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Combined metagenomic and archaeobotanical analyses on human dental calculus: A cross-section of lifestyle conditions in a Copper Age population of central Italy

Alessandra Modi [a], Davide Attolini [b,1], Valentina Zaro [a], Lisa Pisaneschi [b], Gabriel Innocenti [a,c], Stefania Vai [a], David Caramelli [a], Jacopo Moggi Cecchi [a], Andrea Quagliariello [c,**], Marta Mariotti Lippi [b], Martina Lari [a,*]

[a] Department of Biology, University of Florence, Via Del Proconsolo 12, Florence, Italy
[b] Department of Biology, University of Florence, Via La Pira 4, Florence, Italy
[c] Department of Comparative Biomedicine and Food Sciences (BCA), Agripolis, University of Padua, Padua, Italy

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ABSTRACT

Multidisciplinary analyses on ancient dental calculus revealed the possibility to reconstruct habits and diet of ancient human populations, investigate individual health status, as well as provide information on past environments. In the present study we applied both metagenomic and microscopic analysis on ancient human dental calculus in order to obtain a cross-section of the life conditions in a population of central Italy belonging to the Copper Age culture of Rinaldone (IV millennium BCE). The metagenomic profile suggested an agricultural subsistence and a dietary regimen particularly enriched in complex carbohydrates with low soluble fiber. Even bacterial functional profile seems to indicate an almost exclusive carbohydrates intake that could have favoured the occurrence of nutritional stress in the individuals. Exploring the diversity of the plant food consumed, we detected direct evidence of cereals such as wheat and/or barley, and found signals of the use of leaf vegetables, thus providing additional information on human/environment relationship. The presence of oral pathogens, even if at low abundance (<0.1%), can be related to the high consumption of carbohydrates and finds correspondence with the palaeopathological evidence. In conclusion, starting from very minute amounts of ancient dental calculus, our molecular and microscopic analysis jointly provided complementary data in support of past life condition reconstruction in ancient human populations.

1. Introduction

In the last decades, the analyses of dental calculus have provided increasing information about diet, lifestyle, habits, health state, and hygiene of ancient populations. Theoretically, dental calculus contains witnesses of food and all the materials that are introduced in the oral cavity, including inhaled and/or ingested environmental debris and food particles (Radini et al., 2017), as well as biomolecules associated with the oral microbiota and the host (Adler et al., 2013). Usually, calculus does not refer to the entire duration of life but provides data that concern the last years before the death of the individual, an undetermined period depending on numerous factors which are related to the characteristics of the individual and the environmental context where he lived (Nancollas and Johnsson, 1994; Lieverse, 1999). However, calculus could represent a lifelong record in archaeological specimens, due to poor and ineffective oral hygiene activities in the past populations compared to modern days (Warinner et al., 2015).

Regarding food plants, studies for checking how accurately dental calculus records plant consumption evidenced that the amount of microremains from different plants does not reflect their importance in the diet, and not all of the plants are recorded at an individual level; therefore, it is necessary to analyse samples from a high number of individuals for a more accurate reconstruction of plant consumption (Leonard et al., 2015). Numerous factors may control the preservation of

* Corresponding author.
** Corresponding author.
E-mail addresses: andrea.quagliariello@unipd.it (A. Quagliariello), martina.lari@unifi.it (M. Lari).
[1] Present address: Department of Antiquities, Philosophy, History (DAFIST), University of Genoa, Via Balbi 2, Genoa, Italy.

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plant remains (primarily phytoliths and starch grain) in the calculus. An irrefutable reason lies in the fact that not all plants produce phytoliths or have starch deposits in the edible portions. In addition, different starch grains could present different susceptibility to the attack of salivary amylase (Leonard et al., 2015), while the size and shape of phytoliths seem to affect their probability to be included in the dental calculus (Lalueza-Fox et al., 1996).

The analysis of dental calculus often results in a list of plants that have different ecological requirements. During the nomadic lifestyle of the Palaeolithic populations, the diet may be considered as a reliable reflection of the natural environments where they lived, except for portable foods, for example flour (Aranguren et al., 2007). Starting from the Neolithic, the cultivation of the major cereals spreads throughout Europe wherever possible, and cereals may reach an even greater diffusion through commerce. These cultivated plants, which are the basis of human nutrition, are often the most represented in the calculus, but they provide quite limited information on environmental conditions, indicating that they could be cultivated there, but not whether they were able to survive in nature. Even if in smaller amounts, the calculus may also contain evidence of other plants that are part of the local diet. These plants are those able to provide useful information for the reconstruction of the environment. Unfortunately, these plants are often consumed in limited quantities or only seasonally, and the edible parts do not always contain distinguishable starch grains or phytoliths. As result, they are hardly detectable.

More recently, molecular analysis has shown that dental calculus retains an excellent record of the human oral microbiome (Adler et al., 2013; Warinner et al., 2014; Weyrich et al., 2017). Thanks to the growing recognition of the strict relationship between human microbiota and numerous variables (mainly related to disease, diet and lifestyle) we have today the opportunity to use this information to study health status and living conditions in past populations (Adler et al., 2013; Warinner et al., 2014; Weyrich et al., 2017). Additionally, despite DNA within ancient calculus being dominated by microbial DNA (>99%), a very small proportion is derived from other sources, including dietary consumption. For this reason, ancient DNA (aDNA) from dental calculus has the potential to expand our ability to detect the diversity of food consumed in the past. Nevertheless, due to the extremely low proportion of eukaryotic vs microbial DNA in ancient dental calculus, the genetic analysis of dietary components is challenging and methodological approaches has not been adequately validated (Mann et al., 2020).

In this paper, dental calculus was analysed with an integrated approach in order to contribute to the knowledge on dietary practise, health status and humans/environment relationship in the Italian Copper Age. The analysis of the preserved plant microremains was used to directly detect evidence of plant consumption, while ancient DNA sequencing was applied to both reconstruct oral microbiome of individuals and identify genetic records from putative dietary plants. Due to the little presence of dental calculus in the samples (Table S1) we took advantage of a combined protocols to both recover microremains and extract DNA from the same fragment of dental calculus (Modi et al., 2020).

2. The site and the environmental context

The Eneolithic necropolis of Ponte San Pietro (Ischia di Castro) is located in Northern Tuscia (province of Viterbo), close to the Tuscany/Latium border (Fig. 1), in the core area of the Copper Age culture of Rinaldone (3980–2000 cal BCE) (Manfredini et al., 2009; Dolfini, 2010; Negroni Catacchio and Aspesi, 2013; Negroni Catacchio et al., 2014; Bernardini et al., 2021), which extends between the provinces of Grosseto and Viterbo. The necropolis (IV millennium BCE) lays on a tuff lope facing the middle course of the Fiora river, near the confluence of the Olpeta river originating at Lago di Mezzano. It consists of two groups of 13 and 12 burials respectively, which were further divided into subgroups based on the social organization of the local community. The rock tombs are artificial grottos containing collective burials, often with secondary depositions (Negroni Catacchio et al., 2014). The presence of calcareous deposits at the bottom of the rock chambers favoured the preservation of skeletal remains that were attributed to at least 43 individuals (Negroni Catacchio et al., 2014).

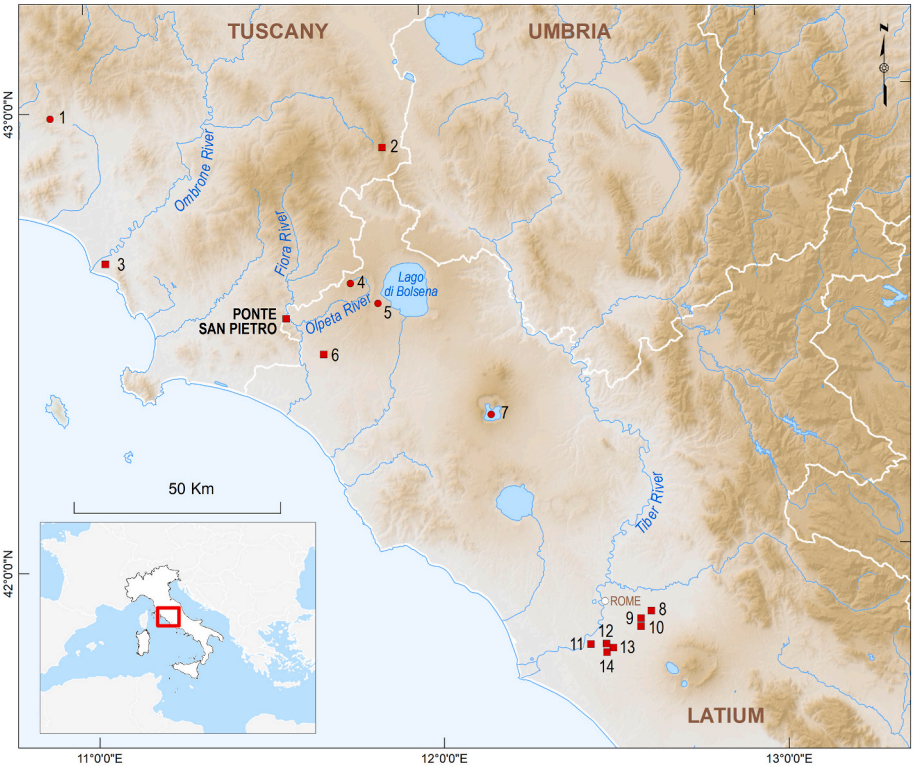

Fig. 1. Ponte San Pietro and other sites mentioned in the text: 1-Lago dell'Accesa; 2-Belverde; 3- Grotta dello Scoglietto; 4- Lago di Mezzano; 5- Lagaccione; 6- Poggio Olivastro; 7- Lago di Vico; 8- Casetta Mistici; 9- Piscina di Torre Spaccata; 10- Osteria del Curato-via Cinquefrondi and Romanina; 11- Torrino-Mezzocammino; 12-Casale Massima; 13-Torre Pagnotta; 14- Torre della Chiesaccia. Squares indicate archaeological sites, circles indicate off-sites sampling points. Map was generated with Esri ArcGIS 10.7.

The Middle Fiora Valley was subjected to intensive human frequentation since the Palaeolithic, as attested by the numerous sites in the plain and the slopes (Fig. 1). During the Neolithic, the settlements were mainly distributed in the plain, while starting from the Eneolithic the sites were preferentially located on the high grounds and they mainly consisted of necropolis (Tagliabue et al., 2010).

Numerous Sites of Community Importance (SCI) are currently in the territory of the N Tuscia, characterized by peculiar fauna and flora. In particular, the "Sistema fluviale Fiora-Olpeta" (River system Fiora-Olpeta, IT6010017*) is an ecological corridor between inland and coast. Other sites, as "Selva of Lamone," and "Mountains of Castro", are characterized by well-preserved mixed oak forests. The riparian mixed forest near Ponte San Pietro is dominated by *Quercus robur* (common oak), *Ulmus* spp (elm) and *Fraxinus* spp (ash). *Alnus glutinosa* (black alder), *Salix* (willow) and *Populus* spp (poplar). are frequent along the riversides. Crop fields occur at the basis of the slopes. Information about the past plant landscape and vegetation history of the area comes from paleobotanical studies carried out at Lago di Mezzano (Sadori, 2018), Lagaccione, near Lago di Bolsena (Magri, 1999) Lago di Vico (Magri and Sadori, 1999) and Lago dell'Accesa (Drescher-Schneider et al., 2007) (Fig. 1). The data indicate a general dominance of deciduous oak forests during the Holocene. Changes in the relative amount of sub-Mediterranean and Mediterranean species or the diffusion of montane species such as *Fagus* (beech) and *Abies* (fir) followed climatic oscillations. Increasing dryness and temperature favoured the diffusion of the Mediterranean species and caused a decrease of montane trees, which was also observed along the coast of Tuscany (Menozzi et al., 2002; Bellini et al., 2009). The occurrence of periods characterised by different rainfall regimes caused alternating decreases and roses of the Lago di Mezzano level (Ramrath et al., 2000).

Regarding the influence of human activity, signals of pasturing and/or firing are provided by microcharcoals and pollen at Lago dell'Accesa starting from the Early Neolithic. At the same site, Cerealia type pollen was discontinuously recorded; the curve starts to be quite continuous at approximately 2600 BCE, suggesting the establishment of crop cultivation (Drescher-Schneider et al., 2007). Cereal pollen was also recorded at Lago di Mezzano (Sadori, 2018), Lagaccione (Magri, 1999), and, later, at Lago di Vico (Magri, 1999). Eneolithic was a period of technological innovations, characterised by the invention of numerous tools that led to different land management and agricultural practices, in addition to social transformations. Nevertheless, carpological remains do not show substantial changes in cereal cultivation compared to Neolithic (Zohary and Hopf, 2000; Carra, 2012). Barley and hulled wheat species (mainly emmer and secondarily einkorn) prevail on the millets (*Panicum miliaceum*, broomcorn millet, and *Setaria italica*, foxtail millet); pulses are scarce. Also, wild fruits were gathered, with an increase of *Cornus mas* (cornelian cherry) fruits. It is to underline, however, that many of the Eneolithic sites, at least in N Italy, are situated on dry ground where the preservation of plant remains is very poor (Rottoli and Castiglioni, 2009).

Regarding the Rinaldone culture, it is principally known through funerary contexts, as Ponte San Pietro (Tagliabue et al., 2010). As a consequence, only a small number of settlements provides information about the lifestyle, plant exploitation and dietary habits of the population. Archaeological, geological and palaeobotanical studies at Poggio Olivastro (VT), a Neo-Eneolithic settlement in the Southern Maremma of Latium (about 15 km SE from Ponte San Pietro), showed a strong connection among rainfall regime, travertine deposition and human activity (Cerilli et al., 2011). The archaeobotanical analysis revealed the presence of cereals (*T. monococcum*, einkorn wheat, *Hordeum*, barley), pulses (*Lathyrus* sp., grass pea, *Lens* sp., lentil), and *Vitis* (grape vine) (Bulgarelli et al., 1993).

A lot of data come from the archaeobotanical studies of the Neo-Eneolithic settlements located at SE of Rome, in central Latium, which offer a witness of everyday life, including the use of food plants. Cereals are the most abundant plant remain, reaching 99% of the total seeds/fruits at Piscina di Torre Spaccata (Celant, 2020), 94% in a silo at Osteria del Curato-via Cinquefrondi and the totality in a fireplace of the same site (Anzidei et al., 2007a; Celant et al., 2020); few caryopses were also found at Torre della Chiesaccia 2 (Celant, 2020). Most of the cereal remains belongs to *Triticum dicoccum* Schübl. (emmer), followed by *T. monococcum* L. and *Hordeum vulgare* L.; one caryopsis from Torresina belongs to *T. aestivum/durum* (naked wheats) (Celant, 2020). Pulses are sporadically recorded: two *Lathyrus* sp. (pea) seeds at Piscina di Torre Spaccata (Costantini and Biasini, 1984), two seeds of *Vicia faba* L. (faba bean) at Torre della Chiesaccia 2 (Celant, 2020) and seeds of *Vicia* sp. from an oven at Osteria del Curato-via Cinquefrondi (Anzidei et al., 2007b; Celant et al., 2020). In addition, a caryopsis of *H. vulgare* and cereal chaff were found inside the ceramic paste of pottery at Romanina; impressions of cereal caryopses, mainly *T. dicoccum,* and pulses cf. *Pisum* were observed on the surface of ceramic artefacts from Casetta Mistici, Torrino-Mezzocammino 1, Torre della Chiesaccia 2, Casale Massima and Osteria del Curato-via Cinquefrondi (Celant, 2020). Other sporadic findings are an uncharred grape pip at Casetta Mistici-via Esperide; two charred endocarps of *Olea europaea* (olive) at Torre Pagnotta and Osteria del Curato-via Cinquefrondi; a kernel of *C. mas* at Casetta Mistici (Celant, 2020).

In Tuscany, the carpological data from the Eneolithic/Bronze Age site of Belverde (SI) indicate the presence of cereals (including naked wheat and barley), millets (*P. miliaceum*), a few pulses (*Pisum* sp., *V. faba*), *C. mas* and *Vitis vinifera* (Bellini et al., 2008). Plant microremains from dental calculus of individuals of the Final Copper-Early Bronze Age burial site of Grotta dello Scoglietto (GR) also indicate the possible consumption of cereals, including *Hordeum/Triticum*, *Avena* (oat) and millets (Mariotti Lippi et al., 2017).

More recently, stable carbon and nitrogen isotope analysis on human and animal bones suggested an important consumption of vegetal proteins, including Fabaceae, in central and southern Italian Copper Age sites (Bernardini et al., 2021).

3. Materials and methods

3.1. Sampling, DNA extraction and sequencing

The dental calculus was collected from 11 individuals buried in 7 tombs of the necropolis of Ponte San Pietro (VT) belonging to the Rinaldone culture and dated between 3750 and 3104 BCE (Negroni Catacchio et al., 2014). Samples were designated with the "PsP" prefix followed by the catalogue number of the skeletal element (Table S1). As reported in previous palaeopathological assessment (Negroni Catacchio et al., 2014), small amounts of dental calculus were present in the selected individuals (Table S1). To verify the occurrence of possible contamination from plant material in analysed ancient dental calculus, control samples were collected from skulls or mandibles surface (Mariotti Lippi et al., 2017) (Table S1).

The calculus samples were collected using a scalpel, stored in 2 mL sterile DNA LoBind tubes (Eppendorf) and treated as described in Modi et al. (2020) (procedure A) in order to simultaneously extract DNA and recover microremains. Briefly, samples were washed with 0.5 M EDTA solution to remove the environmental contaminants, crushed with a sterile micropestel and then digested overnight at 37 °C with 1 mL of extraction buffer (0.45 M EDTA, 0.25 mg/mL proteinase, 0.05% Tween 20). After centrifugation, DNA was extracted from the supernatant as reported in Dabney et al. (2013) and the residual pellet was used for plant microremains analysis. DNA was also extracted from 500 microliters of distilled water recovered after washing the surface of teeth and maxilla in samples PsP_6408 and PsP_6416 (control samples). Twenty microliters of each extract were converted in double-stranded DNA genetic library. Sample-specific barcodes were added to each library via ligation after 10 cycles of PCR prior to sequencing (Meyer and Kircher, 2010) on HiSeq Illumina platform in paired-end mode(2x150 + 8+8 cycles). Rigorous standards were applied to prevent laboratory

contamination (Gilbert et al., 2005; Knapp et al., 2012). Pre-PCR procedures were performed in separate cleanroom facilities, and blank controls were processed in each experimental step. All surfaces and instruments were decontaminated before use with bleach and UV light.

3.2. Bioinformatics analyses

After quality control of the sequenced reads with FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), preprocessing of raw data was performed using EAGER pipeline (Peltzer et al., 2016). Adapters were trimmed and paired-end reads were merged into single sequences in order to exclude all the sequences derived from molecules longer than 100 bp using Clip&Merge v1.7.4 (Peltzer et al., 2016). Only reads with a minimum length of 30 bp were retained. Filtered reads were initially mapped to the human reference genome (Hg19) using BWA v. 0.7.10 (Li and Durbin, 2009) with seeding disabled (-l 16500) and edit distance increased (-n 0.01) (Schubert et al., 2012). Non-human reads were screened for microbiome composition using metaBIT metagenomic pipeline (Louvel et al., 2016) setting default parameters. MetaPhlAn2 was selected as reference database that includes approximately 1 million of marker genes belonging to bacteria, archea, viruses and eukaryotic microbes (Truong et al., 2015). To test whether the microbiome profile could be originated from different sources of human microbiome and/or other exogenous sources, samples were analyzed with SourceTracker (Knights et al., 2011). Comparative metagenomic dataset (Louvel et al., 2016) of possible contaminants, consisted of 704 samples from mouth (n = 382), skin (n = 11), nose (n = 81), vagina (n = 45) and stools (n = 127) published in Human Microbiome Project Consortium (2012), and from soil (n = 15) (Der Sarkissian et al., 2014). Comparative samples were processed following the same pipeline as the calculus samples. The authenticity of the microbial profile was then assessed by analysing the degradation patterns with MapDamage2.0 (Jonsson et al., 2013).

For samples that showed an authentic ancient oral microbiome signature, microbial community structure and its functional potential was analysed in more details with Kraken2 (Wood and Salzberg, 2014) against a custom database composed by complete genomes belonging to bacteria, virus, archaea, mitochondrion and plasmids from NCBI Reference Sequence (RefSeq) updated at December 2020. The selected reference genomes were then masked for low-complexity regions using Dustmasker in order to reduce the impact of spurious classification. Finally, Bracken software was used as well to normalize and get the relative abundance of the identified species (Lu et al., 2017). To authenticate the so obtained microbiome profile, samples were compared through Principal Coordinates Analysis (PCoA) with modern microbiome from different sources: oral (*i.e.*, sub-gingival and supra-gingival plaque and dental calculus), stool, skin, soil, environmental control, laboratory blanks and other published ancient dental calculus (Table S2). Modern microbiome references were analysed following the same step of analysis conducted on ancient samples.

Focusing on the microbiome of individuals from Ponte San Pietro, species showing a relative abundance higher than 0.1% were selected. This threshold was selected based on its capacity to detect more than ~85/90% of the total microbiota profile for each sample. The authenticity of the microbial profile was then assessed by align each sample to the bacterial reference of species with relative abundance >0.1%, retrieved from NCBI databse using Burrows-Wheeler Alignment (bwa) program using the aln algorithm with high stringency (-n 0.1). Consequently, the degradation patterns were analysed with MapDamage2.0 (Jonsson et al., 2013). Each aligned species, was then filtered using PMDtools (*–threshold* 1 *–requiremapq* = 30) and considered for its distance from the reference estimating the edit distance using bedtools (-tag NM) subsequently used to compute the edit distance algorithm (-Δ%) to further confirm sequence antiquity.

We compared our Eneolithic samples to ancient and modern calculus samples (27 and 10, respectively), that were retrieved from the scientific literature (Table S2). Ecological analyses on microbiome data were performed using several *phyloseq* package (McMurdie and Holmes, 2013) on R software. Beta-diversity analysis was performed using UPGMA algorithm (*phangorn* package) (Schliep, 2011) on Bray-Curtis distance at genus level and then converted into a tree class using *ape* package (Paradis and Schliep, 2019). The so constituted clusters were represented using iTOL v.4 (Letunic and Bork, 2019). DESeq2 algorithm was then used to detect differences in species abundance between modern and ancient samples (Love et al., 2014). Mann-Whitney test, corrected for Benjamini & Hochberg, was applied on HUMAnN3 (Franzosa et al., 2018) output for Eneolithic and modern samples to highlight differences in microbial metabolic pathways from metagenomic data.

Finally, additional shotgun data were generated for the two individuals that showed the highest percentage of eukaryotic identification (see Table S4) and examined for plants sequences that could be related to dietary consumption as previously reported in other studies (Warinner et al., 2014; Weyrich et al., 2017). In order to identify possible presence of DNA from food residues among our samples, Kraken2 was applied with a Viridiplantae database consisting of chloroplast and complete genome reference sequences, as suggested by Harbert (2018). To check for possible environmental contamination, reads generated from the two control samples (washing water from the surface skeletal remains) were processed following the same workflow of the reads generated from the dental calculus. Reads present in both laboratory controls (DNA extraction and library negative controls) and control samples were removed, then the most abundant species reads were matched against the respective reference genomes using bwa as previously described for bacterial species. Following recently proposed validation criteria, only eukaryotic species with more than 500 unique reads were selected for evaluation of DNA damage profiles (Mann et al., 2020). Sequences were then aligned to the respective reference and investigated for their cytosine deamination profile using PMDtools and Map Damage2.0 with the same parameters described for bacterial species.

3.3. Plant remain analysis

The pellet samples were treated with a 10% HCl solution for 12 h, washed twice in distilled water and stored in a 50% water-glycerol solution vol/vol The observation was carried out under a light microscope (l.m.) and polarizing l.m., operating at 630 × magnification.

The identification of starch grains was performed based on the morphometric features using literature (Seidemann, 1966) and reference samples from modern plants. Each starch grain or group was counted as one presence.

Phytoliths were described according to their morphology and identified on the basis of literature (Kaplan et al., 1992; Ollendorf, 1992; Rosen, 1992; Ball et al., 1993, 1996; Ponzi and Pizzolongo, 2003; Piperno, 2006; Lu et al., 2009). Nomenclature follows Madella et al. (2005) and David et al. (2019).

The amounts of starch grains and phytoliths in the samples were calculated as the number of remains in a gram of calculus. Due to the laboratory procedure that includes treatments for DNA analysis and plant remain analysis, the calculated values are merely indicative.

A surface of 3–5 cm^2 of skull bone (Table S1) per individual was washed with distilled water and brushed with a toothbrush to verify the presence of possible environmental contaminants on the material (Hart, 2011). The washing water (control samples) was observed under l.m and polarizing l.m.

4. Results

4.1. Metagenomic profiling

After excluding reads mapping on human genome (Table S3), filtered

reads were analysed with the metaBIT pipeline (Louvel et al., 2016) (Table S3). The total number of reads taxonomically assigned by MetaPhlAn2 (Truong et al., 2015) ranged from 124,100 (PsP_6417) to 101 (PsP_6732-A3) after duplicates removing (Table S3). Due to the low number of mapped reads, PsP_6732-A3 was excluded from subsequent analysis. Preliminary analysis conducted with SourceTracker showed different degree of oral microbiome preservation with respect to other contaminant microbial sources (Table S4 and Fig. S1). As observed also in previous studies, a huge percentage of detected species is of unknown source (Ottoni et al., 2019). All the tested oral species presented misincorporation and fragmentation patterns typical of authentic degraded ancient DNA (Fig. S2). Taxonomic, functional, and evolutionary aspects of the microbiome community were therefore explored in more details in 6 out of 11 samples (PsP_6405, PsP_6407, PsP_6408, PsP_6410, PsP_6416 and PsP_6417) because they showed the higher percentage of ancient oral bacteria (Figs. S1 and S2). Results of reads taxonomic identification by Kraken 2 are reported in Table S5). To highlight sample similarity with modern microbiome source (i.e., oral, stool and skin) and with plausible environmental contaminant, we performed PCoA analysis, clearly showing that all the Eneolithic samples from Ponte San Pietro (PSP) cluster with other ancient and modern dental calculus samples, together with other oral sources (Fig. 2).

As expected, more than 99.7% of identified reads belonged to bacteria, while eukaryotic DNA was in the range of 0.04–0.1% (Table S6). Cluster analysis performed on genus level using Bray-Curtis distances on the whole dataset of ancient and modern samples, reported two main clade with further sub-clusters. No clear temporal distinction can be highlighted but most part of the modern samples are located on one cluster (7/10), while ancient samples are differently distributed among clusters. It is interesting to observe that PSP samples are found in close proximity, thus suggesting a very uniform microbial composition (Fig. 3). The reasons behind this sample distribution are explained by difference in microbial relative abundance as highlighted already at high taxonomic rank (*i.e.,* phylum level) as observable in Fig. 3 barplots. The Firmicutes phylum seem to be on average more present in the first cluster, while the second one is characterized by an higher presence of Actinobacteria species. Almost all Eneolithic samples from PSP clustered together except PsP_6410, which shows a composition similar to a sample from industrial revolution, probably linked to a reduction in Actinobacteria abundance than the others. The other PSP samples fall between a hunter-gather and a Neolithic sample. This reduction of Firmicutes abundance among PSP probably affects the overall bacteria biodiversity as observable by alpha diversity analysis, where they show a low value of Shannon index (Fig. S3). At genus level, 22 bacterial genera show a statistically different distribution between ancient and modern samples (Fig. 4). Some of these bacteria show a difference in their presence/absence, while other genera just change their relative abundance between the two groups. Indeed, modern samples seem to be characterize by a lower level of some bacterial genera which were highly represented in the past such as *Olsenella, Pseudomonas, Streptomyces* and *Desulfomicrobium*, among others, while have an increase abundance of others genera like *Rothia*, *Corynebacterium, Haemophilus* and *Neisseria*. Among ancient samples, PSP show the higher incidence of *Olsenella* as recorded also from other data on Neolithic samples from South-Europe (Ottoni et al., 2021).

Focusing on PSP samples, we defined which is the core microbiome at a higher detail, at species level with a detection threshold of 0.1% and 80% of prevalence, observing that their bacterial profile is principally characterized by an elevated percentage of *Olsenella sp. Oral taxon 807*, more than in the other ancient samples, as well as *Actinomyces sp. oral taxon 414* and *Anaerolineaceae bacterium oral taxon 439* (Fig. 5). All the species that constitute the core microbiome have been analysed for their damage profile and edit distance, in order to confirm their antiquity (Table S7 and Fig. S4). Interestingly, if we consider the bacterial species below the 0.1% detection threshold used to describe the core microbiome profile, it is possible to observe the presence of two species linked to periodontal diseases: *Porphyromonas gingivalis* and *Streptococcus mutans* (Fig. S5 A and B).

These differences of the Copper Age PSP samples with respect to modern ones, are obviously reflected in the metabolic profile defined through HUMAnN3 pipeline (Fig. S6). Two different cluster are generated when compared PSP samples metabolic pathways with modern one (Fig. S6). Performing Mann-Whitney analysis on HUMAnN3 result, 248 pathways were obtained as significantly distributed between Copper Age and modern samples. Most part of nowadays pathways are underrepresented within PSP samples and only 5 of the obtained significant pathways are enriched within this group and are related to glutaryl degradation, pyruvate fermentation and C4 photosynthetic carbon

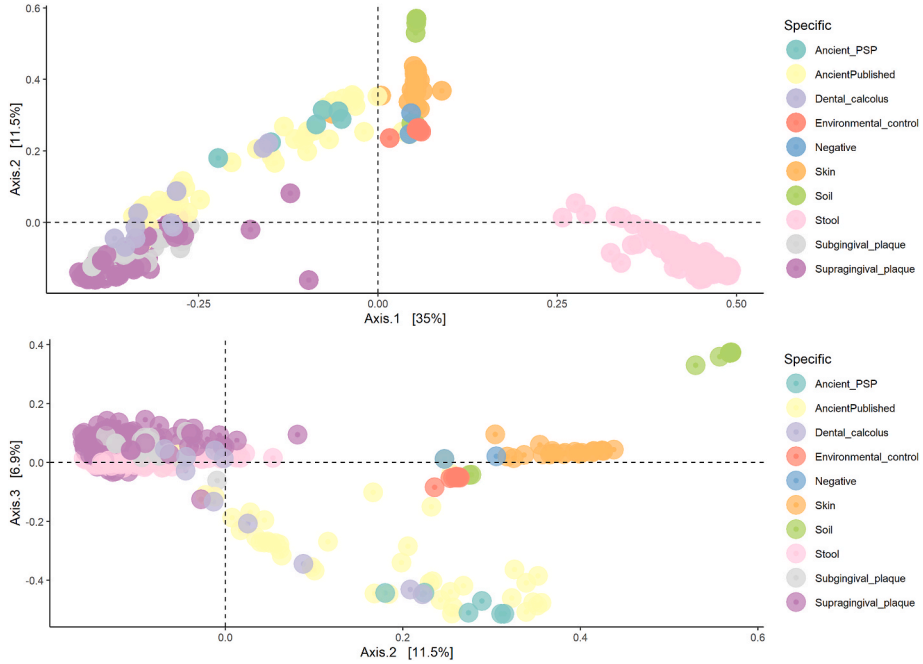

Fig. 2. PCoA analysis on Bray-Curtis distance comparing PSP with other modern and ancient microbiome sources. Full list of samples are reported in Table S2.

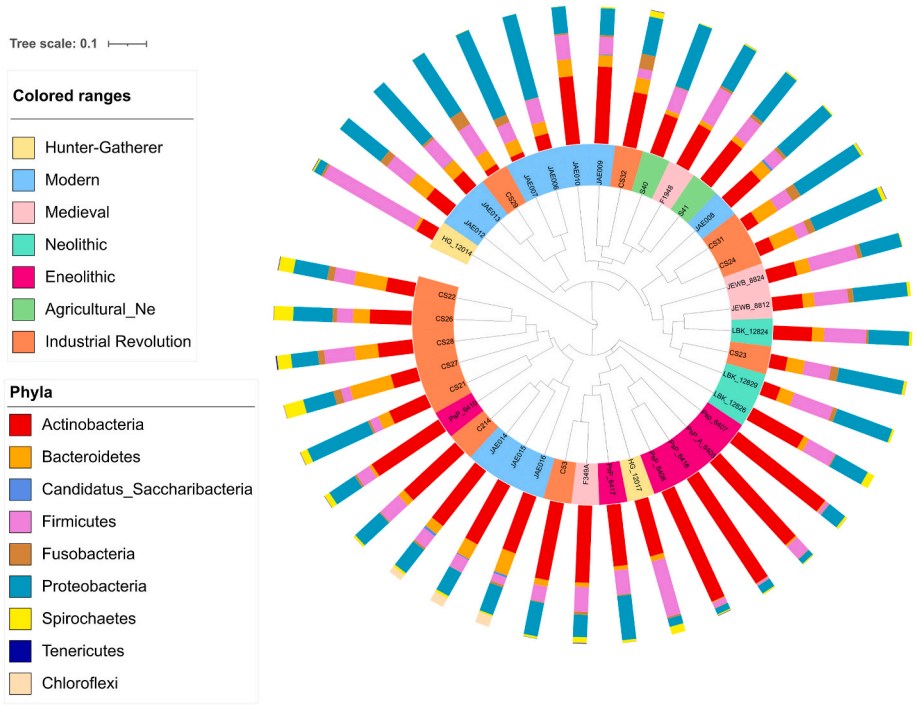

Fig. 3. Cluster analysis on Bray-Curtis distance. Samples' colours identified the historical period. Barplots located on the top of each sample report the distribution of the most abundant phyla. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

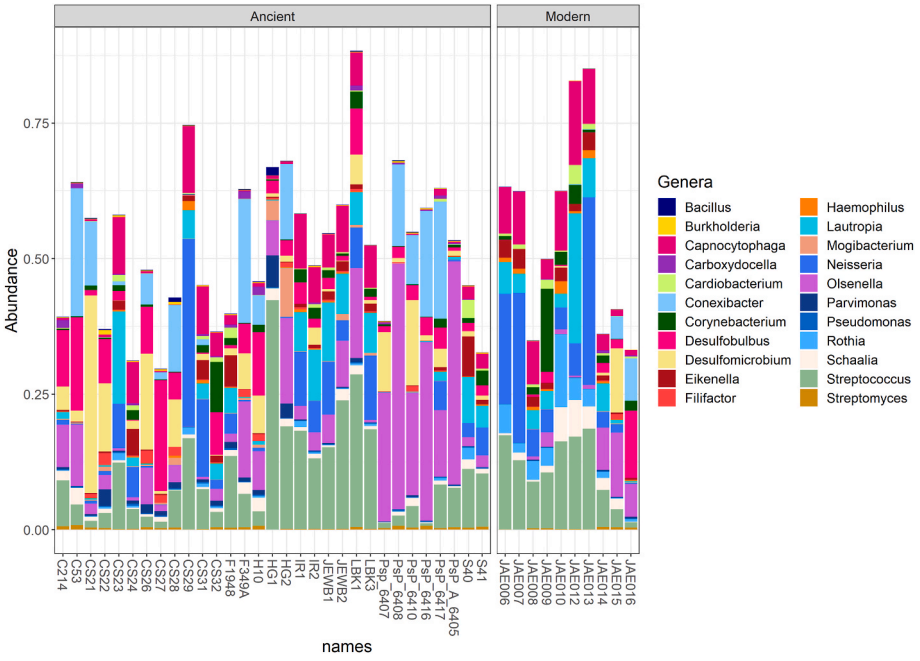

Fig. 4. Significant bacterial genera distribution as identified by DESeq2 analysis between ancient and modern samples.

assimilation (Fig. 6).

Finally, in the two individuals that were sequenced at a higher depth, it was possible to detect plausible signal of ancient DNA associated to *Brassica napus* (oilseed rape), *Beta vulgaris* (beet) and *Cynara cardunculus* (cardoon). These three eukaryotic organisms show a more than 500 unique reads (Table 1) with a good deamination profile and a peak on 1 and 2 of edit distance to the given reference (Fig. S7), as expected for damaged reads but also, possibly, for reads deriving from similar species that are not represented in the database. Notably, no clear deamination profile was observed for other species not native of Mediterranean area, even with similar abundance (Fig. S7). To exclude contamination that could affect eukaryotic identification, reads from the two negative controls processed during DNA extraction and library preparation, as well as reads from two control samples recovered from the surface of the teeth, were analysed against the same plant database. Overall, 39 and 40 plant reads were detected, respectively, in the two control samples. None of these reads was associated with the genera identified in the ancient dental calculus, while only 1 out 17 reads recognized in the negative controls matched the genus *Brassica* (Fig. S8).

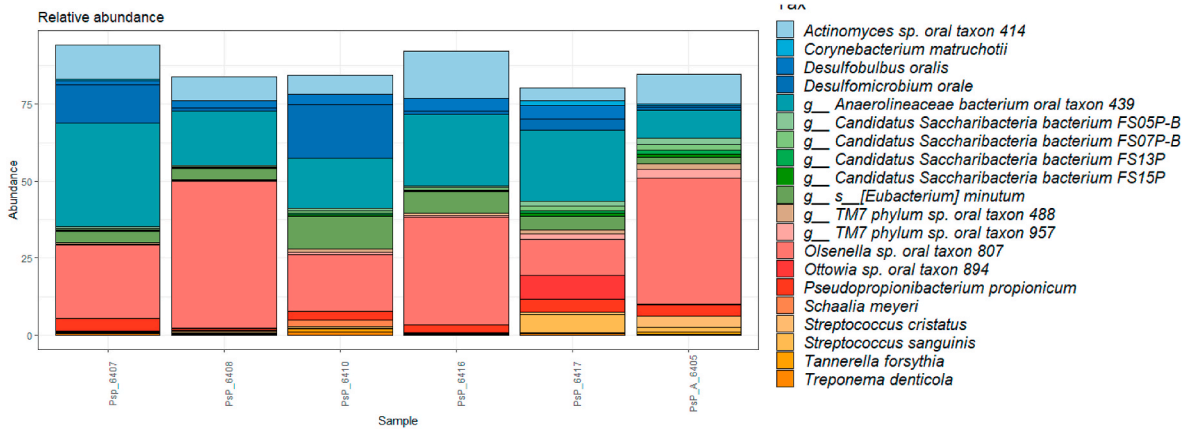

Fig. 5. Core microbiome of the PSP samples at species level.

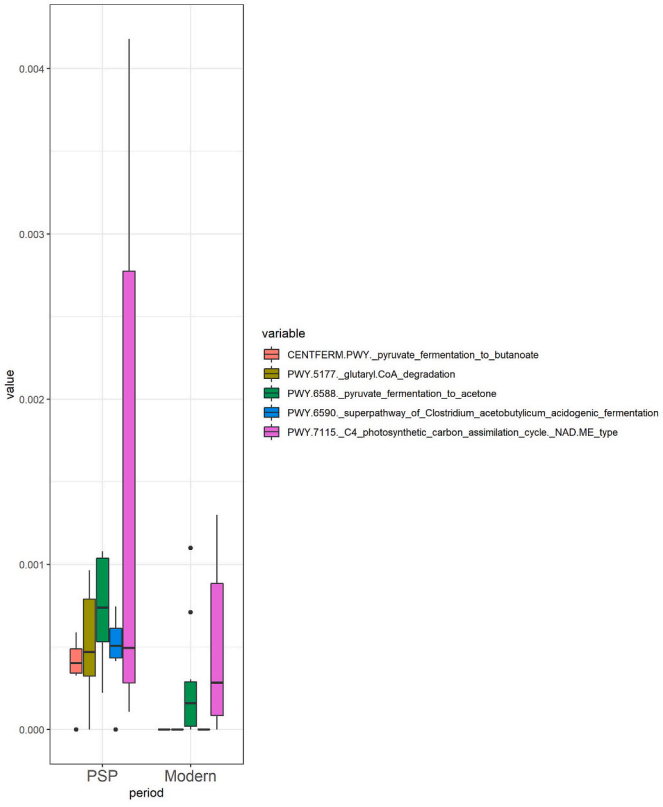

Fig. 6. Significative pathways enriched in PSP with respect to modern samples.

Table 1
Results of plant DNA analysis. Reads and Reads with PMDS >1 indicate the number of reads matched against the respective reference genome and the number of reads with a PMDS score >1. Depth (DoC) and breadth of coverage (>1x) were calculated using BEDTools.

Species	Reads	Reads with PMDS >1	DoC	>1X(%)
Beta vulgaris L.	9387	1587	0.0005	0.025
Brassica napus L.	5171	644	0.00013	0.0083
Cynara cardunculus L.	5373	469	0.00019	0.013

4.2. Identification of the plant remains

The analysis revealed a rather low number of plant microremains (Fig. 7), due to the small size of the samples and a possible loss of material during the lab treatment (Modi et al., 2020). They consisted of starch grains, phytoliths and only two pollen grains.

Starch analysis evidenced grouped and isolated grains (Table 2; Fig. 7). The groups (Fig. 7) were composed of few to almost 90 grains of different sizes, the larger ones with the main axis measuring 28 μm, the smallest 2 μm (Fig. 7). The size distribution of the grains of the two largest groups (Fig. 8) shows the occurrence of some large grains (main axis measuring 20–28 μm) accompanied by a large number of small grains (the most part measuring 3–6 μm) and few medium-size grains (10–16 μm). The large grains are round to oval in outline, with centric, closed or linear hilum, and extinction cross with straight or slightly curved arms that are a little widened at the ends. Such groups of grains may be attributed to *Hordeum* (barley) or *Triticum* (wheat). Their identification at a lower taxonomical level may be attempted, based on the dimension of the grains and the relative amount of large, medium-sized and small grains, following Seidemann (1966). It is to note that grain size is affected by grinding (Ma et al., 2019) and cooking (Henry et al., 2009), two processes that are known to increase the size of the grains and may have occurred to those found in dental calculus. Therefore, the dimension of the largest grains, which had the main axis always below 30 μm, narrowed out the possibilities to barley and/or hulled wheat (*T. monococcum* or *T. dicoccum)*, if we consider improbable the loss of all of the largest grains from the groups. The size distribution of the grains of the largest group (Fig. 8), particularly the relative amount of large grains (i.e. >20 μm, 9%) and medium-size grains (i.e. 10-20 μm, 4.5%), suggests that it most probably belonged to hulled wheat rather than barley. The occurrence of a large prevalence of small grains (≤10 μm, 94%) in the second largest group (Fig. 8) could indicate that it belonged to barley. Regarding the ratio among the grains sizes, the detachment of a small number of large or medium-size grains has a greater weight than that of the same number of small grains, because these last are far more numerous. As a consequence, the discrimination between hulled wheat and barley is not reliable.

Most of the isolated grains showed evident damage by crushing (Fig. 7), such as fissures radiating from the hilum, breaks at the periphery of the grain, evident lamellae and widened faint extinction cross. Their sizes, which ranged from 7 to 40 μm, exceptionally more, have to be considered larger than the original ones. Other grains were apparently undamaged. They were round to oval, ranging from 4 to 24 μm, and showed a centric or a short linear hilum, the latter observed in a part of the largest ones only, with an extinction cross with straight arms (Fig. 7). The origin of the isolated grains is not detectable; they might also come from the disaggregation of groups similar to the ones mentioned above.

Two angular, faceted starch grains (Fig. 7) were found in samples PsP_6416 and PsP_6417. They measured about 16 μm and displayed a centric hilum with short fissures and a quite symmetrical extinction

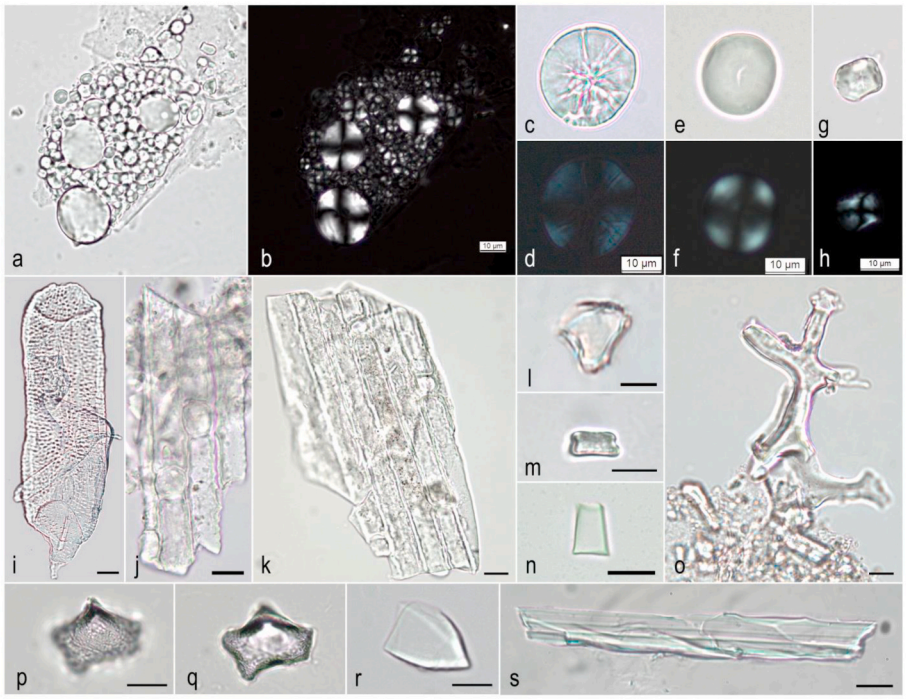

Fig. 7. Starch grains and phytoliths under LM. a) Group of starch grains of different sizes (sample PsP_6410); b) The same group under polarized light; c) Round damaged starch grain (sample PsP_6421); d) The same grain under polarized light; e) Round starch grains (sample PsP_6421); f) The same grain under polarized light; g) Angular starch grain (sample PsP_6417); h) The same grain under polarized light; i) Xylem elements (Tracheary samples PsP_6428); j-k) Fragment of Monocot epidermis (sample PsP_6421); l) Bulliform flabellate phytolith (sample PsP_6417); m) Rondel phytolith (sample PsP_6421); n) Trapeziform phytolith (sample PsP_6408); o) Stellate cell phytolith (sample PsP_6410); p-q) Papilla with polygonal basis (sample PsP_6428): r) Prickle (sample PsP_6412); s) Elongated facetate phytolith (sample). Bar = 10 µm.

Table 2
Quantitative analysis of the plant microremains: starch grains and pollen grains.

STARCH GRAINS	PsP_ 6405	PsP_ 6407	PsP_ 6408	PsP_ 6410	PsP_ 6412	PsP_ 6416	PsP_ 6417	PsP_ 6421	PsP_ 6428	PsP_ 6732 A1	PsP_ 6732 A3
Groups	2		1	2		3				1	1
Round damaged		6	2	4	3	1	3	3	8	3	1
Round to oval		2	5			2			1		1
Angular faceted						1	1				
Spheroidal	1					1		1		1	
Total	3	8	8	6	3	8	4	4	9	5	3
Concentration (n°/ mg)	2,0	31,7	2,3	30,0	20,0	48,5	26,7	26,8	15,0	11,5	40,0
POLLEN GRAINS											
Quercus sp.								1			
Liliaceae s.l.								1			

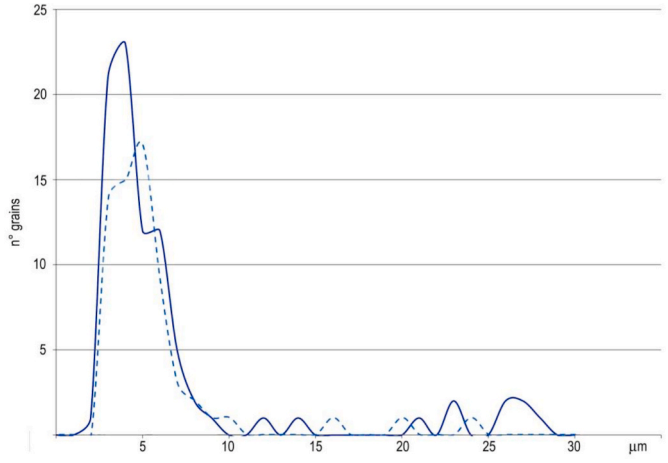

Fig. 8. Size distribution of the starch grains of two groups. Solid line: group of 87 grains; dashed line: group of 65 grains.

cross (Fig. 7). These grains may belong to a millet. Few spherical grains with closed, centric hilum, 8–9 µm in diameter, did not display diagnostic characters.

Phytoliths were referred to about 20 morphotypes (Table 3, Fig. 7). The most frequent are vessels and tracheids or elements of the xylem (Tracheary in Table 3) that did not allow the identification of a definite group of plants (Fig. 7).

The fragments of epidermis of sample PsP_6421 (Fig. 7) consisted of long cells - containing phytoliths with linear or slightly sinuous edges - aligned with short cells, the silica cells measuring less than 10 µm in diameter/main axis. A similar epidermis pattern is typical of the majority of Poaceae (Rudall et al., 2014); in particular, the morphometry of long cells and silica cells is similar to that of the culm or leaves of *Hordeum* and *T. monococcum* (Rosen, 1992; Ball et al., 1993). Few isolated long cells (trapezoid epidermal cell in Table 3) were also found. Isolated cell fragments lacking diagnostic features were not considered.

The bulliform flabellate phytoliths, the rondel and the trapeziform could be attributed to Poaceae (Fig. 7).

A stellate phytolith (Fig. 7) was found in sample PsP_6410. It is a stellate cell belonging to an aerenchyma, a tissue that develops in plants of wet environments. Aerenchymas with stellate cells occur in the stems of Poaceae, Juncaceae and Cyperaceae (Metcalfe, 1960, 1971; Cutler,

Table 3
Quantitative analysis of the plant microremains: phytoliths.

PHYTOLITHS	PsP_ 6405	PsP_ 6407	PsP_ 6408	PsP_ 6410	PsP_ 6412	PsP_ 6416	PsP_ 6417	PsP_ 6421	PsP_ 6428	PsP_ 6732 A1	PsP_ 6732 A3
Acicular					1	1		1			1
Blocky					1						
Bulliform flabellate							1				
Carinate					2						
Cylindric psilate		1	6	4		1	1	3			
Elongated facetate			1	2							1
Epidermis Monocot								3			
Fiber											1
Papilla								1			
Papilla with polygonal basis									1		
Prickle		1	3	1	1		1	1			1
Parallelepipedal			1		1			1			
Polyedral facetate	1		1	3							
Rondel								1			
Trapeziform			1								
Stellate				1							
Tabular psilate				1	1						
Trapezoid epidermal cell			2								
Tracheary		4	5	2	1	2		6	9	3	1
Total	1	6	20	14	8	4	3	17	10	3	5
Concentration (n°/mg)	0,7	23,8	5,8	70,0	53,3	24,2	20,0	113,7	16,7	6,9	66,7

1969) and the leaves of Sparganiaceae and Typhaceae (e.g. Kaul, 1973, 1974), among the families of the Italian Flora. The morphology of the stellate cells found in sample PsP_6410 better fits with those occurring in the stems than in the leaves. The pointed polygonal papilla of sample PsP_6428 (Fig. 7) strongly resembles the papillae of Cyperaceae (Stevanato et al., 2019).

Other morphotypes were not diagnostic, occurring in a large number of plants. However, some of them (e.g. elongated phytoliths and prickles, Fig. 7) are common in numerous Poaceae and Cyperaceae (Metcalfe, 1960, 1971; David et al., 2019; Majumder et al., 2020).

The analysis of the control samples showed that they were free from any plant remains (starch grains and phytoliths), except for a vessel element which was found in sample PsP_6405.

5. Discussion

The Copper Age individuals from Ponte San Pietro (PSP) show an oral microbiota profile which exhibits common traits with other ancient samples, although they constitute a separated sub-cluster. What distinguishes these Eneolithic samples, is not the presence/absence of different bacterial species compared to other ancient samples, but a different distribution in taxa relative abundance. This specific taxonomic and functional pattern suggests that the carbohydrate intake was substantial within PSPs, probably even at the expense of other resources as evidenced by the low presence of numerous bacterial pathways compared to modern samples. This hypothesis found further corroboration from the isotopes analysis conducted on individuals of the same site (Bernardini et al., 2021).

One of the main species that constitute the core microbiome is *Olsenella* sp. which is a Gram positive, non-motile species, highly specialized in carbohydrate fermentation in lactic acid (Kraatz et al., 2011). *Olsenella* spp. have been reported at high level in the gut of nomadic, traditional Mongolian population with seasonal changes in diet regimen (Zhang et al., 2014). Even mice following a diet rich in low soluble fibre demonstrate a bacterial profile that shares elements with that of PSP samples, such as high *Olsenella* abundance and a reduction of Firmicutes phylum (Serino et al., 2012). Other two Actinobacteria species highly represented in the oral microbiota profile of the studied samples are *Corynebacterium* sp. and *Actinomyces* sp. This last is another non-motile, Gram-positive, anaerobe bacteria which is very ubiquitous as it has been found in soil, animal and human microbiota (Dayan et al., 1996; Lee et al., 1996; Li et al., 2018). In humans it is part of the normal microbiota and it is present along the gastrointestinal tract, skin and urinary tract (Li et al., 2018). It plays an important role in human health as it is known to be facultative pathogenic (Valour et al., 2014; Bonnefond et al., 2016). Some *Actinomyces* sp. are involved in the formation of biofilm through the expression of fimbriae which promote intra-bacterial association and allow bacteria to adhere on enamel (Cisar et al., 1979). Also *Actinomyces* can use carbohydrates as substrate to produce energy by glycolysis (Takahashi and Yamada, 1992; Dame-Teixeira et al., 2016). Indeed, *Actinomyces* abundance is associated with sugar availability (Li et al., 2018). Probably, as a consequence of the high presence of *Actinomyces* we detected several *TM7* sp. and Anaerolineaceae sp. These are epiparasitic organisms that require other bacteria as a hosts, often their basibiont is an *Actinomyces* sp. (Bor et al., 2016). *TM7* sp. may exert a beneficial role for their host, such as *Actinomyces*, promoting the formation of biofilm that protects bacteria from saliva flow and from the intervention of the human immune system against them, thus allowing bacteria persistence in the oral cavity (Bedree et al., 2018; Sanz et al., 2017). We can assume that even the presence of *Pseudopropionibacterium propionicum* could be related to the high consumption of carbohydrates in the population diet, considering that it produces proprionic acid from glucose (Hall and Copsey, 2015) and can promote periapical actinomycosis (Siqueira, 2003). It is interesting to note that a very similar microbiota profile characterized by the presence of Anaerolineaceae sp., *P. propionicum* and *Ottowia* sp. has already been observed in ancient samples belonging to prehistoric California (Wada et al., 2018). Taken together, these elements seem to suggest an important, if not an almost exclusive contribution of carbohydrates in the diet of this population, in particular complex carbohydrates rich in fibre.

Moreover, it is possible to observe, within the microbiota of PSP samples, the presence of some sulfate-reducing bacteria (SRB), which are found in high concentration in patients affected by periodontitis, even if the relationship between SRB and periodontal inflammation is still not clear (Langendijk et al., 1999; Kushkevych et al., 2020). Particularly, in our samples both *Desulfobulbus oralis* and *Desulfomicrobium orale* have been detected. The amount of these species is related to the amount of sulphate in the oral cavity, which is affected by dietary habits (Hao et al., 1996). Sulphate is commonly found in several foods including Brassicaceae (Kushkevych et al., 2020), for which we found a plausible molecular signal in the dental calculus of PSP individuals.

The presumed high presence of carbohydrates in the diet of these samples could be linked to the presence of that group of bacteria

belonging to the so-called red complex (i.e., *Porphyromonas gingivalis*, *Tennerella denticola* and *Tennerella forsythia*) (Holt and Ebersole, 2005), which are deeply associated with caries, as well as *Streptococcus mutans* (Lamont et al., 2018). Indeed, high consumption of carbohydrates allows the production of extracellular polymeric substances (EPS) as well as acidic metabolites, thus selecting aciduric microorganisms (Lamont et al., 2018; Takahashi and Nyvad, 2011). Some of these are present at very low abundance within the oral microbiome of the studied subjects.

Finally, if we consider the metabolic pathways analysis, it is possible to observe the presence of an in-depth difference between Copper Age and modern samples, as highlighted by cluster analysis on functional pathways. PSP samples seem to be characterized by a loss of numerous functions present in nowadays samples and a stronger presence of two pathways involved in pyruvate fermentation to butanoate and acetate. Pyruvate is produced mainly by dietary carbohydrates as substrate to generate energy trough fermentation processes (PMID: 31196177). Thus, even in this case, there are elements suggesting that carbohydrates were probably a main resource in the dietary regimen of this population. These functional mechanisms are well known for the human gut but less for the oral microbiome. It is noteworthy, in any case, to highlight that these two pathways, as well as the glutarlyl-CoA degradation founded in PSPs, are involved in the production of two important short-chain fatty acids (SCFA): butyrate and acetate. Both elements are known for their beneficial effect on human health, specifically for their influence on human immune system damping pro-inflammatory cytokines production and stimulating IECs cells (PMID: 23821742; PMID: 26925050). Interestingly, a recent stable carbon and nitrogen isotope analysis on human bones from the same site evidenced a significant consumption of carbohydrates and low intake in animal proteins within PSP individuals (Bernardini et al., 2021).

Metagenomic analysis against a plant database confirmed low DNA yield from putative dietary organisms (Warinner et al., 2014; Weyrich et al., 2017). Coupling deeper sequencing on selected individuals with stringent filtering and authentication criteria (Mann et al., 2020), we were able, however, to detect the signal of at least three putative dietary plants in the dental calculus of PSP individuals (Table 1). We are aware that eukaryotic taxa likely related to ancient dietary consumption are underrepresented in genomic reference databases, included the comparative genome plant dataset used in our analysis. For this reason, identification at species level should be considered cautionary, bearing in mind that molecular signals could also refer to similar species, most possibly of the same genus, that were more diffused in the Mediterranean area in the past times.

The leaves and hypocotyl of *Beta vulgaris* are comestible, eaten boiled or raw. Wild relatives of the domestic *Beta vulgaris* are poorly represented in archaeobotanical assemblages, but there is extensive written documentation of the Roman period. Furthermore, remains of root parenchyma, stalks, and seeds were found in archaeological excavations of Northern Europe attesting the possible dietary use of the plant starting from the Mesolithic (Kubiak-Martens, 1999; Biancardi and Lewellen, 2012). Occasional *Brassica* remains occur in the Neolithic and Bronze Age sites of the Mediterranean area (Zohary and Hopf, 2000; Livarda and Kotzamani, 2013; Frumin et al., 2021). Plants of the genera *Brassica* were exploited for the swollen hypocotyl and leaves and widely employed as oil-crop in Europe starting from the Neolithic (Zohary and Hopf, 2000). *Brassica napus* is the result of a hybrid between *B. rapa* and *B. oleracea* through a process of allopolyploidy that took place about ~7500 years ago (Chalhoub et al., 2014). Therefore, the record of Ponte San Pietro could be the most ancient witness of the consumption of *B. napus*. *Cynara cardunculus* is a plant native to the Mediterranean basin, where it grows on drained soils. Archaeobotanical remains and written sources document its use starting from the Roman Period, when artichoke seems to have been domesticated (van der Veen, 1996; Sonnante et al., 2007). The record of PSP suggests the exploitation of the wild relative since previous times. Indeed, huge wild populations of this plant nowadays occur on the hilly slopes of Northern Latium suggesting that it is indigenous to this area (Pignatti, 1982). The morphometric analysis did not detect microremains referable to the abovementioned plants in the dental calculus of the PSP individuals. Actually, not all the portions and products of plants contain cellular or intracellular structures that may be preserved in the dental calculus. For example, plastids in the leaves contain minimal quantity of starch that is quickly transferred to the storage organs, while the oil may only retain a scarce amount of sediment residue that is improbable to be preserved in dental calculus. Moreover, plants belonging to the Asteraceae family, such as *C. cardunculus*, accumulate inulin and water-soluble carbohydrates as a reserve in the storage organs (Raccuia and Melilli, 2010). The leaves of *Brassica* and *Cynara* contain phytoliths, but they are not diagnostic at this taxonomic level. Conversely, we did not find evidence of DNA from cereals in the metagenomic analysis. Cereals grains are consumed almost exclusively after cooking (prevalently boiled, baked or roasted). In archaeological records cereal caryopses are always charred, and the use of stone tools for grinding cereals seems to indicate that even other cooking procedures were employed by prehistoric human populations before consuming these plants. Cooking causes the swelling of the starch grains and the loss of all the morphological features of the fresh grains, but a small part of the grains may preserve its morphology after cooking and may be identified (Henry et al., 2009). Even a marked reduction in the yield and size of DNA from the source flour occurs, for example, in baked foods after few minutes of baking (Tilley, 2004). Thus, considering the combined degradation effects on DNA due to both food processing and taphonomic processes, the lack of genetic records from cereals in ancient dental calculus is not unexpected. Morphometric and metagenomic analysis of dental calculus are therefore complementary approaches that can be applied to expand the knowledge on dietary practices in well contextualized ancient samples.

The morphometric analysis on plant microremains suggested that most of the starch grains belonged to hulled wheat (*T. monococcum* and *T. dicoccum*) or barley. The precise identification at low taxonomic level was not possible: in fact, it is based on the ratio among large, medium-sized and small grains (Seidemann, 1966), a ratio that may change for the loss of some of the grains of the groups as a consequence of the laboratory treatment or due to the different physicochemical properties and digestibility of the large and small grains (Liu et al., 2007). The use of hulled wheat (perhaps *T. monococcum*) and/or barley is also suggested by the occurrence of fragments of epidermis. Hulled wheat and barley are among the most common findings in the Copper Age archaeological sites of central Italy (Bulgarelli et al., 1993; Celant, 2020). They are considered the three main crops from the Neolithic to Early Bronze Age in the Mediterranean basin (Zohary and Hopf, 2000). Millets are rather rare in Italian Copper Age sites and are mainly attributed to *Panicum* and *Setaria* (Bellini et al., 2008; Rottoli and Castiglioni, 2009), even if recent archaeobotanical studies question the spread of *P. miliaceum* in Europe before the 2nd millennium BCE (Filipovic et al., 2020).

The lack or scarcity of starch grains of pulses is quite common in dental calculus. In this context, it is in agreement with the scarce findings of this foodstuff in the Copper Age sites of central Italy (Bulgarelli et al., 1993; Celant, 2020). It is to note that starch grains constitute only 22–45% of the dry seed of pulses (Hoover et al., 2010). Moreover, pulse starch grains have structure and physicochemical properties different from those of the cereal starch grains and display a different behaviour during heat treatments (Zhou et al., 2004; Ma et al., 2011).

We do not know if cereals were cultivated in the proximity of the site. *T. monococcum* (einkorn) is one of the most resistant wheat species, able to survive where other species cannot; *T. dicoccum* (emmer) tolerates the summer-dry climate, as also barley (Zohary and Hopf, 2000). However, the grains may have been traded and may have arrived at the site from afar.

The most useful evidence -even if scarce-for reconstructing the surrounding environment are provided by *Cynara cardunculus*, detected by metagenomic analysis, that denotes the occurrence of drained soils, and the stellate phytoliths and the papilla of Cyperaceae, that indicate the

presence of plants of wet environments, such as those that grew on the river banks. If we exclude the possibility of environmental contaminants, that seems unlikely considering that the stellate cell we found belongs to the inner tissue of the stems, the introduction of Cyperaceae in the oral cavity might indicate their use for pharmacological purpose (Poulakou-Rebelakou et al., 2010) or even oral healthcare as reported in some present day traditional populations from India (Ganesan, 2008; Biswas and Das, 2012). In this respect, we suggest that the stems of Cyperaceae could be possibly used for removing food residues or relieving toothaches. Interestingly, the PsP_6410 individual where the residues were found, presents multiple caries (Formicola and Garulli, 1988) and the highest abundance of microbial species related to periodontal diseases (Fig. S5).

6. Conclusion

Despite the scarce deposition of dental calculus in the individuals from Ponte San Pietro, our integrated analysis was able to get inside dietary habits and lifestyle of this Copper Age Italian population. The metagenomic analysis here presented represents the first detailed oral microbiome reconstruction of an ancient Italian population. The analysed subjects showed a specific microbial profile that could be associated with an agricultural subsistence, where the plant consumption was prevalent if not almost exclusive in their diet. Particularly, their subsistence relied principally on complex carbohydrate elements rich in fibre that deeply shaped their oral microbiota composition, selecting bacterial species that use glucose as substrate. This feature finds correspondence in the direct evidence of consumption of cereals that we can attribute to hulled wheat and/or barley, while stable isotope analysis on human bones from the same site also suggested the use of Fabaceae (Bernardini et al., 2021). Fibre consumption is normally associated with a healthy status, but probably for the population of PSP this was the only subsistence strategy, thus exposing them to nutritional stress events, as also suggested by the high incidence of dental enamel hypoplasia (Negroni Catacchio et al., 2014). Bacteria associated with caries and periodontal diseases can be related to the high consumption of carbohydrates. A general low incidence of caries is reported in PSP (Negroni Catacchio et al., 2014; Formicola and Garulli, 1988), nevertheless one of the three individuals with multiple caries shows the highest abundance of *P. gingivalis* and *S. mutants* (PsP_6410, Fig. S5), while other samples present abscesses and/or marked dental wear (PsP_6405, PsP_6408).

We also explored the possibility, using molecular signals from ancient plant DNA, to gain much information about the diversity of plant food consumed by the population of PSP. Our analysis confirmed the limits of metagenomics for dietary reconstruction, mainly due to low abundance of eukaryotic DNA in ancient dental calculus and restricted comparative databases. Nevertheless, using increased sequencing depth and a rigorous approach for data authentication and interpretation, we found plausible signals of consumption of leaf vegetables and, possibly, oil-crop in this Copper Age population from central Italy.

Author contributions

M.L. and M.M.L. conceived the study; A.M., L.P., V.Z., and D.A. performed lab work; M.L., M.M.L., A.Q., D.A., A.M., and G.I. performed data analysis; J.M.C. selected the osteological material and provided the anthropological interpretation; S.V. and D.C. contributed tools, materials and reagents; M.L., M.M.L., A.Q., A.M. and D.A. wrote the paper with the input of all co-authors. A.M., D.A., M.M.L. and M.L. contributed equally to the work.

Data availability

Raw data are available at the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under Accession Number PRJNA739881.

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Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.quaint.2021.12.003.

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