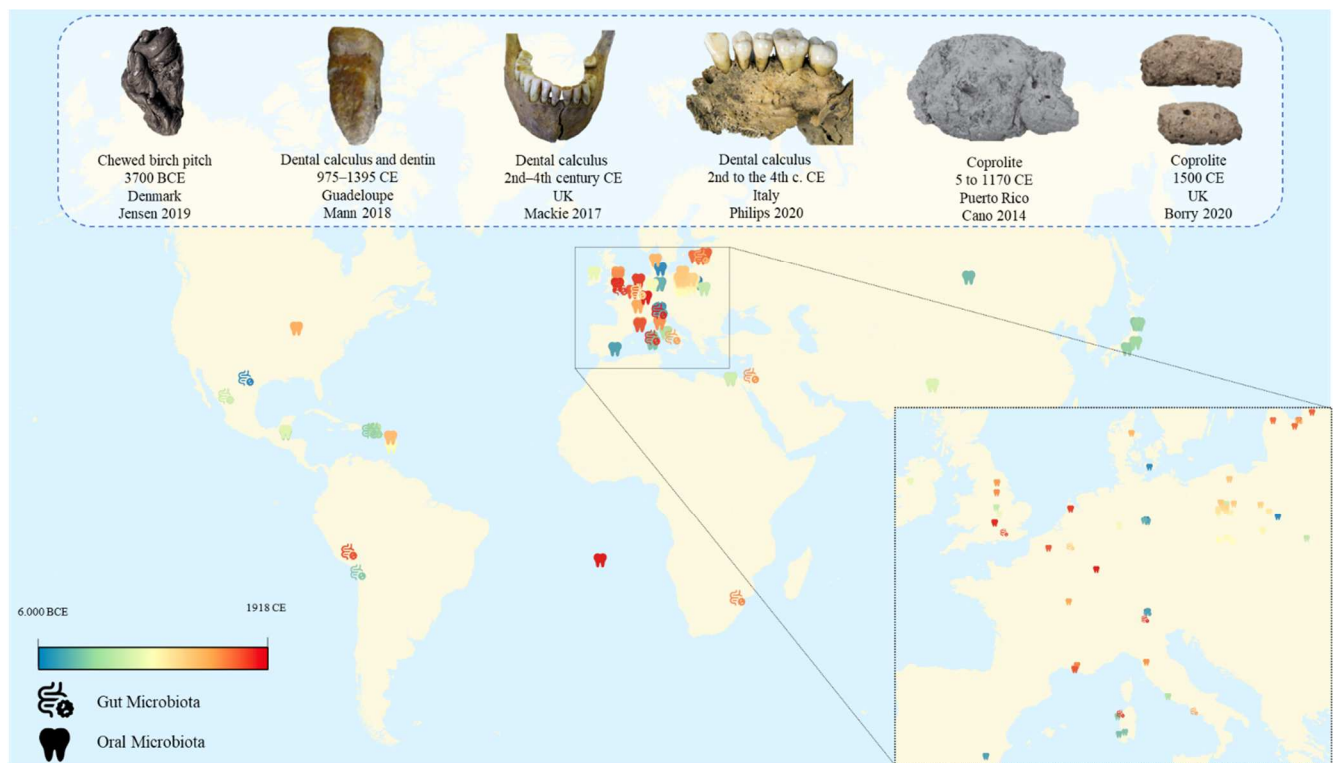
Anse à la Gourde	Guadalupe	975-1395 CE	dental calculus	shotgun metagenomic	[110]
Arbulag Soum, Khövsgöl,	Mongolia	930-1650 BCE	dental calculus	shotgun metagenomic	[110]
Camino del Molino, Caravaca de la Cruz, Murcia	Spain	2340-2920 BCE	dental calculus	shotgun metagenomic	[110]
Kilteasheen	Ireland	600-1300 CE	dental calculus	shotgun metagenomic	[110]
Middenbeemster	Netherlands	1611-1866 CE	dental calculus	shotgun metagenomic	[110]
Norris Farms	Illinois (USA)	1300 CE	dental calculus	shotgun metagenomic	[110]
Samdzong	Nepal	400-650 CE	dental calculus	shotgun metagenomic	[110]
site of Syltholm on the island of Lolland	Denmark	3700 BCE	chewed birch pitch	shotgun metagenomic	[63]
San Pietro di Pozzeveri	Italy	11th to 19th century CE	Dental calculus	16S rRNA gene High-Throughput Sequencing	[51]
La Cueva de los Chiquitos Muertos", Rio Zape, Durango Durango	Mexico	700 CE	coprolites	Shotgun metagenomic	[111]
Similaun mountain	Italy	3400-3100 BCE	intestinal mummified tissue	Shotgun metagenomic	[111]
Radcliffe Infirmary Burial Ground collection	England	1770-1855 CE	dental calculus	mass spectrometry (MS)-based proteomics profiling	[112]
Radcliffe Infirmary Burial Ground collection	England	1770-1855 CE	dental calculus	shotgun metagenomic	[112]
La Cueva de los Chiquitos Muertos", Rio Zape, Durango Durango	Mexico	700 CE	coprolites	Shotgun metagenomic	[113]
Surrey	UK	Post medieval	coprolites	Shotgun metagenomic	[113]
palazzo ducale Sassari	Italy	1800-1850 CE	cesspit sediment	16S and 18S rRNA Gene High-Throughput Sequencing and Shotgun metagenomic	[114]
La Cueva de los Chiquitos Muertos", Rio Zape, Durango Durango	Mexico	700 CE	coprolites	Shotgun metagenomic	[36]
Abusir	Egypt	340-395 CE	Dental calculus	Shotgun metagenomic	[115]
Abusir	Egypt	138-200 CE	Dental calculus	Shotgun metagenomic	[115]
Abusir	Egypt	2196-2045 BCE	Dental calculus	Shotgun metagenomic	[115]
Abusir	Egypt	109-40 BCE	Dental calculus	Shotgun metagenomic	[115]
Abusir	Egypt	369-211 BCE	Dental calculus	Shotgun metagenomic	[115]
Brzeg	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Dzieskanowice	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Gniezno	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Goluń	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Końskie	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Kowalewko	Poland	100-400 CE	teeth	shotgun metagenomic	[116]
Ląd	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Łęgowo	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Markowice	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Maslomęcz	Poland	100-400 CE	teeth	shotgun metagenomic	[116]
Niemcza	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Oblaczkowo	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Opole	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Ostrów Lednicki	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Płońsk	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Poznań-Śródka	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Sowinki	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Warszawa	Poland	900-1400 CE	teeth	shotgun metagenomic	[116]
Bushman Rock Shelter (BRS) in Limpopo Province	South Africa	1460 CE	paleo-faecal sample	Shotgun metagenomic	[117]
western Belize: Chan Chich	Belize	713-885 CE	Dental calculus	Shotgun metagenomic	[118]
western Belize: Chan	Belize	170 BCE-50 CE	Dental calculus	Shotgun metagenomic	[118]

Lu Maccioni (Alghero)	Italy	1126-825 BCE	Dental calculus	Shotgun metagenomic	[118]
Capo Pecora (Arbus)	Italy	1384-936 BCE	Dental calculus	Shotgun metagenomic	[118]
Perdalba (Sardara)	Italy	1400–850 BCE	Dental calculus	Shotgun metagenomic	[118]
Riga	Latvia	1356 CE	Latrine sediment	Shotgun metagenomic	[119]
Jerusalem	Israel/State of Palestine	1400-1500 CE	Latrine sediment	Shotgun metagenomic	[119]
Ebishima shell mound in Iwate prefecture	Japan	1000-400 BCE	Dental calculus	Shotgun metagenomic	[120]
Ikawazu, Aichi prefecture,	Japan	1000-400 BCE	Dental calculus	Shotgun metagenomic	[120]
Miyano, Iwate prefecture	Japan	1000-400 BCE	Dental calculus	Shotgun metagenomic	[120]
Ikenohata-Shichikencho site, Taito-ku, Tokyo	Japan	1000-400 BCE	Dental calculus	Shotgun metagenomic	[120]
Riga	Latvia	1500-1700 CE	Dental calculus	Shotgun metagenomic	[121]
Cesis	Latvia	1600-1700 CE	Dental calculus	Shotgun metagenomic	[121]
Kuldiga	Latvia	1500-1700 CE	Dental calculus	Shotgun metagenomic	[121]
Jelgava	Latvia	1500-1700 CE	Dental calculus	Shotgun metagenomic	[121]

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826 **Figure 1. Geographical localization of ancient human remains analyzed for**
 827 **microbiota studies in the World.** Colour indicate the dating of the analyzed
 828 samples from 6000 BCE to 1918 CE. Oral microbiota and gut microbiota studies are
 829 indicated with two symbols representing a tooth and the gut respectively. The
 830 corresponding references are listed in Table 1. Map generated with QGIS software
 831 [122]. In the upper part of the figure, 6 pictures representative of the original samples
 832 used to study the digestive tract microbiota [39,63,70,109,113,116].



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