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Bioarchaeology of the human microbiome

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Abstract

From prehistory to the present, microbes have played a significant role in the development of human society and culture—from providing essential nutrients and protection through the microbiome, to shaping populations through infectious disease, to producing familiar fermented foods such as cheese, bread, and beer. Yet microbes are generally overlooked in studies of the human past. Only recently has the essential nature of the microbiome in human biology has only recently gained recognition, and public acknowledgement of the importance of microbes is typically limited to clinical infectious disease contexts and the food industry, which utilizes microbes such as *Saccharomyces* yeasts and *Lactobacillus* bacteria in fermentation. Recent advances in DNA technology now make it possible to investigate ancient microbes and their diverse impacts on human biology and culture. Situated at the intersection of biology and archaeology, bioarchaeology is ideally positioned to lead the investigation of the ancient human microbiome and the changing role of microbes in the health and diet of past human populations.

Abstract (Deutsch)

Seit der Vorgeschichte haben Mikroben eine bedeutende Rolle in der Entwicklung menschlicher Gesellschaften und Kulturen gespielt – sei es die Bereitstellung wesentlicher Nährstoffe und der Schutz durch das Mikrobiom, die Beeinflussung von Populationen durch Infektionskrankheiten oder die Herstellung fermentierter Lebensmittel wie Käse, Brot, und Bier. In Studien zur

Menschheitsgeschichte werden Mikroben im Allgemeinen jedoch übersehen. Erst vor kurzem hat die Rolle des Mikrobioms in der Humanbiologie Anerkennung gefunden, jedoch ist die Bedeutung von Mikroben typischerweise auf klinischen Infektionskrankheiten und die Lebensmittelindustrie beschränkt, in der Fermentation-Mikroben wie *Saccharomyces* Hefen und *Lactobacillus* Bakterien eingesetzt werden. Jüngste Fortschritte in der Paleo-DNA Technik ermöglichen es nun, uralte Mikroben und ihre vielfältigen Auswirkungen auf die menschliche Biologie und Kultur detailliert zu untersuchen. In der Schnittstelle von Biologie und Archäologie ist die Bioarchäeologie einzigartig positioniert, um die Bedeutung des alten menschlichen Mikrobioms und die verändernde Rolle der Mikroben in Hinblick auf Gesundheit und Ernährung in vergangenen menschlichen Populationen herauszuarbeiten.

The human microbiome comprises all of the native microorganisms (bacteria, viruses, archaea, fungi, and microeukaryotes), as well as their metabolic products, that are found in and on the human body (Marchesi and Ravel 2015). All surfaces of the human body are covered by microbes, including the skin, the urogenital tract, the mouth, and the intestines. Bacteria make up the bulk of these microbial communities and are the best-understood members of the microbiome. Although it has been widely reported that there are ten times more bacterial cells in us and on us than our own cells, this estimate has recently been revised. The 10:1 ratio is roughly accurate with respect to our nucleated cells, but when considering all cells, including non-nucleated red blood cells, the ratio of microbial cells to human cells falls closer to 1:1 (Sender et al. 2016). Nevertheless, regardless of how our own cells are counted, there is an immense number of microbial cells inhabiting the human body— an estimated 38 trillion—and given the size and essential function of the microbiome, it is not surprising that it is sometimes considered an additional organ (O'Hara and Shanahan 2006; Baquero and Nombela 2012). Together, humans and our microbiome have been collectively described as a superorganism (Hattori and Taylor 2009; Sleator 2010), or more accurately, a holobiont (Gordon et al. 2013; Zilber-Rosenberg and Rosenberg 2008).

Our native microorganisms (microbiota) almost always live in multi-species communities that grow in complex layers of microbes and bacterial “glues” called biofilms. By living in dense communities, microbes can provide protection and nutrients to each other (Grenier and Mayrand 1986), and they establish ecological networks that are at equilibrium within themselves and which interact beneficially with their human host. These microbiota additionally perform a diverse range of essential functions for their human hosts, including aiding in the digestion of refractory plant compounds (e.g., dietary fiber) that humans lack the enzymatic capacity to metabolize (Hehemann et al. 2010), producing essential nutrients such as vitamin K and folic acid (LeBlanc et al. 2013), and training immune cells to recognize and attack invading microbes while at the same time tolerating resident microbiota (Lathrop et al. 2011). These essential functions are evidence of a long history of co-evolution between microbes and their host, with beneficial interactions developing into essential functions, without which humans could not live healthy lives (Sommer and Bäckhed 2013).

However, the microbiome is also involved in disease processes, both directly and indirectly. It is now clear that the microbiota of diseased individuals differ from those of healthy individuals. The altered microbial communities associated with disease are termed “dysbiotic” (Hajishengallis and Lamont 2012), indicating that they are no longer interacting beneficially with the host, and may instead promote inflammation that drives disease processes. Some diseases associated with dysbiotic microbiota are clearly the direct result of microbiome perturbations, such as intestinal *Clostridium difficile* overgrowth following antibiotic treatment (Bien et al. 2013), while others are the result of more complex and subtle changes in the microbiome community. Chronic inflammatory diseases as diverse as periodontal disease (Hajishengallis and Lamont 2012), atherosclerosis (Lockhart et al. 2012), rheumatoid arthritis (Fuggle et al. 2016), Alzheimer’s disease (Wu and Nakanishi 2014), autism spectrum disorder (Mulle et al. 2013), allergies (Hirsch et al. 2017), diabetes (Morris et al. 2016), and depression/anxiety (Foster and McVey Neufeld 2013) have been linked with perturbed, dysbiotic microbiomes, although the exact relationship between microbes and these diseases is uncertain.

Importantly, many chronic inflammatory diseases, collectively termed “diseases of civilization,” are substantially more prevalent today than in the past. Epidemiological records in currently-industrializing nations indicate that the incidence and prevalence of such diseases have risen rapidly in the recent past (Barquera et al. 2015; Dowman et al. 2012), suggesting that industrialization-associated changes drive disease pathogenesis. Microbiota are moreover sensitive to changes in the host environment (David et al. 2014a) that occur during industrialization, including environmental urbanization (Avershina et al. 2015), the introduction or increased abundance of foods (David et al. 2014b), and increasing hygiene (Giacomin et al. 2016). Because the microbiome is important for health, any change in the host environment that disrupts the microbiome may lead to health problems.

Currently, a major focus of the microbiome research community is understanding how the relationship between humans and their microbiome changes in dysbiosis. Thus far, these studies lack long-term (longer than an individual lifetime) data that would enable the investigation of the major social and cultural changes in lifestyle and living habits that influence the microbiome in humans. Bioarchaeological investigations of ancient human microbiota have the potential to provide a deeper understanding of the ancestral human microbiome and how it has changed during human evolutionary history in response to major events.

The Ancient Human Microbiome

Like the soft tissues of the body, most of the microbiome decomposes after death and is thus lost to the archaeological record. However, there are several unusual circumstances under which the microbiome preserves and may be used to explore the human past. Located at the entrance of the digestive tract, the oral microbiome forms a biofilm (dental plaque) on the surface of the teeth. During life, this biofilm naturally mineralizes through the precipitation of salivary minerals to form dental calculus, also known as tooth tartar (Figure 1a). Dental calculus is nearly ubiquitous in skeletal assemblages from agricultural societies, and it is also widely found in non-agricultural populations, including Paleolithic populations (Power et al. 2015) and even members of archaic *Homo* (Henry et al. 2011). Moreover, its densely mineralized nature appears to make it resistant to many postmortem taphonomic processes (Warinner et al. 2015a). At the opposite end of the digestive tract, human feces can also persist over long periods, especially in cold and/or dry environments, preserving a record of the gut microbiome (Tito et al. 2008, 2012). In general, however, ancient feces, also known as paleofeces or coprolites, are neither as prevalent nor as well preserved as dental calculus (Warinner et al. 2015a), although notable exceptions exist (Tito et al. 2008, 2012).

Recent biomolecular studies of ancient dental calculus and preserved feces have demonstrated the wealth of information that can be extracted from such samples (Warinner et al. 2015a, 2015b; Figure 1b), and many new avenues of inquiry can be explored using this information. Moreover, because the gut and oral microbiomes are home to the two most intensely studied and best understood human microbiota, there is great potential to compare ancient and modern microbiomes in order to observe changes through time, to infer the impact of specific activities and behaviors on the microbiome, and to correlate these with other evidence of health and disease determined through archaeological and osteological assessments.

In addition to dental calculus and coprolites, other sources of host-associated microbes in the historical record may provide additional information, but they are not as abundant, and therefore fewer studies have explored their potential. These include both medical collection specimens (preserved in alcohol or formaldehyde) and tissues that have been mummified (naturally or artificially).

The oral biofilm is arguably the best-characterized human biofilm, making it a good target for analysis, and here we will focus primarily on insights that can be gleaned from ancient dental calculus, a substrate that is abundant in skeletal collections and which has shown great potential as a source of historical molecular information.

Dental Calculus Collection Techniques

Collecting dental calculus from skeletons using careful, standardized techniques is critical for generating high-quality data. Figure 2 illustrates a simple collection procedure that uses easily acquired and transported supplies. A basic field sampling kit includes: a dental scaler, forceps, 1.5 mL microcentrifuge tubes, a plastic tube rack, plastic bags, a permanent marker, nitrile gloves, a face mask, aluminum foil, paper towels, and alcohol wipes (Fig. 2a). In the field, sampling should take place at a clean workspace separated from food preparation and other potentially contaminating activities. If work surfaces cannot be effectively cleaned, they may be covered with aluminum foil to provide a clean surface. Tools should be cleaned with alcohol wipes or

commercial bleach (~2-6% NaOCl) between samples, and masks and gloves should be worn during sampling to avoid contamination with respiratory and skin bacteria. It is recommended to wear two sets of gloves so that the outer glove may be changed without exposing the hand between samples. Nitrile gloves are recommended; latex gloves should be avoided for handing samples because latex is a protein contaminant.

To begin sampling, cover the sample tube rack with aluminum foil, label each tube, and shape a piece of aluminum foil into a catchment bowl (Figure 2b). Place the bowl over the rack and puncture it with a microcentrifuge tube, fixing the tube and bowl in place (Figure 2c). Examine the tooth and identify any burial matrix or dental calculus adhering to the tooth. Burial matrix may be removed by gently scraping it away with a dental scaler; unlike dental calculus, burial matrix typically crumbles into a fine powder with the application of slight pressure. Dental calculus deposits may be small (Figure 2d, arrow), moderate in size (Figure 1a), or so large that they nearly obstruct the entire tooth (Figure 3). Using a dental scaler (Figure 2e), remove the dental calculus by applying pressure to the calculus using the broad blade of the scaler, and guide the calculus into the microcentrifuge tube (Figure 2f). If the calculus cracks or breaks unexpectedly, it should be caught by the foil catchment bowl; use forceps to transfer the calculus into the sample microcentrifuge tube.

It is recommended to collect calculus from each tooth separately, and each tooth should be photographed before and after sampling. Proper calculus sampling does not damage the underlying dentition and is non-destructive of dental tissues (Figure 3). The amount of dental calculus required for analysis depends both on the analytical method and on sample preservation. For moderately well preserved and well preserved samples, the following sample sizes are recommended: genetic analysis, 5 mg; protein analysis, 5-10 mg; microfossil analysis, 1-3 mg. In general, collecting a total of 20-50 mg of dental calculus per individual is recommended so that multiple analyses and replicates can be performed and so that some material can be held in reserve for future research.

After collection, samples may be stored at room temperature until further analysis; however, it is important to avoid prolonged exposure to sunlight or temperature fluctuations during storage. Optimal storage conditions are cold, dark, dry, and consistent. Because cultured bacteria and airborne polymerase chain reaction (PCR) products pose a serious contamination risk, ancient samples should never be analyzed or stored in a modern microbiology or molecular biology laboratory. Subsequent analyses should be performed in laboratories designated for ancient biomolecule research.

As noted, to mitigate contamination risk prior to sampling it is helpful to wear nitrile gloves during excavation and at all stages of skeletal handling both in the field and afterwards. Even in cases of previously collected remains and museum collections, in which it is likely that the remains have been heavily handled without such precautions, it is important to reduce further contamination by wearing nitrile gloves at all times during skeletal handling. Contamination prevention is easier and more effective than contamination removal, and thus every effort should be made to reduce contamination risks during excavation, osteological examination, storage, and laboratory analysis.

Advancing Research Through New Technologies

Ancient biomolecular research is rapidly expanding thanks in large part to several recent advances in DNA sequencing and protein mass spectrometry technologies. With respect to ancient DNA (aDNA), the advent of High-Throughput Sequencing (HTS) marks a substantial change in DNA sequencing technology. HTS enables the production of vast amounts of genetic data from degraded samples, while at the same time reducing laboratory time and costs and eliminating certain contamination challenges (Mardis 2008). Prior to HTS, aDNA research largely relied on a genetic approach that combined targeted polymerase chain reaction (PCR) amplification, molecular cloning, and Sanger sequencing. Although this conventional approach has yielded valuable aDNA data, its application has been limited by several shortcomings, including its low-throughput, labor-

intensive nature and the fact that its reliance on relatively long and intact DNA fragments makes it susceptible to modern contamination and amplification dropout.

Over the past five years, conventional DNA sequencing approaches have been largely replaced by HTS technologies, such as Illumina sequencing by synthesis (SBS), which can generate billions of DNA sequences from very short DNA fragments present at low starting concentrations (Mardis 2008). This is a major advantage for studies of the human microbiome because it allows rapid data generation from complex genomic mixtures, also known as metagenomes, that comprise all of the DNA from a microbial community (Vincent et al. 2016); this sheer volume of data is necessary to characterize the complex microbial mixtures characteristic of microbiota, which may contain thousands of taxa, each at a different relative abundance. Importantly, HTS is designed for short DNA fragment sequencing, making it an ideal platform for research on highly degraded aDNA (Dabney et al. 2013). HTS also makes it possible to identify and quantify DNA damage patterns (Ginolhac et al. 2011; Jónsson et al. 2013), allowing confirmation that the sequenced DNA is of ancient origin and not the result of modern contamination (Overballe-Petersen et al. 2012; Dabney et al. 2013). Finally, HTS platforms used for aDNA research are the same as those used to study modern microbiomes, which makes comparing results more meaningful because they are generated at a similar scale and are subject to similar biases.

New developments in protein sequencing technologies are also broadening the repertoire of ancient biomolecular techniques used to understand the human past (Cappellini et al. 2014). Mass spectrometers such as the Thermo Scientific Q Exactive are particularly effective at analyzing highly complex protein mixtures (metaproteomes) using bottom-up shotgun proteomics techniques (Warinner et al. 2014a, 2014b). This approach is well suited to the study of ancient proteins because it does not require intact proteins and it is relatively tolerant of the most common forms of damage typically observed in ancient proteins, such as oxidation of methionine and deamination of asparagine and glutamine (Warinner et al. 2014a).

Although shotgun metaproteomics can be used to some extent for taxonomic identification, the real strength of the method is in characterizing the complex functions performed by both the microbiota and the host (Warinner et al. 2014a; Corthals et al. 2012). In the case of archaeological dental calculus, many of the proteins present are involved in microbial virulence and host immune response, providing clues to the health state of ancient individuals (Warinner et al. 2014a). Ancient proteomics technologies are also particularly useful for identifying dietary components. In addition to providing taxonomic information, protein data can also indicate tissue of origin, such as milk versus meat or seed versus leaf, which cannot be inferred from DNA sequences alone. Within archaeological contexts, this approach has been especially productive in the identification of past dairying practices (Warinner et al. 2014b; Yang et al. 2014).

The excellent preservation of both DNA and proteins in ancient dental calculus suggests that other biomolecules, such as small molecule metabolites, may also preserve within dental calculus. Previous studies of Neanderthal dental calculus using pyrolysis GC-MS have revealed metabolite evidence of wood-fire smoke inhalation and cooked plant consumption (Hardy et al. 2012), and investigations of preserved soft tissues in humans, such as mummified hair, have identified important bioactive compounds, such as cocaine in the hair of coca-chewing ancient Peruvians (Springfield et al. 1993) and nicotine in the hair of ancient Chileans (Echeverría and Niemeyer 2013). Knowledge about past foods and medicines gained from metabolites could be used to trace the introduction of specialized products into historic and prehistoric food supplies, or to confirm that only certain groups of people were consuming a given substance at a particular time and location. Metabolite analysis also has the potential to link the use of specific medications with observed lesions in skeletons to confirm how certain maladies were treated. The Neolithic use of beeswax to fill dental caries, for example, has been studied using this approach (Bernardini et al. 2012).

In addition to dietary and lifestyle-associated metabolites, the small molecule metabolites that are

produced by microbes may be critical for understanding health processes in humans (LeBlanc et al. 2013), and changes in their presence or amount may be important factors in driving chronic diseases (Yu et al. 2014). If evidence of differences in the metabolic profiles of historical and modern microbiomes were found, it could help to explain why certain microbiome-associated diseases are more prevalent today than in the past. Although understudied, metabolites have great potential to reveal important details about past human lifestyle habits and health states for which we otherwise have very little direct evidence.

The Evolution and Ecology of the Oral Microbiome

Overall, ancient dental calculus appears to be similar to its modern counterpart, making it an excellent medium by which to compare the past and the present. This wealth of material can be mined to answer fundamental bioarchaeological questions regarding changes in human health and disease through time in order to better understand the state and context of human health today. However, to begin such studies requires some familiarity with the formation and composition of dental calculus, as well as how it relates to disease today.

The oral biofilm in life: dental plaque

The oral biofilm grows naturally on the teeth during life and is predominantly composed of bacteria. It is the “fuzzy tooth sweater” that can be felt on the surfaces of teeth after a period of time without brushing. There are approximately 700 bacterial species that make up the oral biofilm (Dewhirst et al. 2010), yet nearly half of these cannot yet be grown in the lab (Thompson et al. 2015). This community is highly structured and forms in a series of defined developmental steps (Kolenbrander et al. 2010). Periodically, the mature biofilm rapidly calcifies by crystallization of calcium-phosphate minerals in saliva, entombing the entire biofilm community in an impermeable layer that, much like volcanic ash, preserves the contents exceptionally well. This essentially fossilized biofilm community is the dental calculus that builds up on teeth. During life, this surface is rapidly re-colonized by bacteria from the saliva, a new biofilm grows and matures on top of the old, and the process of calcification and new biofilm growth repeats. The reasons for calculus formation are not well understood (Jepsen et al. 2011), but because of the introduction of modern oral hygiene and calculus-preventing products (Jepsen et al. 2011), the prevalence and buildup of calculus in industrialized societies has declined over the past century (Warinner et al. 2015a; Warinner 2016).

Although the role of the gut microbiome in maintaining human health has received a great deal of attention and study in recent years, (LeBlanc et al. 2013; Lathrop et al. 2011), the contributions of the oral microbiome are less well known. The oral microbiome appears to prevent colonization of certain disease-causing microbes and is involved in nitrite metabolism that produces nitric oxide, an important hormone for controlling blood pressure (Wade 2013). In contrast to its uncertain functions in health, the oral microbiome is well known for its role in disease and is responsible for two of the world’s most common diseases: dental caries (Kassebaum et al. 2014) and periodontal disease (Albandar and Rams 2002). Dental caries is the gradual dissolving of teeth that results from bacterial-produced acids demineralizing the tooth enamel, which today affects nearly 98% of people who do not practice active dental hygiene (Kassebaum et al. 2015). Sugars and starches in the diet contribute to dental caries formation because they are rapidly fermented by oral *Streptococcus* and *Lactobacillus* species, which release lactate, acetate, and other small acids as metabolic by-products. These acids not only dissolve enamel but also inhibit other non-aciduric bacteria that cannot tolerate acidic pH. Over time, through repeated sugar exposure and fermentation, and more dramatic and sustained pH changes, the microbial community shifts, so that the biofilm microbes around carious lesions differ from those on healthy teeth (Peterson 2014).

Periodontal diseases, which include gingivitis and periodontitis, are more complex than dental caries. Although it is initiated by the oral biofilm, the tissue damage ultimately results from destruction of gingival soft tissue and bone (the periodontium) mediated by the host immune system. Gingivitis is inflammation of the gingival tissues, and it occurs in nearly all adults who do not practice active oral hygiene (Albandar and Rams 2002). By contrast, periodontitis is an

advanced form of gingivitis that includes loss of bone that supports the teeth, and it presently affects nearly half of the American adult population (Eke et al. 2015). During disease progression, periodontal inflammation changes the nutrients available for the oral biofilm by increasing proteins available for fermentation. The microbes best able to take advantage of the new nutrients rapidly multiply, and the community composition changes, so that biofilms at sites of periodontal disease differ from those at healthy sites (Abusleme et al. 2013). Using culturing-based studies of the bacteria growing at healthy and periodontitis-affected sites, three species have been identified as significantly associated with clinical disease severity: *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*. These "red complex" periopathogens (Socransky et al. 1998), which appear to play a role in host innate immune system disregulation (Shin et al. 2013; Darveau et al. 1998), have been a major focus of periodontal disease research. Recent HTS studies, however, have revealed more complex community changes under disease states, which has shifted the focus of periodontal research away from individual pathogens as causative agents of disease and towards models of polymicrobial synergy and dysbiosis (Hajishengallis and Lamont 2012).

Increasingly, the oral biofilm is being linked to systemic diseases and disorders and may be indirectly, or even directly, involved in diseases as diverse as cardiovascular disease (Lockhart et al. 2012; Velsko et al. 2015), Alzheimer's disease (Wu and Nakanishi 2014), adverse pregnancy outcomes (Madianos et al. 2013), colon cancer (Kostic et al. 2013; Rubinstein et al. 2013), and rheumatoid arthritis (Fuggle et al. 2016). Many of these "diseases of civilization" have complex and poorly understood etiologies. Therefore, following and understanding changes in the oral microbiome through time may aid our understanding of the rise in prevalence of complex systemic diseases that are otherwise difficult to trace in the archaeological record.

The oral biofilm after death: ancient dental calculus

Investigating ancient dental calculus is a very new and exciting field with great potential to provide novel insights into health, disease, and diet throughout human history. This semi-fossilized oral biofilm preserves a wide range of biomolecules of diverse origins, including oral microbial DNA and proteins, host DNA and proteins, and DNA, proteins and microfossils related to food consumption. DNA recovered from dental calculus is typically well-preserved and originates from known oral biofilm microorganisms, suggesting that it is not prone to contamination with soil bacteria to the same extent as other skeletal tissues, such as tooth dentine (Warinner et al. 2014a). Dental calculus thus functions as a kind of time-capsule that preserves minute traces of daily life accumulated over a lifetime. Table 1 provides selected examples new information gained from recent dental calculus studies that would have been challenging to obtain using conventional archaeological methods.

Basic research on the composition of archaeological dental calculus has demonstrated that it preserves the oral biofilm *in situ*, and that it is nearly identical to modern biofilms in structure and microbial cell morphology (Warinner et al. 2014a). The vast majority of DNA in ancient dental calculus derives from bacteria, but oral archaea and viruses (bacteriophages) are also detectable, as is DNA from respiratory microbes and endogenous opportunistic pathogens, known as pathobionts (Table 1). While these microbes are natural occupants of human airways and usually do not cause harm, they can induce disease under certain conditions and are therefore important to trace through time.

During life, dental calculus acts as a sink for microbes that inhabit the respiratory tract (Warinner et al. 2014a); thus it could potentially be used to investigate the past carriage rates of certain endemic respiratory pathogens, such as *Bordetella pertussis*, the causative agent of whooping cough, or *Corynebacterium diphtheriae*, the causative agent of diphtheria. The distinctive symptoms of whooping cough make it easily identifiable in historical records, which have been used to trace the origins and spread of the disease (Aslanabadi et al. 2015). While both of these diseases are uncommon today in industrialized nations due to extensive vaccination efforts over the past century, they are rising in incidence, particularly in areas with poor vaccination coverage

or with vaccination refusal (Sealey et al. 2016; Sangal and Hoskisson 2016). Detecting these bacteria in ancient dental calculus would provide baseline data on the specific populations and geographic locations affected by these diseases in the past. This information would be valuable to epidemiologists and those working on vaccine development, as it can reveal infection and exposure dynamics, knowledge of which is critical to disease control and prevention.

In addition to microbial DNA, ancient dental calculus also contains human DNA, although it is typically more damaged and fragmented than the microbial DNA (Ozga et al. 2016). Recently, Ozga et al. (2016) successfully reconstructed whole mitochondrial genomes from dental calculus (Table 1). Although dentine was generally found to be a richer source of human mitochondrial DNA than calculus, this study demonstrated that dental calculus can be used for human DNA analysis when other samples are not available or other methods are not feasible. In this way, ancient dental calculus offers a minimally destructive method of sampling human DNA from skeletons and may open up sampling of sensitive collections that are otherwise not available for genetic analysis.

With respect to proteins in dental calculus, most derive from oral microbes, but human salivary and immune response-related proteins are also abundant (Warinner et al. 2014a). This is expected because during life the surfaces of the teeth are constantly bathed in protein-rich saliva and serum-derived gingival crevicular fluid (GCF) that seeps out of the gingival tissue. Additionally, large numbers of white blood cells, especially neutrophils, are recruited to the gingival margin during periodontal disease progression, and many of the host proteins identified within dental calculus are specific to this cell type (Warinner et al. 2014a). The co-occurrence of microbial and immune response proteins in ancient dental calculus presents a unique opportunity to explore disease mechanisms and pathogenesis in the past (Table 1).

In addition to microbial and host biomolecules, dietary DNA and proteins are also present in ancient dental calculus, although in very low abundance relative to the resident microbial molecules (Warinner et al. 2014a). Dental calculus derived genetic evidence of plant and animal consumption has been reported in a medieval European population (Table 1) (Warinner et al. 2014a), and protein evidence for dairy consumption has been identified at numerous sites throughout Europe and western Eurasia (Table 1) (Warinner et al. 2014b). With respect to the latter, the milk proteins of different dairy livestock could be distinguished, allowing the identification of cow, sheep, and goat milk consumption in periods dating as far back as the Bronze Age. Intriguingly, food microbes such as those used to produce cheese are also found in living oral biofilms today (Wade 2013), suggesting they may have been trapped in dental calculus in the past. If it is possible to identify microbes from cheeses or fermented foods such as beer, salami, or kimchee, it may be possible to provide further direct evidence of specific food preparation techniques and consumption habits in past populations.

Microfossils in dental calculus also indicate consumption of specific food items, particularly plants. The exceptional preservation of microfossils in dental calculus, coupled with the fact that some plants produce microfossils with highly distinctive morphology, has allowed researchers to push back the clock on human use of several important plant taxa (Table 1). For example, the identification of wheat starches in the dental calculus of Balkan Mesolithic foragers is rewriting the history of Neolithization in the region (Cristiani et al. 2016). Likewise, the identification of wild barley starches in the dental calculus of Near Eastern Neanderthals has broadened our understanding of the consumption of wild cereals in the Paleolithic (Henry et al. 2011). Such studies demonstrate the power of ancient dental calculus research to contribute to our understanding of major cultural changes.

Future Applications and Prospects

Because the microbiome functions both within itself and with the host, the interactions between the two and their respective influence on the functions of the other are complex and difficult to disentangle. Time-course studies demonstrate how the microbiome and host health status change within the current cultural system and in an individual lifetime (David et al. 2014a,b), but they

cannot predict how long-term changes will affect either microbiome or host, nor how populations change. Ancient dental calculus offers time-course data over generations and in numerous populations, and is therefore critical to answering how microbiomes and humans have co-evolved over time. The questions to ask from a microbial perspective focus on the microbiome and inferring changing interactions with the host from observed changes in the microbiome.

Firstly, we want to know if the microbial communities associated with health and disease have changed over time, and, if so, what those changes are. If the communities associated with health are similar through time, what are those species, and what metabolic activities are dominant in those communities that may protect against disease? If the communities are different, then we can investigate the differences at the community and the metabolic level and offer further insight into what makes a community health-associated and protective against disease. There may be many states of health (Peterson 2014), and different people need different communities to maintain health, which has implications for modern medical care. Likewise, we want to know if communities associated with disease are similar or different. Similarities would suggest that disease processes are unchanged by changing living environments, while differences would suggest that disease processes may be influenced by changing living environments, which would also have implications for modern medical prevention and treatment.

Digging deeper into community changes through time, we want to know specifically what has changed within the microbiome. Are changes the result of the introduction of new species, or the loss of species, as has been shown for the gut microbiome (Obregon-Tito et al. 2015)? Do these changes cause ecological shifts in the microbiome that alter how it interacts with the host by changing the microbial proteins and metabolic products that the host immune cells are exposed to (Kolenbrander et al. 2010)? Shifts in the abundance of specific microbes, where some increase or decrease in number, could similarly change the microbiome metabolic profile and alter host-microbiome interactions. We can also look at how specific microorganisms have changed over time by observing gene acquisition and how the activity of new genes can influence interactions with the host. One example of gene gain influencing oral microbial ecology with a significant impact on human health is seen in *Streptococcus mutans*, an acid-producing oral species strongly linked to dental caries. *Streptococcus mutans* is estimated to have acquired genes to metabolize numerous sugars and starches around ten thousand years ago, at the time of the agricultural revolution (Cornejo et al. 2013), and it is argued that these genes allowed it to take advantage of the newly abundant starches from cultivated grains in the human diet. Other genes of interest include those involved in antibiotic resistance; several recent studies have demonstrated that antibiotic resistance genes predate the use of therapeutic antibiotics (D'Costa et al. 2011; Warinner et al. 2014a), and archaeological samples can provide direct evidence of the presence and distribution of resistance mechanisms in the past.

Because specific strains of certain species are associated with disease, while others are associated with health (Rodrigues et al. 2012; Holt et al. 1999), it is important to investigate strain-level differences in microbial communities. If strain composition has changed, are the strains associated with health and disease today the same as those in the past? If we do not see the same strains in the past, can we determine when they arose, and if there were human lifestyle changes that preceded or accompanied them? Indeed, changes in strains may indicate changes in the oral environment, which could be driven by changes in diet, oral hygiene practices, or interaction with groups of people from distant geographic locations. In some cases, it may be possible to reconstruct population migration histories by tracing the spread of certain microbiome-associated organisms, as has been recently performed for *Helicobacter pylori* (Maixner et al. 2016). One could ask specifically, does appearance of novel organisms correlate with migration or interaction with new populations? Does appearance correlate with a rise of disease in those populations?

On the other hand, we can also investigate if certain immune response molecules are associated with the microbial community, which could help us understand the co-evolution of the microbiome and immune system and provide a long-term record of whether certain microbial communities drive specific immune responses. The immune response connects the outcome of local microbiome-host

interactions with distant systemic organs, and it is the link between microbiome dysbiosis and disease. Because human evolution is much slower than microbial community changes and microbial evolution, a mismatch between the microbiome and the immune response may be the root cause of many microbiome-associated diseases. Tracking the interactions between the two over time may prove particularly informative for understanding the current balance between the microbiome and host response and disease development.

Conclusion

The critical role the human microbiome plays in human health and development and the clear relationship between changing microbiomes and disease highlight the importance of our microbial inhabitants to the human experience. Alterations in the microbiome may reflect large-scale shifts in human lifestyle, from geographic relocation and expanding dietary selections to new social interactions and hygiene changes. Ancient dental calculus not only encases the long-term evolution of the oral microbiome but also provides access to traces of material culture that offer direct evidence of social, cultural, and geographic transitions. Continued exploration of this exciting medium promises to reveal new insights into our past by shedding light on previously inaccessible questions. In the coming years we expect this expanding field will make important contributions to our knowledge of the human past and prove informative for maintaining our health and well-being today. The abundant information that can be collected from the ancient oral microbiome holds great promise to deepen our understanding of human history and to broaden our perspective on what makes us human.

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Table 1. Novel information gained from selected recent studies of ancient dental calculus

Information	Method	Reference
Microbiome		
Genetic identification of oral bacteria	PCR, qPCR, amplicon HTS	Adler et al. 2013; De La Fuente et al. 2012; Warinner et al. 2014a
Demonstration of an intact oral microbiome	Metagenomic HTS	Warinner et al. 2014a; Ziesemer et al. 2015
Disease		
Identification of pathogens involved in ancient periodontal disease	Metagenomic HTS	Warinner et al. 2014a
Periodontal pathogen genome reconstruction (*Tannerella forsythia*)	Metagenomic HTS	Warinner et al. 2014a
Identification of pathogen virulence factors (e.g., *Porphyromonas gingivalis* gingipains)	Protein LC-MS/MS	Warinner et al. 2014a
Characterization of host innate immune response to infection (e.g., neutrophil activity)	Protein LC-MS/MS	Warinner et al. 2014a
Diet		
Identification of plant (e.g., *Brassica*) and animal (e.g., *Sus*) DNA	Metagenomic HTS	Warinner et al. 2014a
Identification of dairy proteins (from *Bos*, *Ovis*, and *Capra*)	Protein LC-MS/MS	Warinner et al. 2014b
Identification of putative medicinal plants	Py-GC-MS; TD-GC-MS	Hardy et al. 2012
Identification of cooked foods	Light microscopy; Py-GC-MS; TD-GC-MS	Buckley et al. 2014; Hardy et al. 2012; Henry et al. 2011
Characterization of food processing and water sources	Light microscopy	Dudgeon and Tromp 2014; Horrocks et al. 2014; Tromp and Dudgeon 2015
Identification of plant microfossils in archaic hominins	Light microscopy	Hardy et al. 2012; Henry et al. 2011, 2012
Identification of plant microfossils in humans	Light microscopy	Buckley et al. 2014; Cristiani et al. 2016; Henry et al. 2011; Humphrey et al. 2014; Mickleburgh and Pagán-Jiménez 2012; Warinner et al. 2014a
Craft activity		
Identification of textile fibers	Light microscopy	Blatt et al. 2011; Warinner et al. 2014a
Ancestry		
Mitochondrial genome reconstruction	DNA sequence-capture HTS	Ozga et al. 2016

Notes:
PCR: polymerase chain reaction
qPCR: quantitative PCR
HTS: high-throughput sequencing
LC-MS/MS: liquid chromatography<n>tandem mass spectrometry
Py-GC-MS: pyrolysis<n>gas chromatography<n>mass spectrometry
TD-GC-MS: thermal desorption<n>gas chromatography<n>mass spectrometry

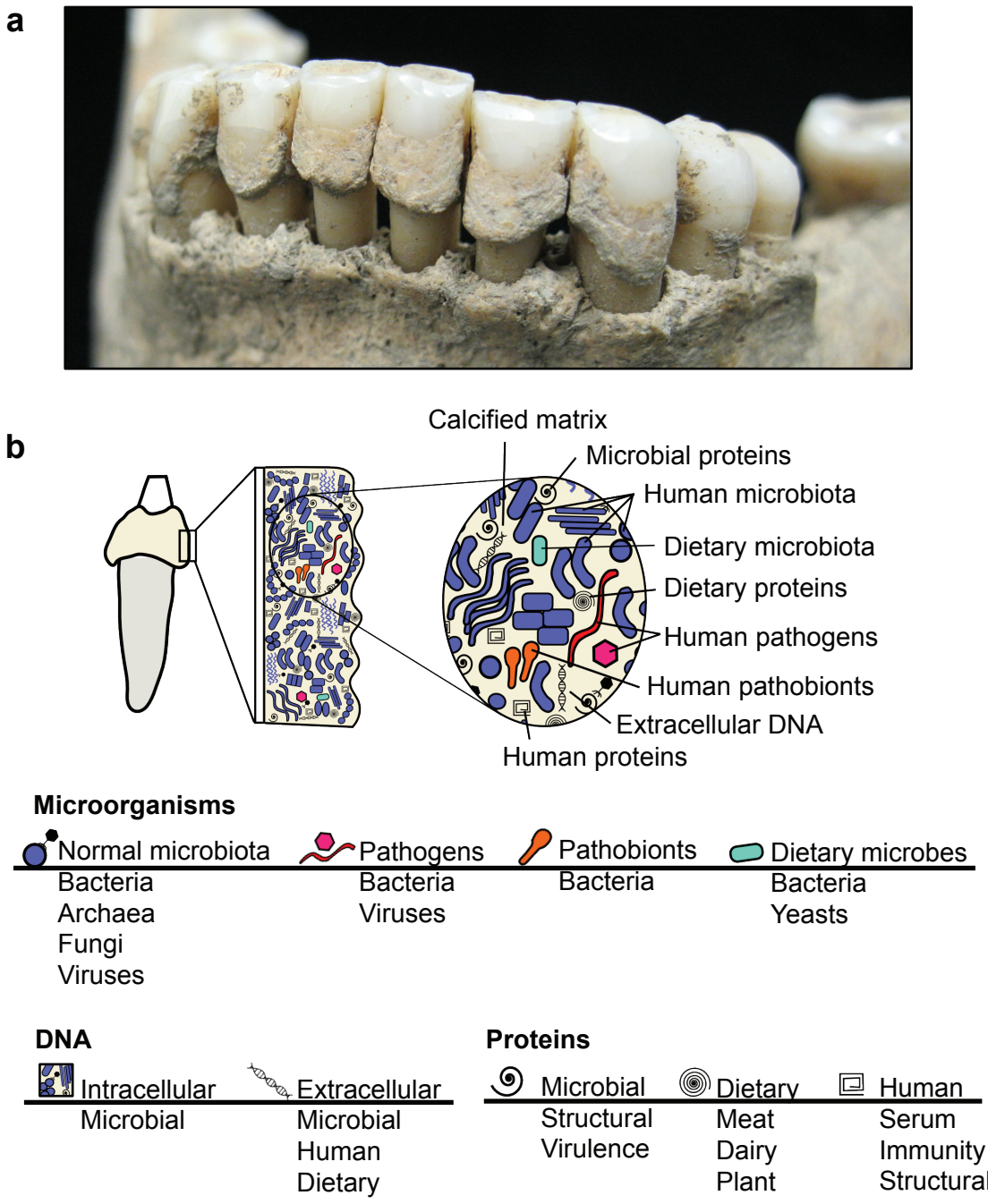

Figure 1. Appearance and microscopic components of ancient dental calculus. (**a**) Heavy calculus deposits on the mandibular dentition of individual B78 from the medieval St. Petri cemetery, Dalheim, Germany, ca. A.D. 1100. (**b**) The wealth of biomolecules in ancient dental calculus include DNA and proteins derived from a broad range of sources, including the human host, normal microbiota, and dietary components.

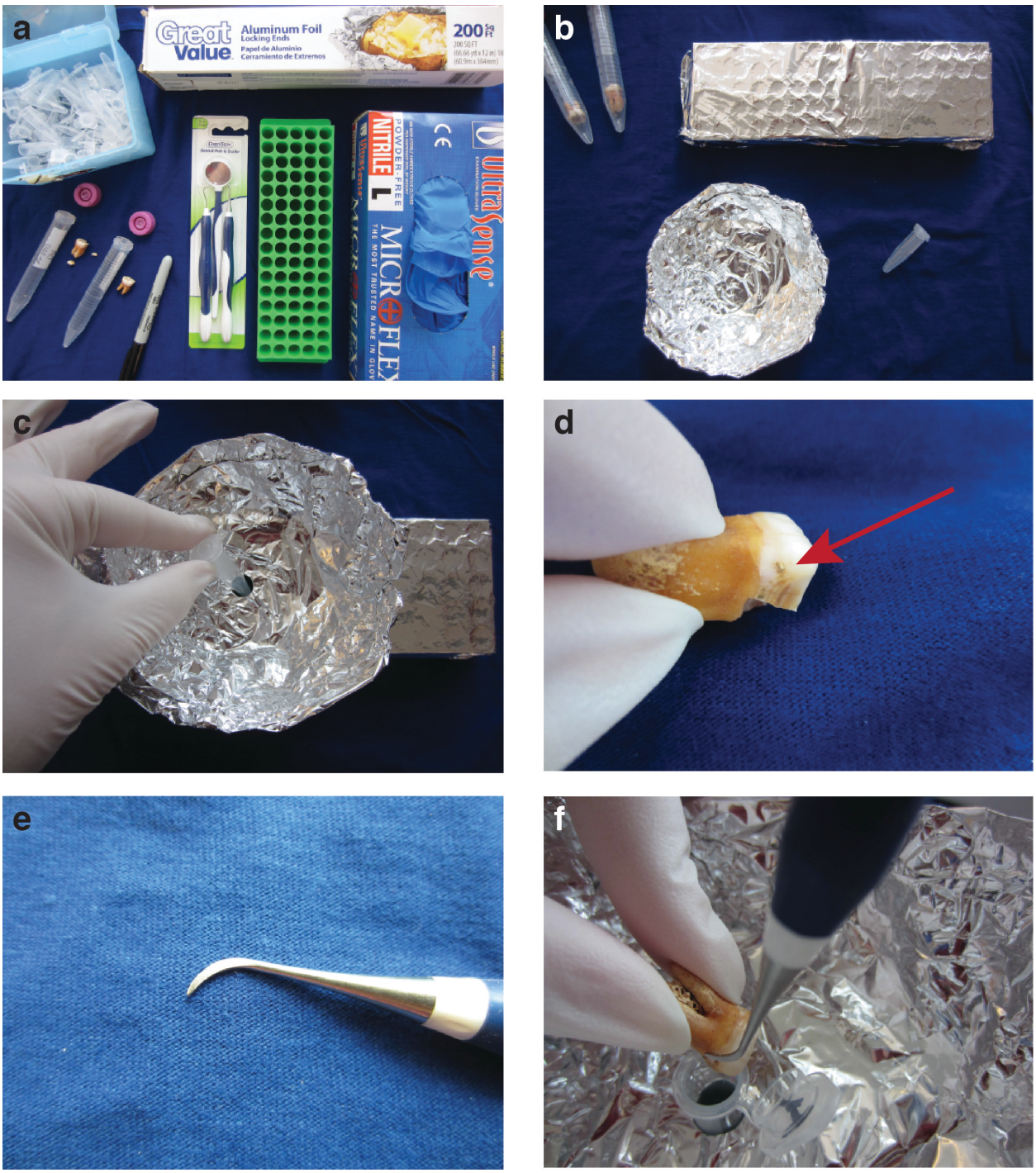

Figure 2. Simple dental calculus sampling protocol. (**a**) Basic field sampling kit (not all supplies are shown; see text for details). (**b**) Preparation of sample rack and aluminum foil catchment bowl. (**c**) Assembly of microcentrifuge tube and catchment bowl. (**d**) Example of a small dental calculus deposit prior to sampling. (**e**) Dental calculus sampling should be performed using a dental scaler, an oral hygiene tool available at many pharmacies and through medical equipment suppliers. (**f**) Demonstration of dental calculus sampling technique.

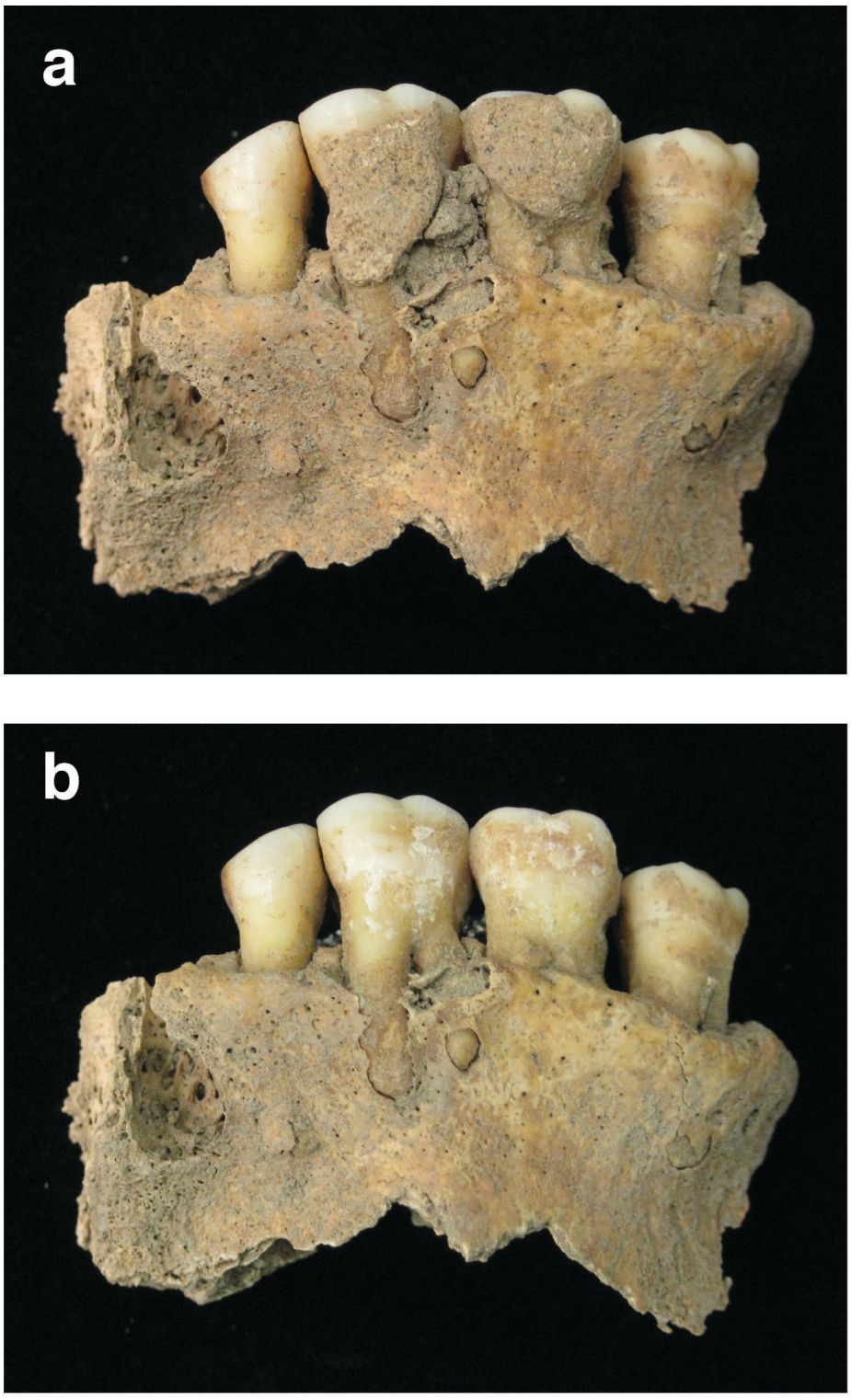

Figure 3. Archaeological dentition (a) before and (b) after dental calculus removal. Removal of dental calculus can be performed on loose teeth (as shown in Fig. 2) or on teeth *in situ* (as shown here) and does not damage the underlying dentition.

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