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Ancient human microbiomes

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ABSTRACT

Very recently, we discovered a vast new microbial self: the human microbiome. Our native microbiota interface with our biology and culture to influence our health, behavior, and quality of life, and yet we know very little about their origin, evolution, or ecology. With the advent of industrialization, globalization, and modern sanitation, it is intuitive that we have changed our relationship with microbes, but we have little information about the ancestral state of our microbiome, and we therefore lack a foundation for characterizing this change. High-throughput sequencing has opened up new opportunities in the field of paleomicrobiology, allowing us to investigate the evolution of the complex microbial ecologies that inhabit our bodies. By focusing on recent coprolite and dental calculus research, we explore how emerging research on ancient human microbiomes is changing the way we think about ancient disease and how archaeological studies can contribute to a medical understanding of health and nutrition today.

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Introduction

Genetic sequencing has revolutionized our understanding of the tree of life and humans' place within it. The development of the Sanger method of DNA sequencing in 1977 and the polymerase chain reaction (PCR) method of DNA amplification in 1983 ushered in an explosion of genetic data that determined the phylogeny of humans and the great apes (Ruvolo, 1997), rejected the biological concept of race in humans (Long and Kittles, 2003), and reconstructed the peopling of the world (Oppenheimer, 2012). The arrival of next generation sequencing (NGS) in the late 1990s facilitated the sequencing of the first complete human genome (Venter et al., 2001), and the subsequent commercial release of this technology in the mid-2000s enabled the genome sequencing of archaic humans, including Neanderthals (Green et al., 2010; Prufer et al., 2014), Denisovans (Krause et al., 2010; Reich et al., 2010; Meyer et al., 2012), and the mitochondrial genome of an archaic hominin classified as *Homo heidelbergensis* (Meyer et al., 2014), resulting in discoveries that have further reorganized and refined the human family tree. These studies have addressed fundamentally important aspects of human evolution. Nevertheless, the human genome encompasses only a fraction of the total genetic diversity found within humans. The collective microbial communities inhabiting the human body, known as the human microbiome, contain a vast amount of genetic and functional diversity far exceeding that of our own nuclear and mitochondrial genomes (Qin et al., 2010). A growing appreciation of the role of microbiomes in host essential life functions, the etiology of disease, and even speciation (Human Microbiome Project Consortium, 2012; Blaser et al., 2013; Brucker and Bordenstein, 2013; McFall-Ngai et al., 2013) challenges conventional views of the biological species concept (Mayr, 1963; Brucker and Bordenstein, 2013) and raises the question of whether or not ancient human microbiomes should also be investigated in order to explore broader issues in human evolution. This paper will discuss the relationship between humans and their microbiomes and review new developments in the emerging field of ancient microbiome research. We argue that only by also exploring our microbiomes both today and in the past can we fully understand what it means to be human.

The human microbiome

Collectively, the microorganisms of the human body include an astounding number of bacteria. Since the late 1970s, it has been known that the number of bacterial cells (~10^{14}) in and on the human body exceeds the number of human cells (~10^{13}) by at least an order of magnitude (Savage, 1977; Peterson et al., 2009; Bianconi et al., 2013). In 2010, it was established that the estimated number

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of unique bacterial genes in our 'accessory genome' (~3,300,000) exceeds the number of our own genes (~22,000) by a factor of 150 (Qin et al., 2010). Despite being 1,000 times smaller than human cells, bacteria still make up about 2% of adult body mass (1.5 kg), making them collectively equivalent in size to the human brain (1.4 kg) or liver (1.6 kg; Molina and DiMaio, 2012), leading some to refer to our resident microbes as an additional human organ (O'Hara and Shanahan, 2006; Baquero and Nombela, 2012). Alternatively, the human–microbial relationship has also been compared to that of a superorganism (Scher and Abramson, 2011), like a colony of bees, or that of a holobiont (Singh et al., 2013), like a coral reef.

In recognition of the need for a collective term to refer to the large number of underexplored and mostly nameless microorganisms inhabiting the human body, Joshua Lederberg coined the term *microbiome* in 2001 to "signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease" (Lederberg and McCray, 2001:8; see also Scher and Abramson, 2011). The term microbiome has also come to be used more restrictively, referring to the collective molecular (especially genomic) data obtained from a community of microorganisms, rather than to the microorganisms themselves (e.g., Hooper and Gordon, 2001), resulting in some confusion in the literature. For the purposes of this review, we will use the term microbiome in accordance with its original published definition by Lederberg as an ecological community of microorganisms, and we will use the term microbiota as a synonym. To refer to the collective genomes of the host and its microbiome, we will use the term hologenome (Zilber-Rosenberg and Rosenberg, 2008). Finally, we will use the terms metagenome and metaproteome to refer to the collective genomic and proteomic information obtained from an environmental sample. These terms have developed in parallel with major technological advances in microbiology and molecular methods for investigating complex microbial systems.

Recent technical advances in microbiome research

Early investigations of human-associated microbes focused on isolating and culturing individual bacterial species and strains. Using these methods, hundreds of human microbiome species have been identified and named (e.g., Dewhirst et al., 2010). However, culture-free molecular analyses of prokaryotic 16S ribosomal RNA (rRNA) and the 16S rRNA gene using methods first developed by Carl Woese (Woese and Fox, 1977) and Norman Pace (Pace, 1997) indicate that at least 60–80% of bacteria inhabiting the human body cannot be cultured in a laboratory (Suau et al., 1999; Hayashi et al., 2002). To access these unculturable members, new molecular methodologies were developed, including terminal restriction fragment length polymorphism (T-RFLP) molecular fingerprinting, single target PCR, multiplex PCR, quantitative PCR (qPCR), fluorescent in situ hybridization (FISH), checkerboard hybridization, and 16S rRNA-based microarrays, among others (Paster and Dewhirst, 2009; Han et al., 2012). The most powerful of these methods combined PCR amplification of 16S rRNA genes from a mixed microbial sample using universal bacterial primers, followed by cloning of PCR products into a plasmid vector, transformation of the plasmids into competent *Escherichia coli* cells, plating and colony picking, plasmid purification, and Sanger sequencing of up to several thousand clones. These sequences could then be clustered by similarity (usually 97.0% or 98.5% sequence similarity) into distinct phylotypes correlating approximately to bacterial species. In 2010, this approach was successfully used to determine 600 prevalent members of the human oral microbiome (Dewhirst et al., 2010); however, characterizing rare or less prevalent oral taxa using this method is not feasible given the high-cost, labor-intensive, and low throughput nature of the cloning and sequencing steps.

The advent of NGS has transformed microbiome investigations by eliminating the need for cloning and allowing the acquisition of millions of 16S rRNA gene sequences at a fraction of the cost per base compared with conventional techniques. Despite shorter read lengths and a shift of focus to individual 16S rRNA gene hypervariable regions rather than the entire gene, the increased sequencing depth has revealed a great diversity of low abundance taxa within the human microbiome, expanding the known taxonomic complexity of the oral microbiome alone by more than an order of magnitude (Keijser et al., 2008; Paster and Dewhirst, 2009). Next generation sequencing has also made possible microbiome metagenomics, the collective study of all genes and genomes within a microbiome. This has enabled microbiome research to move beyond taxonomic questions of 'who's there' to functional questions of 'what they do' (HMP, 2012). Moreover, single cell genome amplification and bioinformatics improvements in sequence assembly from metagenomic datasets are allowing full genome reconstructions of unculturable taxa, providing our first glimpse at the potential function of these enigmatic species (Marcy et al., 2007; Liu et al., 2012). With respect to ancient microbiomes, NGS offers two additional advantages over previous methods. First, being a culture-free method, the analysis can be applied to dead cells, and second, NGS is optimized for fragmented DNA within the size range typical of ancient DNA (<300 bp).

Advances in metagenomic technologies are also being matched in the field of metaproteomics. Previously, the detection of microbial proteins primarily relied on antibody-based approaches, often linked to gel electrophoresis (e.g., Western Blotting). Proteomics-based methods advanced with the use of comparative 2D (pI versus MWt) gels with specific spots eluted and analyzed by soft ionization tandem mass spectroscopy (MS/MS). These 'top-down' approaches are increasingly being replaced by 'bottom-up' approaches also known as shotgun proteomics, which conduct mass spectrometric analyses of peptides from enzymatic digestion of the total protein extract. Such bottom-up approaches allow for higher throughput and are additionally more conducive to ancient protein research, as they do not require intact protein molecules.

These advanced molecular methods are providing unprecedented insights into the function and dysfunction of human microbial systems, giving a path to understanding the role of microbiomes in human health and disease.

Human microbiome function and dysfunction

Since the early 2000s, numerous studies have investigated the structure and function of the human microbiome using a variety of molecular methodologies. A notable boost to these efforts began in 2008 after the initiation of the National Institutes of Health Human Microbiome Project (HMP) in the United States and the Metagenomics of the Human Intestinal Tract (MetaHIT) project in Europe. Whereas human-associated microorganisms were previously viewed at best as passive commensal tag-alongs or nuisances to be scrubbed or flossed away, we now recognize that the human oral, gut, skin, and uritogenital microbiota play critical roles in maintaining host health by performing essential functions in digestion and metabolism (Lozupone et al., 2012; Tremaroli and Backhed, 2012; Yatsunenko et al., 2012), vitamin production (LeBlanc et al., 2013), and immune system education and maintenance (Lee and Mazmanian, 2010; Hooper et al., 2012), as well as by restricting the colonization, growth, reproduction, and virulence expression of exogenous bacterial pathogens through resource competition (Brotman, 2011; Lozupone et al., 2012; Fitz-Gibbon et al., 2013). However, when challenged by poor diet, illness,

stress, antimicrobial drugs, and other environmental disruptions, the ecology of the human microbiome can transition from a mutualistic to a dysbiotic state, contributing to local and systemic illnesses as varied as obesity, type II diabetes, irritable bowel disease, and colon cancer (Rose et al., 2007; Clemente et al., 2012; Devaraj et al., 2013), periodontal disease and dental decay (Marsh, 2003; Pihlstrom et al., 2005; Kumar et al., 2006; Aas et al., 2008), atherosclerosis and endocarditis (Scannapieco et al., 2003; Koren et al., 2011; Koeth et al., 2013), autism, anxiety, and depression (El-Ansary et al., 2013; Foster and Neufeld, 2013).

The healthy human microbiome also plays host to a number of endemic, but potentially acute, opportunistic pathogens, such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Clostridium difficile*, *Propionibacterium acnes*, and *Staphylococcus aureus* (HMP, 2012), some of which are implicated in hospital and community-acquired infections and pose particular risk for the elderly and immunocompromised (Shay, 2002). Alarmingly, multiple antibiotic resistant strains are increasingly being detected in the normal oral and gastrointestinal microbiota of healthy individuals (Ready et al., 2003; Carlet, 2012). This suggests that the use of antibiotic therapy, either through direct clinical application or through indirect growth stimulating or prophylactic application in livestock, impacts non-clinical targets and can result in long-term endogenous reservoirs of antibiotic resistance (Roberts and Mullany, 2010). Additionally, antibacterial therapies can themselves be disruptive to healthy bacterial communities, leading to further complications. Therapeutic courses of broad-spectrum antibiotics, for example, are known to disrupt gut and uritogenital microbiota (Jakobsson et al., 2010; Jernberg et al., 2010), where they may induce antibiotic-associated colitis and bacterial vaginosis, respectively (Willing et al., 2011). As a consequence, there is growing interest in probiotic and prebiotic therapies for treating disrupted microbiomes, but lack of basic knowledge on what constitutes a healthy microbiome, as well as a clearer understanding of the transmission and formation of healthy microbiota, are limiting factors in the development of these therapies (Marsh, 2003; Wade, 2010; Zarco et al., 2012).

Need for paleomicrobiology data

Although considerable effort has been invested in characterizing healthy gut and oral microbiomes, recent investigations of rural, non-Western populations (Lozupone et al., 2012; Yatsunenko et al., 2012) have raised questions about whether the microbiota we currently define as normal have been shaped by recent influences of modern Western diet, hygiene, antibiotic exposure, and lifestyle (Maslowski and Mackay, 2011). The process of industrialization has dramatically reduced our direct interaction with natural environments and fundamentally altered our relationship with food and food production. Situated at the entry point of our food, and the locus of food digestion, the human oral and gut microbiomes have evolved under conditions of regular exposure to a diverse range of environmental and zoonotic microbes that are no longer present in today's globalized food chain. Additionally, the foods themselves have changed from the wild natural products consumed by our hunter-gatherer ancestors to today's urban supermarkets stocked with an abundance of highly processed Western foodstuffs containing artificially enriched levels of sugar, oil, and salt, not to mention antimicrobial preservatives, petroleum-based colorants, and numerous other artificial ingredients. This dietary shift has altered selection pressure on our microbiomes. For example, under the 'ecological plaque hypothesis,' diseases such as dental caries and periodontal disease are described as oral ecological catastrophes of cultural and lifestyle choices (Marsh, 2003).

Although it is now clear that the human microbiome plays a critical role in making us human, in keeping us healthy, and in making us sick, we know remarkably little about the diversity, variation, and evolution of the human microbiome both today and in the past. Instead, we are left with many questions: When and how did our bacterial communities become distinctly human? And what does this mean for our microbiomes today and in the future? How do we acquire and transmit microbiomes and to what degree is this affected by our cultural practices and built environments? How have modern Western diets, hygiene practices, and antibiotic exposure impacted 'normal' microbiome function? Are we still in mutualistic symbiosis with our microbiomes, or are the so-called 'diseases of civilization'—heart disease, obesity, type II diabetes, asthma, allergies, osteoporosis—evidence that our microbiomes are out of ecological balance and teetering on dysbiosis (Stecher et al., 2013)? At an even more fundamental level, who are the members of the human microbiome, how did they come to inhabit us, and how long have they been there? Who is 'our microbial self' (Gonzalez et al., 2011)?

Studies of remote and indigenous communities (Contreras et al., 2010; Yatsunenko et al., 2012; Schnorr et al., 2014) and crowd-sourcing projects such as the American Gut (www.americangut.org), the Earth Microbiome Project (www.earthmicrobiome.org), and uBiome (www.uBiome.com) are attempting to characterize modern microbiomes across a range of contemporary environments. Nevertheless, even the most extensive sampling of modern microbiota will provide limited insight into Pre-Industrial microbiomes. By contrast, the direct investigation of ancient microbiomes from discrete locations and time points in the past would provide a unique view into the coevolution of microbes and hosts, host microbial ecology, and changing human health states through time.

Ancient microbiome research

Upon death, the ecology of the human microbiome transforms dramatically through the process of soft tissue decomposition (Morris et al., 2006). With the exception of frozen and mummified remains, only two microbiomes routinely produce substrates that, under favorable conditions, persist after death in archaeological contexts: fecal material of the gut microbiome may desiccate or mineralize to produce coprolites, and dental plaque of the oral microbiome calcifies in situ during life such that by the time of death it is already in a semi-fossilized state known as dental calculus that resists decomposition and thus continues to preserve after death. The opportunity to investigate ancient microbiomes directly through coprolites and dental calculus allows us to redefine questions of past human health within a much broader framework that includes not only the investigation of epidemic obligate pathogens but also the carriage rates and risks posed by endemic and dormant pathogens, as well as the health and resiliency of the overall microbial community. Finally, in the case of dental calculus, which forms incrementally without remodeling, it may even be possible to reconstruct a life history of disease, including survivorship of past pandemics.

Coprolites

Coprolites are mineralized or desiccated feces. Coprolites have been found dating as far back as 270 Ma (millions of years ago) during the Paleozoic Era (Dentzien-Dias et al., 2013), and many famous examples of dinosaur coprolites are known from the Cretaceous period ca. 145–66 Ma (Chin et al., 1998). Perhaps less well known are the human coprolites and latrine deposits that have been found at archaeological sites dating from the Late Paleolithic

onwards (after ca. 22,000 BP [years before present]; Wendorf et al., 1988; Jenkins et al., 2012). Unlike paleontological coprolites, archaeological coprolites were not initially recognized as an important biological data source and were only irregularly curated throughout the nineteenth and early twentieth centuries. Even now that their potential is recognized, coprolites and preserved intestinal contents remain rare finds, and they tend to preserve intact only under extremely dry or frozen conditions (e.g., Cano et al., 2000; Tito et al., 2008). Additionally, because human coprolites are not fully fossilized and may be fragmentary or amorphous, they are often difficult to identify or recover in situ, and may not be found until downstream screening contexts (Reinhard and Bryant, 2008).

Feces contain an incredible number of microorganisms—more than a billion viable bacterial cells per milligram of stool (Ott et al., 2004). As such, feces are highly biologically active and typically decompose rapidly, except under extraordinary circumstances. The immediate post-depositional environment is crucial to coprolite preservation at the biomolecular level. Latrine deposits and soils, which are useful for dietary and parasite analyses, suffer from the same taphonomic challenges. Calcareous deposits, however, such as those recovered from latrine drainage pipes in Pompeii (Hobson, 2009), may provide a better preservational environment, especially if feces particles become rapidly encrusted with mineral scale, consequently mitigating both the process of decomposition and environmental contamination. Although some coprolites recovered from exceptional contexts (e.g., a mummified colon) may provide insight into personalized health states, most coprolites are not found in association with a particular skeleton. Instead, many coprolites and calcareous deposits are recovered from communal latrine areas or middens, and consequently ancient gut microbiome analysis must be conducted at a population level, rather than on an individual basis.

From the 1930s onward, there have been several attempts to characterize the bacteria within coprolites using both culturing and microscopic techniques. However, these attempts met with limited success due to bacterial cell inviability and the inability to refine bacterial categorization beyond general cellular morphology and a few cell wall chemical properties (Reinhard and Bryant, 1992). This situation improved in the late 1990s with the availability of PCR and molecular cloning techniques. The first studies to extract ancient DNA from coprolites and amplify a portion of the 16S rRNA gene (rDNA) found sequences that were generally consistent with the families and genera expected for gut bacteria. However, because these studies rarely examined more than a few dozen sequence clones per sample (Ubaldi et al., 1998; Cano et al., 2000; Poinar et al., 2001; Luciani et al., 2006; Rollo et al., 2007), there were simply too few data to draw community-level conclusions.

Tito et al. (2008) were the first to perform NGS on ancient coprolites. Using two coprolite samples from the Cueva de los Muertos Chiquitos, near the town of Rio Zape in Durango, Mexico, they generated 45,000 shotgun sequences and demonstrated that the ancient Rio Zape coprolites resembled modern feces in both taxonomic and functional profiles. The fecal microbiomes observed in this study raised the question of whether ancient human microbiota may be more biogeographically structured than they are today, a question that has important implications for current attempts to define a 'core' human microbiome (Arumugam et al., 2011; Huse et al., 2012). Tito et al. (2012) followed up this study with additional targeted 16S rRNA gene deep sequencing of the Rio Zape coprolites, additional coprolites from Hinds Cave, Texas, and mummified human intestinal contents from Caserones, Chile. However, unlike the Rio Zape coprolites, the Hinds Cave coprolites exhibited no resemblance to modern fecal bacteria, and the Caserones sample was most similar to bacterial communities found in composted organic matter. Their results suggested that while some coprolites preserve authentic gut microbiota, as in the case of the Rio Zape samples, coprolites and intestinal contents in general represent open systems that are susceptible to both self-digestion and bacterial infiltration from the burial environment during decomposition.

Recently, shotgun NGS and scanning electron microscopy (SEM) was applied to medieval coprolites sealed within latrine barrels with the aim of retrieving ancient gut microbiome viruses (Appelt et al., 2014). By comparing the resulting metagenomic sequences to the NCBI RefSeq Viral Genomes database, several thousand DNA sequences were identified with homology to known viral families, including a few that infect eukaryotes, but mostly belonging to the Siphoviridae family of double-stranded DNA bacteriophages. Although poorly understood, bacteriophages are estimated to outnumber bacterial cells in the human body by an order of magnitude, and these viruses are thought to play a central role in structuring host-associated bacterial populations and indirectly influencing host health (Fancello et al., 2012; Reyes et al., 2012).

In addition to bacteria and viruses, coprolites also preserve evidence of parasitic infections in the form of microscopically visible helminth eggs, nematode larvae, and adults (Bouchet et al., 2003; Goncalves et al., 2003; Seo et al., 2007, 2008; Shin et al., 2009a,b; Mitchell, 2013). Additionally, parasite proteins (Goncalves et al., 2002, 2004; Mitchell et al., 2008) and DNA (Loreille et al., 2001; Iniguez et al., 2006; Cleeland et al., 2013) can also be detected in coprolites, even in the absence of microscopic parasite remains (Iniguez et al., 2003). Thus, combined microscopic and biomolecular analyses of coprolites provide a wealth of information about the prevalence, transmission, and evolution of parasitic infections, and offer potential insights into gastrointestinal health, water quality, sanitation, and hygiene practices in the past.

Dental calculus

Dental calculus is a calcified bacterial biofilm that forms on the surfaces of teeth, and it is found in all human populations, as well as Miocene apes (12.5–8.5 Ma; Hershkovitz et al., 1997), Neanderthals (Pap et al., 1995; Henry et al., 2011; Hardy et al., 2012), wild chimpanzees (Hardy et al., 2009), and a range of animals (Dobney and Brothwell, 1987; Middleton and Rovner, 1994). Among humans, both in the past and today when professional dentistry care is not available, the incidence of dental calculus is near-ubiquitous among adults by age 30 (White, 1997; Lieverse, 1999), and in our experience we have found that it is not uncommon to observe dental calculus deposits in excess of 100 mg in archaeological assemblages of agricultural populations. In addition to being both common and relatively abundant, dental calculus is also a rich source of ancient biomolecules, with extracted DNA yields up to three orders of magnitude greater than from bone or dentine of the same individual (Warinner et al., 2014a,b; see also Weyrich et al., 2014). This remarkable biomolecular preservation mirrors the structural preservation of archaeological dental calculus, which retains the biological organization, inorganic composition, and remnant organic structures (e.g., cell walls, DNA) observed in the mature dental calculus of living subjects (Warinner et al., 2014a,b). Importantly, dental calculus is unique among ancient microbiome sources in that it does not shed, remodel, or turnover; rather, it forms incrementally through serial deposition and mineralization in situ (White, 1991), making it a layered record of human life history specific to each person.

The excellent preservation of biomolecules within dental calculus is likely due to several factors. First, dental calculus is not a material that is easily colonized or consumed by environmental bacteria. It lacks biological channels, such as Haversian canals,

cannaliculi, or dentinal tubules that typically provide postmortem points of entry and movement for exogenous bacteria within bones and teeth, and it also lacks a rich organic nutrient source, such as bone marrow or dental pulp, to attract and support environmental bacterial growth and facilitate decomposition (Turner-Walker, 2008). A second important factor is the fact that dental calculus begins the process of fossilization long before the host organism dies (Jin and Yip, 2002; Jepsen et al., 2011). As dental plaque biofilms grow, restricted nutrient diffusion to the interior leads to bacterial death and the subsequent deactivation of membrane-bound ATP-dependent ion pumps. High intracellular phosphate concentrations lead to calcium diffusion into the cells in excess of calcium phosphate saturation, resulting in rapid intracellular mineralization (White, 1997) and the interruption of enzymatic and hydrolytic processes associated with decomposition. Meanwhile, extracellular calcium phosphate mineralization proceeds in parallel, effectively entombing bacterial cells within crystal aggregates. Thus, by the time dental calculus is exposed to a postmortem depositional environment it has long been non-vital and biologically inert. Third, the mineral composition and large crystal sizes within dental calculus also likely contribute to the long-term preservation of in situ DNA within this mineralized biofilm. Similar to bone and dentine, calcium phosphates account for 75–80% of the dry weight of mature dental calculus (Schroeder, 1969); however, unlike bone or dentine, these calcium phosphates are present in multiple phases and are typically ordered into a diversity of needle-like and plate-like crystal aggregates. Individual crystallites typically measure 10–300 nm in length and 2–30 nm in width (Schroeder, 1969); this is larger than hydroxyapatite crystals found in bone, which typically measure no more than 55 nm in their largest dimension (Clarke, 2008; Nudelman et al., 2010). Calcium phosphate is known to efficiently bind DNA, and this property has been exploited in molecular biology where calcium phosphate is used as a carrier compound in DNA transfection (Kingston et al., 2003) and as a binding substrate in DNA extraction and purification (Herzer, 2001; Yu et al., 2008). Finally, once mineralized, dental calculus becomes cement-like both in hardness and adhesive strength, making it extremely resistant to decay or removal (White, 1997; Jin and Yip, 2002; Jepsen et al., 2011).

Following electron microscopy imaging of modern dental calculus in the 1960s and 1970s (Schroeder, 1969; Jones, 1972; Lustmann et al., 1976), it was recognized that microorganisms within human, Neanderthal, and extinct primate dental calculus could be imaged and morphologically characterized using SEM (Brothwell, 1972; Dobney and Brothwell, 1986, 1988; Hansen et al., 1991; Dobney, 1994; Vandermeersch et al., 1994; Pap et al., 1995; Arensburg, 1996; Hershkovitz et al., 1997) and later direct optical techniques (Linossier et al., 1996; Charlier et al., 2010). Biomolecular investigations of calculus began with immunohistochemical analysis of *Streptococcus mutans* (Linossier et al., 1996), followed by gold-labeled antibody transmission electron microscopy (TEM) of in situ DNA (Preus et al., 2011) and PCR-based analyses targeting specific oral taxa, including *Actinomyces naeslundii*, *Fusobacterium nucleatum*, *Streptococcus gordonii*, *Porphyromonas gingivalis* and *S. mutans* (De La Fuente et al., 2012; Adler et al., 2013).

Adler et al. (2013) performed the first NGS analysis of ancient dental calculus and demonstrated that dental calculus could be used to characterize ancient oral microbiomes. Amplifying the 16S rRNA gene third hypervariable region (V3), they performed phylum-level comparisons of dental calculus bacteria in Mesolithic, Neolithic, Bronze Age, medieval and modern samples, and reported ecological shifts in Gram-positive members of the oral microbiome corresponding to the origins of agriculture and the Industrial Revolution. Subsequently, Warinner (2014a) used shotgun metagenomic and metaproteomic approaches, in combination with 16S rRNA gene deep sequencing, to reconstruct a species-level taxonomic and protein functional characterization of medieval dental calculus samples. Their study revealed that the oral microbiome has long served as a reservoir for a diverse range of opportunistic pathogens and putative low-level antibiotic resistance genes. From these data they were able to characterize active periodontal disease based on metaproteomic evidence of bacterial virulence factors and host immune activity, and reconstruct the genome of the periodontal pathogen *Tannerella forsythia*. Considering the near ubiquity of dental calculus in the Quaternary paleontological and archaeological records, these studies provide a first glimpse at the potential wealth of evolutionary and health information that ancient oral microbiome research is likely to provide as more geographically and temporally diverse populations are investigated.

Other potential sources of ancient microbiome data

Historic medical specimens Although limited to the past few centuries, medical specimens of human tissue represent an additional source of historic human microbiome samples. Usually preserved in liquid alcohol or formaldehyde or stored in formalin-fixed paraffin-embedded (FFPE) blocks, these samples include anatomical and pathological specimens ranging from whole bodies to complete or partial organs to tissue biopsies. Morphological preservation of these specimens is typically good, but molecular preservation may be adversely affected by specific chemicals used during tissue fixation and storage, such as formalin (Gilbert et al., 2007). Recently, genetic sequences of *Vibrio cholerae*, the causative agent of cholera, were recovered from an alcohol-preserved medical specimen of colon dating to 1849 CE (Devault et al., 2014). Such studies suggest that microbiome characterization from historical medical specimens is feasible. Historic medical specimens may prove to be particularly valuable for accessing microbiome body sites that rarely preserve in the archaeological record, such as the small intestine and proximal colon, as well as the reproductive organs and structures.

Mummified human remains Naturally and artificially mummified remains provide an additional potential source of human microbiota, including those of the skin, lungs, stomach, and other organs and structures. However, despite gross preservation at a macroscopic scale, mummified tissues are still susceptible to the same microscopic and molecular taphonomic processes as other ancient tissues, and attempts to date to analyze microbiota from these sources have yielded mixed results. In analyzing the microbiota of the Tyrolean Ice Man, for example, Rollo et al. (2000, 2007) found that bacteria collected from the skin and stomach were consistent with environmental contamination, while bacteria obtained from the colon yielded 16S rRNA gene sequences consistent with human gut bacteria. Meanwhile, Castillo-Rojas et al. (2008) analyzed gastric samples of a pre-Columbian mummy in Chihuahua, Mexico and successfully PCR-amplified genetic markers for *Helicobacter pylori*, the causative agent of stomach ulcers. Recently, Corthals et al. (2012) applied shotgun proteomics to labial swabs from two 500-year-old Andean mummies and identified several human proteins expressed in saliva. Using targeted PCR, they also recovered several *Mycobacterium* sequences, although whether these sequences originated from pathogenic or environmental taxa is unclear.

Secondary deposition in bone A corollary to the idea that environmental bacteria may infiltrate and alter coprolite microbial communities is the idea that gut microbiome bacteria may leak out of the colon and fecal material and infiltrate bones shortly after death (Bell et al., 1996). The gastrointestinal community composition changes radically after death (Heimesaat et al., 2012), and members of this

community appear in the blood within 24 h postmortem (Saegeman et al., 2009; Heimesaat et al., 2012). Jans (2008) demonstrated that spongiform (microbial) alteration is common in articulated skeletons but rare in disarticulated skeletons, supporting the hypothesis that putrefying bacteria released into the abdominal cavity from the gut are the primary initiators of early stage diagenesis (Turner-Walker, 2008). Hypothetically, these bacteria could then later become entrapped within the bone matrix during taphonomic processes of demineralization and remineralization. Apatite is mobilized but retained and reprecipitated within the bone, apparently around microbial cell membranes (Turner-Walker and Syversen, 2002), potentially leading to long-term molecular preservation. If true, bone could serve as a trap for some gut microbiome bacteria and provide partial access to these bacteria even in the absence of mummified gastrointestinal contents or coprolites. The skeletal remains of infants and young children might serve as an interesting test of this hypothesis, as their gut microbiome is dominated by vaginal and skin bacteria for the first few years of life (Dominguez-Bello et al., 2010; Koenig et al., 2011), and thus should be readily distinguishable. The bacterial composition of bone, however, is complex and likely of heterogeneous origin. Recently analyzed microbial profiles from Neanderthal bone (Zaremba-Niedzwiedzka and Andersson, 2013), permafrost horse bone and dentine (Der Sarkissian et al., 2014), and medieval human bone and dentine (Warinner et al., 2014a,b) were found to be dominated by organisms typical of environmental rather than human microbiome sources. Future studies will be required to determine to what degree mortuary architecture or furniture, such as tomb or coffin use, may increase the amount of human microbiota found within bone, considering that such structures physically retain host-associated bacteria within close proximity of the corpse and limit the exposure of the corpse to environmental bacteria (Duday and Guillon, 2006; Janaway et al., 2009).

Host considerations

In addition to serving as sources of preserved microbiomes, dental calculus and coprolites also contain variable quantities of host DNA, protein, and metabolites. Human DNA within coprolites (presumably from shed epithelial cells of the gastrointestinal tract) may make up as much as 50% of the total DNA in well-preserved samples (Bon et al., 2012). Host biomolecules have been used to validate the coprolite species of origin (Wood et al., 2008; Bon et al., 2012) and to identify and date the presence of humans at early Paleoindian sites where few cultural artifacts are present (Gilbert et al., 2008). Steroidal hormones like estrogen, testosterone, progesterone, and estradiol have been detected in coprolites using radioimmunoassay procedures (Sobolik et al., 1996; Rhode, 2003). Additionally, bacterial cholesterol metabolites, such as coprostanol, can be recovered from ancient coprolites and latrines (Bull et al., 1999), and may be informative of cardiovascular disease risk (Veiga et al., 2005). Finally, considering that cortisol and other steroid hormones can be routinely and non-invasively collected from wildlife fecal samples to study adrenocortical activity (Mostl and Palme, 2002; Millspaugh and Washburn, 2004), human coprolites may also provide insight into the physiological stress and reproduction of ancient populations.

Host DNA within dental calculus is only beginning to be explored (e.g., Kawano et al., 1995; De La Fuente et al., 2012) and seems to account for approximately 0.5% of DNA in ancient dental calculus and 0–2.5% in modern dental plaque (Warinner et al., 2014a,b). There are many possible ways that host DNA could become incorporated into dental calculus given that dental plaque is continuously bathed in host saliva and gingival crevicular fluid, but interestingly host proteins identified within both modern and ancient dental calculus suggest that immunological activity and the release of neutrophil extracellular traps (NETosis) may be the dominant method (Warinner, 2014a). Neutrophils are the primary immune cell type involved in host defense against plaque/calculus bacteria, and during NETosis neutrophils undergo a specialized form of programmed cell death in which they use chromatin DNA as a structural web or net-like material to distribute bactericidal proteins onto advancing plaque deposits (Ryder, 2010; Remijsen et al., 2011; Brinkmann and Zychlinsky, 2012; Branzk and Papayannopoulos, 2013). The result of this process is that host DNA and a wide range of bactericidal proteins become embedded within the dental calculus matrix, but structural and housekeeping proteins of the neutrophil cell are largely absent. As a consequence of this dynamic immunological process, dental calculus represents an important additional source of host biomolecules in the archaeological record that may prove useful for understanding host genomic and proteomic aspects of health and disease.

The ability to recover both human and microbial DNA from the same archaeological substrate provides an exciting potential to investigate the relationships between host genotype and microbiome composition, function, and evolution. While several studies have investigated the role of host genetic variation in determining susceptibility to common diseases (Barnes et al., 2011; Chapman and Hill, 2012), genome wide studies are only beginning to elucidate the role of host genotype in modulating microbiota composition (Xavier and Podolsky, 2007; Benson et al., 2010; Spor et al., 2011). As we improve our understanding of the complex relationship between host genotype and microbiome composition, coprolites and dental calculus will provide new avenues for exploring the environmental and genetic factors that shape host-associated microbial diversity and health states in ancient populations.

Dietary and life history information preserved in microbiome substrates

There is a growing understanding of the role that diet plays in structuring our microbiomes. Not only does the gut microbiome play a major role in digestion, vitamin production, and energy sequestration, but individual dietary choices also influence gut microbiome composition. Differences in the relative proportions of bile-tolerant organisms (e.g., *Bacteroides*) and/or plant polysaccharide metabolizers (e.g., *Prevotella*) have been observed among individuals consuming vegan, vegetarian, and 'omnivorous' diets (Zimmer et al., 2012), as well as between rural and urban populations (Yatsunenko et al., 2012). Moreover, in controlled feeding studies, shifts to predominantly plant- or animal-based diets produced observable changes in the human gut microbiome within as little as 24 h (Wu et al., 2011; David et al., 2014). Despite the plasticity of the gut microbiome, however, microbial composition is constrained by host biology, and dietary effects are typically secondary to host evolutionary relationships in the determination of microbiome structure. For example, despite consuming a diet nearly entirely composed of bamboo, pandas retain a carnivore-like gut microbiome (Ley et al., 2008), which is augmented by cellulose-metabolizing taxa (Zhu et al., 2011).

Diet also plays a role in shaping the composition of oral microbiomes, most notably by the action of dietary sugar in promoting the growth of cariogenic bacteria such as lactobacilli and *S. mutans* (Vågstrand and Birkhed, 2007). Two recent papers have proposed that cariogenic bacteria, such as *S. mutans*, were absent in pre-Neolithic human populations, possibly indicating low carbohydrate diets (Soltysiak, 2012; Adler et al., 2013), while evolutionary genomic analyses of *S. mutans* suggest an expansion in this species approximately 10,000 years ago, coinciding with the onset of

agriculture (Cornejo et al., 2013). Furthermore, research in dental anthropology as well as in vitro studies indicate that diets rich in starch and oil may enhance oral calcification and calculus formation (Lieverse, 1999; Hidaka and Oishi, 2007; Hidaka et al., 2008).

Importantly, coprolites and dental calculus also contain direct evidence of ancient diets. Ancient feces preserve dietary macrofossils, such as undigested or indigestible plant, animal, and fish remains (Callen and Martin, 1969; Fry, 1985; Holden, 1991), microfossils, such as pollen and phytoliths (Reinhard and Bryant, 1992), and dietary DNA molecules (Poinar et al., 1998, 2001; Wood et al., 2008; Bon et al., 2012). Likewise, dental calculus entraps plant microfossils and environmental debris, including plant phytoliths (Fox et al., 1994, 1996), starches (Boyadjian et al., 2007; Henry and Piperno, 2008; Piperno and Dillehay, 2008; Hardy et al., 2009, 2012; Henry et al., 2011), and fibers (Blatt et al., 2011), providing evidence for the consumption of starchy soft plant foods that otherwise rarely preserve in the archaeological record, including tubers, rhizomes, squashes, and legumes (Piperno and Dillehay, 2008; Mickleburgh and Pagán-Jiménez, 2012). Dental calculus has also been shown to preserve DNA and proteins from ancient dietary plant and animal sources (Warinner et al., 2014a,b), such as bread wheat (*Triticum aestivum*) *Brassica* sp., a genus in the cabbage family, and ruminant milk. By preserving both direct evidence of consumed foods and associated microbiomes within a single substrate, we now have the ability to examine the link between diet and microbiota at the level of the individual, and examine how major historical shifts in food acquisition, production, and consumption shaped our microbiomes.

The future of ancient microbiome studies

Ancestral state of the human microbiome

There can be no doubt that modern behavior and dietary changes are altering the microbial ecology of humans. While some of these changes could be beneficial, others are disruptive (Cho and Blaser, 2012) and may be a driving force behind the rapidly increasing rates of chronic inflammatory diseases in developed countries (Jones et al., 2012). Common medical interventions, such as antibiotic therapy, have dramatically reduced infectious disease burdens worldwide. However, rather than being targeted strikes against harmful bacteria alone, such therapies can also act as weapons of mass microbial disruption (Dethlefsen and Relman, 2011; Sommer and Dantas, 2011). Broad-spectrum antibiotic usage is increasingly being linked to more subtle microbial disruptions that, in extreme cases, such as antibiotic-associated diarrhea and pseudomembranous colitis, can result in serious and persistent microbiome disturbances (Lo Vecchio and Zacur, 2012). While we accept this disruption as intuitive, there are few pathways to deciphering how exactly human microbiomes have changed over the past decades, centuries, and millennia.

Within the context of anthropology, researchers have conventionally approached such questions regarding the anatomical and behavioral evolution of modern humans by observing our closest living relatives, the non-human primates, and by partnering with extant traditional peoples engaging in diverse lifeways. However, both approaches have advantages and drawbacks. In the context of microbiome research, even the most traditional peoples have been arguably affected by industrialization and globalization, while those peoples that have been minimally influenced typically live in very restricted environments, such as deep in the Amazon jungle. Additionally, the most recent common ancestor of humans and their closest living primate cousins, the chimpanzees, lived at least 6.5 million years ago and perhaps even earlier (Venn et al., 2014), allowing ample time for distinct microbial evolutionary trajectories.

Ancient microbiome research provides an additional pathway to understanding human biology that cannot be achieved by studies of extant individuals and related species alone. Although reconstructing the ancestral microbiome by studying our ancestors directly is not without challenges (Tito et al., 2012), this approach provides a more direct picture of human-microbe coevolution. Likewise, ancient microbiome sources may reveal to what extent bacteria commonly considered 'pathogenic' in the modern world (for example, *H. pylori*) were endemic indigenous organisms in pre-Industrial microbiomes (Hadley, 2006).

The three paths to reconstructing the ancestral microbiomes are also complimentary. For example, analysis of the gut microbiome from extant, rural peoples in Africa and South America have revealed the presence of a common, potentially commensal, spirochete belonging to the genus *Treponema* (De Filippo et al., 2010; Yatsunenko et al., 2012). Such spirochetes have also been detected in extant hunter-gatherers (Schnorr et al., 2014) and in 1,000-year-old human coprolites from Mexico (Tito et al., 2012), but they are essentially absent from healthy urban populations, and they have not been reported in the gut microbiome of chimpanzees (Moeller et al., 2012). These multiple lines of evidence suggest that this poorly understood spirochete is a member of the ancestral human microbiome, yet not necessarily the broader primate microbiome. Future coprolite research may be able to answer the question of how long this microbe has co-associated with humans, and what niche it fills.

Microbiomes and speciation

Since the availability of DNA sequencing technologies, investigations of the molecular processes of evolution and speciation have focused on the genes and genomes of particular species. However, an increasing appreciation of the role of the microbiome in organism fitness and success (Brucker and Bordenstein, 2012; Ezenwa et al., 2012; McFall-Ngai et al., 2013) is challenging this approach, and there are compelling arguments (Brucker and Bordenstein, 2012) for incorporating the microbiome into the classic biological species concept (Mayr, 1963), in accordance with the hologenome theory of evolution (Zilber-Rosenberg and Rosenberg, 2008). Recently, aspects of this theory have been empirically tested in *Nasonia* wasps, where the microbiome was found to play a central role in hybrid lethality and speciation (Brucker and Bordenstein, 2013). Although research in this area is still in an early phase, the implications of such findings are profound and raise questions about the role of the microbiome in human evolution. Recent evidence of archaic *Homo* genetic introgression into anatomically modern humans (Reich et al., 2010; Sankararaman et al., 2012; Huerta-Sánchez et al., 2014) indicates both direct contact and limited interbreeding among multiple species of the genus *Homo*, and at least one study has indicated transmission of parasites (lice) between archaic and modern humans (Reed et al., 2004). Dental calculus has been documented in primates dating as far back as the Miocene (Hershkovitz et al., 1997), and a 50,000-year-old coprolite associated with Neanderthals was recently discovered in Spain (Sistiaga et al., 2014), demonstrating that microbiome substrates are available for analysis. Investigating these ancient microbiomes may yield important insights into the evolution of the human lineage and clarify how *Homo sapiens* came to be the only species within our genus to survive into the Holocene.

Evolution of human microbial commensals and pathogens

The advent of NGS, coupled with DNA hybridization capture techniques, has enabled the complete genome reconstruction of a

number of historic pathogens infecting humans (*Yersinia pestis*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, and *T. forsythia*) from a variety of ancient sources, including archaeological bone, dentine, and dental calculus, as well as museum-curated herbarium specimens (Bos et al., 2011; Donoghue, 2013; Warinner et al., 2014a,b). As our technological and bioinformatic capabilities grow, it will become possible to move beyond this handful of pathogens to recover the genomes of myriad commensal and endemic microbiota.

Importantly, coprolites and especially calculus represent reliable and abundant sources of directly datable bacterial genomes from a wide variety of microorganisms including archaea, bacteria, and fungi. Not only can these complete ancient genomes provide important phylogenetic data, but they also provide the means for calibrating molecular clocks of bacterial evolution. While current bacterial dating methods primarily rely on the geological record (e.g., speciation because of island formation) or medical specimen collection dates (Bromham and Penny, 2003), these approaches limit dating to substrates that are either extremely old (on a geological time scale, with very wide temporal error ranges) or fairly young (generally within the past century). Bacterial genomes recovered from Quaternary deposits, therefore, represent a key middle ground. Moreover, as microbiomes generally reflect complex ecological communities, coprolites and calculus provide key substrates for analyzing the rate of horizontal gene transfer, as well as the evolution of mechanisms underlying anti-microbial resistance (D'Costa et al., 2011; Warinner et al., 2014a,b).

Conclusion

Characterizing ancient human microbiomes is more complex than a simple binary present to past comparison and will instead require a time-series approach linked to major moments in human development and innovation, from migrations out of Africa, admixture with archaic humans, refining tool technologies, domesticating, and industrializing. Our goal now should be to discover if and how each of these pivotal moments in human history and prehistory reflect moments where our relationship with microbes was changed. Fortuitously, substrates such as dental calculus appear to preserve well and are nearly as ubiquitous as the skeletal material itself, with globally diverse distributions through time. There is now a wealth of ancient human microbiome information available to us, which is providing a more complete picture of human biology and evolution. The future for ancient microbiome research is very bright indeed.

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