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Reconstructing Neandertal behavior, diet, and disease using ancient DNA

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Abstract

Recent genomic data has revealed multiple interactions between Neandertals and humans, but there is currently little genetic evidence about Neandertal behavior, diet, or health. We shotgun sequenced ancient DNA from five Neandertal dental calculus specimens to characterize regional differences in Neandertal ecology. At Spy, Belgium, Neandertal diet was heavily meat based, and included woolly rhinoceros and wild sheep - animals characteristic of a steppe environment. In El Sidrón, Spain, no meat was detected in the dental calculus, but dietary components including mushrooms, pine nuts, and moss reflected forest gathering. Differences in diet were also linked to an overall shift in the oral bacterial community (microbiota) in Neandertals, suggesting that meat consumption contributed to significant variation between Neandertal microbiota. Evidence for self-medication was identified in one El Sidrón Neandertal with a dental abscess, who also likely suffered from a chronic gastrointestinal pathogen (*Enterocytozoon bieneusi*). Lastly, we characterized a nearly complete genome of the archaeal commensal *Methanobrevibacter oralis* in Neandertals – the oldest draft microbial genome generated to date at ~48,000 years old (10.2 depth). DNA preserved within dental calculus represents an important new resource of behavioral and health information for ancient hominid specimens, as well as a unique long-term study system for microbial evolution.

Main Text:

Neandertals remain one of our closest extinct hominid relatives, and have been shown to have co-existed and occasionally interbred with anatomically modern humans (AMHs) across Eurasia in the Late Pleistocene. Neandertals went extinct in Europe around 42,000 years ago, while the extinction process across the rest of Eurasia is less clear (*1*, *2*). Isotopic and archaeological data through the last glacial cycle (~120-12,000 years ago) suggests that Neandertals were as carnivorous as polar bears or wolves (*3*, *4*), with a diet heavily based on large terrestrial herbivores, such as reindeer, woolly mammoth, and woolly rhinoceros (*4*, *5*). In contrast, microwear analysis of tooth surfaces from Neandertals in different ecological settings, suggests diets were likely guided by local food availability (*6*). Furthermore, phytoliths and proteins preserved in calcified dental plaque (calculus) indicate Neandertal diets included many plants, including some potentially cooked prior to consumption or used for medicinal purposes (*7*, *8*). As a result, Neandertal diet and behavior remains a topic of considerable debate, with important questions remaining about the specific animals and plants actually consumed, or potential impacts on Neandertal health or disease.

While genomic studies continue to reveal evidence of interbreeding between AMHs and Neandertals across Eurasia (*9*), little is known about the health consequences of these interactions. The genetic analysis of Neandertal dental calculus represents an opportunity to examine this issue, and to reconstruct Neandertal diet, behavior, and health (*10*, *11*, *12*). Here, we report the first genetic analysis of dental calculus from five Neandertals from El Sidrón cave in Spain (n=2), Spy cave in Belgium (n=2) and Breuil Grotta in Italy (n=1), alongside a historic chimpanzee (n=1) and a modern human (n=1) for comparison (Table S1). To provide increased

91 resolution of Neandertal diseases, we also deeply sequenced dental calculus from the
92 best preserved specimen, El Sidrón 1 (>147 million reads), which was also suffering
93 from an dental abscess (*13*).
94 Recent reports have identified significant size-based amplification biases
95 when amplicon approaches are applied to ancient microbial DNA (*i.e.* when using
96 16S ribosomal RNA (rRNA) amplicon sequencing of ancient bacterial taxa) (*14*).
97 Hence, we examined the Neandertal dental calculus specimens using both Illumina
98 16S rRNA amplicon (V4 region) (*15*) and metagenomic shotgun sequencing
99 approaches. Blank control samples (EBCs) were sequenced to monitor contamination,
100 and a stringent filtering strategy was applied to remove contaminant sequences in
101 both datasets using QIIME (amplicon) or MEGAN5 (shotgun) (*16*, *17*). The
102 Neandertal sample from Breuil Grotta failed to produce amplicons, and was removed
103 from downstream analysis. As expected, the amplicon datasets were not
104 representative of the biodiversity revealed by shotgun sequencing (Figures S3-S4,
105 S12, and S15-S16; Tables S2 and S7), and clustered together phylogenetically
106 irrespective of sample age, while containing disproportionately large proportions of
107 non-oral and environmental contaminant microorganisms (Figure 1, Table S2 and
108 S7). Clearly, amplicon data from ancient specimens can be biased by DNA damage
109 leading to preferential amplification of contaminating modern DNA and/or
110 prokaryotic sequences with short ribosomal sequences (*14*). As a result, the
111 metagenomic shotgun sequencing approach was used to analyze the Neandertal
112 specimens.
113 The shotgun datasets consisted of short DNA fragments (<70 bp) which
114 complicated accurate bacterial species identification using standard software, such as
115 MG-RAST and DIAMOND (Figure S14) (*17*, *18*). To circumvent this problem, we

116 used a novel metagenomic alignment tool that rapidly identifies species from shorter
117 fragment lengths using a BLASTX like algorithm (MEGAN ALignment Tool with
118 BLASTX-like alignments; MALT-X) (*19*). After benchmarking the tool (Figure S8-
119 S10 and S14) (*17*), we applied MALTX to the shotgun datasets and checked that the
120 bacterial diversity accurately matched the subset of 16S rRNA fragments they
121 contained (which are typically utilized to identify bacterial taxa); we also successfully
122 repeated this test for two previously published ancient calculus datasets (Figure 1 and
123 S13; Table S6) (*20*). Bioinformatic filtering was applied to remove laboratory and
124 environmental contaminants from the samples, revealing that the Spy Neandertals
125 were the most heavily impacted by environmental contamination (Figure S15-S17;
126 Table S7) (*17*). Indeed, Spy I clustered within the biodiversity observed in the modern
127 sample (Figure 1), contained high diversity analogous to environmental samples
128 (Figure S20), and presented DNA damage patterns characteristic of modern
129 contamination (Figure S22), so was therefore discarded from further analyses (*17*).
130 The three robust Neandertal datasets (El Sidrón 1, El Sidrón 2, and Spy II) contained
131 on average 93.76% bacterial, 5.91% archaeal, 0.27% eukaryotic, and 0.06% viral
132 identifiable sequences, similar to previously published biodiversity of human dental
133 calculus (Figure 2A and S17) (*12*). In addition, the six dominant bacterial phyla in the
134 modern human mouth (Actinobacteria, Firmicutes, Bacteroidetes, Fusobacteria,
135 Proteobacteria, and Spirochaetes) were also dominant in each of the Neandertals, with
136 an average of 222 bacterial species per individual (Figure 2A and S17) (*17*).
137 We first examined Neandertal diets using the eukaryotic diversity preserved
138 within dental calculus, after filtering spurious results (*17*). Calculus from the Spy II
139 individual contained high numbers of reads mapping to rhinoceros (*Ceratotherium*
140 *simum*) and sheep (*Ovis aries*), as well as the edible 'grey shag' mushroom

141 (*Coprinopsis cinerea*) (Table 1). Bones of woolly rhino and mouflon sheep were
142 present in Spy Cave along with other mammalian fauna (*21*, *22*), and both species are
143 thought to have been part of the Neandertal diet at Spy and the nearby site of La
144 Naulette (*23*). The dietary profile for El Sidrón Neandertals was markedly different,
145 and contained no sequences matching large herbivores – no animal bone bearing
146 contexts were associated with the recovery of the El Sidron Neanderthal remains.
147 However, reads mapping to edible mushrooms ('split gill'; *Schizophyllum commune*),
148 pine nuts (*Pinus koraiensis*), forest moss (*Physcomitrella patens*), and poplar
149 (*Populus trichocarpa*) were identified (Table 1). Sequences mapping to plant fungal
150 pathogens were also observed (*Zymoseptoria tritici, Phaeosphaeria nodorum,*
151 *Penicillium rubens,* and *Myceliophthora thermophile*), suggesting the El Sidrón
152 Neandertals may have been consuming molded herbaceous material. The intracellular
153 eukaryotic pathogen, *Enterocytozoon bieneusi,* which causes severe diarrhea (*24*),
154 was also observed in El Sidrón 1, a young male that also had a dental abscess and
155 may have used bitter medicinal compounds for self-medication (*25*). Our findings
156 support earlier suggestions of self-medication, as he was the only individual whose
157 calculus contained the natural antibiotic producing *Penicillium*, and poplar, whose
158 bark, roots, and leaves contains the natural pain killer, salicylic acid (i.e. the active
159 ingredient in aspirin) (*26*).
160 To examine how the oral microbial patterns in Neandertals were linked to
161 dietary composition, we compared the data to a wide range of dental calculus
162 specimens from ancient humans with varying diets, including ancient Later Stone Age
163 (LSA) African gatherers; African Pastoralist Period individuals with high meat
164 consumption (*27*); European hunter-gatherers with diets that included a wide range of
165 protiens; and early European farmers with diets largely based around carbohydrates

166 and milk consumption (see SI for archaeological descriptions of dietary information)
167 (*17*). We used UPGMA to cluster Bray Curtis distances obtained by comparing
168 shotgun sequenced oral microbiota at an equal depth, and revealed four distinct
169 groups: foragers with limited meat consumption (El Sidrón Neandertals, chimpanzee,
170 and LSA African gatherers); hunter-gatherers or pastoralists with a frequent meat diet
171 (Spy Neandertal, African pastoralists, and European hunter-gatherers); ancient
172 agriculturalists (European farming individuals); and modern humans (Figure 2B).
173 This analysis identifies a split between hunter-gatherers and agriculturalists, as
174 previously observed (*11*), but also reveals two distinct hunter-gatherer clades,
175 differentiated by the amount of meat consumed in their diet. Meat consumption
176 appears to have impacted early hominid microbiota and health, similar to differences
177 observed between carnivorous and herbivorous mammals (*28*). This finding also
178 indicates that dental calculus may be used to directly infer the dietary behavior of
179 ancient humans or hominids.
180 We then examined the Neandertal microbial diversity for signs of disease.
181 Neandertal microbiota were more similar to the historic chimpanzee sample than
182 modern humans, and contained less pathogenic Gram-negative species (18.9% Gram-
183 negatives in Neandertals, compared to 77.6% in the modern human; Figure S21),
184 which are associated with secondary enamel colonization, increased plaque formation,
185 and periodontal disease (*29*). The lower levels of these immunostimulatory Gram-
186 negative taxa in Neandertals may be related to the reduced presence of Fusobacteria
187 taxa (Figure S17), as this keystone group facilitates the binding of Gram-negative
188 microorganisms to the primary colonizers that bind to tooth enamel (e.g.
189 *Streptococcus, Actinomyces* and *Methanobrevibacter* species).

190 Several oral pathogens could be identified within the shotgun data, although
191 the short ancient sequences and diverse metagenomic background complicated
192 identification. We established a number of exclusion criteria to verify the authenticity
193 of short sequences, including the assessment of ancient DNA damage, phylogenetic
194 position, and bioinformatic comparisons to differentiate close relatives (*17*).
195 Pathogens that passed the exclusion criteria included the caries-associated pathogen
196 *Streptococcus mutans*, which was identified in all Neandertals (0.08% to 0.18%)
197 (Table S10-S11). All three members of the 'red complex' pathogens associated with
198 modern periodontal disease were identified in at least one Neandertal
199 (*Porphyromonas gingivalis*: 0-0.52%; *Tannerella forsythia* 0.05-2.4%; and
200 *Treponema denticola* 0-1.87%), although all three pathogens were not identified in
201 any single individual. These oral pathogens support the isolated evidence of
202 Neandertal dental caries, periodontal disease, dental calculus, and associated tooth-
203 picking to relieve dental pain observed from specimens at Krapina (Croatia), Shanidar
204 (Iraq) and Cova Forada (Spain) (*30–32*). The microorganisms known to cause
205 exacerbated whooping cough infections (*Bordetella parapertusssis* and *Pasteurella*
206 *multocida*) (Table S9) were also detected in El Sidrón 1; however, only a limited
207 number of *B. parapertussis*-specific reads were identified (i.e. only 212 reads, mostly
208 in a region containing hypothetical proteins, mapped more efficiently to *B.*
209 *parapertussis* than *Bordetella petrii*, an environmental isolate). Similarly, pathogens
210 closely related to common oral flora (*Neisseria gonorrhoeae*, *Streptococcus*
211 *pyogenes*, and *Corynebacterium diptheriae*) were identified but could not be
212 unambigously distinguished from closely related commensal oral taxa (Figure S23).
213 These findings highlight the need for rigorous criteria when identifying pathogenic
214 strains from ancient metagenomic data (*17*).

215 We also examined the commensal microorganisms in Neandertals in greater
216 detail. Within the deeply sequenced El Sidrón 1 oral microbiome, we were able to
217 recover eight draft ancient microbial genomes with >1-fold depth, corresponding to
218 the most prevalent microbial taxa (Table 2). We were particularly interested in an
219 archaeal species that dominated the oral metagenome of El Sidrón 1 (14.7%; Figure
220 S17), but was present in lower proportions in the other Neandertal specimens (1.4%
221 and 1.2% in El Sidrón 2, and Spy II, respectively). The large differences in G/C
222 content between bacteria and archaea facilitated efficient read mapping of the
223 archaeal sequences (Table 2), which mapped closest to the modern human-associated
224 *Methanobrevibacter oralis* JMR01 strain. We were able to produce the first draft
225 ancient archaeal genome, *Methanobrevibacter oralis neandertalensis* (10.3-fold
226 depth; 44.7% of 2.1 Mbps; Figure 3), which is also the oldest draft microbial genome
227 to date at ~48,000 years old (*33*). The DNA damage (33% C-T; 36% G-A) and
228 fragment length distribution (average 58.67 bp) of *M. oralis neandertalensis* were
229 consistent with an ancient microbial genome (Table 2).
230 Within the *M. oralis neandertalensis* genome, 1,929 coding sequences could
231 be matched to modern human *M. oralis*, while 136 in the latter appeared to be absent
232 (6.5% of all coding sequences in *M. oralis*) (Table S16). The absent loci included
233 analogs of genes encoding antiseptic resistance (*qacE*) and those required for
234 regulation of maltose metabolism (*sfsA*), likely reflecting the impacts of modern oral
235 hygiene and relative dearth of carbohydrates in ancient Neandertal diets, respectively.
236 As expected, bacterial immunity loci were also variable, as regions encoding for
237 CRISPR Cas2 and Cas6 in modern *M. oralis* were missing from *M. oralis*
238 *neandertalensis*, and the Cas1 CRISPR system in *M. oralis neandertalensis* could
239 only be partially assembled (Table S16). The ratio of non-synonymous to

240 synonymous mutations (d_N/d_S) between translatable *M. oralis neandertalensis* protein
241 coding sequences and modern human *M. oralis* suggested that 58% were under
242 purifying selection (d_N/d_S<0.1) (Table 3) (*17*). Only 4% appeared to be under positive
243 selection (d_N/d_S>1.2), and included the conjugal transfer gene, *traB*, which aids in the
244 uptake of foreign DNA (i.e. plasmid transfer) (*34*) and *mutT*, which is involved in
245 DNA mismatch repair (*35*). These findings suggest that much of the genome is under
246 purifying selective pressure, potentially due to the conserved environmental
247 conditions in the hominid mouth over time (*36*). Overall, it appears that modern
248 human *M. oralis* genome adaptation has occurred primarily through the uptake of new
249 DNA sequences, rather than adaptive gene mutation.

250 Phylogenetic analysis of seven modern *Methanobrevibacter* genomes revealed
251 that *M. oralis neandertalensis* is sister taxa to the modern human *M. oralis* strain
252 (JMR01), and together, this clade is sister to *Methanobrevibacter smithii* (a
253 commensal found in the modern gut) (Figure 3B). Molecular dating using a strict
254 clock model indicates the divergence between *M. oralis neandertalensis* and modern
255 human *M. oralis* strains occurred around 126,000 years ago (95% highest posterior
256 density interval of 112-143K years) (Figure 3B), long after the genomic divergence of
257 Neandertals and modern humans (450-750 kyr) (*37*). This suggests that microbial
258 species were transferred during subsequent interactions, potentially during early
259 human-Neandertal interbreeding in Africa (*38*).

260 Preserved dental calculus represents an important new resource of behavioral
261 and health information for ancient hominid specimens, as well as a unique long-term
262 study system for microbial evolution. A prime example is the potential link between
263 the elevated levels of Fusobacteria taxa in modern humans and the increased diversity
264 of Gram-negative immunostimulatory taxa. The latter are strongly associated with a

265 wide range of modern Western diseases, revealing the potential power of ancient

266 DNA to inform modern medical research.

267

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365 **Figures and Legends**

366 **Figure 1: Comparison of 16S amplicon and shotgun datasets obtained from**
367 **ancient, historic, and modern dental calculus samples.** Filtered and unfiltered 16S
368 rRNA amplicon and shotgun datasets, as well as 16S rRNA shotgun sequences
369 identified using GraftM, were compared using UPGMA clustering of Bray Curtis
370 distances from a chimpanzee (red), Neandertals (El Sidrón 1 (green), El Sidrón 2
371 (light green), Spy I (grey), Spy II (blue)), and a modern human (orange).

Figure 2: Bacterial community composition at the phyla level of oral microbiota from chimpanzee, Neandertal, and modern human samples. Oral microbiota from shotgun datasets of a wild-caught chimpanzee (A), Neandertals (n=3; B), and a modern human (C) are presented at the phyla level. Phyla names were simplified for clarity, and unidentified reads were excluded. Gram-positive (blue) and Gram-negative (red) phyla are differentiated by color. (D) UPGMA clustering of Bray Curtis values obtained from 22 oral rarefied metagenomes is displayed. Definitions for abbreviations can be found in the SI.

Figure 3: 48,000 yBP archeal draft genome and phylogeny of *Methanobrevibacter oralis neandertalensis*.

(A) Ancient sequences mapping to *Methanobrevibacter oralis* JMR01 are displayed in a Circos plot (black), alongside the depth of coverage obtained (red). The reference sequence is displayed (grey) with the GC content of the reference sequence calculated in 2500 bp bins (green). (B) A Methanobrevibacter phylogeny was constructed from whole genome alignments in RAxML with 100 bootstraps. The date for the split between *M. oralis* strains and *M. smithii* strains was calculated from whole genomes in BEAST using a strict clock model.

Table 1: Dietary information preserved in calculus.

DNA sequences mapping to eukaryotic species are shown as a proportion of the total eukaryotic reads identified within each sample. Eukaryotic sequencing identified in the extraction blank controls and the Spy I Neandertal, which is heavily contaminated with modern DNA, are shown to the right.

Table 2: Draft microbial genomes present in El Sidrón 1.

Eight draft microbial genomes were obtained from the deeply sequenced El Sidrón 1 specimen by mapping with ancient DNA parameters using bwa aln. The sequences coverage, GC content, sequencing depth, and damage profile (average fragment length and base pair modifications calculated from MapDamage v2) are displayed for each genome.

Table 3: Purifying and positive selection in *M. oralis neandertalensis.*

The ratio of nonsynonymous to synonymous (d_N/d_S) mutations was calculated for coding regions with sufficient coverage and that were conserved between *M. oralis* and *M. oralis neandertalensis.* Genes that have undergone definite purifying (d_N/d_S = 0) or positive selection (d_N/d_S > 1.2; grey) are displayed if the function of the gene was annotated. Hypothetical proteins and those not matching to the *M. oralis* genome during BLAST searches are not shown.

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Author Contributions

AGM, KWA, DC, VD, MF, MF, NG, WH, KH, KH, PH, JK, CLF, MR, AR, PS, AS, DU, and JW provided samples and interpretations of associated archaeological goods; LSW, KD, and AC designed experiments; LSW performed experiments; LSW, SD, EH, JS, BL, JB, LA, and AGF performed bioinformatics analysis and interpretation on the data; DHH developed bioinformatics tools; NG, JK, and GT analyzed medical relevance of data; LSW and AC wrote the paper; and all authors contributed to editing the manuscript.

Author Information

Raw and analyzed datasets and the scripts utilized for this analysis are available in the Online Ancient Genome Repository (OAGR) (currently available at: https://www.oagr.org.au/experiment/view/16/?token=9LCF0GKSL7DHO3FPR4YBSZCGYD0ASJ). The Australian Research Council supported this work, and the authors declare no competing financial interests. Requests for materials should be addressed to laura.weyrich@adelaide.edu.au.