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Ancient and Contemporary Analyses of the Impact of the Agricultural Transition on the Human Oral Microbiome

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Thesis Declaration

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30/11/2018

Muslih. A. Abdul-Aziz   Date

"You are what you eat" "Tell me what you eat and I'll tell you what you are"

Jean Anthelme Brillat-Savarin, French gastronome, 1862

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Thesis Abstract

Communities of bacteria inhabit different parts of the human body and influence human metabolic, immune and even nervous systems, playing key roles in human health and disease. This microbial world and its genomes were recently discovered and was termed the "microbiome". Recent research suggests that human cultural changes such as the human transition to agriculture and the subsequent Industrial revolution changed the human microbiome. These changes may underpin many modern metabolic diseases, such as diabetes and heart disease, and explain why modern metabolic disease is more prevalent in humans not currently living a hunter-gatherer lifestyle. However, limited geographical sampling has resulted in an incomplete understanding of the impact of these human cultural changes on the microbiome. Expanding sampling to lesser-studied locations, such as Africa and the Near East has the potential to improve our understanding of the roles that microbiota play in our health.

In this thesis, I obtain ancient DNA from calcified dental plaque (calculus) to describe the first oral microbiomes from ancient Near Eastern individuals over a 7000-year span. This ground-breaking data allowed me to explore the impact of agriculture at the earliest sites of agriculture in ancient Egypt and the Levant. First, I explore how research into the nascent field of microbiome has evolved over the past two decades and how microbiome research may be influenced by new interactions between the human genome and microbiome. Second, I examine the impact of widely used and novel ancient DNA extraction and library preparation methods on microbiome composition and assess methods that could allow future researchers to obtain higher yields of ancient DNA from dental calculus samples from poorly preserved samples. Third, I use ancient and historic shotgun oral metagenomes obtained from dental calculus remains of individuals from Africa, the Near East and Asia to explore the oral microbiome of ancient hunter-gatherers and agriculturalists to examine how the transition to agriculture could have impacted modern health. I also compare these individuals with those from Europe, which are better studied, to reveal the impact of environment and diet on the microbiome. While I find few significant differences between ancient hunter-

gatherers and agriculturalists in the composition of species present within ancient oral microbiomes, the largest difference observed was in functions present within the two groups. This suggests that diet may drive functional differences in oral microbiomes between ancient hunter-gatherers and agriculturalists, while composition may be influenced by other factors, such as microbial ecology in the oral cavity, host genetics, oral hygiene, and environment. Finally, using saliva samples, I examine differences in the oral microbiomes of two contemporary African populations with different sustenance strategies (hunter-gatherers and farmers) living in close proximity in Central Africa and compare them to other westernised and non-westernised populations. My results show that Industrialisation results in lower oral microbial diversity and an increase in potentially oral disease-causing bacteria. This suggests non-Industrialised individuals likely had a balance between beneficial bacteria and disease-causing bacteria in their mouths, while microbiota from Industrialised individuals may be in a state of imbalance, with the presence of certain specific disease-causing bacteria shifting the overall community composition to a state of disease. These results will be of great value to our understanding of how disease, changes in human diet, and environment impact our oral microbiome, while further enriching our understanding of human prehistory.

Acknowledgments

There's an African proverb that says "It takes a village to raise a child". My experience over the past three years has taught me that it takes a village to train and develop a PhD student too. I'd like to use this section of my thesis to thank everyone who has been part of my "village" over the past three years.

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The problem [with genetic research] is, we're just starting down this path, feeling our way in the dark. We have a small lantern in the form of a gene, but the lantern doesn't penetrate more than a couple of hundred feet. We don't know whether we're going to encounter chasms, rock walls or mountain ranges along the way. We don't even know how long the path is.

Francis S. Collins, Director - U.S. National Institutes of Health (NIH)
(Quoted in J. Madeleine Nash, et al., 'Tracking Down Killer Genes', Time magazine (17 Sep 1990))

Introduction

Overview

The advent of next generation sequencing over the last three decades has revolutionised the field of genetics into genomics. The successful sequencing of the human genome led to a growth in the understanding of human genetics and advanced the development of more efficient sequencing technologies at a fraction of the cost. This revolution was also expected to reveal mechanisms behind a number of human diseases by explaining their underlying genetic blueprint, thus leading to new therapeutic targets and thereby resulting in game changing treatment for diseases such as, cancer and Alzheimer's. However, this has not been the case, as the human genome was found to have substantially lower than expected numbers of protein-coding genes (20,000 – 25,000), leading to the search for other sources and mechanisms that drive human health or disease. This search led to the discovery of communities of microbes (*i.e.* the microbiome) that inhabit diverse niches of human bodies and influence human metabolic and immune systems.

This thesis aims to explore the human oral microbiome and how it has been influenced in the past and present by human cultural changes. In this introductory chapter, I introduce the human microbiome and factors that influence it, followed by a brief review on what is currently known about the impact of cultural shifts on microbiome composition and function. I then introduce the nascent field of paleomicrobiome research, how it allows us to go beyond the study of contemporary human microbiomes towards the study of ancient microbiomes and the challenges that this new field faces. This is then followed by recent research on how human genetics impacts the microbiome. I then end with a short explanation of the aims of this thesis and an overview of the chapters included in this thesis.

The human microbiome

These microbial communities are defined as the 'microbiome,' and the collective genomes of all microbial species and their environment is termed the 'microbiome' (Lederberg and McCray 2001; Marchesi and Ravel 2015). With a larger repertoire of 150 times more functional genes compared to the human genome (Qin et al. 2010), the microbiome has been shown to play key roles in human health (Knight 2018), from birth (Gregory, 2011), early childhood (Rodríguez et al., 2015), adulthood (Yatsunenko et al., 2012; Zapata &

Quagliarello, 2015), aging (Ogawa et al. 2018; Zapata and Quagliarello 2015), and even post mortem (Can et al., 2014; Metcalf et al., 2015). The microbiome has also co-evolved with their human host and changes in its composition can to lead to disease (Kodaman et al. 2014; Ley et al. 2008). Many of these diseases are non-communicable metabolic diseases (NCDs), formerly known as "diseases of affluence" such as obesity, type 2 diabetes, and inflammatory bowel disease (IBD) (Tuohy and Del Rio 2014) that have been associated with the disruption of the normal composition of the microbial communities (Parekh, Balart, and Johnson 2015). NCDs are now on the rise globally and cause 60% of global early deaths, where most are now in the developing world and will cost the world economy an estimated $47 Trillion USD by 2030 (Legetic et al. 2016). Research on the human microbiome and on factors that influence it to cause disease will be crucial in finding therapies for these NCDs in order to save lives and wealth globally. The human microbiome is a complex ecological system that is influenced by a number of diverse factors, such as cultural and environmental changes through lifestyle and diet (Voreades, Kozil, and Weir 2014), host genetics (Gomez et al. 2017; Goodrich et al. 2014), and environment (Rothschild et al. 2018). In the following sections, I briefly discuss what is known so far on the human microbiome.

The impact of diet on the microbiome

The influence of diet on the human microbiome has been a major area of interest within the field of microbiome research. Initial studies on the effect of diet on the human microbiome has largely focused on the gut microbiome; the microbial community within the human colon. These studies compared the gut microbiome of individuals with different diets to assess how animal based or plant based diets impacted the composition of the gut microbiome (Fallucca et al. 2014; De Filippo et al. 2010; Hinnig et al. 2018; Sonnenburg and Bäckhed 2016; Wu et al. 2011). These studies showed that the microbiome responds quite rapidly to dietary change. Research by Walker et al. (2011) showed a shift within 3-4 days when 14 over-weight men were placed on a controlled diet of either animal or plant products for 10 weeks. Walker et al. (2011) showed that plant based diets high in resistant starch increased the proportion of *Firmicutes* bacteria. However, the changes observed were rapidly reversed as the subjects returned to an animal based protein diet. Even

more rapid changes in the gut microbiome composition were observed by David et al. (2014), where an animal based diet was showed to alter microbiome composition in a single day (24 hours), resulting in significant changes, such as an increase in the abundance of bile-tolerant microorganisms, *Alistipes*, *Bilophila* and *Bacteroides*, as well as in bacterial species linked to IBD, such as *Bilophila wadsworthia* (David et al. 2014; Devkota et al. 2012). As these microbial taxa increased in these individuals, there was also a concurrent decrease in the levels of *Firmicutes* that metabolize dietary plant polysaccharides, such as *Ruminococcus*, highlighting trade-offs between microbial taxa involved in carbohydrate and protein fermentation in a manner similar to that observed in the microbiomes of mammal carnivores and herbivores (Muegge et al. 2011). These findings indicate that dietary changes can rapidly shift microbiome composition.

Furthermore, Moeller et al. (2014) showed that the contemporary human microbiome deviated from its ancestral state relative to the microbiomes of wild apes and points towards major cultural shifts in human evolutionary history, starting with the advent of cooking of food with fire through to the Agricultural, and more recently, the Industrial revolution, that resulted in drastic changes to human lifestyle, especially within the human diet. These changes likely played major roles in the disruption of the human ancestral microbiome to the state we see today, which may underlie the "diseases of affluence". As little is known about the advent of cooking food with fire, in the following sections, I focus on the latter two of these cultural shifts, the Agricultural transition and the Industrial revolution and what is currently known about their impact on the human microbiome.

The Agricultural transition

Arising out of Africa around 200,000 years ago, anatomically modern humans (AMH) subsisted on hunting of animals and gathering of wild plants (Martin and Sauerborn 2013). This hunter-gatherer lifestyle underwent a drastic change around 10,000 years ago, towards a sedentary lifestyle dominated by domesticated plants and animals (Langlie et al. 2014). This transition to agriculture, which is also known as the Agricultural transition, changed the trajectory of human history. The ability to grow crops and domesticate animals reduced variation in diet and encouraged a sedentary lifestyle, which had an immense impact on human

health and disease (Harper and Armelagos 2013). In addition, certain lifestyle practices, such as cohabitation with animals, high density living, and large populations, facilitated the spread of zoonotic diseases from animal hosts to humans (Weiss 2001; Wolfe, Dunavan, and Diamond 2007). The Agricultural transition also altered human genomic DNA, resulting in changes in genetic variants associated with height, the ability to digest lactose in adulthood, and skin pigmentation (Mathieson et al. 2015).

Recently research has explored differences in the gut microbiomes of hunter-gatherer and agriculturalist populations to explore how the shift towards agriculture changed the human microbiome. Researchers compared the gut microbiome of contemporary hunter-gatherers with those of agriculturalists and showed that hunter-gatherer groups, such as the Hadza (Tanzania) (Schnorr et al. 2014), Guahibo (Venezuela) (Clemente et al. 2015), Yanomami (Venezuela) (Clemente et al. 2015), BaAka (Central African Republic) (Gomez et al. 2016), and Matses (Peru) (Obregon-Tito et al. 2015), had high gut microbial diversity and an enrichment in gut microbial taxa, such as *Treponema* and *Bacteroidetes*, that were characteristic of their foraging lifestyle. However, agriculturalist groups, such as the Bantu (Central African Republic) (Gomez et al. 2016), Malawians (Clemente et al. 2015), and Tunapuco (Peru) (Obregon-Tito et al. 2015), compared with the hunter-gatherers, had gut microbiomes enriched in Firmicutes and microbial functions related to degradation of plant based sugars, such as carbohydrate and xenobiotic metabolism.

However, it is important to note that grouping human populations into either hunter-gatherers or agriculturalists is not quite as simple. Much research into the influence of diet on the microbiome has focused on the use of the terms, hunter-gatherer and forager to describe various non-agriculturalist populations that consumed a diet mostly sourced from the environment. This definition is quite broad and there are no true hunter-gatherer communities today that consume their diet based on hunting and gathering alone (Crittenden and Schnorr 2017). To illustrate this variety in hunter-gatherer diets, it is notable that the gut microbiome of the indigenous hunter-gather group, the Inuit from the Canadian Artic was found to be broadly similar to that of individuals with a western diet (Dubois et al. 2017). This is hypothesized to be due to the similarity in the diets between modern western populations and the Inuit diet consisting of protein rich marine and terrestrial

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mammals, as well as reduced amounts of plants and berries. Similarly, ancient hunter-gatherers in Morocco had a starch rich diet consisting of acorn and pine nuts (Humphrey et al. 2014), while honey is a substantial part of the contemporary African hunter-gather group, the Hadza's diet (Marlowe et al. 2014; Schnorr et al. 2014) both foods are not commonly considered as components of hunter-gatherer diets. Hunter-gatherer populations have also been shown to transition their diets to include diverse, unexpected food sources, as a result of food stress driven by seasonal changes in rainfall (Speth 1987), resulting in some hunter-gatherer populations subsisting on horticulture for short periods of time. Environmental and temporal context is crucial in understanding the dietary patterns of hunter-gather populations and how those patterns influence the gut microbiome.

In contrast to the gut, the impact of diet on the oral microbiome has not been as well studied. A few studies have revealed that differences in the levels of microbial diversity and composition in the human oral microbiome between hunter-gather populations and agriculturalists are similar to that observed in the gut microbiome. Studies on hunter-gatherers, such as the Batwa (Uganda) (Li et al. 2014), indigenous Alaskans (United States) (Li et al. 2014), and hunter-gatherer groups in the Philippines (Lassalle et al. 2017), identified significant differences in their oral microbiome, characterized by higher diversity and an increase in beneficial microbial taxa, such as *Bifidobacterium*, compared to agriculturalists from Sierra Leone, the Democratic Republic of Congo (Li et al. 2014; Nasidize et al. 2011), and the Philippines (Lassalle et al. 2017). They reported differences in community structure and levels of microbial diversity between the two lifestyles, concluding that the adoption of agriculture has also had an impact on the human oral microbiome.

Even though we have begun to characterize differences in microbiomes according to unique subsistence strategies, many of these studies are confounded by environmental factors, i.e. limited geographic locations. For example, it is difficult to disentangle if differences between the gut microbiome of a hunter-gather group and an agriculturalist from different parts of the world are due to their diet or environment. In order to control for environment, it is crucial to study the microbiomes of populations with different subsistence strategies but the same environment. It is also important to note that the handfuls of contemporary hunter-gatherer populations that have been studied are not analogs to a Paleolithic past

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(Crittenden and Schnorr 2017). Furthermore, in order to understand how these changes have impacted the modern microbiome, we need to examine individuals who are past ancestors of modern populations. A bold new tool in unveiling the evolutionary past of the human oral microbiome is the use of ancient DNA (aDNA) in paleomicrobiome research.

The Industrial revolution

The Agricultural transition was more recently followed by Industrialization. Starting in the mid 1800's and characterized by the mechanization of food production, the availability of low fiber, sugar based foods, antibiotics (in its later stages), and increased level of toxins in the environment. Industrialization, in its later stages, also led to urbanization and globalization, resulting in an increase in population density and the intermixing of populations from different parts of the world (Steckel 1999; Szreter 2004). As such, western populations from economically "developed" countries, such as those in Europe, the United States, Canada, Australia and New Zealand, are widely regarded as Industrialized. This rapid Industrialization over the past two centuries has resulted in better health outcomes and increased lifespans in many populations (Steckel 1999; Szreter 2004). However, rapid Industrialization has also been linked to increases in diseases of affluence, such as heart disease, obesity, and type 2 diabetes (Basch, Samuel, and Ethan 2013; Tuohy and Del Rio 2014). Recently, research has identified strong links between these diseases and changes in the microbiome (Belkaid and Hand 2014; Parekh, Balart, and Johnson 2015), and further studies have also suggested that these changes may originate from Industrialization (Logan, Jacka, and Prescott 2016).

While early microbiome research suggested that these changes were isolated to the gut, recent evidence suggests that similar Industrial changes occurred within the oral microbiome (Adler et al. 2013; Clemente et al. 2015). Much of this research was done by comparing the oral microbiome of hunter-gatherer groups, such as the Yanomami (Venezuela) (Clemente et al. 2015), Batwa (Uganda) (Nasidze et al. 2011), Native Alaskans (United States) (Li et al. 2014), and those in semi-urban environments of Tanzania (Bisanz et al. 2015), Sierra Leone, and Democratic Republic of Congo (Nasidze et al. 2011), to those living a Western,

Industrialized lifestyle (Li et al. 2014; Nasidze et al. 2009). Industrialized populations are generally found to have lower microbial diversity due to their homogenous diets and lifestyles, compared to non-Industrialized populations, and had higher numbers of microbial taxa linked to caries and periodontal disease (Clemente et al. 2015; Li et al. 2014; Nasidze et al. 2009, 2011). However, many of these studies had small sample sizes and only compared a single non-Industrialized community to that of a single Industrialized population, and was also limited in geographical span, raising questions to the broader impacts of Industrialization on diverse human populations.

Expanding the focus of oral microbiome studies over larger geographic areas and diverse human populations is required to further characterize the diversity of the human microbiome. However, an investigation into how microbial diversity has changed through time is also required. In addition to examining contemporary populations, ancient human populations will provide us with evolutionary snapshots of changes to the microbiome before, during, and after past cultural shifts, such as the Agricultural transition and the Industrial revolutions. Studying these evolutionary changes through the prism of microbiome change will provide us with a better understanding on how they impacted human health and disease resulting in potentially more effective microbial based therapies. Studies using ancient microbial DNA through the field of paleomicrobiome research can provide us with these evolutionary snapshots.

Paleomicrobiome research

Paleomicrobiome research is a recently established field of research focused on the study of the microbiome of historical and pre-historic populations. This field of research is an off-shoot of the field of aDNA research or paleogenomics. In this section, I introduce aDNA and discuss its use in paleomicrobiome research through exploring ancient dental calculus, followed by what is currently known about the ancient oral microbiome. I then end the section with challenges faced by this nascent field of research.

Ancient DNA

Ancient DNA (aDNA), *i.e.* fragile, short, damaged, and degraded DNA fragments extracted from fossilised bone and tooth samples of prehistoric populations, has revolutionised the understanding of evolutionary genomics. From the unveiling of the Neanderthal genome (Prüfer et al. 2014) to expansion of the understanding of prehistoric migrations in Europe (Haak et al. 2015), ancient DNA has provided researchers with an unparalleled ability to unveil past evolutionary processes (Orlando and Cooper 2014). These advances happened in parallel with research work to explore ancient microbial DNA from fossilised material, which led to the birth of paleomicrobiome research. Paleomicrobiome research uses aDNA extracted from microbial sources, such as preserved faeces (*e.g.* coprolites; Tito et al. 2012), calcium carbonate (Frisia et al. 2017), and dental calculus (Adler et al. 2013; De La Fuente, Flores, and Moraga 2013; Warinner et al. 2014). Coprolites and dental calculus can be used to reconstruct the gut and oral microbiomes of ancient populations respectively. Sequencing aDNA in coprolites led to the characterisation of the gut microbiome of ancient populations of North and South America (Tito et al. 2012). However, coprolite can be contaminated by microbial DNA from microbes in the environment post disposal, making it difficult to disentangle contaminant environmental DNA from endogenous DNA. This has led many researchers to focus on the use of dental calculus to unravel the oral microbiome of pre-historic populations.

Dental Calculus

Dental calculus is a calcified form of the dental plaque, a biofilm that forms on teeth and gum surfaces. Dental calculus formation is dependent on a number of factors, including salivary flow, poor hygiene, host genetics, or diet, with a carbohydrate rich diet resulting in a large calculus deposits (Arensburg 1996; Lieverse 1999). Dental calculus can form above the gum line (supragingival) or below the gum line (subgingival) (Fig. 1&3). While the exact process of the formation of dental calculus is currently an area of active research, it is widely agreed that an oral biofilm eventually calcifies into dental calculus during the lifetime of an individual through the co-aggregation of bacterial taxa, such as *Streptococcus* and *Actinomyces* on the tooth surface with a layer of saliva (Conroy

and Sturzenberger 1968; Marsh 2004). This co-aggregation process is promoted by substances that support bacterial growth, such as glucose polysaccharide and dextran (Cantarel, Lombard, and Henrissat 2012; Palmer et al. 2003). This first biofilm layer is then followed by other bacterial species that form a bridge between the first layer and subsequent bacterial species that adhere to the biofilm (Mark Welch et al. 2016) (Fig. 2). The layer by layer formation of dental calculus points towards an ecological system where specific bacteria interact with each other either on a basis of cooperation or competition, influenced by diet via its effect on saliva flow and pH in the oral cavity (Kolenbrander and London 1993). However, how these taxa involved in the formation of biofilm layers are impacted by various factors, such as diet, environment, host genetics, and the overall state of host health, is still unknown. This has led to a number of studies exploring the oral microbiome of prehistoric and ancient populations in order to elucidate how interactions within this complex microbial community and its host may result in health or disease.
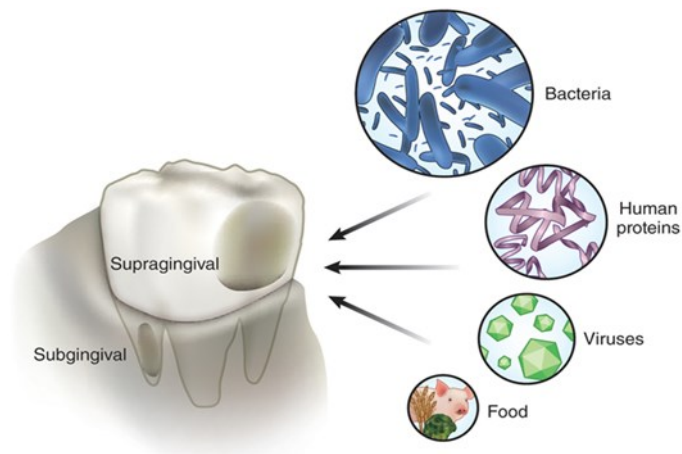
Figure 1. Dental calculus can be formed both above and below the gum line. It consists primarily of microbes but can also include small amounts of human proteins, viruses and food material. Reproduced from (Metcalf, Ursell, and Knight 2014).
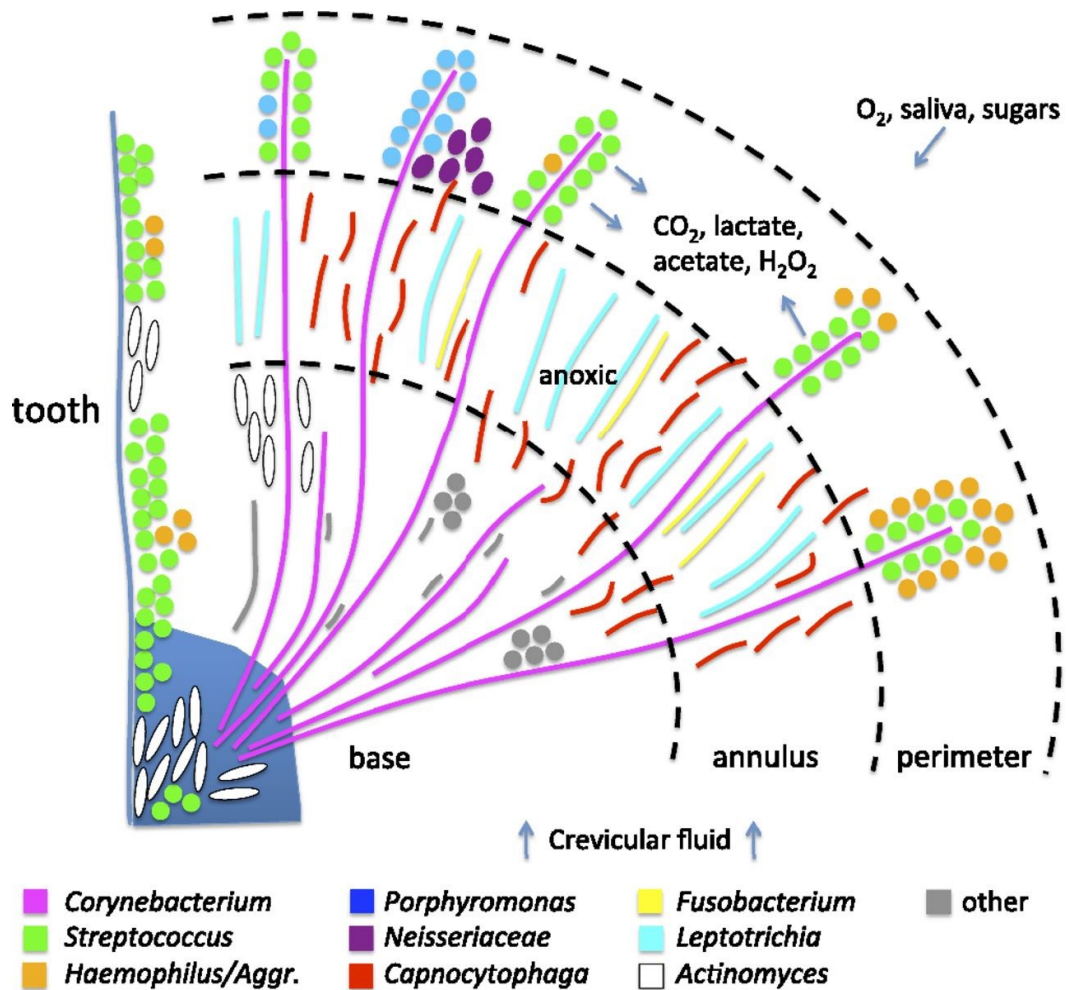
Figure 2. Proposed biofilm structure of identified bacteria in dental plaque. Reproduced from (Mark Welch et al. 2016).

While researchers have explored the presence of aDNA in dental calculus for some time (De La Fuente et al. 2013; Preus et al. 2011). Adler et al. (2013) were the first to use dental calculus to show that the transition to agriculture shifted the oral microbiome composition in Europeans to a state of imbalance which has been shown as a cause of disease (Herrero et al. 2018). A subsequent paper by Warinner et al. (2014) further characterized the ancient oral microbiome in a diseased state and further confirmed Adler et al (2013)'s findings on the presence of pathogens associated with the aetiology of periodontal disease in dental calculus. More recently, Farrer et al. (2018) used dental calculus to identify associations between microbiome composition, health and socio-economic status in medieval and post medieval British individuals. These studies have helped unravel how the human oral microbiome was impacted by dietary and cultural shifts through time and

explore the roles of these changes on the onset of oral and systemic disease. However, recent research on dental calculus, excluding a handful of African samples explored by Weyrich et al. (2017), has primarily been focused on samples from Europe due to better sample preservation and easy accessibility to samples. In order to understand the ancient oral microbiome of prehistoric populations on a global level, it is crucial to expand geographical sampling to study the oral microbiome of prehistoric non-European populations such as those of Asia, including the Near East and Africa.

Current challenges within paleomicrobiome research

As any field of research, paleomicrobiome research comes with its own unique set of challenges. Since paleomicrobiome research relies on the analysis of extracted endogenous aDNA from fossilised dental calculus or coprolite samples, sample collection, use of effective and efficient lab methods, analysis tools, and contamination are the most important challenges faced by this field.

Sample collection

Access to samples with adequate amounts of dental calculus deposits is a main challenge of any aDNA research. Currently in paleomicrobiome research, most samples examined thus far has been limited to samples collected from well preserved locations in Europe (Wade 2015) with sparse research on samples from other important regions in human evolutionary past, such as Africa and the Near East. This is due to difficulty in accessing archaeological sites and materials in these regions but also the poor preservation state of samples from these regions, as well as their warmer and wetter climatic conditions that does not promote DNA preservation (Adler et al. 2011; Kistler et al. 2017; Pinhasi et al. 2015).

Figure 3. Dental calculus on an ancient tooth sample. Sample collection from the Natural History Museum, London, UK. (Photograph taken by Laura Weyrich and Alan Cooper; ACAD records)

Due to the relatively early state of paleomicrobiome research using dental calculus, there is also a relatively lower level of awareness about dental calculus as a bioarchaeological specimen compared to bone and teeth samples, and some museum curators are not often able to discriminate between calculus deposits on tooth and jaw samples as opposed to soil debris. This has resulted in the unwitting disposal of dental calculus in some cases, further limiting the number of dental calculus samples available for study.

Lab methodology

As an extension of the field of paleogenomics, paleomicrobiome research has mostly used lab methods/protocols (Meyer and Kircher 2010; Rohland and Hofreiter 2007) developed for extraction and library preparation of aDNA from bone and tooth samples primarily from well preserved locations in Europe (Warinner et al. 2015; Warinner et al. 2014; Weyrich et al. 2017; Weyrich, Dobney, and Cooper 2015). While most of these methods have been successful in the extraction of aDNA from very old samples to reconstruct the genomes of the Neanderthals (Prüfer et al. 2014) and Denisovan (Meyer et al. 2012) as well as in obtaining the oral microbiome of Neanderthals (Weyrich et al. 2017), their impact and that of recently developed methods (Dabney et al. 2013; Gansauge et al. 2017;

Gansauge and Meyer 2013) on the aDNA obtained from dental calculus and its microbiome composition have not been tested. Furthermore, as the majority of studies have been based on well preserved samples from Europe, little is known on how effective these methods would be for dental calculus samples from poorly preserved environments such as the Near East and Africa.

Assessing and dealing with contamination.

Due to the relatively lower levels of aDNA obtained from preserved material compared to modern samples, as well as its short and damaged nature, aDNA can be easily contaminated by larger DNA fragments from macro and micro non-target organisms present in the environment (exogenous DNA) (Korlević et al. 2014; Willerslev and Cooper 2005). Typical sources include the soil from where a sample is buried, museum curators who handle the samples, laboratory reagents, researchers conducting DNA extractions, and air-borne microbes (Glassing et al. 2016; de Goffau et al. 2018; Mogul et al. 2018; Salter et al. 2014; S. Weiss et al. 2014). This contamination can occur at various stages of analysis and can lead to erroneous conclusions (Eisenhofer et al. 2018; Eisenhofer and Weyrich 2017; Wall and Kim 2007). In order to mitigate these challenges, the aDNA field has established a number of strategies to help reduce contamination (Cooper and Poinar 2000; Eisenhofer et al. 2018).

The first is the use of adequate physical barriers (e.g. latex gloves, long-sleeved clothes, and face-masks) to reduce the potential of introducing exogenous DNA into ancient samples as they are handled at archaeological sites and museums (Fig. 4). This is followed by strictly conducting research on ancient samples in ultra clean, dedicated aDNA facilities that are physically isolated from other molecular biology laboratories in order to prevent the introduction of PCR products into ancient samples (Cooper and Poinar 2000). To help reduce airborne contamination from outside the laboratory, the aDNA facilities have positive air pressure coupled with a HEPA filtered ventilation system. Dedicated entry rooms are made available for researchers to enter and change into disposable sterile, full body suits, gloves, face-masks and boots to further limit the introduction of DNA from their own body. Within the facility, surfaces are regularly disinfected with ~5% bleach (sodium hypochlorite) and irradiated with ultra-violet (UV) bulbs which help limit

exogenous DNA already within the laboratory (Llamas et al. 2017; Woyke et al. 2011).

Figure 4. Working in an aDNA laboratory at the Australian Centre for Ancient DNA. (Photograph by Raphael Eisenhofer and Oscar Estrada Santamaria; ACAD records)

Once the ancient sample are ready for processing through the respective laboratory protocols (Fig. 5), they are first decontaminated by the physically removing the exterior of the sample or by soaking the sample in bleach or EDTA (Ethylenediaminetetraacetic acid) to remove DNA contamination on the exterior (Boessenkool et al. 2017; García-Garcerà et al. 2011; Kemp and Smith 2005; Christina Warinner, Rodrigues, et al. 2014). As the ancient samples go through the protocols, it is essential to add negative controls in order to monitor exogenous DNA that might be introduced into the ancient samples (Cooper and Poinar 2000;
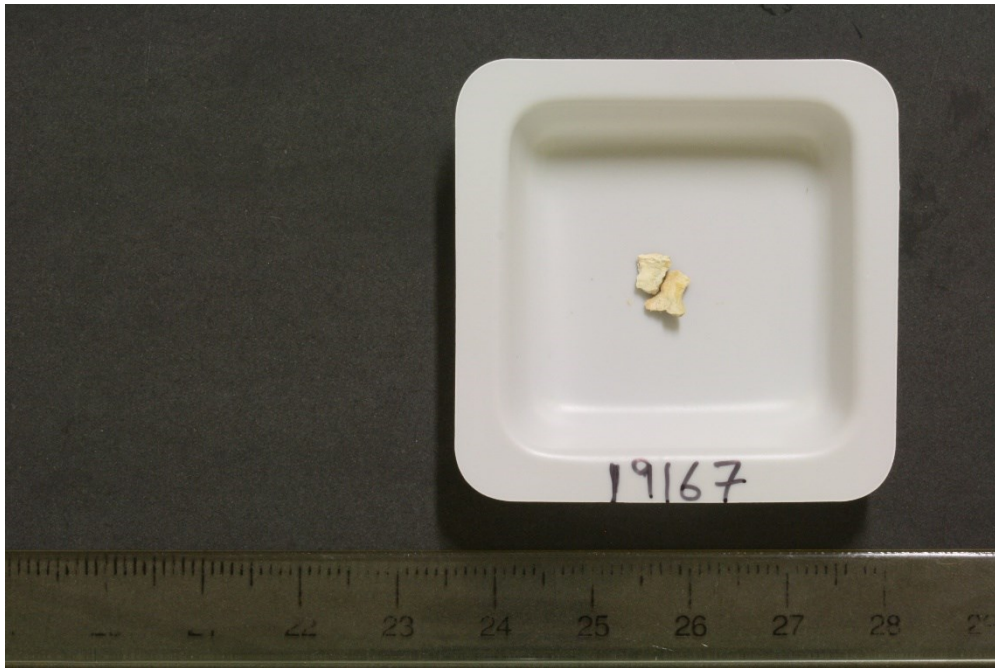
Figure 5. Dental calculus after sampling off of tooth in the Ancient DNA laboratory. (Photograph by Muslih. Abdul-Aziz; ACAD records)

Llamas et al. 2017). This inclusion of extraction blank controls (empty tubes without sample DNA) in every DNA extraction allows for the capture of exogenous DNA present in the laboratory environment that might originate from reagents or inadvertently introduced by the researcher (Glassing et al. 2016; de Goffau et al. 2018; Salter et al. 2014). These controls should also be sequenced alongside the ancient samples to allow for bioinformatic analysis of the contamination (Eisenhofer et al. 2018).

Following sequencing, a comparison of sequences found in ancient samples to those in negative controls is crucial in order to reduce the impact of contamination on subsequent analyses and conclusions. Furthermore, ancient DNA characteristics (short fragment sizes, cross linkage between DNA molecules, and increased cytosine deamination at the terminal ends of the molecule (Hofreiter et al., 2001; Willerslev and Cooper, 2005; Linderholm, 2015) can be used to confirm of the authenticity of the DNA fragments. Tools, such as MapDamage, can assess the fragment length and elevation in cytosine deamination at the terminal ends of sequenced fragments to confirm the presence of authentic aDNA above levels of exogenous, contaminant DNA (Ginolhac et al. 2011; Jónsson et al. 2013). Even with these laborious steps to limit contaminant DNA in paleomicrobiome and the

wider field of aDNA research, contaminant DNA can still be present in some samples post sequencing. This may be due to the introduction of contaminant DNA through reagents in the laboratory steps, as no method exists that completely eradicates contamination.

Therefore, bioinformatic tools are required to assess the level of non-endogenous DNA contamination in the sequenced data to identify and filter out contaminant DNA (Warinner et al. 2017). In additional, it also makes it important to be cautious in the interpretation of results (Warinner et al. 2017; Weyrich et al. 2017).

Sequencing and Analysis

Once access to aDNA molecules from dental calculus is obtained, the challenges do not end. Sequencing and data analysis steps pose their own unique challenges in reconstructing the ancient oral microbiome from a sample. DNA sequencing has reduced in cost and improved in accuracy and throughput, however, the right choice of sequencing technology is crucial. Previous research had used relatively cheaper 16S rRNA barcoding coupled with the Roche 454 sequencing platform to reconstruct the oral microbiome of ancient populations (Adler, et al. 2013). However, research by Ziesemer et al. (2015) revealed systematic amplification bias with the use of 16S rRNA barcoding in paleomicrobiome research and recommended the use of more accurate but much more expensive whole genome shot gun sequencing. As the number of dental calculus samples being used in paleomicrobiome research studies increases, even with cheap sequencing costs, it will become cost prohibitive for many labs to perform large scale paleomicrobiome research. However, the use automation for sample processing in the laboratory (Gansauge et al. 2017), efficient new ways to screen for dental calculus samples that have the best chances for successful sequencing of aDNA, and techniques such as the multiplexing multiple samples into one sequencing lane (Kircher, Sawyer, and Meyer 2012; Ranjan et al. 2016) may help mitigate these challenges.

Once sequencing data is obtained, the next step is to accurately assign aDNA fragments to a microbial taxon using an effective and efficient assignment software. A well curated and expansive database of microbial taxa is also crucial, as the microbial taxa are identified within sequenced data by searching for similar sequences within curated, known databases. While many assignment tools have been developed for modern microbiome research, only a handful have been applied to aDNA, such as MALT (Herbig et al. 2016), DIAMOND (Buchfink, Xie, and Huson 2014) and MetaPhlAan (Truong et al. 2015). However, many of these tools face limitations such as inefficient alignment algorithms and in effective ways to deal with ever increasing microbial databases. This is as a result of the reduction in sequencing costs that have made an increasing number of microbial genomes available, thereby resulting in an increase in the size of databases needed for microbial assignments (Velsko et al. 2018). This requirement for computing space and power has resulted in prohibitive costs which can restrict many research laboratories from conducting paleomicrobiome research. However, new and effective tools that assign reads to genomes effectively and efficiently while minimising computational resources required for database storage and access, potentially through new data compression techniques, could help mitigate this challenge.

Aims

This thesis explores the impact of a key human cultural change, the transition to agriculture, on the human oral microbiome. I hypothesize that this cultural change began the alteration of the human oral microbiome from an ancestral state, resulting in a compositionally imbalanced disease promoting oral microbiome present within Industrialised populations today.

The aims of this thesis are to:

- Assess the impact of currently used and recently developed aDNA laboratory protocols on dental calculus samples and explore the impact of these methods on the reconstruction of the ancient oral microbiome composition.
- Explore the oral microbiome of ancient hunter-gatherers and agriculturalists from diverse global environments in Europe, the

Near East, Asia and Africa to examine the impact of the transition towards agriculture on the human oral microbiome.

- Explore the oral microbiome of two contemporary African populations with different subsistence strategies living in the same environment and compare them with western populations to assess the impact of agriculture and subsequent industrialisation on the human oral microbiome.

Thesis overview

Through four manuscripts, this thesis addresses the questions and challenges introduced in this introductory chapter. The overarching theme of this thesis is to explore how dietary differences and lifestyle changes impact the oral microbiome by exploring the oral microbiome of globally diverse modern and ancient populations with different lifestyles and diets. Ultimately, this thesis seeks to expand the focus of microbiome research beyond well preserved, easily accessible samples from Europe to globally diverse populations.

Chapter I: *Exploring Relationships between Host Genome and Microbiome: New Insights from Genome-Wide Association Studies.*

The human genome has been shown to influence the microbiome but not much is known about this influence to date. In this first chapter, I introduce the human microbiome and review recent advances in the new research area of human genome - microbiome interactions, including those that use genome-wide association studies to examine the interactions. I find this new field is restricted from its complete potential at this stage due to the use of small sample sizes that are not statistically powerful enough, lack of group replication studies, and inadequacy of studies to confirm the mechanisms behind recent findings. I also discuss the importance of understanding long-term interactions between the human genome and microbiome, as well as the potential repercussions of disrupting this relationship. In addition, I suggest new research avenues that may further deepen the understanding of the shared evolutionary history of the human genome and microbiome.

Chapter II: *Ancient DNA extraction and library preparation methodology impacts on ancient microbiome within dental calculus of varying preservation states.*

In this chapter, I compare widely used protocols with recently published extraction and library preparation methods on dental calculus samples of varying preservation states. I find that microbial composition in well preserved dental calculus is not influenced by the methods. However, I find that poorly preserved samples are significantly impacted and that the single stranded library preparation method allowed access to ultrashort endogenous DNA fragments resulting in a more robust oral microbiome composition following subtractive filtering.

Chapter III: *Global ancient dental calculus assessment examining ancient oral microbiota responses to agriculture.*

In this chapter, I examined aDNA from ancient and historic dental calculus samples to reveal the oral microbiome of ancient hunter-gatherers and agriculturalists, including poorly preserved dental calculus samples from Africa and Asia (from the Near East to the Pacific). I also tested the impact environment and diet has on the oral microbiome; my results indicate differences in both taxonomic and functional composition between ancient hunter-gatherer and agriculturalist samples. My findings also suggest that, although diet pays a role in the functional and taxonomic differences between the ancient hunter-gatherer and agriculturalist microbiome other factors also appear play a significant role.

Chapter IV: *Industrialisation dramatically impacts oral microbiome on a multi-continental scale.*

In this chapter, I return to research on contemporary human microbiomes. I examine the oral microbiome obtained from saliva samples from two African populations, the Batwa, rainforest hunter-gatherers, and the Bakiga, traditional subsistence farmers, living in close proximity in Uganda. I used 16S ribosomal RNA gene sequencing for a robust reconstruction of their oral microbiome signal and found similarity in microbial diversity and composition between the two populations. I then compared these Ugandan microbiotas with published data from non-Industrialised populations such as a hunter-gatherer group in South America, rural Africans, and Industrialised populations from the western world. Ugandan individuals were found to have the most diverse oral microbiome of any human

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population and differentiated significantly from Industrialised populations in community structure. I also find that oral microbiome of individuals living non-Industrial lifestyles clustered in a diverse manner according to their geographic location/environment. For example, I find the Ugandan hunter-gatherer oral microbiome to be significantly different from that of the South American hunter-gatherer group in both microbial diversity and community structure although they both had a similar lifestyle and both clustered away from Industrialised populations. However, I find Industrialised populations from various geographic locations/environments clustered together. This research further describes the global diversity of the human microbiome and the influence cultural changes such as Industrialisation have had and will have towards diminishing the global human microbiome diversity as more countries develop and shift towards an industrialised lifestyle.

References

1. Adler, C.J. J., W. Haak, D. Donlon, and A. Cooper. 2011. "Survival and Recovery of DNA from Ancient Teeth and Bones." *Journal of Archaeological Science* 38(5): 956–64. http://linkinghub.elsevier.com/retrieve/pii/S0305440310004097 (July 25, 2018).

2. Adler, Christina J, Keith Dobney, Laura S Weyrich, John Kaidonis, Alan W Walker, Wolfgang Haak, Corey JA Bradshaw, Grant Townsend, Arkadiusz Sołtysiak, Kurt W Alt, Julian Parkhill, Alan Cooper, et al. 2013. "Sequencing Ancient Calcified Dental Plaque Shows Changes in Oral Microbiota with Dietary Shifts of the Neolithic and Industrial Revolutions Europe PMC Funders Group." *Nat Genet* 45(4): 450–55. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3996550/pdf/emss-57665.pdf (September 3, 2018).

3. Arensburg, B. 1996. "Ancient Dental Calculus and Diet." *Human Evolution* 11(2): 139–45. http://link.springer.com/10.1007/BF02437397 (July 6, 2018).

4. Basch, Corey H., Lalitha Samuel, and Danna Ethan. 2013. "Obesity, Diabetes and Heart Disease: Effects of Globalization on Population Health, Preventive Efforts, and the Importance of Social Change." *International Journal of Health Promotion and Education* 51(4): 185–97. http://www.tandfonline.com/doi/abs/10.1080/14635240.2013.787732 (April 26, 2018).

5. Belkaid, Yasmine, and Timothy W Hand. 2014. "Role of the Microbiota in Immunity and Inflammation." *Cell* 157(1): 121–41. http://www.sciencedirect.com/science/article/pii/S0092867414003456 (July 15, 2014).

6. Bisanz, Jordan E et al. 2015. "Microbiota at Multiple Body Sites during Pregnancy in a Rural Tanzanian Population and Effects of Moringa-Supplemented Probiotic Yogurt" ed. H. L. Drake. *Applied and Environmental Microbiology* 81(15): 4965–75. http://www.ncbi.nlm.nih.gov/pubmed/25979893 (November 27, 2017).

7. Boessenkool, Sanne et al. 2017. "Combining Bleach and Mild Predigestion Improves Ancient DNA Recovery from Bones." *Molecular Ecology Resources* 17(4): 742–51. http://doi.wiley.com/10.1111/1755-0998.12623 (November 3, 2016).

8. Buchfink, Benjamin, Chao Xie, and Daniel H Huson. 2014. "Fast and Sensitive Protein Alignment Using DIAMOND." *Nature methods* 12(1): 59–60. http://dx.doi.org/10.1038/nmeth.3176 (November 16, 2017).

9. Cantarel, Brandi L, Vincent Lombard, and Bernard Henrissat. 2012. "Complex Carbohydrate Utilization by the Healthy Human Microbiome." *PloS one* 7(6): e28742. http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0028742 (January 20, 2016).

10. Clemente, J. C. et al. 2015. "The Microbiome of Uncontacted Amerindians." *Science Advances* 1(3): e1500183–e1500183. http://advances.sciencemag.org/cgi/doi/10.1126/sciadv.1500183 (April 18, 2015).

11. Conroy, C W, and O P Sturzenberger. 1968. "The Rate of Calculus Formation in Adults." *Journal of Periodontology* 39(3): 142–44. https://onlinelibrary-wiley-com.proxy.library.adelaide.edu.au/doi/pdf/10.1111/prd.12151 (September 1, 2018).

12. Cooper, a, and H N Poinar. 2000. "Ancient DNA: Do It Right or Not at All." *Science (New York, N.Y.)* 289(5482): 1139.

13. Crittenden, Alyssa N., and Stephanie L. Schnorr. 2017. "Current Views on Hunter-Gatherer Nutrition and the Evolution of the Human Diet." *American Journal of Physical Anthropology* 162(S63): e23148. http://doi.wiley.com/10.1002/ajpa.23148 (July 28, 2017).

14. Dabney, J. et al. 2013. "Complete Mitochondrial Genome Sequence of a Middle Pleistocene Cave Bear Reconstructed from Ultrashort DNA Fragments." *Proceedings of the National Academy of Sciences* 110(39): 15758–63. http://www.pnas.org/content/110/39/15758.abstract (January 10, 2016).

15. David, Lawrence A. et al. 2014. "Diet Rapidly and Reproducibly Alters the Human Gut Microbiome." *Nature* 505(7484): 559–63.

http://www.nature.com/articles/nature12820 (October 13, 2018).

16. Devkota, Suzanne et al. 2012. "Dietary-Fat-Induced Taurocholic Acid Promotes Pathobiont Expansion and Colitis in Il10 −/− Mice." *Nature* 487(7405): 104–8. http://www.nature.com/articles/nature11225 (October 13, 2018).

17. Dubois, Geneviève, Catherine Girard, François-Joseph Lapointe, and B Jesse Shapiro. 2017. "The Inuit Gut Microbiome Is Dynamic over Time and Shaped by Traditional Foods." 5. https://microbiomejournal.biomedcentral.com/track/pdf/10.1186/s40168-017-0370-7?site=microbiomejournal.biomedcentral.com (November 21, 2017).

18. Eisenhofer, Raphael, Laura Weyrich, Raphael Eisenhofer, and Laura S. Weyrich. 2018. "Proper Authentication of Ancient DNA Is Still Essential." *Genes* 9(3): 122. http://www.mdpi.com/2073-4425/9/3/122 (September 3, 2018).

19. Fallucca, Francesco, Carmen Porrata, Sara Fallucca, and Mario Pianesi. 2014. "Influence of Diet on Gut Microbiota, Inflammation and Type 2 Diabetes Mellitus. First Experience with Macrobiotic Ma-Pi 2 Diet." *Diabetes/Metabolism Research and Reviews* 30(S1): 48–54. http://doi.wiley.com/10.1002/dmrr.2518 (May 11, 2017).

20. De Filippo, Carlotta et al. 2010. "Impact of Diet in Shaping Gut Microbiota Revealed by a Comparative Study in Children from Europe and Rural Africa." *Proceedings of the National Academy of Sciences of the United States of America* 107(33): 14691–96. http://www.ncbi.nlm.nih.gov/pubmed/20679230 (October 13, 2018).

21. Frisia, Silvia et al. 2017. "The Influence of Antarctic Subglacial Volcanism on the Global Iron Cycle during the Last Glacial Maximum." *Nature Communications* 8: 15425. http://www.nature.com/doifinder/10.1038/ncomms15425 (November 22, 2018).

22. Gansauge, Marie-Theres et al. 2017. "Single-Stranded DNA Library Preparation from Highly Degraded DNA Using T4 DNA Ligase." *Nucleic Acids Research* 45(10): gkx033. https://academic.oup.com/nar/article-abstract/45/10/e79/2948436 (July 25, 2018).

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23. Gansauge, Marie-Theres, and Matthias Meyer. 2013. "Single-Stranded
    DNA Library Preparation for the Sequencing of Ancient or Damaged
    DNA." *Nature Protocols* 8(4): 737–48.
    http://www.nature.com/doifinder/10.1038/nprot.2013.038 (July 25, 2018).

24. García-Garcerà, Marc et al. 2011. "Fragmentation of Contaminant and
    Endogenous DNA in Ancient Samples Determined by Shotgun
    Sequencing; Prospects for Human Palaeogenomics." *PloS one* 6(8):
    e24161. http://www.ncbi.nlm.nih.gov/pubmed/21904610 (January 5,
    2018).

25. Ginolhac, Aurelien et al. 2011. "MapDamage: Testing for Damage
    Patterns in Ancient DNA Sequences." *Bioinformatics* 27(15): 2153–55.
    https://academic.oup.com/bioinformatics/article-
    lookup/doi/10.1093/bioinformatics/btr347 (July 25, 2018).

26. Glassing, Angela et al. 2016. "Inherent Bacterial DNA Contamination of
    Extraction and Sequencing Reagents May Affect Interpretation of
    Microbiota in Low Bacterial Biomass Samples." *Gut pathogens* 8(1): 24.
    http://gutpathogens.biomedcentral.com/articles/10.1186/s13099-016-
    0103-7 (October 20, 2016).

27. de Goffau, Marcus C. et al. 2018. "Recognizing the Reagent
    Microbiome." *Nature Microbiology* 3(8): 851–53.
    http://www.nature.com/articles/s41564-018-0202-y (August 3, 2018).

28. Gomez, Andres et al. 2016. "Gut Microbiome of Coexisting BaAka
    Pygmies and Bantu Reflects Gradients of Traditional Subsistence
    Patterns." *Cell Reports* 14(9): 2142–53.
    http://www.cell.com/article/S2211124716300997/fulltext (February 26,
    2016).

29. ———. 2017. "Host Genetic Control of the Oral Microbiome in Health
    and Disease." *Cell Host & Microbe* 22(3): 269–278.e3. https://www-
    sciencedirect-
    com.proxy.library.adelaide.edu.au/science/article/pii/S1931312817303463
    (September 8, 2018).

30. Goodrich, Julia K. et al. 2014. "Human Genetics Shape the Gut
    Microbiome." *Cell* 159(4): 789–99.
    http://www.cell.com/article/S0092867414012410/fulltext (November 6,

2014).

31. Haak, Wolfgang et al. 2015. "Massive Migration from the Steppe Was a Source for Indo-European Languages in Europe." *Nature* 522(7555): 207–11. https://www.nature.com/nature/journal/v522/n7555/pdf/nature14317.pdf (January 21, 2016).

32. Harper, Kristin N., and George J. Armelagos. 2013. "Genomics, the Origins of Agriculture, and Our Changing Microbe-Scape: Time to Revisit Some Old Tales and Tell Some New Ones." *American Journal of Physical Anthropology* 152: 135–52. http://doi.wiley.com/10.1002/ajpa.22396 (December 22, 2015).

33. Herbig, Alexander et al. 2016. bioRxiv *MALT: Fast Alignment and Analysis of Metagenomic DNA Sequence Data Applied to the Tyrolean Iceman*. Cold Spring Harbor Labs Journals. http://biorxiv.org/content/early/2016/04/27/050559.abstract (April 28, 2016).

34. Hinnig, Patrícia et al. 2018. "Dietary Patterns of Children and Adolescents from High, Medium and Low Human Development Countries and Associated Socioeconomic Factors: A Systematic Review." *Nutrients* 10(4): 436. http://www.mdpi.com/2072-6643/10/4/436 (April 7, 2018).

35. Humphrey, Louise T et al. 2014. "Earliest Evidence for Caries and Exploitation of Starchy Plant Foods in Pleistocene Hunter-Gatherers from Morocco." *Proceedings of the National Academy of Sciences of the United States of America* 111(3): 954–59. http://www.ncbi.nlm.nih.gov/pubmed/24395774 (June 17, 2018).

36. Jónsson, Hákon et al. 2013. "MapDamage2.0: Fast Approximate Bayesian Estimates of Ancient DNA Damage Parameters." *Bioinformatics* 29(13): 1682–84. http://www.ncbi.nlm.nih.gov/pubmed/23613487 (July 25, 2018).

37. Kemp, Brian M., and David Glenn Smith. 2005. "Use of Bleach to Eliminate Contaminating DNA from the Surface of Bones and Teeth." *Forensic Science International*.

38. Kircher, Martin, Susanna Sawyer, and Matthias Meyer. 2012. "Double Indexing Overcomes Inaccuracies in Multiplex Sequencing on the Illumina Platform." *Nucleic acids research* 40(1): e3.

http://www.ncbi.nlm.nih.gov/pubmed/22021376 (June 15, 2017).

39. Kistler, Logan et al. 2017. "A New Model for Ancient DNA Decay Based on Paleogenomic Meta-Analysis." *Nucleic Acids Research* 45(11): 6310–20. https://academic.oup.com/nar/article-abstract/45/11/6310/3806656 (July 25, 2018).

40. Knight, Rob. 2018. "Expanding Our Understanding of the Role of the Microbiome in Health and Disease." *Archives of Medical Research* 48(8): 663–65. http://www.ncbi.nlm.nih.gov/pubmed/29429588 (April 10, 2018).

41. Kodaman, Nuri et al. 2014. "Disrupted Human-Pathogen Co-Evolution: A Model for Disease." *Frontiers in genetics* 5(August): 290. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4142859&tool=pmcentrez&rendertype=abstract.

42. Kolenbrander, P. E., and J. London. 1993. "Adhere Today, Here Tomorrow: Oral Bacterial Adherence." *Journal of Bacteriology*.

43. De La Fuente, C., S. Flores, and M. Moraga. 2013. "DNA from Human Ancient Bacteria: A Novel Source of Genetic Evidence from Archaeological Dental Calculus." *Archaeometry* 55(4): 767–78. http://doi.wiley.com/10.1111/j.1475-4754.2012.00707.x (February 11, 2016).

44. Langlie, BrieAnna S, Natalie G Mueller, Robert N Spengler, and Gayle J Fritz. 2014. "Agricultural Origins from the Ground up: Archaeological Approaches to Plant Domestication." *American journal of botany* 101(10): 1601–17. http://www.amjbot.org/content/101/10/1601.full (February 25, 2016).

45. Lassalle, Florent et al. 2017. "Oral Microbiomes from Hunter-Gatherers and Traditional Farmers Reveal Shifts in Commensal Balance and Pathogen Load Linked to Diet." *Molecular Ecology* 27(1): 182–95. http://www.ncbi.nlm.nih.gov/pubmed/29165844 (November 27, 2017).

46. Lederberg, J., and A.T. McCray. 2001. "Ome Sweet 'Omics-a Genealogical Treasury of Words." *Scientist*.

47. Legetic, Branka et al. 2016. *Economic Dimensions of Noncommunicable Diseases in Latin America and the Caribbean*. World Health Organization. Regional Office for the Americas. iris.paho.org/xmlui/handle/123456789/28501 (October 22, 2018).

48. Ley, Ruth E et al. 2008. "Evolution of Mammals and Their Gut Microbes." *Science (New York, N.Y.)* 320(5883): 1647–51. http://www.sciencemag.org/content/320/5883/1647.abstract (July 22, 2014).

49. Li, Jing et al. 2014. "Comparative Analysis of the Human Saliva Microbiome from Different Climate Zones: Alaska, Germany, and Africa." *BMC Microbiology* 14(1): 316. http://www.scopus.com/inward/record.url?eid=2-s2.0-84924137629&partnerID=tZOtx3y1 (October 16, 2015).

50. Lieverse, Angela R. 1999. "Diet and the Aetiology of Dental Calculus." *International Journal of Osteoarchaeology Int. J. Osteoarchaeol* 9(4): 219–32. (January 5, 2018).

51. Llamas, Bastien et al. 2017. "From the Field to the Laboratory: Controlling DNA Contamination in Human Ancient DNA Research in the High-Throughput Sequencing Era." *STAR: Science & Technology of Archaeological Research*.

52. Logan, Alan C, Felice N Jacka, and Susan L Prescott. 2016. "Immune-Microbiota Interactions: Dysbiosis as a Global Health Issue." *Current allergy and asthma reports* 16(2): 13. http://www.ncbi.nlm.nih.gov/pubmed/26768621 (January 18, 2016).

53. Marchesi, Julian R., and Jacques Ravel. 2015. "The Vocabulary of Microbiome Research: A Proposal." *Microbiome* 3(1): 31. http://www.microbiomejournal.com/content/3/1/31 (July 31, 2015).

54. Mark Welch, Jessica L. et al. 2016. "Biogeography of a Human Oral Microbiome at the Micron Scale." *Proceedings of the National Academy of Sciences of the United States of America* 113(6): E791-800. http://www.ncbi.nlm.nih.gov/pubmed/26811460 (January 15, 2018).

55. Marlowe, Frank W et al. 2014. "Honey, Hadza, Hunter-Gatherers, and Human Evolution." http://dx.doi.org/10.1016/j.jhevol.2014.03.006 (October 13, 2018).

56. Marsh, P. D. 2004. "Dental Plaque as a Microbial Biofilm." *Caries Research* 38(3): 204–11.

57. Martin, Konrad, and Joachim Sauerborn. 2013. "Origin and Development of Agriculture." In *Agroecology*, Dordrecht: Springer Netherlands, 9–48.

http://link.springer.com/10.1007/978-94-007-5917-6_2 (June 17, 2018).

58. Mathieson, Iain et al. 2015. "Genome-Wide Patterns of Selection in 230 Ancient Eurasians." *Nature* advance on. http://dx.doi.org/10.1038/nature16152 (November 24, 2015).

59. Metcalf, Jessica L, Luke K Ursell, and Rob Knight. 2014. "Ancient Human Oral Plaque Preserves a Wealth of Biological Data." *Nature genetics* 46(4): 321–23. http://www.ncbi.nlm.nih.gov/pubmed/24675519.

60. Meyer, Matthias et al. 2012. "A High-Coverage Genome Sequence from an Archaic Denisovan Individual." *Science (New York, N.Y.)* 338(6104): 222–26. http://www.ncbi.nlm.nih.gov/pubmed/22936568 (June 15, 2017).

61. Meyer, Matthias, and Martin Kircher. 2010. "Illumina Sequencing Library Preparation for Highly Multiplexed Target Capture and Sequencing." *Cold Spring Harbor Protocols*.

62. Muegge, Brian D. et al. 2011. "Diet Drives Convergence in Gut Microbiome Functions across Mammalian Phylogeny and within Humans." *Science (New York, N.Y.)* 332(6032): 970–74. http://www.ncbi.nlm.nih.gov/pubmed/21596990 (March 15, 2018).

63. Nasidze, Ivan et al. 2009. "Global Diversity in the Human Salivary Microbiome." *Genome research* 19(4): 636–43. http://genome.cshlp.org/content/19/4/636.short (November 16, 2015).

64. ———. 2011. "High Diversity of the Saliva Microbiome in Batwa Pygmies" ed. Francisco Rodríguez-Valera. *PLoS ONE* 6(8): e23352. http://dx.plos.org/10.1371/journal.pone.0023352 (August 30, 2017).

65. Obregon-Tito, Alexandra J. et al. 2015. "Subsistence Strategies in Traditional Societies Distinguish Gut Microbiomes." *Nature communications* 6: 6505. http://www.nature.com/ncomms/2015/150325/ncomms7505/full/ncomms7505.html (December 5, 2015).

66. Ogawa, Taiji et al. 2018. "Composition of Salivary Microbiota in Elderly Subjects." *Scientific Reports* 8(1): 414. http://www.nature.com/articles/s41598-017-18677-0 (January 15, 2018).

67. Orlando, Ludovic, and Alan Cooper. 2014. "Using Ancient DNA to Understand Evolutionary and Ecological Processes." *Annual Review of Ecology, Evolution, and Systematics* 45(1): 573–98.

http://www.annualreviews.org/doi/abs/10.1146/annurev-ecolsys-120213-091712 (February 10, 2016).

68. Palmer, Robert J., Sharon M. Gordon, John O. Cisar, and Paul E. Kolenbrander. 2003. "Coaggregation-Mediated Interactions of Streptococci and Actinomyces Detected in Initial Human Dental Plaque." *Journal of Bacteriology*.

69. Parekh, Parth J, Luis A Balart, and David A Johnson. 2015. "The Influence of the Gut Microbiome on Obesity, Metabolic Syndrome and Gastrointestinal Disease." *Clinical and translational gastroenterology* 6: e91. http://dx.doi.org/10.1038/ctg.2015.16 (December 14, 2015).

70. Pinhasi, Ron et al. 2015. "Optimal Ancient DNA Yields from the Inner Ear Part of the Human Petrous Bone." *PloS one* 10(6): e0129102. http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0129102 (September 9, 2015).

71. Prüfer, Kay et al. 2014. "The Complete Genome Sequence of a Neanderthal from the Altai Mountains." *Nature* 505(7481): 43–49. http://www.ncbi.nlm.nih.gov/pubmed/24352235 (September 26, 2018).

72. Qin, Junjie et al. 2010. "A Human Gut Microbial Gene Catalogue Established by Metagenomic Sequencing." *Nature* 464(7285): 59–65. http://www.ncbi.nlm.nih.gov/pubmed/20203603 (October 22, 2018).

73. Ranjan, Ravi et al. 2016. "Analysis of the Microbiome: Advantages of Whole Genome Shotgun versus 16S Amplicon Sequencing." *Biochemical and Biophysical Research Communications* 469(4).

74. Rohland, Nadin, and Michael Hofreiter. 2007. "Ancient DNA Extraction from Bones and Teeth." https://www-nature-com.proxy.library.adelaide.edu.au/articles/nprot.2007.247.pdf (September 24, 2018).

75. Rothschild, Daphna et al. 2018. "Environment Dominates over Host Genetics in Shaping Human Gut Microbiota." *Nature* 555(7695): 210–15. http://www.nature.com/doifinder/10.1038/nature25973 (March 18, 2018).

76. Salter, SJ Susannah J et al. 2014. "Reagent and Laboratory Contamination Can Critically Impact Sequence-Based Microbiome Analyses." *BMC Biology* 12(1): 87. http://www.biomedcentral.com/1741-7007/12/87 (October 10, 2016).

77. Schnorr, Stephanie L et al. 2014. "Gut Microbiome of the Hadza Hunter-Gatherers." *Nature communications* 5: 3654. http://www.nature.com/ncomms/2014/140415/ncomms4654/full/ncomms4654.html.

78. Sonnenburg, Justin L, and Fredrik Bäckhed. 2016. "Diet–microbiota Interactions as Moderators of Human Metabolism." *Nature* 535(7610).

79. Speth, John D. 1987. "Early Hominid Subsistence Strategies in Seasonal Habitats." *Journal of Archaeological Science* 14(1): 13–29. https://www.sciencedirect.com/science/article/pii/S0305440387800031 (September 8, 2018).

80. Steckel, Richard. 1999. *Industrialization and Health in Historical Perspective*. Cambridge, MA. http://www.nber.org/papers/h0118.pdf (April 26, 2018).

81. Szreter, S. 2004. "Industrialization and Health." *British Medical Bulletin* 69(1): 75–86. https://academic.oup.com/bmb/article-lookup/doi/10.1093/bmb/ldh005 (April 26, 2018).

82. Tito, Raul Y. et al. 2012. "Insights from Characterizing Extinct Human Gut Microbiomes." *PLoS ONE* 7(12): 1–8.

83. Truong, Duy Tin et al. 2015. "MetaPhlAn2 for Enhanced Metagenomic Taxonomic Profiling." *Nature Methods* 12(10).

84. Tuohy, Kieran, and Daniele Del Rio. 2014. *Diet-Microbe Interactions in the Gut : Effects on Human Health and Disease*. Elsevier Science. https://books.google.com.au/books?id=MQR0AwAAQBAJ&pg=PA53&lpg=PA53&dq=diseases+of+affluence+microbiome&source=bl&ots=iw4ftdxRrn&sig=xFcZT6WptcTNOh1QOwQ3LVU0W6c&hl=en&sa=X&ved=0ahUKEwing7PA7tbaAhUBXLwKHaMoBTIQ6AEIdDAH#v=onepage&q=diseases%2520of%2520affluence%25 (April 26, 2018).

85. Velsko, Irina Marie et al. 2018. "Selection of Appropriate Metagenome Taxonomic Classifiers for Ancient Microbiome Research." *bioRxiv*: 260042. https://www.biorxiv.org/content/early/2018/02/05/260042 (February 11, 2018).

86. Voreades, Noah, Anne Kozil, and Tiffany L. Weir. 2014. "Diet and the Development of the Human Intestinal Microbiome." *Frontiers in Microbiology* 5: 494.

http://journal.frontiersin.org/article/10.3389/fmicb.2014.00494/abstract (October 13, 2018).

87. Wade, Lizzie. 2015. "Breaking a Tropical Taboo." *Science.*

88. Walker, Alan W et al. 2011. "Dominant and Diet-Responsive Groups of Bacteria within the Human Colonic Microbiota." *The ISME journal* 5(2): 220–30. http://www.ncbi.nlm.nih.gov/pubmed/20686513 (October 21, 2018).

89. Warinner, C. et al. 2015. "Direct Evidence of Milk Consumption from Ancient Human Dental Calculus." *Scientific Reports* 4(1): 7104. http://www.nature.com/articles/srep07104.

90. Warinner, Christina, João F Matias Rodrigues, et al. 2014. "Pathogens and Host Immunity in the Ancient Human Oral Cavity." *Nature genetics* 46(4): 336–44. http://dx.doi.org/10.1038/ng.2906.

91. Warinner, Christina, João F Matias Rodrigues, et al. 2014. "Pathogens and Host Immunity in the Ancient Human Oral Cavity." *Nature Publishing Group* 46. http://www.nature.com.proxy.library.adelaide.edu.au/articles/ng.2906.pdf (September 3, 2018).

92. Warinner, Christina et al. 2017. "A Robust Framework for Microbial Archaeology." *Annual Review of Genomics and Human Genetics* 18(1): 321–56. https://doi.org/10.1146/annurev-genom-091416-035526 (May 2, 2017).

93. Weiss, R A. 2001. "The Leeuwenhoek Lecture 2001. Animal Origins of Human Infectious Disease." *Philosophical Transactions of the Royal Society B-Biological Sciences* 356(1410): 957–77.

94. Weyrich, Laura S., Keith Dobney, and Alan Cooper. 2015. "Ancient DNA Analysis of Dental Calculus." *Journal of Human Evolution* 79: 119–24. https://ac-els-cdn-com.proxy.library.adelaide.edu.au/S0047248414002541/1-s2.0-S0047248414002541-main.pdf?_tid=3e735bda-9676-4977-b507-61c4a0c7c047&acdnat=1522637436_38a86a3a6b77342dcad9a3e7b605a431 (April 2, 2018).

95. Weyrich, Laura S et al. 2017. "Neanderthal Behaviour, Diet, and Disease Inferred from Ancient DNA in Dental Calculus." *Nature Publishing*

Group 544.

https://www.nature.com/nature/journal/v544/n7650/pdf/nature21674.pdf

(November 1, 2017).

96. Wolfe, Nathan D, Claire Panosian Dunavan, and Jared Diamond. 2007.

"Origins of Major Human Infectious Diseases." *Nature* 447(7142): 279–

83. http://www.ncbi.nlm.nih.gov/pubmed/17507975.

97. Woyke, Tanja et al. 2011. "Decontamination of MDA Reagents for Single

Cell Whole Genome Amplification." *PLoS ONE*.

98. Wu, Gary D. et al. 2011. "Linking Long-Term Dietary Patterns with Gut

Microbial Enterotypes." *Science*.

99. Zapata, Heidi J., and Vincent J. Quagliarello. 2015. "The Microbiota and

Microbiome in Aging: Potential Implications in Health and Age-Related

Diseases." *Journal of the American Geriatrics Society* 63(4): 776–81.

http://doi.wiley.com/10.1111/jgs.13310.

100.   Ziesemer, Kirsten A et al. 2015. "Intrinsic Challenges in Ancient

Microbiome Reconstruction Using 16S RRNA Gene Amplification."

Scientific reports 5: 16498.

http://www.nature.com/srep/2015/151113/srep16498/full/srep16498.html

(February 8, 2016).

"No man is an island entire of itself; every man

is a piece of the continent, a part of the main"

John Donne, English poet (1572-1631)

Chapter I

Exploring Relationships between Host Genome and Microbiome: New Insights from Genome-Wide Association Studies

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By signing the Statement of Authorship, each author certifies that:

i.   the candidate's stated contribution to the publication is accurate (as detailed above);

ii.   permission is granted for the candidate to include the publication in the thesis; and

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Exploring Relationships between Host Genome and Microbiome: New Insights from Genome-Wide Association Studies

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As our understanding of the human microbiome expands, impacts on health and disease continue to be revealed. Alterations in the microbiome can result in dysbiosis, which has now been linked to subsequent autoimmune and metabolic diseases, highlighting the need to identify factors that shape the microbiome. Research has identified that the composition and functions of the human microbiome can be influenced by diet, age, sex, and environment. More recently, studies have explored how human genetic variation may also influence the microbiome. Here, we review several recent analytical advances in this new research area, including those that use genome-wide association studies to examine host genome–microbiome interactions, while controlling for the influence of other factors. We find that current research is limited by small sample sizes, lack of cohort replication, and insufficient confirmatory mechanistic studies. In addition, we discuss the importance of understanding long-term interactions between the host genome and microbiome, as well as the potential impacts of disrupting this relationship, and explore new research avenues that may provide information about the co-evolutionary history of humans and their microorganisms.

Keywords: microbiome, GWAS, ancient DNA, microbiome GWAS, model organisms, microbiota, symbiosis

INTRODUCTION

Our view of the microbial community within us has shifted drastically in the last few decades from simplistic commensalism to complex mutualisms. As a comprehensive understanding of this microbial community expands, we are beginning to unravel the impact of microbes on human health. This diverse microbial community is defined as the 'microbiota,' and the collective genomes of all microbial species and their environment is termed the 'microbiome' (Marchesi and Ravel, 2015). Microbiota play key roles in human health throughout life (Gregory, 2011; Yatsunenko et al., 2012; Rodríguez et al., 2015; Zapata and Quagliarello, 2015), and dysbiosis, defined as an imbalance of the composition of the microbial components of the microbiota (Karlsson et al., 2013; Parekh et al., 2015) has now been linked with various metabolic diseases such as obesity, type 2 diabetes and inflammatory bowel disease (IBD).

Our improved ability to examine the microbiome is a result of a revolution in DNA sequencing technologies and techniques, and the reapplication of pre-existing concepts from ecology. Exponential advances in DNA sequencing technology, from low-throughput Sanger sequencing

to high-throughput next generation sequencing, have allowed us to obtain huge amounts of sequence data at a fraction of the cost (Cho and Blaser, 2012; van Dijk et al., 2014). Furthermore, the availability of cheap computational power, coupled with free and readily available open source software tools, has resulted in larger capacities for in-depth analysis of sequenced data to characterize microbial communities. In addition, the applications of concepts from ecology, such as diversity indices [Alpha diversity and Beta diversity (Whittaker, 1972)], have enabled us to better categorize and understand the composition and diversity of the microbial world within us (Blaser, 2014).

Two separate methods are commonly applied to examine human microbiota: metabarcoding or shotgun sequencing. Metabarcoding typically uses the 16S ribosomal RNA (rRNA) encoding gene to characterize the species structure of bacterial communities in various environments, and has been the gold standard in microbiome research due to the ubiquity of the 16S rRNA gene amongst prokaryotes and the availability of large reference databases (Woese et al., 1990). However, shotgun metagenomic sequencing, or sequencing DNA fragments at random from the sample, is increasing in popularity, as it can be used for both species profiling and functional analysis. Both approaches have limitations. For example, 16S rRNA sequencing can be biased due to uneven amplification of bacterial 16S rRNA genes or primer biases. While challenges with identifying low abundance community members with shotgun metagenomic sequencing and the cost of large-scale multiplexing at sufficiently high depths make this method prohibitive for many labs, shotgun metagenomic sequencing has the potential to provide information about the microbial community and the host simultaneously (Poretsky et al., 2014).

From an evolutionary perspective, microbiome studies have revealed compositional differences in microbiota between great apes, archaic hominins, and modern humans, with a marked reduction in microbial diversity observed within modern humans. This loss of bacterial diversity in modern humans is postulated to be a result of lifestyle changes (Cho and Blaser, 2012). Dietary changes brought about by agriculture altered the human microbiota considerably over 7,500 years ago, and changed again with the recent movement toward animal-based and fat-rich western diets (Adler et al., 2013). This reduction in diversity is thought to be partially responsible for the dysbiosis observed in modern human microbiomes that is now associated with various metabolic and autoimmune diseases (Blaser and Falkow, 2009; Adler et al., 2013; Moeller et al., 2014; Logan et al., 2016). Furthermore, changes in the human microbiome through time and the linked impacts on human health highlight the importance of studying the co-evolution of the human microbiome and how those evolutionary changes in microbial composition may have interacted with our genomes (Linderholm, 2015).

In parallel with these recent discoveries in microbiome research, the last two decades of genomic research have expanded our understanding of how human genomic differences result in phenotypic changes that impact human health and well-being. The advent of large genome wide association studies (GWAS) on a population level have allowed us to better understand the relationships between common genetic variants and diseases, such as Alzheimer's, type 2 diabetes and obesity (Imamura and Maeda, 2011; Fall and Ingelsson, 2014; Rao et al., 2014). However, these two research fields, microbiome research and human population genetics, have only recently begun to explore the human genome and microbiome simultaneously, to examine how their interaction influences our health and disease. Our understanding of their interactive roles in our health and disease remains in its infancy (Gilbert et al., 2016; Wang and Jia, 2016). This review highlights in chronological order advances linking host genetic variation with microbiome composition and more recent research using GWAS.

MICROBIOMES

The Human Microbiome Project (HMP) determined that different body sites are distinct niches for various bacteria, resulting in differences in microbiome composition throughout the body (The Human Microbiome Project, 2012). The human gut, due to its large surface area and role in nutrition and energy homeostasis, is home to a plethora of microbes. Its microbiome has been the most studied due to its easy access for sampling and critical importance for health. The gut microbiome has been shown to play essential roles in the metabolism of complex polysaccharides. These complex polysaccharides are used to synthesize short-chain fatty acids such as butyrate, acetate, and propionate that are used as signaling molecules in the communication and development of host innate immune system (Jacobs and Braun, 2014). This system can breakdown due to changes in microbiome composition or pathogenic microorganism colonization that hijacks and alters these pathways, contributing to the etiology of metabolic and infectious diseases, such as type 2 diabetes, IBD, obesity (Carding et al., 2015), and *Clostridium difficile* infection (CDI; Bien et al., 2013). In addition to the influence of the gut microbiome on the immune system, short-chain fatty acids produced by the gut microbiota have also been shown to impact the brain and nervous system (Rhee et al., 2009; Thomas et al., 2012).

Microbiomes at different body sites interact and can influence one another. This interaction between microbiomes at different sites in the body, such as the oral and gut microbiomes, was indicated in a study by Ding and Schloss (2014) where specific microbes abundant in the gut were more likely to be present in the oral cavity (saliva and supragingival plaque). This predictive relationship between the oral and gut microbiota is not surprising, as the oral cavity is the gateway to the gut for various microbes (Dewhirst et al., 2010). The advent of modern dentistry has exposed the presence of pathogenic bacteria in the oral cavity. However, our understanding of the role played by non-pathogenic commensal bacteria is relatively recent. Recent research has revealed over 600 bacterial species or phylotypes present at different sites in the oral cavity (Dewhirst et al., 2010), including many commensal species that help in host defense by colonizing prime locations and creating an inhospitable environment for secondary colonization of pathogenic bacteria.

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Disruption of the composition of this healthy oral microbiome has been observed to result in inflammation and diseases such as periodontitis and the proliferation of bacterial species, such as *Streptococcus mutans*, which play crucial roles in the etiology of dental caries (cavities) (Johansson et al., 2015; Kumar and Mason, 2015). This is exemplified by recent research showing that the oral microbiomes of patients with dental caries are distinct in composition and abundance of *Streptococcus* compared to that of healthy individuals (Johansson et al., 2015).

In order to study host genome and microbiome interactions, it is crucial to examine other factors that influence microbiome composition. Recent advances in microbiome research has shown that microbial composition can be heavily influenced by the environment through diet and lifestyle (David et al., 2014). Western populations have homogeneous diets and lifestyles resulting in lower microbial diversity. Microbiomes from non-western and indigenous populations are currently being explored as a means to examine how unique lifestyles impact the human microbiome. Studies examining microbiota in African hunter-gatherers such as the Hadza (Schnorr et al., 2014) and South American indigenous peoples (Yatsunenko et al., 2012) have revealed a broader picture of the human gut microbiome – one that is more diverse and complex. Sex has also been shown to influence microbial composition with a number of recent studies correlating specific microbial composition to specific sexes (Markle et al., 2013; Ding and Schloss, 2014; Blekhman et al., 2015). In many studies, such as those on the microbiomes of the Hadza and Hutterites (a North American isolated community with shared diet and non-standard cultural practices), differences in microbial compositions between the sexes are related to the dissimilarities in societal roles played by men and women (Schnorr et al., 2014; Davenport et al., 2014, 2015). Age is another factor that influences microbial composition. In infants, the gut microbiome is highly unstable and can resemble that of the mother, but shifts toward a more robust adult microbiome within 2–3 years of life (Yatsunenko et al., 2012). This shift may be due to changing diets due to the increase in age. In a murine model system, Langille et al. (2014) demonstrated differences in microbial composition between old and young mice, and identified specific bacterial genera, such as *Alistipes*, that were more prevalent in older mice. Furthermore, two recent publications using large datasets ($N > 1000$) confirmed these factors, as well as 69 others that fall in categories, such as medication, blood parameters, health status, anthropometric features, and lifestyle that correlated with variation in microbiome composition (Falony et al., 2016; Zhernakova et al., 2016). These differences in age, sex, populations, culture, and environment will need to be addressed in studies exploring human genome–microbiome interactions.

EXPLORING HOST GENOME–MICROBIOME INTERACTIONS

Animal Models

Animal models provide an avenue to probe these interactions at a depth that is not possible using human-based studies.

Studies using zebrafish (Kanther and Rawls, 2010; Milligan-Myhre et al., 2011), *Drosophila* (Kuraishi et al., 2013), and mice have been crucial for laying the foundations of microbiome research (Kostic et al., 2013). Many early animal-based microbiome studies focused on the symbiosis between microbial communities and their hosts. Sharon et al. (2010) illustrated the role that commensal microbiota play in the mating preference of *Drosophila melanogaster*. In zebrafish, the influence of the host genome on the microbiome was confirmed by an experiment involving transplantation of microbiota between germ-free zebrafish and mice. The transplanted microbiota adapted to resemble the host's normal microbiota, demonstrating that the host genome selects for specific microbes to suit certain niches within an organism (Rawls et al., 2006).

Murine Models

Microbiome research in mice has been ground-breaking, as germ-free mice have played crucial roles in many baseline experiments. Murine models have shown that gut microbiota are influenced by the genetic background of the mice (Esworthy et al., 2010), and that gut microbial composition should be viewed as a complex polygenic trait (Benson et al., 2010). Mice have a large number of orthologous genes and similarities in microbiome composition to humans, and quantitative trait loci (QTL) analyses in mice have revealed specific gene regions that modulate microbiome composition (Srinivas et al., 2013). Benson et al. (2010) reported 18 QTLs that were associated with specific bacterial taxa. Furthermore, McKnite et al. (2012) used genetic mapping to link gut microbiota of laboratory crossed mice to immune genes that modulate microbiome composition.

Human-Based Studies

Murine model studies have consistently shown that host genotypes are important in regulating microbiota composition (Campbell et al., 2012; Hildebrand et al., 2013; Bongers et al., 2014). While murine models demonstrate the importance of genotypes in regulating the composition of microbiota, they cannot be viewed as replacements for human-based studies due to the inherent complexity involved in extrapolating murine results to humans (Seok et al., 2013). Turnbaugh et al. (2009) and Yatsunenko et al. (2012) used dizygotic and monozygotic twins to examine the heritability of the gut microbiome while controlling for environmental factors. Although both studies concluded that there were no statistically significant differences between monozygotic and dizygotic twins, they were both underpowered due to their small sample sizes and lack of statistical rigor. Consequently, a follow up studies using larger sample sizes of the same twin datasets by Goodrich et al. (2014) and more recently by Goodrich et al. (2016a) revealed the measurable influence of host genetic variation on microbial composition, showing *Christensenellaceae* to be the most heritable taxon, while *Bacteroidetes* was more influenced by the environment. Goodrich et al. (2016a) also showed that highly heritable taxa correlated with higher levels of temporal stability, highlighting the importance of these taxa to the host.

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A candidate gene approach has also been explored (Jacobs and Braun, 2014). Research by Folseraas et al. (2012) and Knights et al. (2014) examined the role of gut microbiome composition and host genetic loci associated with IBD and related diseases in humans. Folseraas et al. (2012) showed that the gene FUT2 is associated with a signficant increase in the abundance of Firmicutes and significant decrease of Proteobacteria, while Knights et al. (2014) correlated the NOD2 gene with Enterobactericea, pointing toward the impact of these two genotypes and their associated bacterial taxa as risk factors for IBD and IBD related diseases.

While these studies provided valuable insight into how the microbiome can be inherited like other phenotypic traits, their study design using heritability is limited, as it cannot identify statistically significant genetic loci on a genome-wide level that interact with gut microbiota composition.

The Advent Of Microbiome – GWAS Research

Recently, researchers have begun to apply techniques from GWAS to microbiome research. GWAS seek to identify relevant common genetic variants associated with various disease phenotypes, by simultaneously screening thousands of the most common variants in the human genome and these phenotypes (Bush and Moore, 2012). In humans, GWAS have successfully linked genetic variation to various disease phenotypes, including type 2 diabetes, obesity, and heart disease (Visscher et al., 2012). However, very few studies have used GWAS to explore the interaction between human genetics and microbiome composition.

Three recent studies have broke new ground by using GWAS to study the influence of genetic variation on microbiome composition. Blekhman et al. (2015) reported the first microbiome GWAS study based on genomic and microbiome data mined from the HMP. Microbiome data from 15 sites on the body were correlated with human genomic data inferred from "contaminating" human DNA of the body sites to expose the role that host genetic variation plays in microbiome composition across various sites in the body. These inferences on the host were made possible due to the large amounts of host DNA found, which were variable at different body sites. A subsequent publication by Davenport

et al. (2015) analyzed the gut microbiome and host genetic data from Hutterites, a North American isolated community with shared diet and identical cultural practices, to control for environmental confounders. The third and most recent study by Goodrich et al. (2016a) revisited the inheritance of the gut microbiota in twins and examined the link between host genetics and gut microbiome composition in 1,248 individuals, including mono- and di-zygotic twin pairs. All three studies (Blekhman et al., 2015; Davenport et al., 2015; Goodrich et al., 2016a) revealed that specific bacterial taxa, such as *Bifidobacterium*, are inheritable and correlate with specific host genotypes. Goodrich et al. (2016a) linked *Bifidobacteria* which metabolizes lactose in the gut with the LCT gene locus which encodes for the enzyme lactase that hydrolyses lactose. They revealed that lactase 'non-persisters' harbored lower levels of *Bifidobacterium* relative to lactase 'persisters,' possibly due to the higher availability of lactose in the gut of lactase non-persisters. Blekhman et al. (2015) and Davenport et al. (2015) also found that immunity-related genes, such as interleukin-2 (IL2) influenced the modulation of microbiome composition via pathways that may then result in complex diseases, although the exact mechanism remain unknown. Goodrich et al. (2016a) also updated and summarized an earlier list by Spor et al. (2011) of number of other host genetic loci that are suspected to be associated with the microbiome that was primarily based on mouse QTL research.

Furthermore, these studies used gene expression data to illustrate that genetic loci linked to microbiome composition have a functional role in metabolic diseases, such as obesity (Blekhman et al., 2015; Davenport et al., 2015).

Microbiome – GWAS Methodology

Thus far, many of the most recent microbiome GWAS studies on human hosts used either Alpha diversity indices (α-diversity; within population diversity) or Beta diversity indices (β-diversity; between population diversity) of microbial composition as phenotypes, and correlated the diversity with common genetic variants (SNPs). **Table 1** details the methods used in microbiome GWAS in human hosts published to date, based on our knowledge. Blekhman et al. (2015) utilized a traditional GWAS statistical software package, PLINK (Purcell et al., 2007), and completed regression

TABLE 1 | Methods used in human microbiome Genome wide association studies (GWAS).

Method	Software tool	Sample size	Microbiome (sampling site)	Number of SNP/ Genes correlated	Reference	Notes
Additive linear mixed model	PLINK (v1.07) Purcell et al., 2007; Chang et al., 2015	93	Microbiome composition at various body sites (10)	83*	Blekhman et al., 2015	*Pathway based analysis was used in lieu of genes.
Genome-wide efficient mixed-model association (GEMMA)	GEMMA (v0.94) Zhou and Stephens, 2012	127	Gut microbiome	187*	Davenport et al., 2015	*SNP
Microbiome GWAS	Microbiome GWAS Hua et al., 2015	1,248	Gut microbiome	142*	Goodrich et al., 2016a	*Genes *GEMMA (v0.94) was also used.

analysis with an additive linear mixed model, followed by multiple test corrections to identify SNPs associated with microbiome composition at genome-wide significances. However, Davenport et al. (2015) and Goodrich et al. (2016a) used a genome-wide efficient mixed model implemented in the GEMMA software tool in their studies (Zhou and Stephens, 2012). These studies demonstrated that β-diversity is a more appropriate metric than α-diversity for microbiome GWAS, as it represents the microbial community more comprehensively and reduces the temporal variability observed with α-diversity resulting in an increase in statistical power (Hua et al., 2015). Microbiome GWAS (Hua et al., 2015) is a recently developed statistical software package that uses β-diversity indices calculated using both unweighted or weighted UniFrac and genome-wide SNPs, and allows for controlling confounders as covariates in a computationally efficient framework. Microbiome GWAS has been tested using both simulations and on a lung cancer dataset to identify microbiota associated with lung cancer risk in Europeans (Hua et al., 2015). More recently, Microbiome GWAS was applied to the expanded UK twins dataset ($n = 1,248$) (Goodrich et al., 2016a). This statistical package has the capability to correct for skewness and kurtosis in the score statistics that are a result of small sample sizes. Another recently published software package is MiRKAT (Zhao et al., 2015), which is a kernel regression based test to find associations between genome-wide SNPs and β-diversity computed using a generalized UniFrac (Chen et al., 2012). MiRKAT is limited by its computationally inefficient framework, and requires long run time and a large amount of processing power (Hua et al., 2015).

These microbiome – GWAS wide association studies form the initial attempts to elucidate genome–microbiome interaction; however, a number of limitations currently exist. GWAS typically require large sample sizes in order to obtain statistically significant results and account for small effect sizes that can be attributed to variants correlated with phenotypes (Hayes, 2013). The studies of Blekhman et al. (2015) and Davenport et al. (2015) both had small sample sizes (~100) that likely resulted in underpowered statistical analyses, especially following the multiple test corrections that were required for the results to be statistically significant. Blekhman et al.(2015) combined various individual SNPs into groups based on their association with similar biological pathways to increase statistical power, while Davenport et al. (2015) performed multiple test corrections on SNPs within each genome-wide association study to reduce the number of SNPs undergoing multiple test corrections. Nevertheless, both of these approaches do not fit the required statistical rigor observed in traditional GWAS studies (Barsh et al., 2012). Another challenge in microbiome – GWAS studies is the need for replicate cohorts. While Blekhman et al. (2015) used the Twins UK dataset (Moayyeri et al., 2012), Davenport et al. (2015) lacked a replication cohort to confirm their results. Improving statistical power by accounting for excess zeros in OTU counts of metagenomic samples (Xu et al., 2015), as well as larger host sample sizes and replication cohorts, are crucial if we are to obtain a more reliable understanding of genome–microbiome interactions. Furthermore, while most studies of this type identify genetic loci that appear to interact with the genome, all are yet to be confirmed by mechanistic or functional studies.

EVOLUTIONARY PERSPECTIVE ON GENOME–MICROBIOME INTERACTIONS

Due to changes in the human microbiome and genome over long periods of time, an evolutionary perspective on the microbiome is important in interpreting these effects on human health and disease. Several groups have examined modern populations to infer the influences of different evolutionary histories on the human microbiome. Moeller et al. (2014) showed how the human microbiome evolved by sequencing the gut microbiome of chimpanzees, bonobos, gorillas, and modern humans. More recently, they reported the presence of shared microbial composition across all four host species, suggesting the existence of an ancestral hominid microbiome (Moeller et al., 2016). In addition, Moeller et al. (2014) showed that the microbiome of our hominid ancestors was more diverse and more stable compared to that of modern humans. Disruptive dietary and environmental changes, such as the transition to agriculture, may have likely contributed to the loss of such diversity (Adler et al., 2011). Moeller et al. (2014) observed that bacterial taxa, such as *Bacteroidetes*, are associated with animal fat, rich diets, and high protein intake, and increase in abundance in modern humans. In contrast, taxa responsible for the degradation of complex polysaccharides, such as *Methanobrevibacter*, underwent decreases in abundance. The authors also compared their data to people in remote Venezuela, rural Malawi, and industrial United States, demonstrating a gradient of diminished microbiotic diversity driven by environment and diet (Moeller et al., 2014, 2016).

Similar findings have also been observed in other studies, Schnorr et al. (2014) and Schnorr (2015) explored the gut microbiome of the Hadza people, an Indigenous group of Tanzanian Hunter-gatherers. Researchers observed high bacterial diversity in this group compared to Western populations, as well as higher abundances of plant polysaccharide metabolizing taxa, such as *Prevotella* and *Treponema*. These taxa likely provide the Hadza with short chain fatty acids, such as butyrate and propionic acid, which positively influence immune and nervous systems by regulating inflammation (Rhee et al., 2009; Thomas et al., 2012). Other researchers hypothesized that the diversity and composition of the Hadza's microbiome contribute to their low rates of nutritional and metabolic diseases (Blurton Jones et al., 1992). Similarly, De Filippo et al. (2010) and Yatsunenko et al. (2012) have shown the presence of a decreasing gradient of microbial diversity and abundance from contemporary Hunter-gatherers, rural farming communities to western populations. These studies suggest that our changing diets and lifestyles are responsible for our "missing microbes,"

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and the resulting metabolic and autoimmune diseases (Blaser and Falkow, 2009).

Ancient DNA

Expanding the focus of microbiome studies to non-western and indigenous populations around the world has helped us to understand the diversity of the human microbiome. However, these studies do not provide us the information necessary to understand evolutionary changes through time. The expansion of microbiome research from contemporary to ancient human populations has provided us with evolutionary snapshots of change through time, allowing for the examination of the human microbiome before, during, and after cultural shifts, such as the agricultural transition (Neolithic Revolution), the Industrial Revolution, and the advent of modern globalized and highly processed food (Adler et al., 2013). Studying these evolutionary snapshots provide information on how these communities have changed and adapted in real time, and improve our understanding of how these changes have influenced human health and disease.

In recent years, ancient DNA (aDNA) research has unveiled genomes of archaic hominins, such as Neanderthals (Prüfer et al., 2014), Denisovans (Meyer et al., 2012) as well as prehistoric human populations (Mathieson et al., 2015). Ancient DNA refers to damaged DNA extracted from fossil remains, and is characterized by short fragment sizes, cross linkage between DNA molecules, and increased cytosine deamination due to the taphonomic effects of the environment and time (Hofreiter et al., 2001; Willerslev and Cooper, 2005; Linderholm, 2015). Typical samples for extraction of aDNA are obtained from locations in the human body that offer the most protection from exogenous DNA (i.e., that from both the environment and microbes). The samples that have been most successful for the retrieval of hominin aDNA are bones (femur and petrosal) (Meyer et al., 2014; Pinhasi et al., 2015) and teeth (Adler et al., 2011).

While the vast majority of research in this field has focused on the extraction and analysis of genomic data for human population history, the last 5 years has seen cutting-edge research expose the potential of ancient human microbiome DNA (Adler et al., 2013; Warinner et al., 2015). This has primarily been done using dental calculus, a calcified bacterial biofilm (dental plaque) that forms on teeth. Due to the process of dental calculus formation that encapsulates microbial DNA protecting it from external contamination, it is possible to extract large amounts of microbial aDNA that are relatively uncontaminated by the environment. Dental calculus research has already provided insights into changes in the oral microbiome during large cultural shifts in time, including transitions to agriculture and industrialization (Adler et al., 2013).

Research to analyze the microbial diversity within dental calculus began with de la Fuente et al. (2013) using species-specific PCR amplification, followed by Adler et al. (2013) using 16S metabarcode sequencing and Warinner et al. (2014) using metagenomic shotgun sequencing. Ancient dental calculus revealed how the human microbiome has adapted through time, as observed through 34 ancient calculus samples in Europe over the past 8,000 years (Adler et al., 2013). The authors were able to show that both the transition to agriculture (∼7,500 years ago) followed by the availability of processed food, antibiotics, and toxins in the environment following the Industrial Revolution (∼150 years ago) resulted in decreased bacterial diversity and an increase in oral microbiota dysbiosis.

aDNA has also been used to explore the evolutionary history of specific microorganisms, such as the causative agents of tuberculosis (Bos et al., 2014), plague (Bos et al., 2011), leprosy (Inskip et al., 2015), and gastritis (Maixner et al., 2016). While important, these studies have not revealed how the microbiome in these individuals has shifted. Microbiome-based studies, using aDNA from dental calculus of ancient populations, can provide us with a unique perspective on the evolution of entire bacterial communities, and their coevolution with their hosts, rather than individual taxa (Adler et al., 2013; Metcalf et al., 2014; Warinner et al., 2014). The growing availability of genomic information from ancient humans (Haak et al., 2015; Mathieson et al., 2015; Skoglund et al., 2015) provides a unique opportunity to examine co-evolutionary interactions through time. For example, several studies have described the human genomic changes that occurred during the agricultural transition, including alterations in both immune and metabolic genes that allowed early farming communities to adapt to new diets and environments (Allentoft et al., 2015). These loci, such as those relating to lactase persistence and immune responses, are similar to loci identified in modern day microbiome – GWAS studies Goodrich et al. (2016b), suggesting that there may be a link between genomic and microbiome alterations.

FUTURE DIRECTIONS

In order to obtain a detailed understanding of these interactions, larger sample sizes and independent replication cohorts are required to confirm the associations that are detected. This will be beyond the singular capacity of many research laboratories and will require large collaborations, as seen in the medical genetics community where GWAS studies have been applied to specific human diseases. As the list of microbiome associated genetic loci grows, there is also the need for functional studies to understand the mechanisms that underlie genome–microbiome interactions. Gnotobiotic mice and human cell lines hold promise toward that aim and are part of the NIH Integrated HMP (iHMP/HMP 2.0), announced in 2014 as the successor to the HMP. The Integrated HMP has initiated the transition from high throughput screening to longitudinal and detailed mechanistic studies [Integrative Hmp (iHMP) Research Network Consortium, 2014]. In addition, studying the co-evolution of the genome and microbiome in ancient humans using ancient DNA will provide a comprehensive overview of the changes to the interaction over time. Further research will also require multidisciplinary expertise that is missing in recent publications. Microbiologists and population

geneticists will need to work together with bioinformaticians to accurately design experiments, perform rigorous statistical tests, and analyze results. With these steps, a clear understanding of the interactions between our genome and the microbiome will open new avenues for numerous medical therapies. This research will truly bring us into the age of personalized medicine, with the ability to modify our microbiome in light of our genome, to prevent disease and maintain human health.

AUTHOR CONTRIBUTIONS

MA-A wrote the manuscript; LW and AC commented and edited the manuscript. All authors read and approved the final version for submission.

REFERENCES

Adler, C. J., Dobney, K., Weyrich, L. S., Kaidonis, J., Walker, A. W., Haak, W., et al. (2013). Sequencing ancient calcified dental plaque shows changes in oral microbiota with dietary shifts of the Neolithic and Industrial revolutions. *Nat. Genet.* 45, 450–455. doi: 10.1038/ng.2536

Adler, C. J., Haak, W., Donlon, D., and Cooper, A. (2011). Survival and recovery of DNA from ancient teeth and bones. *J. Archaeol. Sci.* 38, 956–964. doi: 10.1016/j.jas.2010.11.010

Allentoft, M. E., Sikora, M., Sjögren, K.-G., Rasmussen, S., Rasmussen, M., Stenderup, J., et al. (2015). Population genomics of Bronze Age Eurasia. *Nature* 522, 167–172. doi: 10.1038/nature14507

Barsh, G. S., Copenhaver, G. P., Gibson, G., and Williams, S. M. (2012). Guidelines for genome-wide association studies. *PLoS Genet.* 8:e1002812. doi: 10.1371/journal.pgen.1002812

Benson, A. K., Kelly, S. A., Legge, R., Ma, F., Low, S. J., Kim, J., et al. (2010). Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. *Proc. Natl. Acad. Sci. U.S.A.* 107, 18933–18938. doi: 10.1073/pnas.1007028107

Bien, J., Palagani, V., and Bozko, P. (2013). The intestinal microbiota dysbiosis and *Clostridium difficile* infection: is there a relationship with inflammatory bowel disease? *Ther. Adv. Gastroenterol.* 6, 53–68. doi: 10.1177/1756283X12454590

Blaser, M. J. (2014). The microbiome revolution. *J. Clin. Invest.* 124, 4162–4165. doi: 10.1172/JCI78366

Blaser, M. J., and Falkow, S. (2009). What are the consequences of the disappearing human microbiota? *Nat. Rev. Microbiol.* 7, 887–894. doi: 10.1038/nrmicro2245

Blekhman, R., Goodrich, J. K., Huang, K., Sun, Q., Bukowski, R., Bell, J. T., et al. (2015). Host genetic variation impacts microbiome composition across human body sites. *Genome Biol.* 16:191. doi: 10.1186/s13059-015-0759-1

Blurton Jones, N. G., Smith, L. C., O'Connell, J. F., Hawkes, K., and Kamuzora, C. L. (1992). Demography of the Hadza, an increasing and high density population of Savanna foragers. *Am. J. Phys. Anthropol.* 89, 159–181. doi: 10.1002/ajpa.1330890204

Bongers, G., Pacer, M. E., Geraldino, T. H., Chen, L., He, Z., Hashimoto, D., et al. (2014). Interplay of host microbiota, genetic perturbations, and inflammation promotes local development of intestinal neoplasms in mice. *J. Exp. Med.* 211, 457–472. doi: 10.1084/jem.20131587

Bos, K. I., Harkins, K. M., Herbig, A., Coscolla, M., Weber, N., Comas, I., et al. (2014). Pre-Columbian mycobacterial genomes reveal seals as a source of New World human tuberculosis. *Nature* 514, 494–497. doi: 10.1038/nature13591

Bos, K. I., Schuenemann, V. J., Golding, G. B., Burbano, H. A., Waglechner, N., Coombes, B. K., et al. (2011). A draft genome of *Yersinia pestis* from victims of the Black Death. *Nature* 478, 506–510. doi: 10.1038/nature10549

Bush, W. S., and Moore, J. H. (2012). Chapter 11: genome-wide association studies. *PLoS Comput. Biol.* 8:e1002822. doi: 10.1371/journal.pcbi.1002822

Campbell, J. H., Foster, C. M., Vishnivetskaya, T., Campbell, A. G., Yang, Z. K., Wymore, A., et al. (2012). Host genetic and environmental effects on mouse intestinal microbiota. *ISME J.* 6, 2033–2044. doi: 10.1038/ismej.2012.54

Carding, S., Verbeke, K., Vipond, D. T., Corfe, B. M., and Owen, L. J. (2015). Dysbiosis of the gut microbiota in disease. *Microb. Ecol. Health Dis.* 26:26191.

Chang, C. C., Chow, C. C., Tellier, L. C., Vattikuti, S., Purcell, S. M., and Lee, J. J. (2015). Second-generation PLINK: rising to the challenge of larger and richer datasets. *Gigascience* 4:7. doi: 10.1186/s13742-015-0047-8

Chen, J., Bittinger, K., Charlson, E. S., Hoffmann, C., Lewis, J., Wu, G. D., et al. (2012). Associating microbiome composition with environmental covariates using generalized UniFrac distances. *Bioinformatics* 28, 2106–2113. doi: 10.1093/bioinformatics/bts342

Cho, I., and Blaser, M. J. (2012). Applications of next-generation sequencing the human microbiome: at the interface of health and disease. *Nat. Rev. Genet.* 13, 260–270. doi: 10.1038/Nrg3182

Davenport, E. R., Cusanovich, D. A., Michelini, K., Barreiro, L. B., Ober, C., and Gilad, Y. (2015). Genome-wide association studies of the human gut microbiota. *PLoS ONE* 10:e0140301. doi: 10.1371/journal.pone.0140301

Davenport, E. R., Mizrahi-Man, O., Michelini, K., Barreiro, L. B., Ober, C., and Gilad, Y. (2014). Seasonal variation in human gut microbiome composition. *PLoS ONE* 9:e90731. doi: 10.1371/journal.pone.0090731

David, L. A., Maurice, C. F., Carmody, R. N., Gootenberg, D. B., Button, J. E., Wolfe, B. E., et al. (2014). Diet rapidly and reproducibly alters the human gut microbiome. *Nature* 505, 559–563. doi: 10.1038/nature12820

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J. B., Massart, S., et al. (2010). Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc. Natl. Acad. Sci. U.S.A.* 107, 14691–14696. doi: 10.1073/pnas.1005963107

de la Fuente, C., Flores, S., and Moraga, M. (2013). DNA from human ancient bacteria: a novel source of genetic evidence from archaeological dental calculus. *Archaeometry* 55, 767–778. doi: 10.1111/j.1475-4754.2012.00707.x

Dewhirst, F. E., Chen, T., Izard, J., Paster, B. J., Tanner, A. C. R., Yu, W. H., et al. (2010). The human oral microbiome. *J. Bacteriol.* 192, 5002–5017. doi: 10.1128/JB.00542-10

Ding, T., and Schloss, P. D. (2014). Dynamics and associations of microbial community types across the human body. *Nature* 509, 357–360. doi: 10.1038/nature13178

Esworthy, R. S., Smith, D. D., and Chu, F.-F. (2010). A strong impact of genetic background on gut microflora in mice. *Int. J. Inflamm.* 2010:986046. doi: 10.4061/2010/986046

Fall, T., and Ingelsson, E. (2014). Genome-wide association studies of obesity and metabolic syndrome. *Mol. Cell. Endocrinol.* 382, 740–757. doi: 10.1016/j.mce.2012.08.018

45

Falony, G., Joossens, M., Vieira-Silva, S., Wang, J., Darzi, Y., Faust, K., et al. (2016). Population-level analysis of gut microbiome variation. *Science* 352, 560–564. doi: 10.1126/science.aad3503

Folseraas, T., Melum, E., Rausch, P., Juran, B. D., Ellinghaus, E., Shiryaev, A., et al. (2012). Extended analysis of a genome-wide association study in primary sclerosing cholangitis detects multiple novel risk loci. *J. Hepatol.* 57, 366–375. doi: 10.1016/j.jhep.2012.03.031

Gilbert, J. A., Quinn, R. A., Debelius, J., Xu, Z. Z., Morton, J., Garg, N., et al. (2016). Microbiome-wide association studies link dynamic microbial consortia to disease. *Nature* 535, 94–103. doi: 10.1038/nature18850

Goodrich, J. K., Davenport, E. R., Beaumont, M., Jackson, M. A., Knight, R., Ober, C., et al. (2016a). Genetic determinants of the gut microbiome in UK twins. *Cell Host Microbe* 19, 731–743. doi: 10.1016/j.chom.2016.04.017

Goodrich, J. K., Davenport, E. R., Waters, J. L., Clark, A. G., and Ley, R. E. (2016b). Cross-species comparisons of host genetic associations with the microbiome. *Science* 352, 532–535. doi: 10.1126/science.aad9379

Goodrich, J. K., Waters, J. L., Poole, A. C., Sutter, J. L., Koren, O., Blekhman, R., et al. (2014). Human genetics shape the gut microbiome. *Cell* 159, 789–799. doi: 10.1016/j.cell.2014.09.053

Gregory, K. E. (2011). Microbiome aspects of perinatal and neonatal health. *J. Perinat. Neonatal Nurs.* 25, 154–158. doi: 10.1097/JPN.0b013e3182169346

Haak, W., Lazaridis, I., Patterson, N., Rohland, N., Mallick, S., Llamas, B., et al. (2015). Massive migration from the steppe was a source for Indo-European languages in Europe. *Nature* 522, 207–211. doi: 10.1038/nature14317

Hayes, B. (2013). Overview of statistical methods for genome-wide association studies (GWAS). *Methods Mol. Biol.* 1019, 149–169. doi: 10.1007/978-1-62703-447-0_6

Hildebrand, F., Nguyen, T. L. A., Brinkman, B., Yunta, R. G., Cauwe, B., Vandenabeele, P., et al. (2013). Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. *Genome Biol.* 14:R4. doi: 10.1186/gb-2013-14-1-r4

Hofreiter, M., Serre, D., Poinar, H. N., Kuch, M., and Pääbo, S. (2001). Ancient DNA. *Nat. Rev. Genet.* 2, 353–359. doi: 10.1038/35072071

Hua, X., Song, L., Yu, G., Goedert, J. J., Abnet, C. C., Landi, M. T., et al. (2015). MicrobiomeGWAS: a tool for identifying host genetic variants associated with microbiome composition. *bioRxiv* doi: 10.1101/031187

Imamura, M., and Maeda, S. (2011). Genetics of type 2 diabetes: the GWAS era and future perspectives [Review]. *Endocr. J.* 58, 723–739. doi: 10.1507/endocrj.EJ11-0113

Inskip, S. A., Taylor, G. M., Zakrzewski, S. R., Mays, S. A., Pike, A. W. G., Llewellyn, G., et al. (2015). Osteological, biomolecular and geochemical examination of an early anglo-saxon case of lepromatous leprosy. *PLoS ONE* 10:e0124282. doi: 10.1371/journal.pone.0124282

Integrative Hmp (iHMP) Research Network Consortium. (2014). The Integrative Human Microbiome Project: dynamic analysis of microbiome-host omics profiles during periods of human health, and disease. *Cell Host Microbe* 16, 276–289. doi: 10.1016/j.chom.2014.08.014

Jacobs, J. P., and Braun, J. (2014). Immune and genetic gardening of the intestinal microbiome. *FEBS Lett.* 588, 4102–4111. doi: 10.1016/j.febslet.2014.02.052

Johansson, I., Witkowska, E., Kaveh, B., Lif Holgerson, P., and Tanner, A. C. R. (2015). The microbiome in populations with a low and high prevalence of caries. *J. Dent. Res.* 95, 80–86. doi: 10.1177/0022034515609554

Kanther, M., and Rawls, J. F. (2010). Host-microbe interactions in the developing zebrafish. *Curr. Opin. Immunol.* 22, 10–19. doi: 10.1016/j.coi.2010.01.006

Karlsson, F., Tremaroli, V., Nielsen, J., and Bäckhed, F. (2013). Assessing the human gut microbiota in metabolic diseases. *Diabetes Metab. Res. Rev.* 62, 3341–3349. doi: 10.2337/db13-0844

Knights, D., Silverberg, M. S., Weersma, R. K., Gevers, D., Dijkstra, G., Huang, H. H., et al. (2014). Complex host genetics influence the microbiome in inflammatory bowel disease. *Genome Med.* 6:107. doi: 10.1186/s13073-014-0107-1

Kostic, A. D., Howitt, M. R., and Garrett, W. S. (2013). Exploring host-microbiota interactions in animal models and humans. *Genes Dev.* 27, 701–718. doi: 10.1101/gad.212522.112

Kumar, P. S., and Mason, M. R. (2015). Mouthguards: does the indigenous microbiome play a role in maintaining oral health? *Front. Cell. Infect. Microbiol.* 5:35. doi: 10.3389/fcimb.2015.00035

Kuraishi, T., Hori, A., and Kurata, S. (2013). Host-microbe interactions in the gut of *Drosophila melanogaster*. *Front. Physiol.* 4:375. doi: 10.3389/fphys.2013.00375

Langille, M. G., Meehan, C. J., Koenig, J. E., Dhanani, A. S., Rose, R. A., Howlett, S. E., et al. (2014). Microbial shifts in the aging mouse gut. *Microbiome* 2:50. doi: 10.1186/s40168-014-0050-9

Linderholm, A. (2015). Ancient DNA: the next generation - chapter and verse. *Biol. J. Linn. Soc.* 117, 150–160. doi: 10.1111/bij.12616

Logan, A. C., Jacka, F. N., and Prescott, S. L. (2016). Immune-microbiota interactions: dysbiosis as a global health issue. *Curr. Allergy Asthma Rep.* 16:13. doi: 10.1007/s11882-015-0590-5

Maixner, F., Krause-Kyora, B., Turaev, D., Herbig, A., Hoopmann, M. R., Hallows, J. L., et al. (2016). The 5300-year-old *Helicobacter pylori* genome of the Iceman. *Science* 351, 162–165. doi: 10.1126/science.aad2545

Marchesi, J. R., and Ravel, J. (2015). The vocabulary of microbiome research: a proposal. *Microbiome* 3:31. doi: 10.1186/s40168-015-0094-5

Markle, J. G. M., Frank, D. N., Mortin-Toth, S., Robertson, C. E., Feazel, L. M., Rolle-Kampczyk, U., et al. (2013). Sex differences in the gut microbiome drive hormone-dependent regulation of autoimmunity. *Science* 339, 1084–1088. doi: 10.1126/science.1233521

Mathieson, I., Lazaridis, I., Rohland, N., Mallick, S., Patterson, N., Roodenberg, S. A., et al. (2015). Genome-wide patterns of selection in 230 ancient Eurasians. *Nature* 528, 499–503. doi: 10.1038/nature16152

McKnite, A. M., Perez-Munoz, M. E., Lu, L., Williams, E. G., Brewer, S., Andreux, P. A., et al. (2012). Murine gut microbiota is defined by host genetics and modulates variation of metabolic traits. *PLoS ONE* 7:e39191. doi: 10.1371/journal.pone.0039191

Metcalf, J. L., Ursell, L. K., and Knight, R. (2014). Ancient human oral plaque preserves a wealth of biological data. *Nat. Genet.* 46, 321–323. doi: 10.1038/ng.2930

Meyer, M., Fu, Q., Aximu-Petri, A., Glocke, I., Nickel, B., Arsuaga, J.-L., et al. (2014). A mitochondrial genome sequence of a hominin from Sima de los Huesos. *Nature* 505, 403–406. doi: 10.1038/nature12788

Meyer, M., Kircher, M., Gansauge, M.-T., Li, H., Racimo, F., Mallick, S., et al. (2012). A high-coverage genome sequence from an archaic Denisovan individual. *Science* 338, 222–226. doi: 10.1126/science.1224344

Milligan-Myhre, K., Charette, J. R., Phennicie, R. T., Stephens, W. Z., Rawls, J. F., Guillemin, K., et al. (2011). Study of host-microbe interactions in zebrafish. *Methods Cell Biol.* 105, 87–116. doi: 10.1016/B978-0-12-381320-6.00004-7

Moaayeri, A., Hammond, C. J., Hart, D. J., and Spector, T. D. (2012). The UK adult twin registry (twins UK resource). *Twin Res. Hum. Genet.* 16, 144–149. doi: 10.1017/thg.2012.89

Moeller, A. H., Caro-Quintero, A., Mjungu, D., Georgiev, A. V., Lonsdorf, E. V., Muller, M. N., et al. (2016). Cospeciation of gut microbiota with hominids. *Science* 353, 380–382. doi: 10.1126/science.aaf3951

Moeller, A. H., Li, Y., Mpoudi Ngole, E., Ahuka-Mundeke, S., Lonsdorf, E. V., Pusey, A. E., et al. (2014). Rapid changes in the gut microbiome during human evolution. *Proc. Natl. Acad. Sci. U.S.A.* 111, 16431–16435. doi: 10.1073/pnas.1419130111

Parekh, P. J., Balart, L. A., and Johnson, D. A. (2015). The influence of the gut microbiome on obesity, metabolic syndrome and gastrointestinal disease. *Clin. Transl. Gastroenterol.* 6:e91. doi: 10.1038/ctg.2015.16

Pinhasi, R., Fernandes, D., Sirak, K., Novak, M., Connell, S., Alpaslan-Roodenberg, S., et al. (2015). Optimal ancient DNA yields from the inner ear part of the human petrous bone. *PLoS ONE* 10:e0129102. doi: 10.1371/journal.pone.0129102

Poretsky, R., Rodriguez-R, L. M., Luo, C., Tsementzi, D., and Konstantinidis, K. T. (2014). Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics. *PLoS ONE* 9:e93827. doi: 10.1371/journal.pone.0093827

Prüfer, K., Racimo, F., Patterson, N., Jay, F., Sankararaman, S., Sawyer, S., et al. (2014). The complete genome sequence of a Neanderthal from the Altai mountains. *Nature* 505, 43–49. doi: 10.1038/nature12886

Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M. A. R., Bender, D., et al. (2007). PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* 81, 559–575. doi: 10.1086/519795

Rao, A. T., Degnan, A. J., and Levy, L. M. (2014). Genetics of Alzheimer disease. *AJNR Am. J. Neuroradiol.* 35, 457–458. doi: 10.3174/ajnr.A3545

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Rawls, J. F., Mahowald, M. A., Ley, R. E., and Gordon, J. I. (2006). Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. *Cell* 127, 423–433. doi: 10.1016/j.cell.2006.08.043

Rhee, S. H., Pothoulakis, C., and Mayer, E. A. (2009). Principles and clinical implications of the brain-gut-enteric microbiota axis. *Nat. Rev. Gastroenterol. Hepatol.* 6, 306–314. doi: 10.1038/nrgastro.2009.35

Rodríguez, J. M., Murphy, K., Stanton, C., Ross, R. P., Kober, O. I., Juge, N., et al. (2015). The composition of the gut microbiota throughout life, with an emphasis on early life. *Microb. Ecol. Health Dis.* 26:26050. doi: 10.3402/mehd.v26.26050

Schnorr, S. L. (2015). The diverse microbiome of the hunter-gatherer. *Nature* 518, S14–S15. doi: 10.1038/518S14a

Schnorr, S. L., Candela, M., Rampelli, S., Centanni, M., Consolandi, C., Basaglia, G., et al. (2014). Gut microbiome of the Hadza hunter-gatherers. *Nat. Commun.* 5:3654. doi: 10.1038/ncomms4654

Seok, J., Warren, H. S., Cuenca, A. G., Mindrinos, M. N., Baker, H. V., Xu, W., et al. (2013). Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc. Natl. Acad. Sci. U.S.A.* 110, 3507–3512. doi: 10.1073/pnas.1222878110

Sharon, G., Segal, D., Ringo, J. M., Hefetz, A., Zilber-Rosenberg, I., and Rosenberg, E. (2010). Commensal bacteria play a role in mating preference of Drosophila melanogaster. *Proc. Natl. Acad. Sci. U.S.A.* 107, 20051–20056. doi: 10.1073/pnas.1009906107

Skoglund, P., Mallick, S., Bortolini, M. C., Chennagiri, N., Hünemeier, T., Petzl-Erler, M. L., et al. (2015). Genetic evidence for two founding populations of the Americas. *Nature* 525, 104–108. doi: 10.1038/nature14895

Spor, A., Koren, O., and Ley, R. (2011). Unravelling the effects of the environment and host genotype on the gut microbiome. *Nat. Rev. Microbiol.* 9, 279–290. doi: 10.1038/nrmicro2540

Srinivas, G., Möller, S., Wang, J., Künzel, S., Zillikens, D., Baines, J. F., et al. (2013). Genome-wide mapping of gene–microbiota interactions in susceptibility to autoimmune skin blistering. *Nat. Commun.* 4:2462. doi: 10.1038/ncomms3462

The Human Microbiome Project (2012). Structure, function and diversity of the healthy human microbiome. *Nature* 486, 207–214. doi: 10.1038/nature11234

Thomas, R. H., Meeking, M. M., Mepham, J. R., Tichenoff, L., Possmayer, F., Liu, S., et al. (2012). The enteric bacterial metabolite propionic acid alters brain and plasma phospholipid molecular species: further development of a rodent model of autism spectrum disorders. *J. Neuroinflamm.* 9:153. doi: 10.1186/1742-2094-9-153

Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Ley, R. E., Sogin, M. L., et al. (2009). A core gut microbiom in obese and lean twins. *Nature* 457, 480–484. doi: 10.1038/nature07540.A

van Dijk, E. L., Auger, H., Jaszczyszyn, Y., and Thermes, C. (2014). Ten years of next-generation sequencing technology. *Trends Genet.* 30, 418–426. doi: 10.1016/j.tig.2014.07.001

Visscher, P. M., Brown, M. A., McCarthy, M. I., and Yang, J. (2012). Five years of GWAS discovery. *Am. J. Hum. Genet.* 90, 7–24. doi: 10.1016/j.ajhg.2011.11.029

Wang, J., and Jia, H. (2016). Metagenome-wide association studies: fine-mining the microbiome. *Nat. Rev. Microbiol.* 14, 508–522. doi: 10.1038/nrmicro.2016.83

Warinner, C., Rodrigues, J. F. M., Vyas, R., Trachsel, C., Shved, N., Grossmann, J., et al. (2014). Pathogens and host immunity in the ancient human oral cavity. *Nat. Genet.* 46, 336–344. doi: 10.1038/ng.2906

Warinner, C., Speller, C., Collins, M. J., and Lewis, C. M. (2015). Ancient human microbiomes. *J. Hum. Evol.* 79, 125–136. doi: 10.1016/j.jhevol.2014.10.016

Whittaker, R. H. (1972). Evolution and measurement of species diversity. *Taxon* 21, 213–251. doi: 10.2307/1218190

Willerslev, E., and Cooper, A. (2005). Ancient DNA. *Proc. R. Soc.* 272, 3–16. doi: 10.1098/rspb.2004.2813

Woese, C. R., Kandler, O., and Wheelis, M. L. (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc. Natl. Acad. Sci. U.S.A.* 87, 4576–4579. doi: 10.1073/pnas.87.12.4576

Xu, L., Paterson, A. D., Turpin, W., and Xu, W. (2015). Assessment and selection of competing models for zero-inflated microbiome data. *PLoS ONE* 10:e0129606. doi: 10.1371/journal.pone.0129606

Yatsunenko, T., Rey, F. E., Manary, M. J. M. M. J., Trehan, I., Dominguez-Bello, M. G., Contreras, M., et al. (2012). Human gut microbiome viewed across age and geography. *Nature* 486, 222–227. doi: 10.1038/nature11053

Zapata, H. J., and Quagliarello, V. J. (2015). The microbiota and microbiome in aging: potential implications in health and age-related diseases. *J. Am. Geriatr. Soc.* 63, 776–781. doi: 10.1111/jgs.13310

Zhao, N., Chen, J., Carroll, I. M., Ringel-Kulka, T., Epstein, M. P., Zhou, H., et al. (2015). Testing in microbiome-profiling studies with MiRKAT, the microbiome regression-based kernel association test. *Am. J. Hum. Genet.* 96, 797–807. doi: 10.1016/j.ajhg.2015.04.003

Zhernakova, A., Kurilshikov, A., Bonder, M. J., Tigchelaar, E. F., Schirmer, M., Vatanen, T., et al. (2016). Population-based metagenomics analysis reveals markers for gut microbiome composition and diversity. *Science* 352, 565–569. doi: 10.1126/science.aad3369

Zhou, X., and Stephens, M. (2012). Genome-wide efficient mixed-model analysis for association studies. *Nat. Genet.* 44, 821–824. doi: 10.1038/ng.2310

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"In truth, the laboratory is the forecourt of the temple of philosophy, and whoso has not offered sacrifices and undergone purification there has little chance of admission into the sanctuary" - Thomas Huxley, English biologist

Cyril Bibby (1971). "T. H. Huxley on Education", p.166, Cambridge University Press

Chapter II

Ancient DNA Extraction and Library Preparation Methodology Impacts on Ancient Microbiome within Dental Calculus of Varying Preservation States

Statement of Authorship

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Name of Principal Author (Candidate)	Muslihudeen Abdul-Aziz
Contribution to the Paper	Designed the experiment. Performed all the laboratory work, data analysis and interpretation of results. Wrote the manuscript.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 29/11/18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:
  i.   the candidate's stated contribution to the publication is accurate (as detailed above);
  ii.   permission is granted for the candidate to include the publication in the thesis; and
  iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Alan Cooper
Contribution to the Paper	Provided funding. Assisted with the collection of samples. Discussed and edited the manuscript.
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Contribution to the Paper	Provided funding, Co-designed the experiments. Advised on data analysis and interpretation of results, and discussed and edited the manuscript.

Signature		Date	21/11/18

Ancient DNA Extraction and Library Preparation Methodology Impacts on Ancient Microbiome within Dental Calculus of Varying Preservation States

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Abstract

The ability to obtain ancient DNA from dental calculus has allowed us to peer into the evolutionary past of microbial communities. The advent of new extraction and library preparation methods coupled with next generation sequencing has led to the reconstruction of a Neanderthal oral microbiome that was ~48,000 years old. However, there has been little exploration on the impact of these extraction and library preparation methods on microbiome composition obtained from dental calculus, as these methods were initially designed for extraction of ancient DNA from a single species using ancient teeth and bone. Here, we compare widely used and recently published extraction and library preparation methods on dental calculus samples of varying preservation states. We find that we obtain shorter, more damaged DNA fragments with lower GC content using a single stranded library preparation method. We also find that microbial composition is not influenced by extraction or library preparation methods in well preserved samples. However, we find that methodology does impact composition in poorly preserved samples, as the single stranded library preparation method drastically increases the ability to reconstruct oral microbial taxa, such as *Methanobrevibacter*, *Streptococcus*, *Olsenella* and *Fretibacterium* from poorly preserved samples. This research will improve future work examining ancient microbiomes from global locations where ancient DNA preservation is poor, thereby improving the understanding of the global diversity of the ancient human oral microbiomes.

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Introduction

From the sequencing of the genomes of Neanderthals and Denisovans to the unveiling of the oral microbiomes of pre-historic populations, the last two decades have heralded a revolution in our understanding of evolutionary processes that underpin human health and disease (Hagelberg, Hofreiter, and Keyser 2015). These new discoveries have been made possible by recent methodological advancements in efficiently extracting ancient DNA, *i.e.* methods that improve the visibility of short damaged and fragmented DNA from ancient specimens, whilst reducing non-endogenous DNA contamination. These advancements coupled with next generation sequencing technologies have created a new and exciting era in the field of paleogenomics.

New sources of ancient DNA, such as calcified dental plaque (calculus), were found to have large amounts of ancient DNA (Warinner et al. 2014) and are becoming increasingly utilised in paleogenomics to explore ancient microbial communities (microbiomes). Dental calculus is a mineralised bacterial biofilm that forms on the surface of teeth with the involvement of both saliva and gingival cervical fluid (Conroy and Sturzenberger 1968). Dental calculus accumulates throughout the life of an individual and stays relatively unperturbed following death, allowing access to the oral microbiome composition of ancient individuals throughout their lives (Warinner et al. 2014; Weyrich, Dobney, and Cooper 2015). Modern research has shown that shifts in oral microbial composition result in changes in human oral and systematic health (Herrero et al. 2018), suggesting that dental calculus should be a valuable tool for reconstructing ancient human health. Therefore, robustly accessing DNA within dental calculus is critical to examine how oral microbiomes have changed through time and to identify the source of those changes, such as cultural transitions, dietary change, and environmental shifts (Shaw et al. 2017). This ability has heralded a new era in field of paleomicrobiome research. Recent ground-breaking research examining ancient DNA within dental calculus has reconstructed the oral microbiome of Neanderthals (Weyrich et al. 2017) and pre-historic human individuals (Adler et al. 2013; De La Fuente 2013; Mann et al. 2018; Warinner et al. 2014).

Many methodologies currently applied in paleomicrobiology originate from paleogenomics approaches that examine ancient DNA from a single species and

the need to be able to extract even shorter and more damaged ancient DNA from further back in time, such as in the genomic reconstructions from Neanderthal (Green et al. 2010; Prüfer et al. 2014) and Denisovan bones (Meyer et al. 2012). These ground-breaking endeavours employed an efficient library preparation method that relied on ligated specific, barcoded adaptor oligos to extracted DNA (Meyer and Kircher 2010). These methods were also designed to limit contamination, that is still a pertinent issue within ancient DNA research (Cooper and Poinar 2000). The methods, complimented with next generation sequencing technologies, allowed for quick and efficient ancient DNA sequencing (van Dijk et al. 2014). The most widely used extraction method is the method developed by Rohland and Hofreiter (2007a; (QG method)) to maximize the recovery of ancient DNA by using ethylenediaminetetraacetic acid (EDTA) and proteinase K to digest the bone or tooth sample, followed by purification using a binding buffer consisting of silica and a high concentration of guanidinium thiocyanate to ensure efficient DNA release and reduce inhibition of DNA amplification. Due to its effectiveness and ease of use, this method is still widely used in the ancient DNA field and has been favourably compared to previously used extraction methods in the ancient DNA research field (Rohland and Hofreiter 2007b). This extraction method was further improved upon by Dabney et al. (2013; (PB method)). The PB method allows access to even shorter fragment sizes (<50 bp) to be routinely obtained by altering the DNA binding buffer by adding sodium acetate and isopropanol and replacing the guanidinium thiocyanate with guanidine hydrochloride. The method also included an increase in the ratio of the volume of binding buffer to that of the extraction buffer and the use of silica spin columns instead of silica suspensions used in the QG method. While the PB method allowed access to shorter fragments following the extraction step, most of these shorter fragments were previously lost when the fragments were prepared for double-stranded library preparation due to the size of the primers required.

The library preparation step is also crucial to access ancient DNA after DNA extraction. The most widely used library preparation method in ancient DNA research was developed by Meyer and Kircher (2010), which is also known as the double stranded library method (DSL). The DSL method is a fast and reliable method that allowed for the efficient and high throughput library preparation of

double stranded ancient DNA fragments even at low levels and it is the most commonly used method. However, a large number of ancient DNA fragments are older, more degraded, and are present in a single strand configuration, and the DSL method was unable to access and tag them. This lead to the development of the single stranded library (SSL) preparation method (Gansauge and Meyer 2013). This method allowed access to single stranded DNA molecules by ligating a biotinylated adapter oligonucleotide to heat denatured DNA, capturing both single and double stranded DNA molecules and substantially increasing the amount of DNA available for analysis. While the initial SSL method was expensive, an updated method developed by Gansauge et al. (2017) is more cost effective and produces equivalent results.

Several studies have assessed how different extraction and library preparation methods can influence the construction of ancient DNA data sets. Dabney et al. (2013) compared the QG extraction method (Rohland and Hofreiter 2007a) against the PB extraction method (Dabney et al. 2013), coupled with a single stranded library method (Gansauge and Meyer 2013). Their results using ancient cave bear bones showed that shorter DNA fragments were obtained with the PB extraction method coupled with SSL. Gamba et al. (2016) compared the two extraction methods (QG and PB methods) using only the DSL library method on ancient equine bone samples. They reported shorter fragment sizes with the PB method coupled with the DSL method and found that QG method relatively increased the clonality (duplication rate/rate of non-unique fragments) of DNA fragments. Barlow et al. (2016) did a comprehensive comparison of the two extraction methods, QG and PB, coupled with two library preparation methods (DSL & SSL (Gansauge and Meyer 2013)) on ancient brown bear bones and also showed that PB method coupled with the SSL resulted in an increase in shorter DNA fragments. Barlow et al. (2016) also showed that while extraction methods had no impact on GC content, the library preparation method had a significant impact on samples prepared with the DSL method, as they had higher GC content compared to samples prepared with the SSL method (Gansauge and Meyer 2013). Together, these studies have shown that the use of the PB extraction method alongside the SSL method allows ancient DNA researchers to access and increase shorter DNA fragments that are likely to be ancient.

However, there has been little research on the suitability of current laboratory methods on accurately retrieving oral microbiomes, especially within samples from different geographic regions and in various states of preservation, *e.g.* outside cooler European climes that support good sample preservation. In dental calculus research, there has been little exploration on the impact of the extraction and library preparation methods on microbial community structure, as well as Guanine-Cytosine (GC) content and clonality (duplication rate/rate of non-unique fragments) across numerous species within a single sample. GC content and clonality are both crucial to ensuring the uniqueness and microbial diversity of the resulting DNA sequences. Since dental calculus is formed of microbial biofilm community consisting of various microbial species with varying taphonomy (preservation states) (Adler et al. 2013; Mann et al. 2018), GC content (Jesse Dabney and Meyer 2012; Papudeshi et al. 2017; Quail et al. 2012), and abundances, it is quite possible that the process of extraction and library preparation might impact their accurate reconstruction. Here, we explore the impact of two extraction methods (QG method and PB method) with two library preparation techniques (DSL and SSL (Gansauge, Gerber, Glocke, Korleví, et al. 2017) on the reconstruction of ancient oral microbiomes from well and poorly preserved dental calculus samples.

Material and Methods

Ancient dental calculus sample collection

We obtained ethics approval for this study from the University of Adelaide Human Research Ethics Committee (H-2012-108). Ancient dental calculus samples (total n = 6) were collected from Hungary (n = 3) and Niger (n = 3). Both samples were of similar age (7,000 ± BP). The samples from Hungary were well preserved (W), as mitochondrial DNA was previously successfully extracted from the tooth samples of the same individuals (Haak et al. 2015). However, the samples from Niger were poorly preserved (P) with multiple previous unsuccessful attempts at extracting ancient DNA from bone and tooth material (Table 1; Fig. 1; Fig.S1). Supragingival dental calculus deposits were dislodged from the surface of tooth samples using a sterile dental pick. Gentle pressure was applied in parallel to the tooth surface in order to avoid enamel damage as previously described (Weyrich,

Dobney, and Cooper 2015). Collected fragments were then stored in sterile sealed zip bags for transportation to the ancient DNA facility at the Australian Centre for Ancient DNA (ACAD), University of Adelaide, Australia. Sample metadata was also collected at this stage (Table S1).

Table 1. Samples used in the analysis, their preservation state, origin and sample age.

SampleID	Sample	Location	Site	Age (yBP)
18393	Poorly Preserved 1	Niger	Gobero	7200-4200
18398	Poorly Preserved 2	Niger	Gobero	7200-4200
18400	Poorly Preserved 3	Niger	Gobero	7200-4200
18416	Well Preserved 1	Hungary	Balatonszarszo	7500-7000
18421	Well Preserved 2	Hungary	Alsonyek	6900-6400
18427	Well Preserved 3	Hungary	Alsonyek	6900-6400

Decontamination Protocols

We performed all sample processing and laboratory procedures prior to PCR amplification at the specialized ancient DNA facility at the University of Adelaide. The facility was designed to allow ancient DNA research to be performed in a low contamination environment by using positive air pressure in the general facility, regularly cleaned with 3% sodium hypochlorite (Bleach) and irradiated with ultraviolet light each night. To further limit the introduction of modern contaminant DNA, all experiments were performed within ultraviolet light-treated, still-air hoods located in isolated, still-air rooms. All personnel accessed the facility using a dedicated single access room and wore disposable full body suits, gloves and face masks.
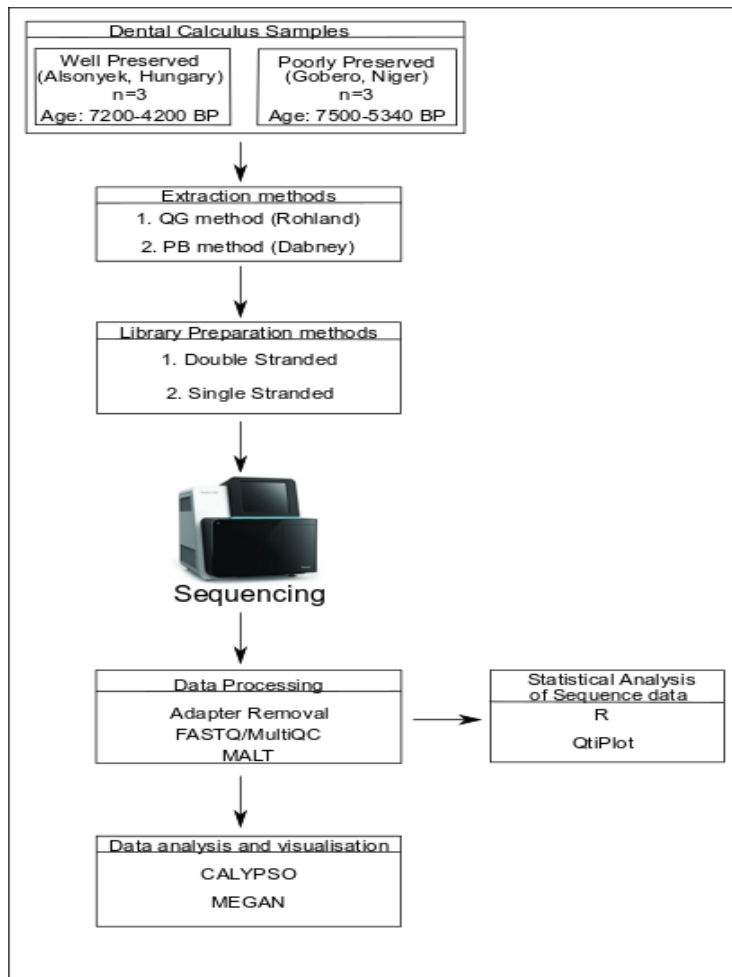
Figure 1. Experimental design for the comparison of two DNA extraction methods and two library preparation methods on well and poorly preserved ancient dental calculus samples from Hungary and Niger.

Sample decontamination, DNA extraction, library preparation and sequencing

In order to minimize environmental contamination, we first decontaminated the dental calculus samples using Ultraviolet (UV) irradiation for 15 minutes on each side, followed by soaking in two mL of 5% sodium hypochlorite for three minutes, rinsing in 90% ethanol for a minute, and drying at room temperature for two minutes. Samples were then immediately placed into a sterile plastic tubes and crushed into powder on the side of the tube with sterile tweezers.

We then performed two different extractions (QG and PB) on the same sample. The QG method was done using an in-house adapted strategy, as previously described (SI Text 1) (Brotherton et al. 2013), but with decreased buffer volumes (1.72 ml lysis (extraction) buffer (1.6 ml EDTA; 100 µl SDS; 20 µl 20 mg ml^{-1}

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proteinase K) and 3 ml of guanidine DNA-binding buffer (QG buffer from Qiagen (Germany)) (Weyrich et al. 2017). The PB method (SI Text 2) was modified based on the DNA extraction method developed by Dabney et al. (2013), where sodium acetate (420 μl) and isopropanol (30%) were added and guanidinium thiocyanate in the QG method was replaced with guanidine hydrochloride in the binding buffer (PB buffer from Qiagen (Germany)). The method also includes an increase in the ratio of the volume of binding buffer (14 ml of guanidine DNA-binding buffer) to that of the lysis buffer (1 ml lysis buffer (900 μl EDTA; 80 μl ddH$_2$0; 20 μl 20 mg ml^{-1} proteinase K) and further modified with the use of silica suspension instead of silica columns. We produced two DNA extracts for each sample, reflecting both the QG and PB extraction methods.

For each extraction method, we introduced two negative control samples that were processed alongside the samples and sequenced. For environmental controls, we also separately extracted and prepared libraries for soil samples obtained from the archaeological sites from where the Hungarian samples were collected.

Both extracts from either extraction method were then used to create two (DSL and SSL) libraries. First, we applied the DSL (SI Text 3), as previously described (Meyer and Kircher 2010) and used in (Weyrich et al. 2017), and included unique combinations of 7 bp forward and reverse barcodes. Libraries were amplified using 13 cycles of PCR amplification were employed for the first amplification step with P5/P7 barcoded adapters, followed by an additional 13 cycles for the addition of a GAII-index and sequencing primers. The second library method was the SSL method (SI Text 4), based on the modified protocol previously described by (Gansauge, Gerber, Glocke, Korleví, et al. 2017) with minor modifications, such as the use of a silica suspension instead of silica columns and smaller volumes for the smaller dental calculus samples compared to those used previously for bone and tooth material (Gansauge, Gerber, Glocke, Korleví, et al. 2017). 13 cycles of PCR amplification were completed using unique combinations of the GAII-index and sequencing primers.

The constructed libraries were then purified using Ampure XP (Beckman Coulter, USA), quantified using an Agilent TapeStation (Agilent Technologies,

USA), and pooled at equimolar concentrations. Final pools were quantified using an Applied Biosystems Real Time qPCR machine. We then sequenced all the libraries on the Illumina NextSeq platform (Illumina, USA) using the 2 x 150 bp configuration. Experimental design is illustrated in Figure 1.

Bioinformatic analysis of taxonomic composition

Sequenced data was converted into FASTQ file format using the Illumina bcl2fastq (v1.8.4) software. It was then trimmed, demultiplexed and collapsed using AdapterRemoval v2 (Schubert, Lindgreen, and Orlando 2016) based on the unique P5/P7 barcoded adapters. Only collapsed reads (<300 bp) were used to limit the impacts of modern DNA contamination. The quality of the resulting sequences was analysed using FastQC, and fragment length, GC content, and clonality were further statistically analysed in QtiPlot (v.0.9.9) and R (v.3.5.1) using the paired t-test. Normality was evaluated using the Shapiro-Wilk test. Taxonomic composition was generated from sequenced data using the nucleotide alignment option in the MEGAN Alignment Tool (MALTn; v. 0.3.8) (Herbig et al. 2016). MALTn aligned DNA reads from samples against an in-house database created using 47,696 archaeal and bacterial genome assemblies from the NCBI Assembly database (Eisenhofer and Weyrich In preparation 2018). The resulting alignment based blast-text files were then converted in to RMA files using the blast2rma script included with the program MEGAN (v 6.11.1) (Huson et al. 2016) with the following last common ancestor (LCA) parameters: Weighted-LCA=80% , minimum bitscore = 42, minimum E-value=0.01, minimum support percent=0.1.

The resulting RMA files constituted microbial composition and were then analysed in MEGAN. Within MEGAN samples were assessed for ancient DNA authenticity by comparison to extraction blank controls. Ancient DNA authenticity was assessed by the estimation of cytosine deamination using MapDamage (Jónsson et al. 2013) on a bacterial species, *Anaerolineaceae oral taxon 439*, that is widely abundant in the ancient dental calculus samples. The MapDamage cytosine deamination estimation was also used to assess the level of ancient DNA damage between methods. Post-sequencing, we applied a conservative approach called subtractive filtering by filtering out microbial taxa found in laboratory controls from ancient samples. This was done as background contamination has

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been shown to impact the integrity of the microbial composition (Eisenhofer et al., 2018) and the filtration of background contamination has been shown to improve the reconstruction of microbiomes from ancient samples (Weyrich et al. 2017)

Subsequent statistical analysis was then performed by exporting species-level data into Calypso v. 8.68 (Zakrzewski et al. 2017). Specifically, filtered, species-level taxonomic composition data was exported from MEGAN as a BIOM file and then converted into Calypso V3 files using the Calypso converter. We then removed rare taxa (less than 0.01%) to reduce noise and improve statistical power, following which we then normalised the dataset using total sum normalisation (TSS) combined with square root transformation (Hellinger transformation) on the dataset. This approach normalises count data by dividing feature read counts by the total number of reads in each sample and converts raw feature counts to relative abundance. Calypso was then used to perform alpha diversity using Shannon diversity and beta diversity analysis using a Bray Curtis dissimilarity index, which was visualised on a PCoA plot. ANOVA and ANOSIM were used within Calypso to test for statistical significance in composition between groups.

Results

SSL preparation allowed access to shorter DNA fragment lengths in dental calculus samples

We first assessed the impact of extraction and library preparation methods on the fragment lengths of ancient DNA extracted from dental calculus samples, as previously examined for bone and teeth (Barlow et al. 2016; Gamba et al. 2016). Using the DSL method, we compared the impact of the two extraction methods (QG and PB) on average fragment length. In both well preserved and poorly preserved samples, we find significant differences between the PB and QG extraction method with shorter average fragments observed within the PB method (59.8 bp) compared to the QG method (108.8 bp) (Paired t-test ($p < 0.05$)). In well preserved samples, we obtained significantly shorter average fragments lengths using PB extraction method (63 bp) than in the QG method (110 bp) (Paired t-test ($p < 0.05$)). Poorly preserved samples also had generally shorter average fragment lengths using the PB extraction method (63.3 bp) compared to the QG extraction method (107 bp); however, they were not significant in contrast to differences

observed in well preserved samples (Paired t-test (p > 0.05)) (Fig. 2A; Fig S7). This suggests that preservation state may impact the accuracy of fragment length assessment, potentially due to environmental contamination.
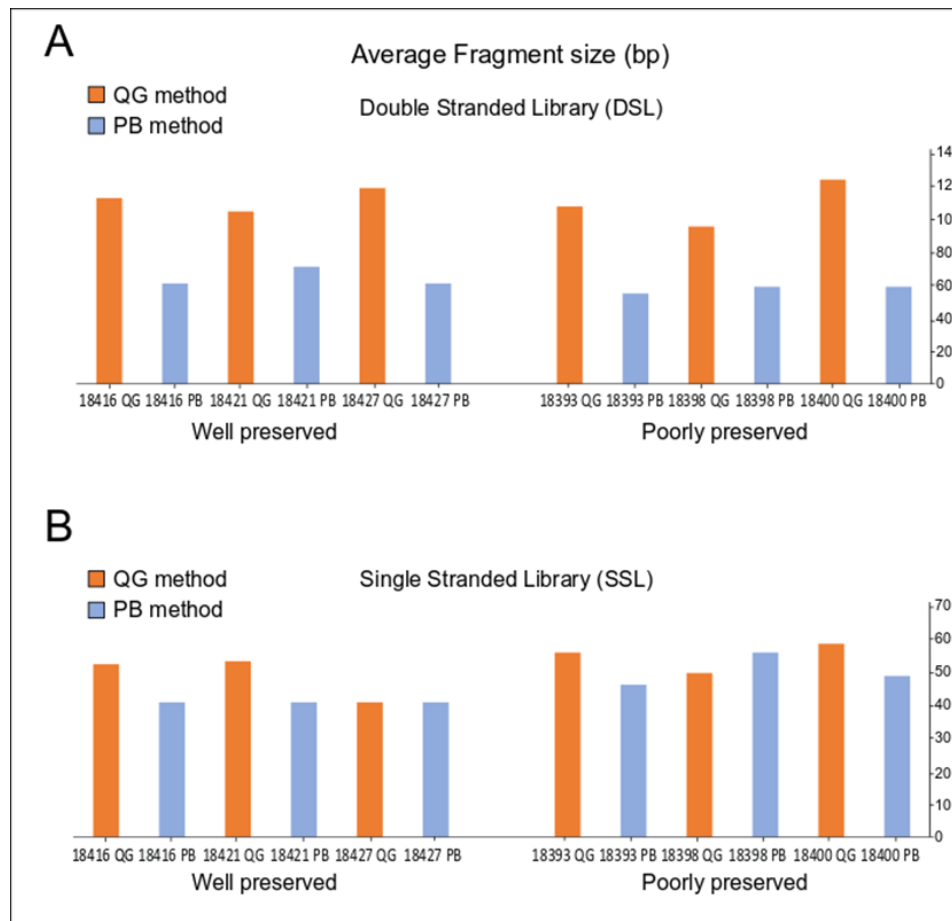
Figure 2. Bar plots showing the effect of different DNA extraction and library methods on total mean fragment length recovery from six well and poorly preserved ancient dental calculus samples. Library method is indicated above bar plots, and DNA extraction method below (paired t-test, p < 0.05).

Using the SSL preparation method, QG and PB methods recovered equivalent distributions of DNA fragment lengths when we examined all samples (QG - 116.6 bp; PB - 102.5 bp) (Paired t-test (p > 0.05). In well preserved samples, we found no significant differences in average fragment length between the two extraction methods using the SSL method (QG - 46.6 bp; PB - 55 bp); this was also

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true in poorly preserved samples (QG - 61.6 bp; PB - 56.6 bp) (Paired t-test (p > 0.05) (Fig. 2B). This result is similar to what was previously observed in bone and teeth samples and suggests that shorter DNA fragments can be obtained using a PB and DSL method over the QG and DSL method in well preserved samples.

We next assessed the impact of the two library preparation protocols, DSL and SSL, on fragment lengths irrespective of extraction method. We found that the SSL method provided significantly shorter average fragment lengths (54.8 bp) across both extraction methods compared to the DSL (84.3 bp) (Paired t-test (p < 0.05)). This was also true in both well preserved (DSL - 86.8 bp; SSL - 50.5 bp) (Paired t-test (p < 0.05)) and poorly preserved samples (DSL - 81.8 bp; SSL - 59.6 bp) (Paired t-test (p < 0.05)) (Fig. 2 & Fig. S5). Overall, our results show that the SSL library preparation method allows for access to shorter DNA fragments in ancient dental calculus samples.

SSL preparation results in reduced GC content in both well preserved and poorly preserved dental calculus samples

We then assessed the impact of extraction methods on GC content of ancient DNA extracted from dental calculus samples. Using only the DSL method, we found no significant differences in GC content when we looked at samples of both preservation states, (QG - 54.6 %; PB - 52.8 %) (Paired t-test (p > 0.05)) (Fig. 3A). As well as in only well preserved samples (QG - 52.6 %; PB - 52.6 %) (Paired t-test (p > 0.05)) and in poorly preserved samples (QG - 53%; PB - 55 %) (Paired t-test (p > 0.05)). Using the SSL method, we also found no significant differences between extraction methods on GC content in all samples (QG - 42.6 %; PB - 41.8 %) (Paired t-test (p > 0.05)) (Fig. 3B), only well preserved samples (QG - 43.6 %; PB - 42 %) (Paired t-test (p > 0.05)), or only poorly preserved samples (QG - 41.6 %; PB - 41.6 %) (Paired t-test (p > 0.05)). These results reflect similar results by Barlow et al. (2016) and suggest that the DNA extraction method has little impact on GC content.
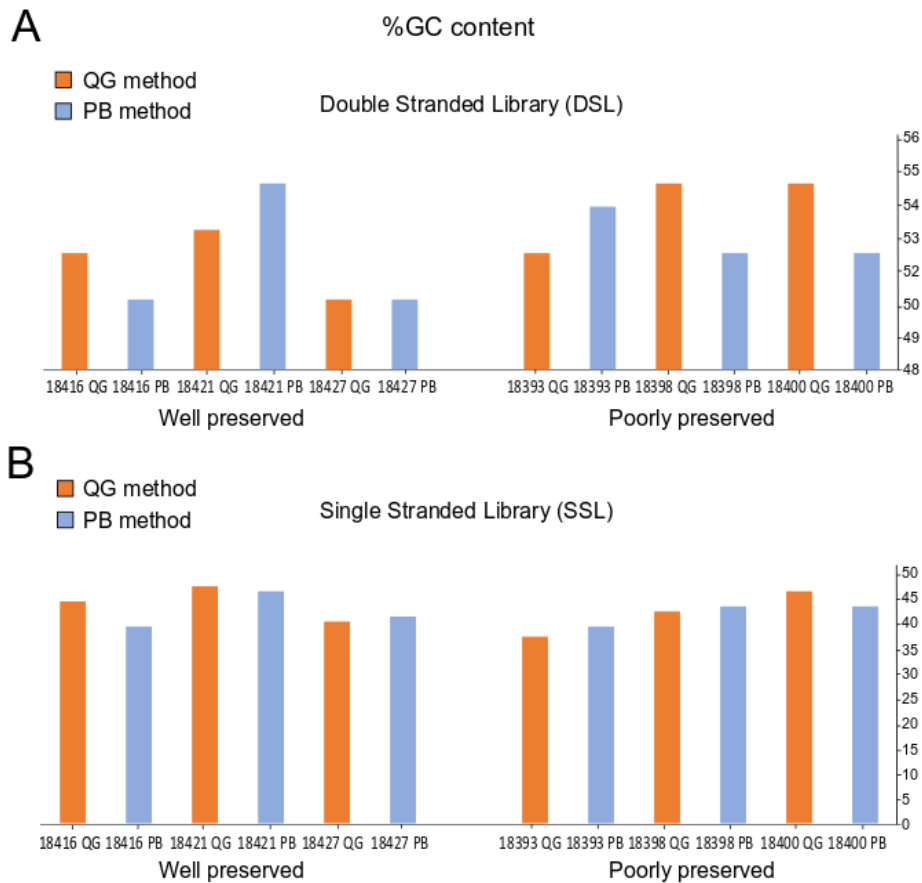
Figure 3. Bar plots showing the effect of different DNA extraction and library methods on GC content (%) of six well and poorly preserved ancient dental calculus samples. Library method is indicated above bar plots, and DNA extraction method below (paired t-test, p < 0.05).

We then examined the GC content using different library preparation methods, DSL and SSL. GC content was significantly lower when SSL was used on samples of both preservation states (DSL - 53.5%; SSL - 42.2 %) (Paired t-test (p < 0.05)) (Fig. 3). This result was also replicated when only well preserved (DSL - 52.6%; SSL - 42.8 %) and only poorly preserved (DSL - 54.3%; SSL - 41.6 %) samples were tested (Paired t-test (p < 0.05). Overall, our results show that the SSL library preparation method significantly decreases the GC content of ancient DNA from dental calculus, which is similar to results found using bone and tooth samples (Barlow et al. 2016).
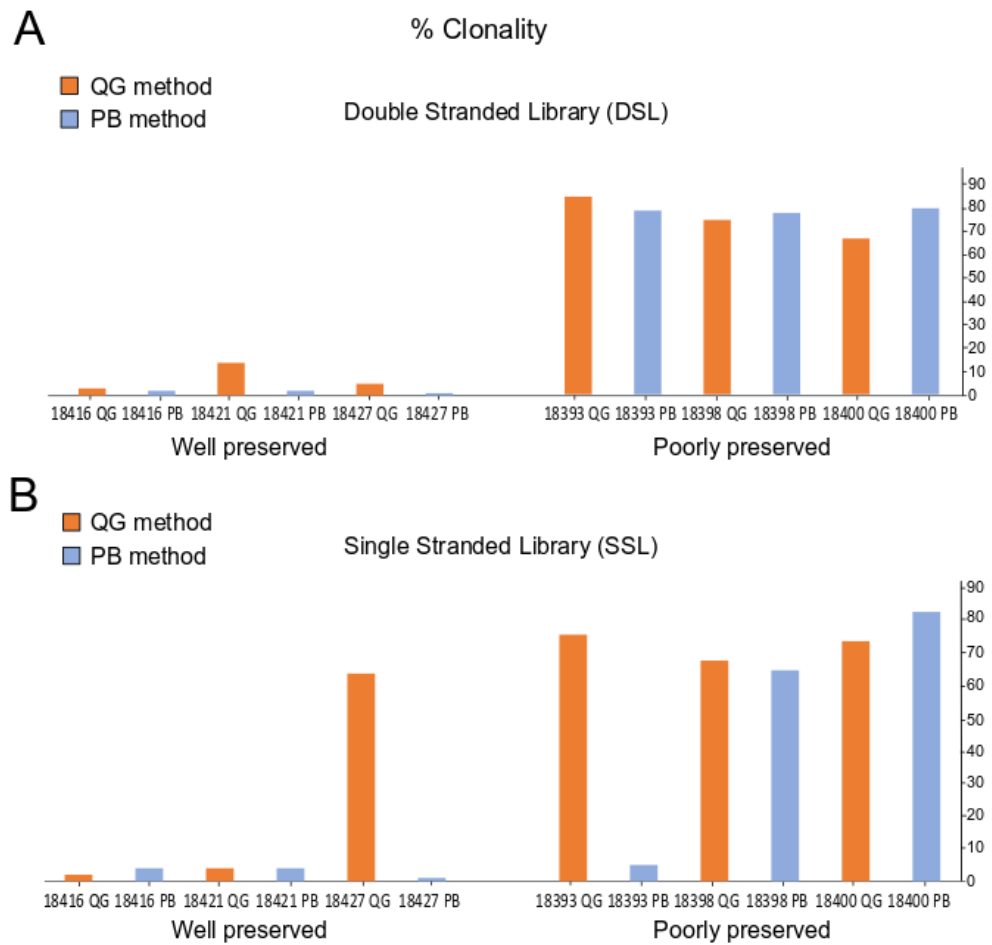
Figure 4. Bar plots showing the effect of different DNA extraction and library methods on rate of clonality/duplication of six well and poorly preserved ancient dental calculus samples. Library method is indicated above bar plots, and DNA extraction method below (paired t-test, $p < 0.05$).

Extraction and library preparation methods minimally impact clonality

The impact of the two extraction and library preparation methods on clonality was also explored. Using only the DSL method, we found slightly higher clonality when using the QG method (41.5%) compared to the PB method (40.3 %) in all samples, as previously observed, although a significant difference was not observed (Paired t-test ($p > 0.05$)). Clonality was only marginally increased in the QG vs PB extraction protocols in well preserved samples (QG - 7.3 %; PB - 1.6 %) (Paired t-test ($p > 0.05$)), and was marginally higher in PB compared to QG in poorly preserved samples (QG - 75 %; PB - 79 %) (Paired t-test ($p > 0.05$)). While poorly preserved samples had higher clonality as expected, the extraction method

did not appear to contribute to clonality in DSL prepared libraries prepared from either extraction method. Using the SSL method, we also found no significant differences between extraction methods on clonality in samples of both preservation states (QG - 48 %; PB - 27 %) (Paired t-test (p > 0.05)) (Fig. 4B). well preserved samples (QG - 23 %; PB - 3 %) (Paired t-test (p > 0.05) or poorly preserved samples (QG - 72.6 %; PB - 51 %) (Paired t-test (p > 0.05)). Together, these results suggest that extraction method has little impact on the clonality of ancient dental calculus libraries.

We then examined how library preparation methods, DSL and SSL, impact clonality. We found no significant differences between the two library preparation methods in samples of both preservation states (DSL - 40.9 %; SSL - 37.5 %) (Paired t-test (p > 0.05)) (Fig. 4), and similar results were obtained when we tested only well preserved (DSL - 4.5 %; SSL - 13.1 %), and then only poorly preserved samples (DSL - 77.3%; SSL - 61.8 %) (Paired t-test (p > 0.05)). Overall, our results show that library preparation method has minimal impacts on clonality in ancient calculus specimens.

Ancient DNA damage was enriched in samples using the SSL method

We next explored the impact of the DNA extraction methodology on the ability to obtain damaged reads through MapDamage, a program that estimates cytosine deamination that occurs on the terminal ends of ancient DNA fragments and indicates authentic ancient DNA. Using only the DSL method, we compared the impact of the two extraction methods, QG and PB methods. We find in well preserved samples that PB method had slightly higher cytosine deamination rates on average, although not significantly so (PB 22 % vs QG 15%) (Paired t-test (p > 0.05)) (Fig. S3). In poorly preserved samples, fewer than the required 1000 reads mapped to the bacterial species, *Anaerolineaceae oral taxon 439*, so we did not have results for these samples using the DSL method. Using the SSL method, we also found no significant differences between extraction methods on cytosine deamination rates in samples of both preservation states (QG - 26 %; PB - 26 %) (Paired t-test (p > 0.05) (Fig. S3 &S4), and also when we tested only well preserved samples (QG - 25 %; PB -28 %) (Paired t-test (p > 0.05) (Fig.S3) or tested only poorly preserved samples (QG - 27 %; PB - 25 %) (Paired t-test (p > 0.05)) (Fig.

S4). Overall, this suggests that DNA extraction methodology may not impact the number of sequences with deamination obtained from an ancient dental calculus extract.

We then compared DNA deamination rates in different library preparation methods, DSL and SSL. As we did not obtain terminal cytosine deamination rates for poorly preserved samples with DSL method, we were unable to compare them with the SSL method. Terminal deamination rates were significantly higher when SSL was used on well preserved samples (DSL - 18 %; SSL - 26 %) (Paired t-test ($p < 0.05$)) (Fig. S3). Overall, our results show that the SSL method significantly increases access to more damaged reads in well preserved samples, but we were unable to examine this in the DSL library preparations.

SSL method increases alpha diversity within poorly preserved dental calculus samples

We then assessed the impact of the two extraction methods on oral microbiome diversity within samples (alpha diversity). Using the DSL method, we found no significant difference between the two extraction methods when all our samples of both preservation conditions were compared together (ANOVA ($p > 0.05$)) (Fig. S2A). In well preserved samples, we also found that extraction method did not significantly impact the alpha diversity (ANOVA ($p > 0.05$)) (Fig. S2A), as was the same for poorly preserved samples (ANOVA ($p > 0.05$)) (Fig. S2A). Using the SSL method, we found no significant difference between the two extraction methods when samples of both preservation conditions were compared together (ANOVA ($p > 0.05$)) (Fig. S2A), as was the same in only well preserved samples, (ANOVA ($p > 0.05$)) (Fig. S2A), or in only poorly preserved samples (ANOVA ($p > 0.05$)) (Fig. S2A). This suggests that extraction methods have no impact on the level of microbial diversity.

We then assessed the impact of the two library preparation methods, DSL and SSL irrespective of extraction method on alpha diversity. Here too, we found no significance difference in alpha diversity between the two library preparation methods in samples of both preservation states (ANOVA ($p > 0.05$)) (Fig. S2A) and when we tested well preserved samples (ANOVA ($p > 0.05$)) (Fig. S2A). However, the SSL method significantly increased alpha diversity compared to the DSL method when we examined only poorly preserved samples (ANOVA ($p <$

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0.05)) (Fig. S2A). This suggests that the SSL method allows for a more robust reconstruction of the ancient microbiome in poorly preserved samples thereby allowing access to microbial diversity that would otherwise be lost if the DSL method was used.

SSL preparation affects beta-diversity of poorly preserved samples

We then assessed the impact of the two extraction methods on oral microbiomes diversity between samples (beta diversity). Using the DSL method, we found no significant difference in beta diversity between the two extraction methods when samples of both preservation conditions were compared together (ANOSIM ($p > 0.05$)) (Fig. S2B), or when we tested only well preserved samples, (ANOSIM ($p > 0.05$)) (Fig. S2B), or only poorly preserved samples (ANOSIM ($p > 0.05$)) (Fig. S2B). This suggests that there was no advantage in using one extraction method over the other. Using the SSL method, we also found no significant differences between the two extraction methods when all our samples of both preservation conditions were compared samples of both preservation conditions together (ANOSIM ($p > 0.05$)) (Fig. S2B), or when we tested only well preserved samples (ANOSIM ($p > 0.05$)) (Fig. S2B) and only poorly preserved samples (ANOSIM ($p > 0.05$)) (Fig. S2B). Overall this suggests that extraction methods had no impact on beta diversity irrespective of the preservation state of the sample.

We then assessed the impact of the two library preparation methods, DSL and SSL irrespective of extraction method on beta diversity. Here, when we tested all our samples together, we found that there is no significance between the two library preparation methods (ANOSIM ($p > 0.05$)) (Fig. S2B); this was also the same in well preserved samples (ANOSIM ($p > 0.05$)) (Fig. S2B). However, the SSL method had a significantly different beta diversity in poorly preserved samples, as the SSL prepared samples clustered further away from the controls compared to the DSL method (ANOSIM ($p < 0.05$)) (Fig. S2B). This suggests that the SSL method may provide for access to shorter, single stranded fragments that allow for a robust reconstruction of the ancient oral microbiome in poorly preserved samples.

Extraction method and library preparation method significantly impact the taxonomic profile of poorly preserved dental calculus samples

Following our assessment of alpha and beta diversity, we then assessed the impact of the two extraction and library preparation methods on oral microbiomes composition using taxa bar plots of the top 20 most dominant taxa. Using the DSL method, in only well preserved samples, the extraction method had little impact on microbial composition that was dominated by taxa, such as *Streptococcus, Olsenella, Methanobrevibacter* and *Fretibacterium* (ANOVA ($p > 0.05$)) (Fig. S2C). However, extraction method did significantly impact microbial composition in poorly preserved samples (ANOVA ($p < 0.05$)) (Fig. S2C). We found that the QG samples were enriched in known laboratory contaminants such as *Enterococcus, Pseudomonas* and *Acinetobacter*, while the PB samples whose were enriched in known oral taxa such *Streptococcus, Olsnella and Methanobrevibacter.* This suggests that PB method improves access to endogenous DNA in poorly preserved samples. Using the SSL method, we then explored only well preserved samples and found that extraction method had little impact on microbial composition (ANOVA ($p > 0.05$)) (Fig. S2C). Again, this was not the case with poorly preserved samples, where QG samples significantly enriched in know contaminants such as *Enterococcus* and *Pseudomonas*, while PB samples comprised of known oral microbiomes species such as *Methanobrevibacter, Olsnella* and *Fretibacterium.*

We then assessed the impact of the two library preparation methods, DSL and SSL irrespective of extraction method on microbial composition. In well preserved samples, there was no significance between the two library preparation methods (ANOVA ($p > 0.05$)) (Fig. S2C). However, the SSL method had significantly altered several taxa in poorly preserved samples by increasing the abundance of taxa that are known members of the oral microbiome such as *Streptococcus, Olsnella and Methanobrevibacter* (The Human Microbiome Project 2012), while samples processed with the DSL method were enriched in known laboratory contaminant taxa (Salter et al. 2014), such as *Enterococcus* and *Pseudomonas (*ANOVA ($p < 0.05$)) (Fig. S2C). This suggests that SSL method is best suited for the reconstruction of ancient oral microbiomes from poorly preserved samples.

Subtractive filtering improved the reconstruction of oral microbiomes from poorly preserved samples.
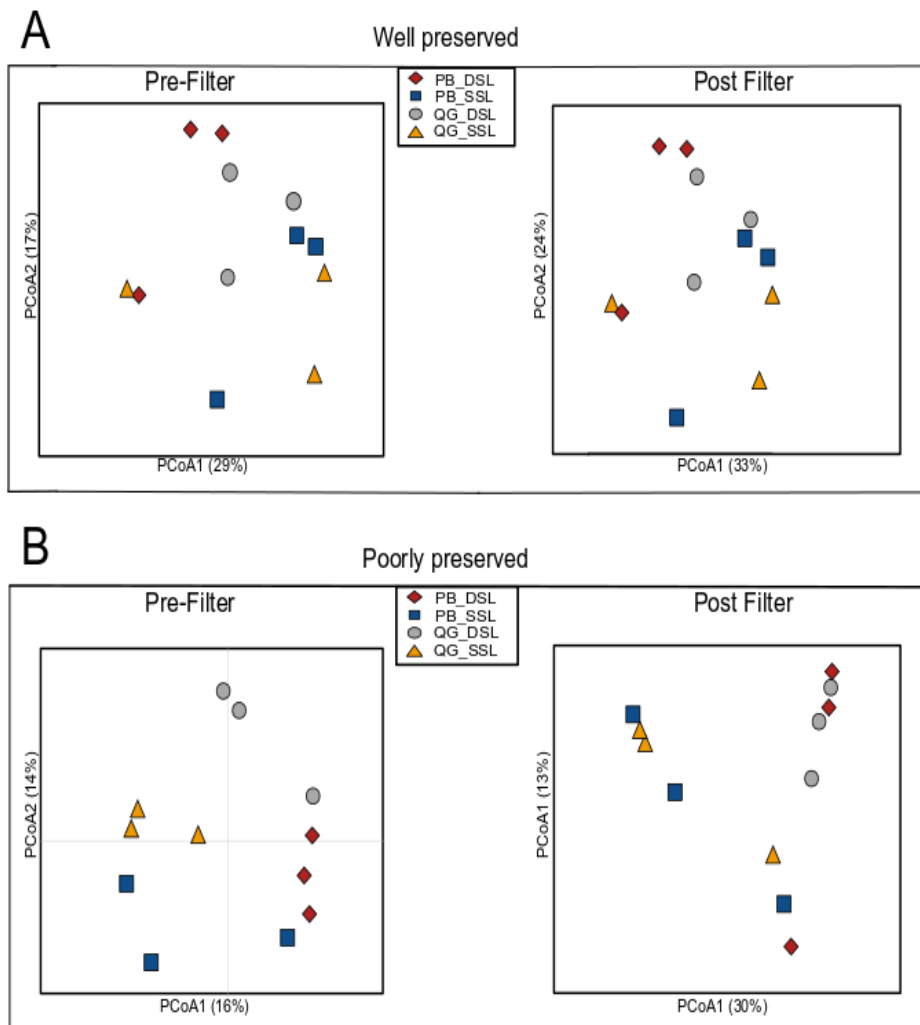
Figure 5. Microbial composition based on a PCoA plot of Bray-Curtis similarity between the two DNA extraction methods and two library preparation methods on: A. Well preserved ancient dental calculus samples, before and after subtractive filtering. B. Poorly preserved ancient dental calculus samples, before and after subtractive filtering (ANOVA; $p < 0.05$).
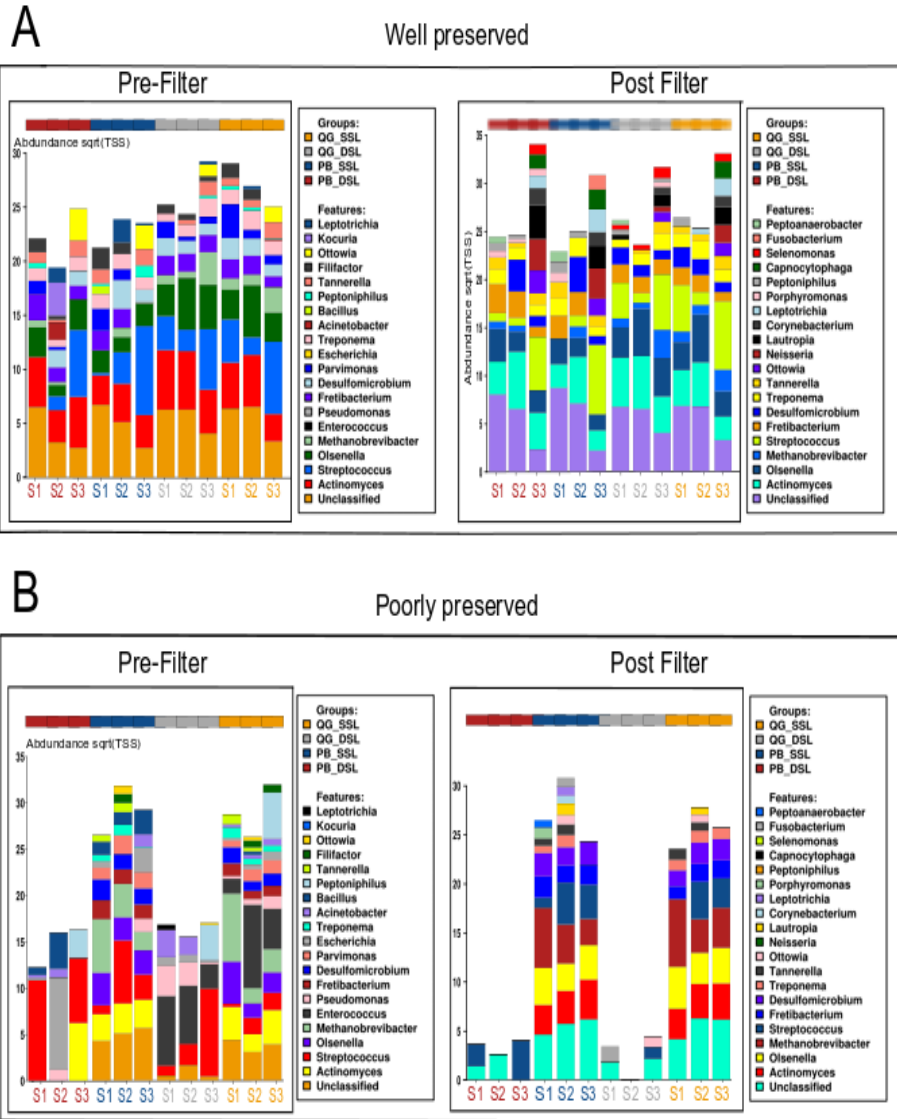
Figure 6. Taxa bar plot of top 20 genera in microbial composition between the two DNA extraction methods and two library preparation methods on: A. Well preserved ancient dental calculus samples, before and after subtractive filtering. B. Poorly preserved ancient dental calculus samples, before and after subtractive filtering.
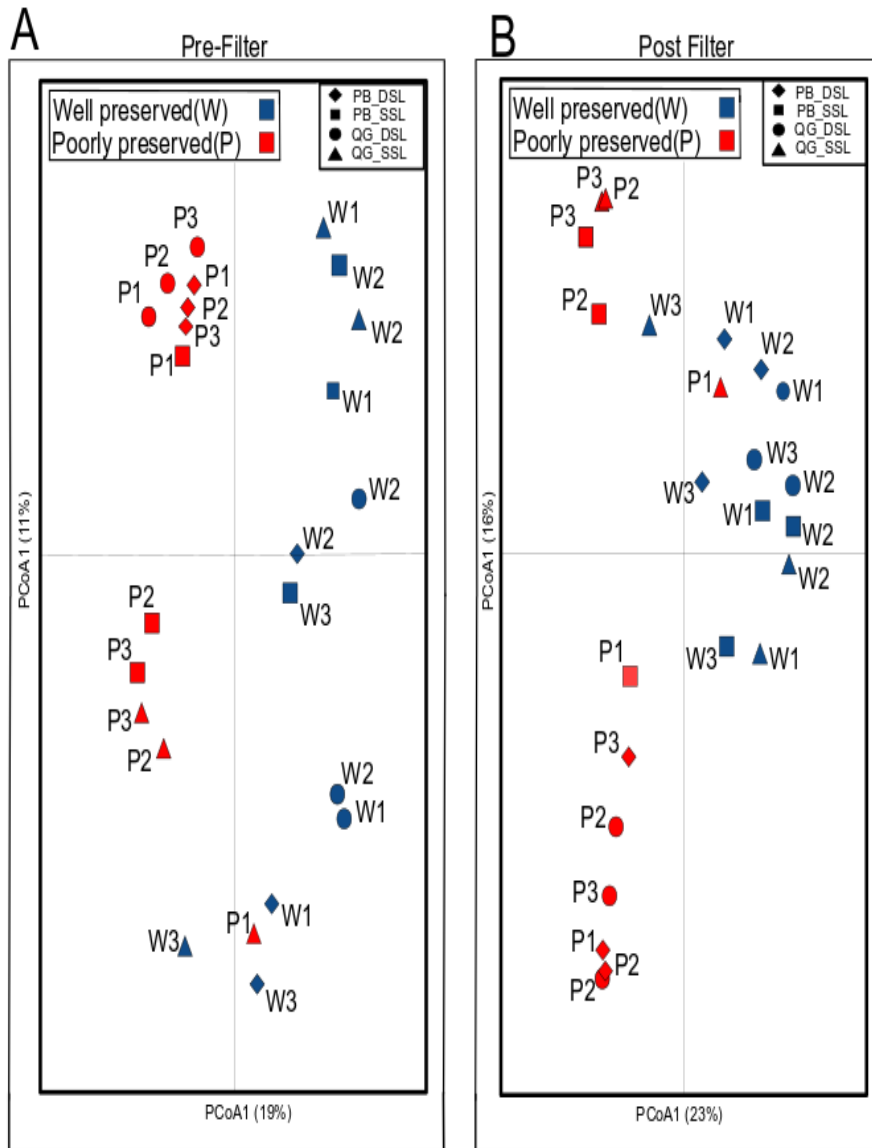
Figure 7. Microbial composition based on a PCoA plot of Bray-Curtis similarity between the two DNA extraction methods and two library preparation methods on: A. Well preserved (W) and poorly preserved (P) ancient dental calculus samples ancient dental calculus samples analyzed together before subtractive filtering. B. Well preserved (W) and poorly preserved (P) ancient dental calculus samples ancient dental calculus samples analyzed together after subtractive filtering.
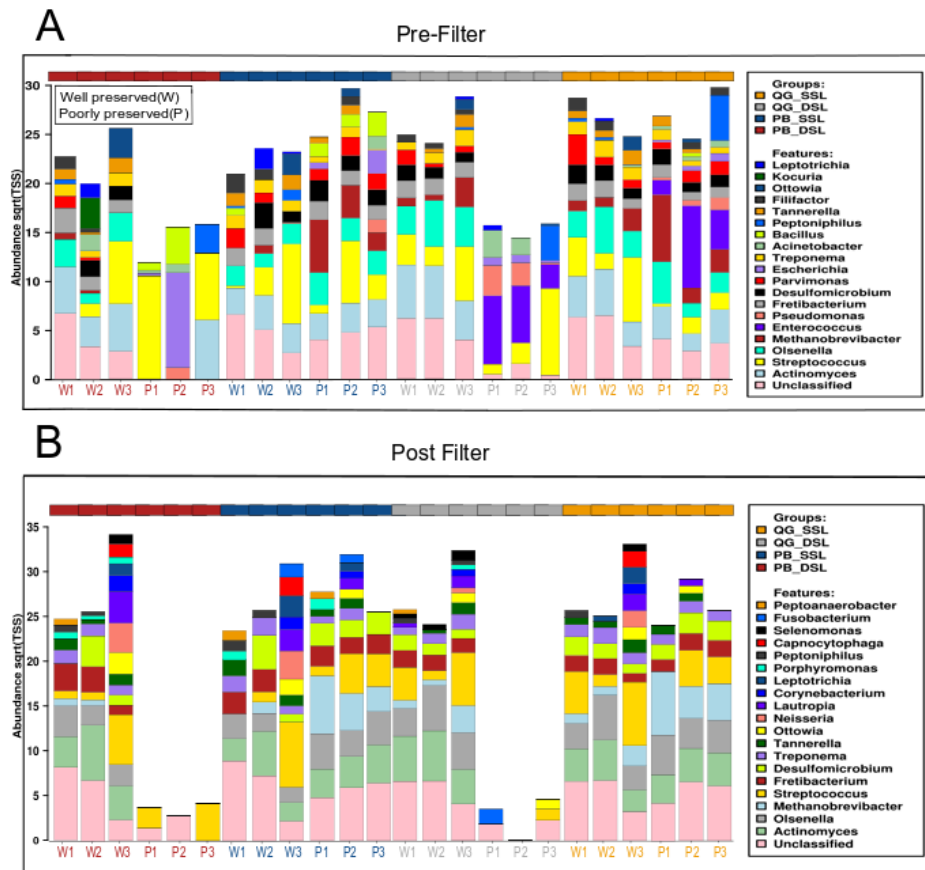
Figure 8. Taxa bar plot of top 20 genera in microbial composition between the two DNA extraction methods and two library preparation methods on: A. Well preserved (W) and poorly preserved (P) ancient dental calculus samples ancient dental calculus samples analyzed together before subtractive filtering. B Well preserved (W) and poorly preserved (P) ancient dental calculus samples ancient dental calculus samples analyzed together after subtractive filtering.

Contamination in ancient data sets is known to confound alpha and beta-diversity, as well as taxonomic analysis (Eisenhofer, Cooper, and Weyrich 2017). Therefore, we applied subtractive filtering, a conservative approach that filters microbial taxa present in laboratory controls from our samples during downstream bioinformatic analysis. We also analysed the two groups of samples with varying preservation states together and found similar results before and after performing subtractive filtering showing that samples of varying preservation states can be analysed together (Fig. 7 & Fig. 8). Subtractive filtering had a small but significant impact on the microbial composition of well-preserved samples processed using both extraction and library preparation methods (ANOSIM, ANOVA ($p < 0.05$)) (Fig. 5A & 6A). However, subtractive filtering had a larger and more significant

impact on poorly preserved samples extracted using both extraction and library preparation methods (ANOSIM, ANOVA (p < 0.05)) (Fig. 5B & 6B). In the poorly preserved samples, post subtractive filtering significantly depleted known contaminant taxa, such as *Acinetobacter*, *Bacillus, Escherichia, Enterococcus* and *Pseudomonas* (Fig. 6B). While subtractive filtering resulted in the loss of 4% of reads across all methods for well-preserved samples, this was as high as 70% in poorly preserved samples due to the high amounts of contamination in these samples, except those processed using the SSL method where only 4% of reads were filtered out. It appeared that the SSL method was able to preferentially select for shorter and more endogenous ancient DNA fragments resulting in a more robust reconstruction of the ancient oral microbiome with lower levels of laboratory contaminant taxa.

Discussion

Previous studies explored the impact of various laboratory protocols on ancient DNA extracted from ancient bone and teeth, but none have explored its impact on ancient DNA extracted from dental calculus and oral microbiome compositions. Our results confirm that shorter, more damaged DNA fragments with lower GC content are obtained using the SSL library preparation method. We find that microbial composition is minimally influenced by extraction or library preparation methodology in well preserved samples, but that SSL library preparation method significantly impacts alpha and beta diversity estimates in poorly preserved samples. The SSL also allowed access to ultrashort endogenous DNA fragments and an increase in microbial diversity once laboratory contaminants were filtered from the data set, allowing for a robust reconstruction of the ancient oral microbiomes in poorly preserved samples.

We observed that the PB extraction method resulted in an increase in shorter fragment sizes in well preserved samples, as observed by Gamba et al (2016). This is probably due to the use of guanidine hydrochloride and an increase binding buffer to extraction buffer that resulted in an increase in shorter fragments. Furthermore, we obtained even shorter fragment length distributions when SSL library preparation method was used in both poorly preserved and well preserved samples, again as observed in (Barlow et al. 2016). These shorter fragments were

also more damaged compared to fragments obtained from the DSL library preparation method. The SSL library preparation method allowed retrieval of ultra-short fragments by enabling access to shorter DNA fragments that were not accessible to the DSL method. However, the increased costs and number of steps and reagents involved have limited the ability of many researchers to use SSL approach routinely, opting for regular use of the DSL method. In this case, our results show it would be beneficial to use the PB extraction method alongside the DSL method, if the samples are well preserved. The PB method is an effective way to obtain shorter fragment sizes using the DSL method, even if a SSL method is not a feasible option. Overall, our results show that the SSL library preparation method is more effective in obtaining shorter fragments, in dental calculus as it is in bone and teeth.

We also found that GC content did not vary significantly between the two extraction methods, however, it was found to be significantly lower with SSL library preparation method, which suggests that library preparation methods have a stronger effect on GC content compared to extraction method. Furthermore, Barlow et al. (2016) previously reported that the DSL method resulted in intersample variation in GC content, while the SSL method (Gansauge and Meyer 2013) was reported to provide a more stable decrease in GC content. However, our data did not reflect any substantial intersample variation in GC content. GC content can be strongly influenced by enzymes used in the library preparation method (Dabney and Meyer 2012; Mann et al. 2018), and this may explain differences that we observed in GC content between SSL and DSL libraries. Overall, our results show that library preparation methods have an impact on the GC content of metagenomic libraries, with the SSL method resulting in a decrease in GC content. These results are similar to that found by (Barlow et al. 2016) where SSL method resulted in lower GC content in animal bones and tooth samples.

Clonality was higher in poorly preserved samples due to low amounts of DNA present in the sample as expected from degraded samples (Nieves-Colón et al. 2018). Furthermore, two rounds of amplification were performed on poorly preserved samples in order to obtain adequate DNA concentrations for sequencing compared to a single round for the well preserved samples. Beyond preservation states, we found that the methods we used had little impacts on clonality. Contrary to our results, Gamba et al. (2016) observed that clonality was higher in samples

extracted using the QG method for both preservation states irrespective of library preparation method. The discrepancy may be due to differences in the number of amplification steps performed before sequencing (Barlow et al. 2016; Weyrich et al. 2017).

We were able to reconstruct robust oral microbiomes composition from well preserved ancient samples due to the ability to access short ancient DNA fragments. However, poorly preserved samples where endogenous DNA content was low resulted in the oral microbial signal being overwhelmed by laboratory contaminants except in poorly preserved samples that underwent a SSL method. Most poorly preserved samples either overlapped or clustered with controls. This was expected as poorly preserved samples had higher contamination due to lower endogenous reads being swamped by contamination resulting microbial composition profiles that reflected that of laboratory controls. We also find that the extraction method had no impact on microbial composition. We posit that, as the PB method differs from the QG method only in the composition of the binding buffer and increased ratio between extraction and binding buffers that this may explain the lack of influence on composition. Likewise, we found that choice of library preparation method had no impact on well preserved samples but the SSL preparation method improved composition in poorly preserved samples. As ancient DNA has a higher probability to be single stranded due to damage, poorly preserved samples which have larger amounts of damaged DNA would benefit from the use of the SSL method which would allow access to these damaged single stranded fragments that would have not been accessible to the DSL library preparation method in poorly preserved samples due to extensive damage. The DSL method used on poorly preserved samples also lead to an increase as a result of the amplification of mostly double stranded contaminants.

The microbial composition of poorly preserved samples were greatly improved using subtractive filtering. Low number of endogenous DNA fragments were inundated by contaminated non-endogenous DNA fragments that were salvaged by removing the contaminated reads using laboratory controls specific to the extraction or library method used to process the poorly preserved sample. However, subtractive filtering will only work if enough endogenous DNA is legitimately present, which will be challenge with many poorly preserved samples. However, the SSL library preparation method helped improve access to

endogenous DNA, and this method, coupled with subtractive filtering, would help reconstruct ancient oral microbiomes from poorly preserved dental calculus samples.

Here, we compared various extraction and library preparation methods for their impact on the compositions of reconstructed ancient oral microbiomes. Based on our results, we propose the use of SSL library preparation method coupled with use of laboratory controls and effective subtractive filtration for the reconstruction of ancient oral composition data from poorly preserved dental calculus samples. This finding will allow paleomicrobiome research to expand the study of ancient microbiomes beyond well preserved locations in Europe to more global locations that have challenging preservation environments. This advance will therefore help further expand our understanding of the global diversity of the ancient human oral microbiome and the impact of various factors including environment, host genetics and diet on its composition.

References

1. Adler, Christina J et al. 2013. "Sequencing Ancient Calcified Dental Plaque Shows Changes in Oral Microbiota with Dietary Shifts of the Neolithic and Industrial Revolutions." *Nature genetics* 45(4): 450–5, 455e1. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3996550&tool=pmcentrez&rendertype=abstract.

2. Barlow, Axel et al. 2016. "Massive Influence of DNA Isolation and Library Preparation Approaches on Palaeogenomic Sequencing Data." *bioRxiv*: 075911. https://www.biorxiv.org/content/early/2016/09/19/075911.full.pdf+html (July 25, 2018).

3. Brotherton, Paul et al. 2013. "Neolithic Mitochondrial Haplogroup H Genomes and the Genetic Origins of Europeans." *Nature communications* 4: 1764. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3978205&tool=pmcentrez&rendertype=abstract (February 15, 2016).

4. Conroy, C W, and O P Sturzenberger. 1968. "The Rate of Calculus Formation in Adults." *Journal of Periodontology* 39(3): 142–44. https://onlinelibrary-wiley-com.proxy.library.adelaide.edu.au/doi/pdf/10.1111/prd.12151 (September 1, 2018).

5. Cooper, a, and H N Poinar. 2000. "Ancient DNA: Do It Right or Not at All." *Science (New York, N.Y.)* 289(5482): 1139.

6. Dabney, J. et al. 2013. "Complete Mitochondrial Genome Sequence of a Middle Pleistocene Cave Bear Reconstructed from Ultrashort DNA Fragments." *Proceedings of the National Academy of Sciences* 110(39): 15758–63. http://www.pnas.org/content/110/39/15758.abstract (January 10, 2016).

7. Dabney, Jesse, and Matthias Meyer. 2012. "Length and GC-Biases during Sequencing Library Amplification: A Comparison of Various Polymerase-Buffer Systems with Ancient and Modern DNA Sequencing Libraries."

BioTechniques 52(2): 87–94. https://www.future-science.com/doi/10.2144/000113809 (June 15, 2017).

8. van Dijk, Erwin L., Hélène Auger, Yan Jaszczyszyn, and Claude Thermes. 2014. "Ten Years of Next-Generation Sequencing Technology." *Trends in Genetics* 30(9): 418–26. http://www.cell.com/article/S0168952514001127/fulltext (August 7, 2014).

9. Eisenhofer R, Weyrich LS. "Assessing Alignment-Based Taxonomic Classification of Ancient Microbial DNA."

10. Eisenhofer, Raphael, Alan Cooper, and Laura S Weyrich. 2017. "Reply to Santiago-Rodriguez et Al.: Proper Authentication of Ancient DNA Is Essential." *FEMS Microbiology Ecology* 93(5). http://www.ncbi.nlm.nih.gov/pubmed/28369385 (September 3, 2018).

11. Gamba, Cristina et al. 2016. "Comparing the Performance of Three Ancient DNA Extraction Methods for High-Throughput Sequencing." *Molecular Ecology Resources* 16(2): 459–69. http://doi.wiley.com/10.1111/1755-0998.12470 (October 12, 2015).

12. Gansauge, Marie-Theres, Tobias Gerber, Isabelle Glocke, Petra Korlević, et al. 2017. "Single-Stranded DNA Library Preparation from Highly Degraded DNA Using T4 DNA Ligase." *Nucleic Acids Research* 45(10): gkx033. https://academic.oup.com/nar/article-abstract/45/10/e79/2948436 (July 25, 2018).

13. Gansauge, Marie-Theres, and Matthias Meyer. 2013. "Single-Stranded DNA Library Preparation for the Sequencing of Ancient or Damaged DNA." *Nature Protocols* 8(4): 737–48. http://www.nature.com/doifinder/10.1038/nprot.2013.038 (July 25, 2018).

14. Green, Richard E et al. 2010. "A Draft Sequence of the Neandertal Genome." *Science (New York, N.Y.)* 328(5979): 710–22. http://www.ncbi.nlm.nih.gov/pubmed/20448178 (September 26, 2018).

15. Haak, Wolfgang et al. 2015. "Massive Migration from the Steppe Was a Source for Indo-European Languages in Europe." *Nature* 522(7555): 207–11. https://www.nature.com/nature/journal/v522/n7555/pdf/nature14317.pdf (January 21, 2016).

16. Hagelberg, Erika, Michael Hofreiter, and Christine Keyser. 2015. "Ancient DNA: The First Three Decades." *Philosophical Transactions of the Royal Society B: Biological Sciences*.

17. Herbig, Alexander et al. 2016. bioRxiv *MALT: Fast Alignment and Analysis of Metagenomic DNA Sequence Data Applied to the Tyrolean Iceman*. Cold Spring Harbor Labs Journals. http://biorxiv.org/content/early/2016/04/27/050559.abstract (April 28, 2016).

18. Herrero, E.R. et al. 2018. "Dysbiotic Biofilms Deregulate the Periodontal Inflammatory Response." *Journal of Dental Research* 97(5): 547–55. http://journals.sagepub.com/doi/10.1177/0022034517752675 (September 3, 2018).

19. Huson, Daniel H. et al. 2016. "MEGAN Community Edition - Interactive Exploration and Analysis of Large-Scale Microbiome Sequencing Data." ed. Timothée Poisot. *PLoS computational biology* 12(6): e1004957. http://dx.plos.org/10.1371/journal.pcbi.1004957 (June 28, 2016).

20. Jónsson, Hákon et al. 2013. "MapDamage2.0: Fast Approximate Bayesian Estimates of Ancient DNA Damage Parameters." *Bioinformatics* 29(13): 1682–84. http://www.ncbi.nlm.nih.gov/pubmed/23613487 (July 25, 2018).

21. De La Fuente, C., S. Flores, and M. Moraga. 2013. "DNA from Human Ancient Bacteria: A Novel Source of Genetic Evidence from Archaeological Dental Calculus." *Archaeometry* 55(4): 767–78. http://doi.wiley.com/10.1111/j.1475-4754.2012.00707.x (February 11, 2016).

22. Mann, Allison E. et al. 2018b. "Differential Preservation of Endogenous Human and Microbial DNA in Dental Calculus and Dentin." *Scientific Reports* 8(1): 9822. http://www.nature.com/articles/s41598-018-28091-9 (July 3, 2018).

23. ———. 2018a. "Differential Preservation of Endogenous Human and Microbial DNA in Dental Calculus and Dentin." *Scientific Reports* 8(1): 9822. http://www.nature.com/articles/s41598-018-28091-9 (August 10, 2018).

24. Meyer, Matthias et al. 2012. "A High-Coverage Genome Sequence from an Archaic Denisovan Individual." *Science (New York, N.Y.)* 338(6104):

222–26. http://www.ncbi.nlm.nih.gov/pubmed/22936568 (June 15, 2017).

25. Meyer, Matthias, and Martin Kircher. 2010. "Illumina Sequencing Library Preparation for Highly Multiplexed Target Capture and Sequencing." *Cold Spring Harbor Protocols* 2010(6): pdb.prot5448. http://www.cshprotocols.org/cgi/doi/10.1101/pdb.prot5448 (July 11, 2014).

26. Nieves-Colón, Maria A. et al. 2018. "Comparison of Two Ancient DNA Extraction Protocols for Skeletal Remains from Tropical Environments." *American Journal of Physical Anthropology* 166(4): 824–36. http://doi.wiley.com/10.1002/ajpa.23472 (September 5, 2017).

27. Papudeshi, Bhavya et al. 2017. "Optimizing and Evaluating the Reconstruction of Metagenome-Assembled Microbial Genomes." *BMC Genomics* 18(1): 915. http://www.ncbi.nlm.nih.gov/pubmed/29183281 (January 5, 2018).

28. Prüfer, Kay et al. 2014. "The Complete Genome Sequence of a Neanderthal from the Altai Mountains." *Nature* 505(7481): 43–49. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4031459&tool=pmcentrez&rendertype=abstract.

29. Quail, Michael et al. 2012. "A Tale of Three next Generation Sequencing Platforms: Comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq Sequencers." *BMC Genomics* 13(1): 1. http://dx.doi.org/10.1186/1471-2164-13-341.

30. Rohland, Nadin, and Michael Hofreiter. 2007a. "Ancient DNA Extraction from Bones and Teeth." https://www-nature-com.proxy.library.adelaide.edu.au/articles/nprot.2007.247.pdf (September 24, 2018).

31. ———. 2007b. "Comparison and Optimization of Ancient DNA Extraction." *BioTechniques* 42(3): 343–52. http://www.ncbi.nlm.nih.gov/pubmed/17390541 (June 15, 2017).

32. Salter, SJ Susannah J et al. 2014. "Reagent and Laboratory Contamination Can Critically Impact Sequence-Based Microbiome Analyses." *BMC Biology* 12(1): 87. http://www.biomedcentral.com/1741-7007/12/87 (October 10, 2016).

33. Schubert, Mikkel, Stinus Lindgreen, and Ludovic Orlando. 2016.

"AdapterRemoval v2: Rapid Adapter Trimming, Identification, and Read Merging." *BMC Research Notes* 9(1): 88. http://www.biomedcentral.com/1756-0500/9/88 (August 26, 2018).

34. Shaw, Liam et al. 2017. "The Human Salivary Microbiome Is Shaped by Shared Environment Rather than Genetics: Evidence from a Large Family of Closely Related Individuals." *mBio* 8(5): e01237-17. http://www.ncbi.nlm.nih.gov/pubmed/28900019 (September 18, 2017).

35. The Human Microbiome Project. 2012. "Structure, Function and Diversity of the Healthy Human Microbiome." *Nature* 486: 207–14. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3564958&tool=pmcentrez&rendertype=abstract.

36. Warinner, Christina, João F Matias Rodrigues, et al. 2014. "Pathogens and Host Immunity in the Ancient Human Oral Cavity." *Nature genetics* 46(4): 336–44. http://dx.doi.org/10.1038/ng.2906.

37. Warinner, Christina, Camilla Speller, and Matthew J. Collins. 2014. "A New Era in Palaeomicrobiology: Prospects for Ancient Dental Calculus as a Long-Term Record of the Human Oral Microbiome." *Philosophical Transactions of the Royal Society B: Biological Sciences* 370(1660): 20130376–20130376. http://rstb.royalsocietypublishing.org/cgi/doi/10.1098/rstb.2013.0376 (April 4, 2018).

38. Weyrich, Laura S., Keith Dobney, and Alan Cooper. 2015a. "Ancient DNA Analysis of Dental Calculus." *Journal of Human Evolution* 79: 119–24. https://ac-els-cdn-com.proxy.library.adelaide.edu.au/S0047248414002541/1-s2.0-S0047248414002541-main.pdf?_tid=3e735bda-9676-4977-b507-61c4a0c7c047&acdnat=1522637436_38a86a3a6b77342dcad9a3e7b605a431 (April 2, 2018).

39. Weyrich, Laura S et al. 2017. "Neanderthal Behaviour, Diet, and Disease Inferred from Ancient DNA in Dental Calculus." *Nature Publishing Group* 544. https://www.nature.com/nature/journal/v544/n7650/pdf/nature21674.pdf (November 1, 2017).

40. Weyrich, Laura S, Keith Dobney, and Alan Cooper. 2015b. "Ancient

DNA Analysis of Dental Calculus." *Journal of Human Evolution* 79: 119–24. http://dx.doi.org/10.1016/j.jhevol.2014.06.018 (September 24, 2018).

41. Zakrzewski, Martha et al. 2017. "Calypso: A User-Friendly Web-Server for Mining and Visualizing Microbiome-Environment Interactions." *Bioinformatics (Oxford, England)* 33(5): 782–83. http://www.ncbi.nlm.nih.gov/pubmed/28025202 (July 5, 2018).

Supplementary Figures
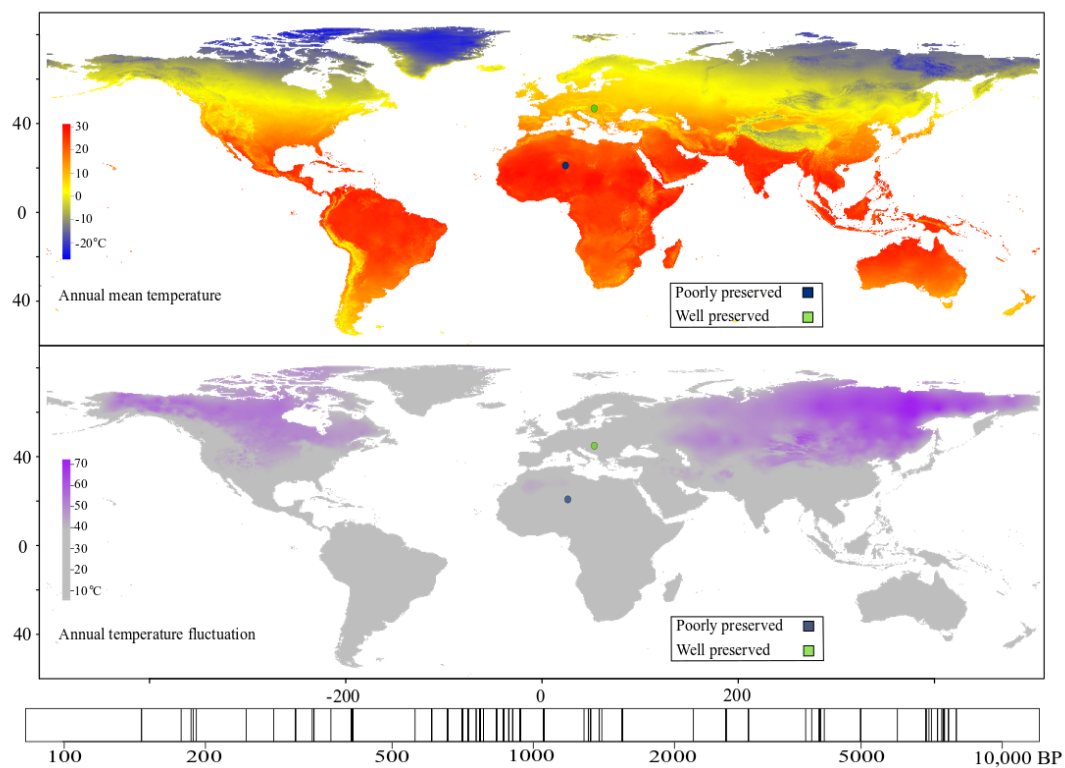
Figure S1. Locations of 6 well preserved (n=3) and poorly preserved (n=3) samples used in the analysis, including the global variation in mean temperature, temperature fluctuation of their respective collection sites.
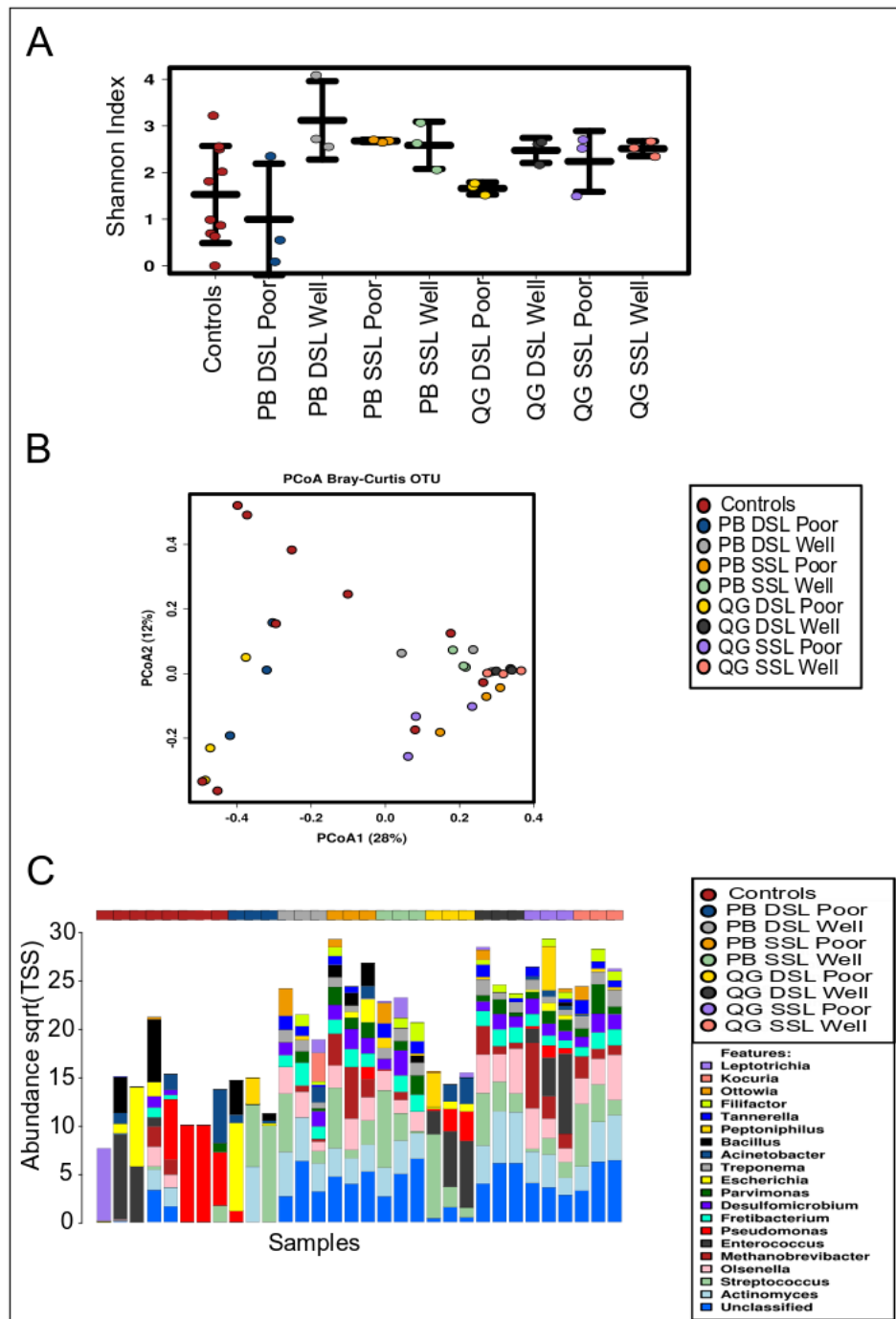
Figure S2. A. Shannon diversity index showing the impact of different DNA extraction and library methods on Alpha diversity between samples with controls. B. PCoA plot of Bray-Curtis similarities showing the effect of different DNA extraction and library methods on Beta diversity between samples with controls. C. Taxa bar plot of top 20 genera in microbial composition showing the impact of different DNA extraction and library methods on samples compared with controls. The levels of microbial diversity and microbiota composition in ancient dental calculus samples were significantly different from controls (ANOVA & ANOSIM (p < 0.05))
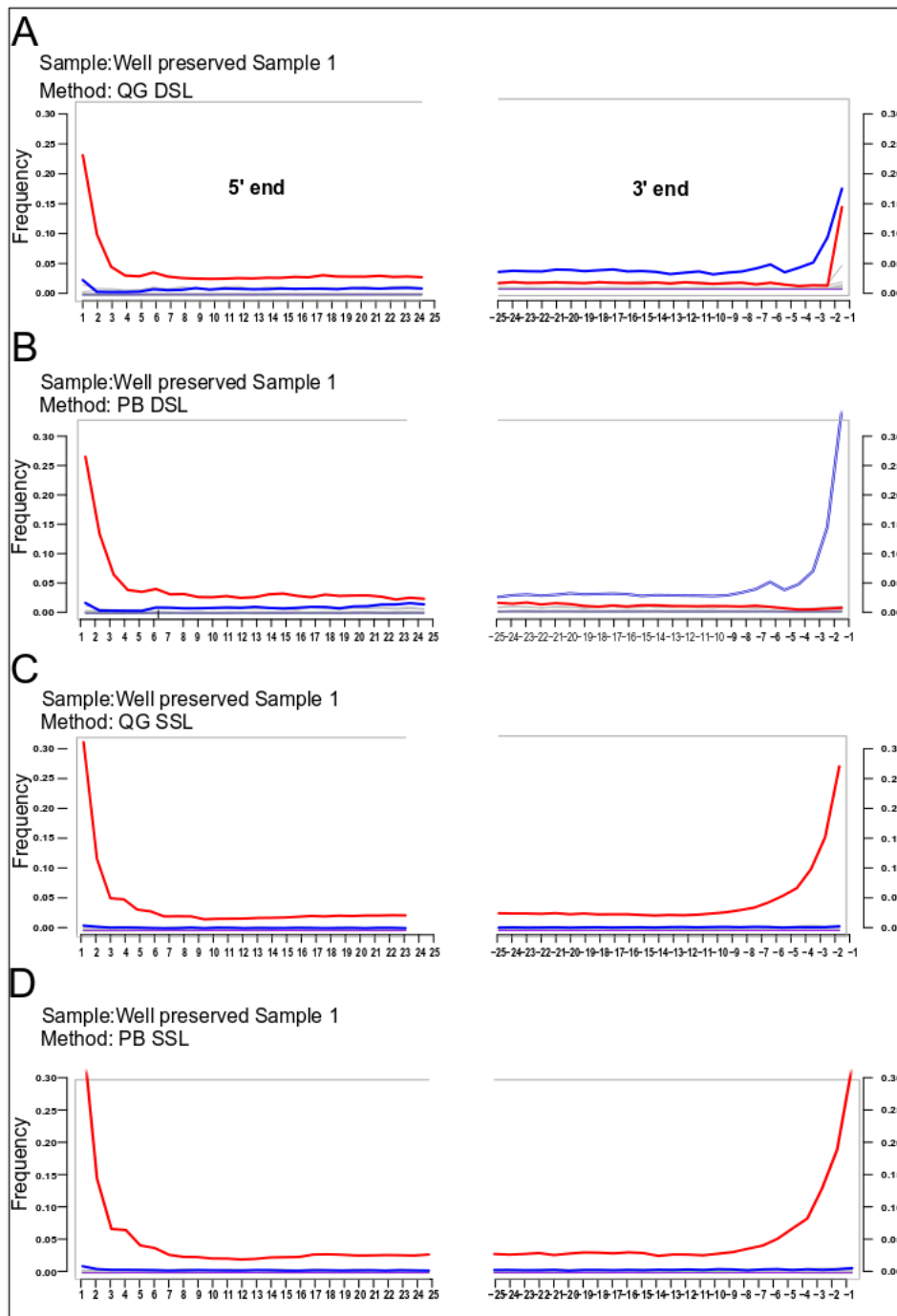
Figure S3A. Ancient DNA damage analysis based on MapDamage 2.0 using *Anaerolineaceae bacterium oral taxon 439* compared between the two DNA extraction methods and two library preparation methods on well preserved ancient dental calculus samples. We find that the single stranded library preparation method irrespective of extraction method used had higher levels of ancient DNA damage (terminal cytosine deamination) compared to double stranded library preparation method.
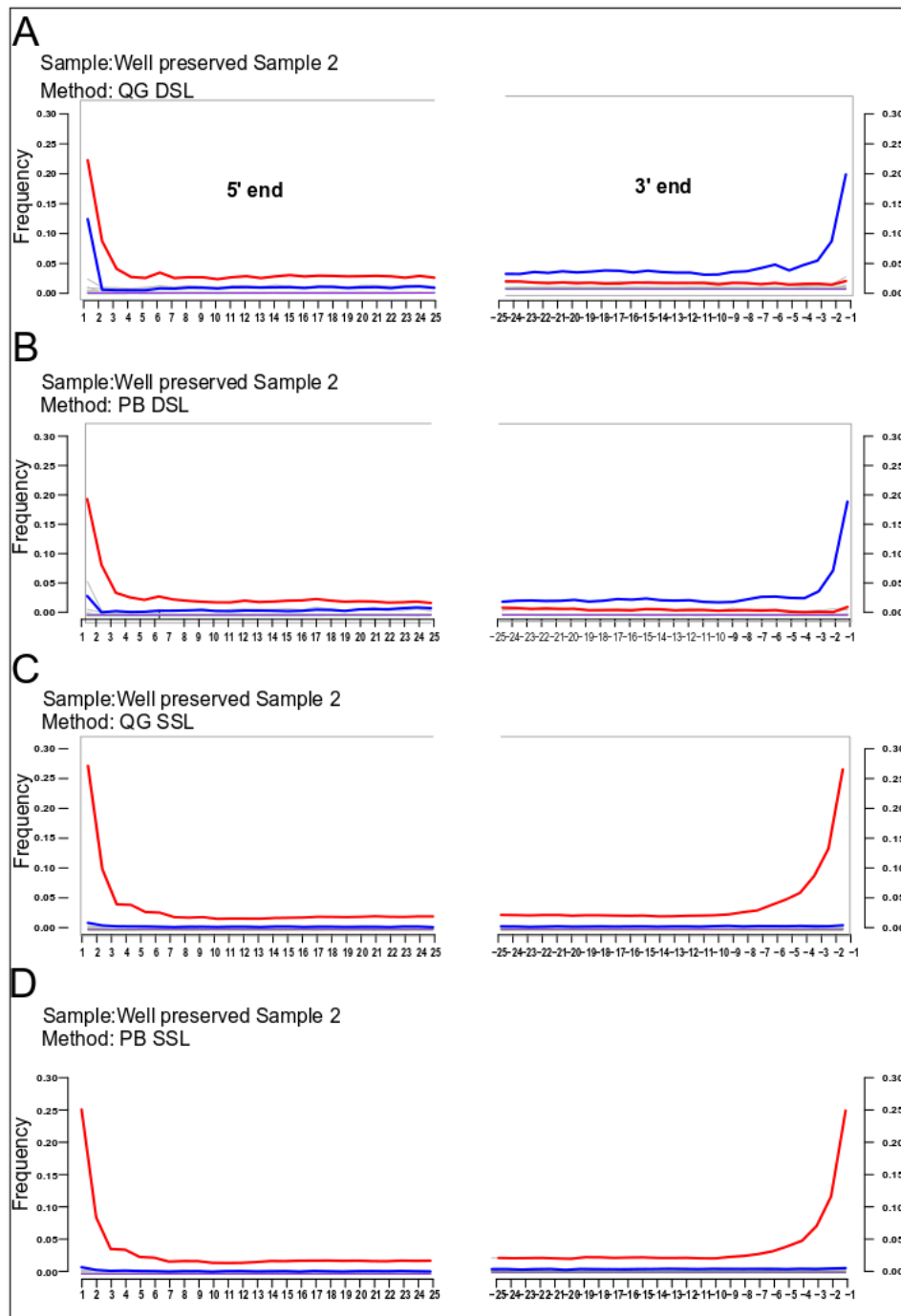
Figure S3B. Ancient DNA damage analysis based on MapDamage 2.0 using *Anaerolineaceae bacterium oral taxon 439* compared between the two DNA extraction methods and two library preparation methods on well preserved ancient dental calculus samples. We find that the single stranded library preparation method irrespective of extraction method used had higher levels of ancient DNA damage (terminal cytosine deamination) compared to double stranded library preparation method.
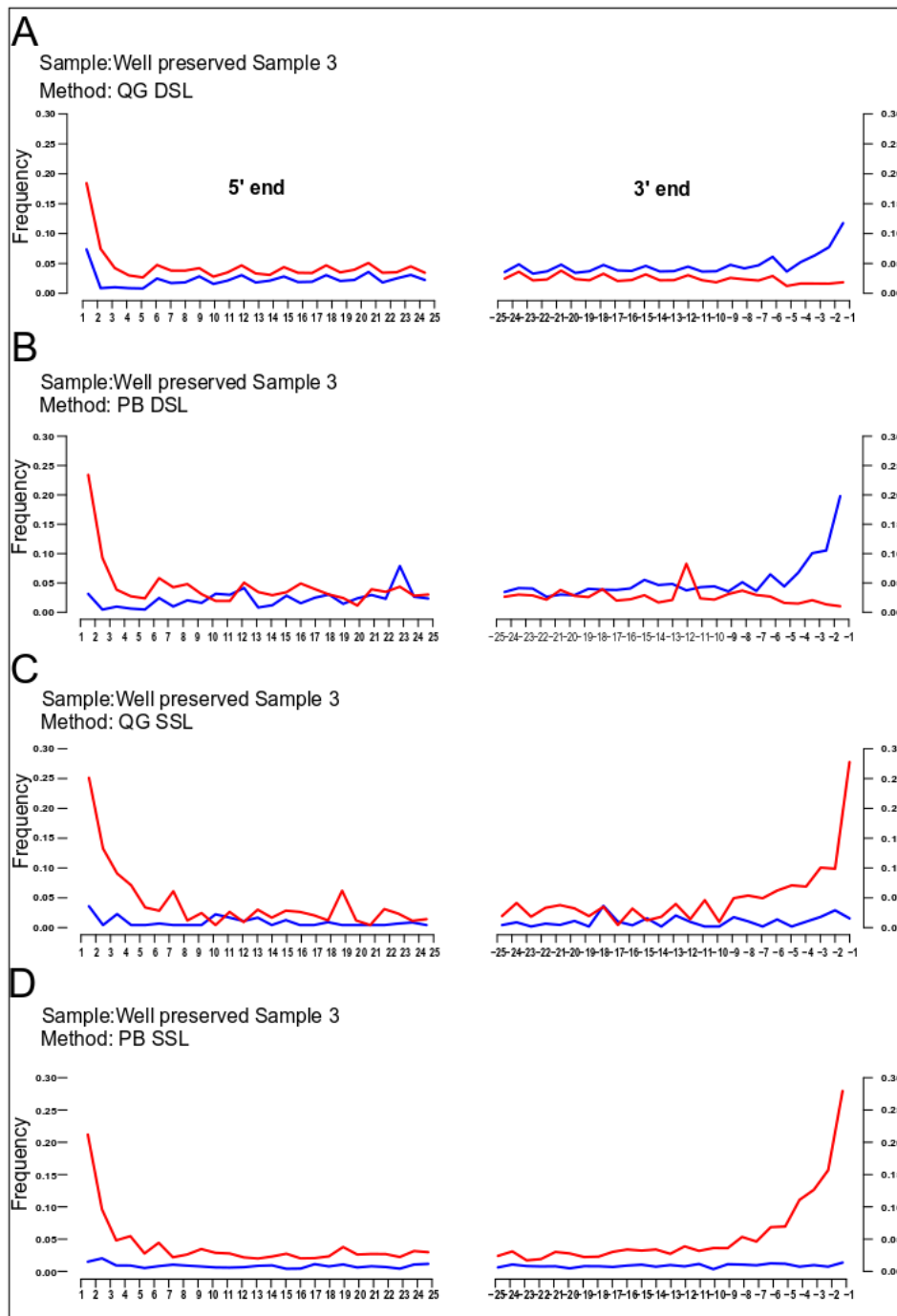
Figure S3C. Ancient DNA damage analysis based on MapDamage 2.0 using *Anaerolineaceae bacterium oral taxon 439* compared between the two DNA extraction methods and two library preparation methods on well preserved ancient dental calculus samples. We find that the single stranded library preparation method irrespective of extraction method used had higher levels of ancient DNA damage (terminal cytosine deamination) compared to double stranded library preparation method.
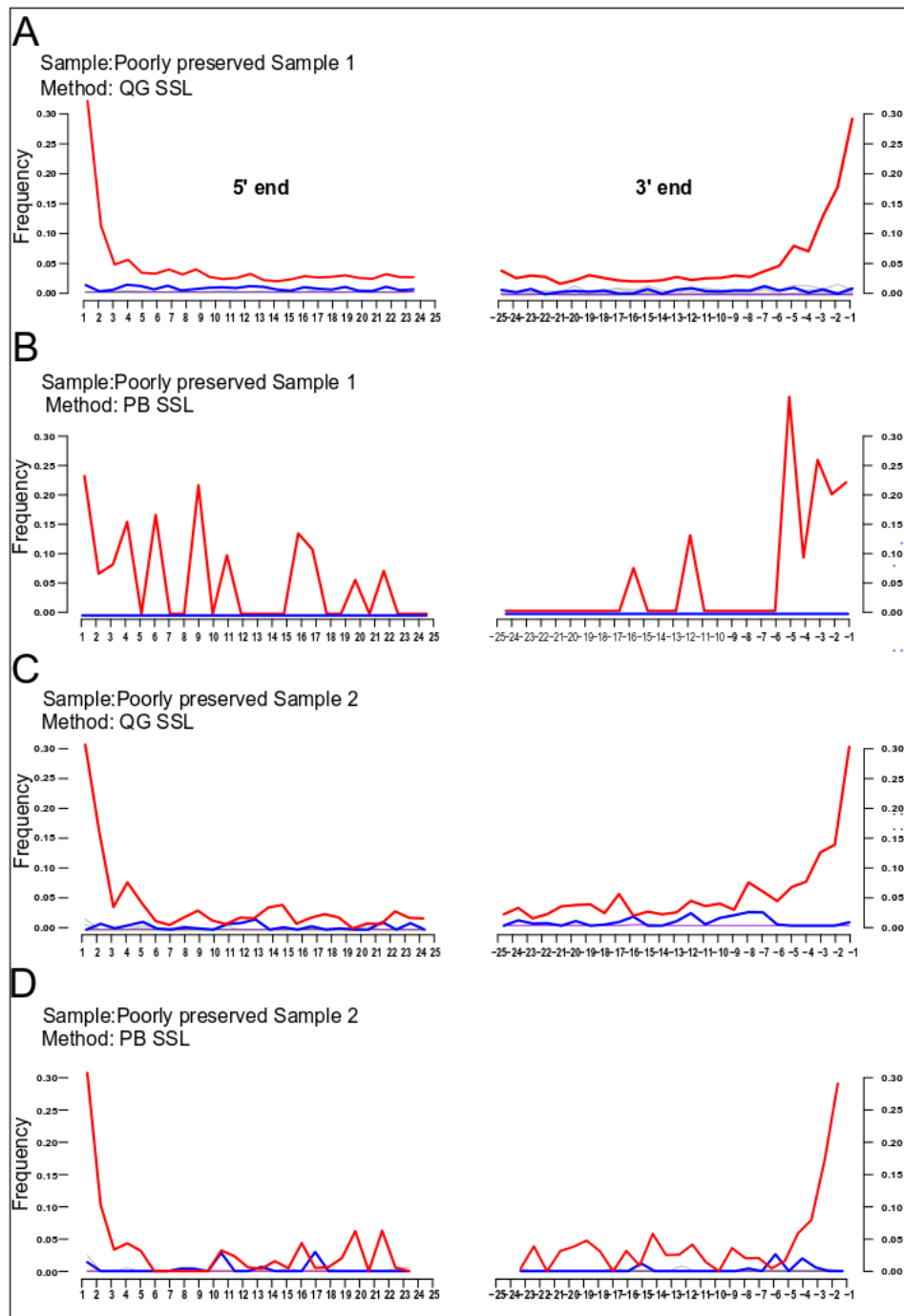
Figure S4A. Ancient DNA damage analysis based on MapDamage 2.0 using *Anaerolineaceae bacterium oral taxon 439* compared between the two DNA extraction methods and two library preparation methods on poorly preserved ancient dental calculus samples. We find that the single stranded library preparation method irrespective of extraction method used had higher levels of ancient DNA damage (terminal cytosine deamination) compared to double stranded library preparation method. Due to the low number of reads mapping to *Anaerolineaceae bacterium oral taxon 439*, the bacteria used as reference for Map Damage in the poorly preserved samples using double stranded library (DSL) method, we were unable to obtain Map Damage results for these samples.

Figure S4B. Ancient DNA damage analysis based on MapDamage 2.0 using *Anaerolineaceae bacterium oral taxon 439* compared between the two DNA extraction methods and two library preparation methods on poorly preserved ancient dental calculus samples. We find that the single stranded library preparation method irrespective of extraction method used had higher levels of ancient DNA damage (terminal cytosine deamination) compared to double stranded library preparation method. Due to the low number of reads mapping to *Anaerolineaceae bacterium oral taxon 439*, the bacteria used as reference for Map Damage in the poorly preserved samples using double stranded library (DSL) method, we were unable to obtain Map Damage results for these samples.

Table S1. Metadata on samples used in the analysis.

SampleID	Sample	TotalReads	Clonality	FragLengthAv	GC_contentPerct	LibraryP ▼	Extractior	Methodology
18393p_Gobero_DC2_PB	Poorly Preserved 1	46500	79	54	53	DSL	PB	PB_DSL
18398p_Gobero_DC3_PB	Poorly Preserved 2	62493	78	58	53	DSL	PB	PB_DSL
18400p_Gobero_DC1_PB	Poorly Preserved 3	15510	80	57	53	DSL	PB	PB_DSL
18416p_Hungary4_PB	Well Preserved 1	1069458	2	60	51	DSL	PB	PB_DSL
18421p_Hungary11_PB	Well Preserved 2	1115816	2	70	56	DSL	PB	PB_DSL
18427p_Hungary7_PB	Well Preserved 3	1796842	1	60	51	DSL	PB	PB_DSL
18393_Gobero_DC2_QG	Poorly Preserved 1	38154	85	106	55	DSL	QG	QG_DSL
18398_Gobero_DC3_QG	Poorly Preserved 2	21165	75	94	56	DSL	QG	QG_DSL
18400_Gobero_DC1_QG	Poorly Preserved 3	100913	67	122	56	DSL	QG	QG_DSL
18416_Hungary4	Well Preserved 1	1076156	3	111	53	DSL	QG	QG_DSL
18421_Hungary11	Well Preserved 2	1444150	14	103	54	DSL	QG	QG_DSL
18427_Hungary7	Well Preserved 3	1928694	5	117	51	DSL	QG	QG_DSL
18393PB_SSL	Poorly Preserved 1	2308	5	52	39	SSL	PB	PB_SSL
18398PB_SSL	Poorly Preserved 2	33054	65	63	43	SSL	PB	PB_SSL
18400PB_SSL	Poorly Preserved 3	277168	83	55	43	SSL	PB	PB_SSL
18416PB_SSL	Well Preserved 1	5657100	4	46	39	SSL	PB	PB_SSL
18421PB_SSL	Well Preserved 2	4566559	4	46	46	SSL	PB	PB_SSL
18427PB_SSL	Well Preserved 3	10185382	1	46	41	SSL	PB	PB_SSL
18393QG_SSL	Poorly Preserved 1	706286	76	63	37	SSL	QG	QG_SSL
18398QG_SSL	Poorly Preserved 2	76958	68	56	42	SSL	QG	QG_SSL
18400QG_SSL	Poorly Preserved 3	29055	74	66	46	SSL	QG	QG_SSL
18416QG_SSL	Well Preserved 1	17377666	2	59	44	SSL	QG	QG_SSL
18421QG_SSL	Well Preserved 2	4939739	4	60	47	SSL	QG	QG_SSL
18427QG_SSL	Well Preserved 3	303465	64	46	40	SSL	QG	QG_SSL

Supplementary Text. Laboratory protocols for the extraction and library preparation methods used. **A.** QG Method Protocol **B.** PB Method Protocol **C.** DSL Protocol **D.** SSL protocol.

A. Laboratory protocol for the QG extraction method

Modified by L. Weyrich and A. Farrer (Weyrich et al. 2017)

Prepare all reagents before opening samples – Keep stock solutions sterile

Day 1

No DNA room

   * Clean hood according to entry protocols

  Water Aliqouts
1. Ultrapure water in 3x 25ml in 50ml tube, 3x 50ml tube, 10x 1.5ml tube

  Silica Solution

1. Add 6 g of silica to 50 ml of water and vortex
2. Leave to settle for 1 hour
3. Pipette off~40 ml of the suspended silica matrix to new 50ml Falcon tube – leave larger silica particles
4. Leave to settle overnight
5. Pipette or pour off supernatant (leaving about 10 ml of silica suspension at the bottom) – leave medium silica particles
6. Store in the fridge, in the dark (Keeps for 1 month)
7. Store in five 1.5ml Eppendorf tubes

  TLE buffer

1. Mix:
   a. 500 μl Tris HCL (1M)
   b. 10 μl EDTA (0.5M)
   c. 50 ml Water

  80 % Ethanol

- 30 ml for 15 samples
  - 6 ml Water (water first)
  - 24 ml Absolute Ethanol

  Digestion buffer

1. Make a master mix without Protease K

Reagent	Per sample
EDTA (0.5 M)	1.6 ml

SDS (10%)	100 ul
Protease K (20 mg/ml)	20 ul

2. <u>Mix gently by inverting – don't form bubbles</u>

3. Clean hood according to exit protocols

Dental Calculus room

4. Move to DC room with digestion buffer (without Protease K)
5. Store Protease K aliquots in the freezer
6. Turn on the incubator (55°C) and put in digestion buffer (50ml MM) to fully dissolve

PC main lab

1. Explore samples on Sample Database / Add samples to the Database
2. Take samples from the Freezer
3. Add extracted entry and give two extraction blanks ACAD numbers
4. Take small boats
5. Clean photo stand with decon
6. Switch on PC to Photo program
7. Put DC samples in the boat and add a scale
8. Place DC samples into UV fryer (Switch on when samples all done)
9. *UV irradiate the dental calculus samples for 15 minutes each side*

Bone room

1. Clean counters and tools with paper towel soaked in bleach
2. Prepare Tweezers, Petri Dishes, Decon Bath
3. Prepare 2 ml screw cap tubes (x sample no.) (Label: ACAD number, description, date, initials) – Include 2 EBC's
4. Prepare the hood for samples and set up timer
5. Prepare 5% bleach – 12mlH20 +15ml bleach in 50ml tube
6. Bring in samples one by one
7. Open up the first 2ml tube as EBC1
8. Soak sample in **5 % bleach** (3 min), new petri dish
9. Soak sample in **80 % ethanol** (2 min), new petri dish lid
10. Dry on kimwipe
11. Place into 2 ml screw cap tube
12. Crush sample with tweezers
13. Place tweezers in decon bath (water + splash of decon)
14. Sterilise work space, get clean tweezers
15. Bring the next sample
16. Open up the last 2 ml tube as EBC2
17. Decon wash tweezers.
18. Place tools in UV oven and clean counters
19. Take tubes to the UV table in the PC main lab

Dental Calculus room

1. Add Protease K to the digestion buffer master mix
2. Add 1.72 ml to each sample tube (860ul x2)
    a. Be quick
    b. Slide tip along edge of tube
    c. Don't double press pipette (causes bubbles)
3. Screw lids on tight
4. Place in rotary mixer and incubator (55C)
5. Leave overnight

Day 2

No DNA room

* Clean according to entry cleaning protocol

Modified QG Binding buffer

Reagents	Quantity for 4 ml	Start with 50 ml QG Buffer
QG Buffer	3.7 **ml**	50 **ml**
Water	61.4 **μl**	833.7 **μl**
NaCl (5M)	20.0 **μl**	271.8 **μl**
Triton-X 100	52.1 **μl**	707.6 **μl**
NaOAc (3M)	222.4 **μl**	3021.9 **μl**

- Rock to mix (no bubbles form)

Dental Calculus room

Silica Binding

Do not use bleach during this protocol – Guanidinium present

1. 3 ml Binding Buffer into 15 ml Falcon tube (per sample)
2. Add 100 μl Silica suspension (per sample)
3. Remove samples from incubator
4. Turn incubator off and leave door open
5. Centrifuge samples (3 min, 14,500 rpm)
6. Transfer supernatant to falcon tubes (don't transfer pellet or solids)
    a. Store pellet
7. Tighten falcon tube lids
8. Rotary mixer for > 1 hr.

DNA purification and elution

1. Set heat block to 37 °C
2. Remove tubes from rotary mixer
3. Centrifuge samples (5 min, 4,550 rpm, main lab) -> Prepare 1.5 ml tubes
4. Pour off supernatant into QG buffer tubes x2
5. Add 900 μl of 80 % ethanol
6. Re-suspend pellet (long reach pipette)
7. Transfer to 1.5 ml tube

8. Centrifuge (1 min, 14,000 rpm)
9. Pipette off supernatant
10. Add 900 μl of 80 % ethanol
11. Re-suspend pellet (vortex)
12. Centrifuge (1 min, 14,000 rpm)
13. Pipette off supernatant
14. Place samples in heat block to dry (15 min, lid open, cover with kimwipe) - Prepare tubes for next step
15. Remove samples
16. Set heat block to 50 °C
17. Add 100 μl of TLE
18. Re-suspend pellet (vortex)
19. Place tubes on heat block for 10 min
    a. Label 1.5 ml screw-top with ACAD numbers and date
20. Centrifuge (1 min, 14,000 rpm)
21. Supernatant to 1.5 ml screw-top tube
22. Repeat steps 17 – 21

Store extracts in freezer

B. Laboratory protocol for the PB extraction method

Prepare all reagents before opening samples – Keep stock solutions sterile

<u>Day 1</u>

No DNA room

 * Clean hood according to entry protocols

 Water Aliqouts
 1. Ultrapure water in 3x 25ml in 50ml tube, 3x 50ml tube, 10x 1.5ml tube

 Silica Solution

 2. Add 6 g of silica to 50 ml of water and vortex
 3. Leave to settle for 1 hour
 4. Pipette off~40 ml of the suspended silica matrix to new 50ml Falcon tube – leave larger silica particles
 5. Leave to settle overnight
 6. Pipette or pour off supernatant (leaving about 10 ml of silica suspension at the bottom) – leave medium silica particles
 7. Store in the fridge, in the dark (Keeps for 1 month)
 8. Store in five 1.5ml Eppendorf tubes

 TLE buffer

 9. Mix:
    a. 500 μl Tris HCL (1M)
    b. 10 μl EDTA (0.5M)
    c. 50 ml Water

 80 % Ethanol

- 30 ml for 15 samples
    - 6 ml Water (water first)
    - 24 ml Absolute Ethanol

Digestion buffer

10. Make a master mix without Protease K

Reagent	Per sample
EDTA (0.5 M)	900ul
ddH20	80 ul
Protease K (20 mg/ml)	20 ul

11. <u>Mix gently by inverting – don't form bubbles</u>

12. Clean hood according to exit protocols
\# <u>Switch off the light and switch on the UV light.</u>

Dental Calculus room

13. Move to DC room with digestion buffer (without Protease K)
14. Store Protease K aliquots in the freezer
15. Turn on the incubator (55°C) and put in digestion buffer (50ml MM) to fully dissolve

PC main lab

16. Explore samples on Sample Database / Add samples to the Database
17. Take samples from the Freezer
18. Add extracted entry and give two extraction blanks ACAD numbers
19. Take small boats
20. Clean photo stand with decon
21. Switch on PC to E.. Photo program
22. Put DC samples in the boat and add a scale
23. Place DC samples into UV fryer (Switch on when samples all done)
24. *UV irradiate the dental calculus samples for 15 minutes each side*

Bone room

25. Clean counters and tools with paper towel soaked in bleach
26. Prepare Tweezers, Petri Dishes, Decon Bath
27. Prepare 2 ml screw cap tubes (x sample no.) (Label: ACAD number, description, date, initials) – Include 2 EBC's
28. Prepare the hood for samples and set up timer
29. Prepare 5% bleach – 12mlH20 +15ml bleach in 50ml tube
30. Bring in samples one by one

31. Open up the first 2ml tube as EBC1
32. Soak sample in **5 % bleach** (3 min), new petri dish
33. Soak sample in **80 % ethanol** (2 min), new petri dish lid
34. Dry on kimwipe
35. Place into 2 ml screw cap tube
36. Crush sample with tweezers
37. Place tweezers in decon bath (water + splash of decon)
38. Sterilise work space, get clean tweezers
39. Bring the next sample
40. Open up the last 2 ml tube as EBC2
41. Decon wash tweezers.
42. Place tools in UV oven and clean counters
43. Take tubes to the UV table in the PC main lab
44. Clean the Bone room with water + bleach and swipe the floor
45. Vacum the room with the wetvac.
46. Take tubes to the DC room
47. Add Protease K to the digestion buffer master mix
48. Add 1 ml to each sample tube (1000ul)
    a. Be quick
    b. Slide tip along edge of tube
    c. Don't double press pipette (causes bubbles)
    d. Screw lids on tight
    e. Place in rotary mixer and incubator (55C)
49. Leave overnight (~24hrs)

Day 2

No DNA room

* Clean according to entry cleaning protocol

Modified PB Binding buffer

Reagents	Quantity for 1 sample
PB Buffer	13.57 **ml**
Tween-20	7 **µl**
NaOAc (3M)	420 **µl**

- Rock to mix (no bubbles form)

Dental Calculus room

 Silica Binding

 Do not use bleach during this protocol – Guanidinium present

50. 14.06 ml Binding Buffer into 15 ml Falcon tube (per sample)
51. Add 100 µl Silica suspension (per sample)
52. Remove samples from incubator
53. Turn incubator off and leave door open
54. Centrifuge samples (3 min, 14,500 rpm) and bone (2min 4500rpm)
55. Transfer supernatant to falcon tubes (don't transfer pellet or solids)

98

a. Store pellet
56. Tighten falcon tube lids
57. Rotary mixer for > 1 hr.

DNA purification and elution

58. Set heat block to 37 °C
59. Remove tubes from rotary mixer
60. Centrifuge all samples (5 min, 4,550 rpm, main lab) -> Prepare 1.5 ml tubes
61. Pour off supernatant into PB buffer waste tubes x2
62. Add 900 μl of 80 % ethanol
63. Re-suspend pellet (long reach pipette)
64. Transfer to 1.5 ml tube
65. Centrifuge (1 min, 14,000 rpm)
66. Pipette off supernatant
67. Add 900 μl of 80 % ethanol
68. Re-suspend pellet (vortex)
69. Centrifuge (1 min, 14,000 rpm)
70. Pipette off supernatant
71. Place samples in heat block to dry (15 min, lid open, cover with kimwipe) - Prepare tubes for next step
72. Remove samples
73. Set heat block to 50 °C
74. Add 100 μl of TLE
75. Re-suspend pellet (vortex)
76. Place tubes on heat block for 10 min
a. Label 1.5 ml screw-top with ACAD numbers and date
77. Centrifuge (1 min, 14,000 rpm)
78. Supernatant to 1.5 ml screw-top tube
79. Repeat steps 74 – 78

Store extracts in freezer

C. Laboratory protocol for the DSL method

1- Clean hood + UV start (20mins)
2- Prep. Repair Master Mix (MM)

4) Repair reactions 40ul final volume	
	ul
10x NEB2 buffer (or Tango buffer)	4
25 mM each (100mM total) dNTPs	0.4
10mM ATP	4
dH20	8.1
T4 PNK (10 U/ul)	2
T4 DNA Polymerase (3 U/ul)	1.5
Total volume	20
Transfer 20 uL to PCR strip tubes, then add DNA extracts	
Pipette mix gently + flash spin	
Incubate on thermal cycler (25°C)	15min

3- Transfer 20ul of MM to new PCR tubes + spin down
4- Transfer 20ul sample to PCR tubes with MM
5- Pipette mix gentle and flash spin
6- Incubate on thermocycler :

        a.   Use block A : change PNK program to 15mins at 25C : no heat block

7- <u>Set up a new 1.5ml tube and put 300ul of ERC buffer in.</u>

8- Bring in Minlute columns and label them

Minelute cleanup (Enzymatic Reaction Cleanup kit)		
200ul PB Buffer	60s	13000rpm
750ul Buffer PE	60s	13000rpm
	60s	top speed

9- Switch on the heat block to 50C

10- Put away enzymes and DNA extracts from previous set up

11- Add your DNA template from the previous step and Pippette up and down.

12- Transfer to solution (~400ul) to the spin column

13- Let sit for 1min

14- Centrifuge for 1min @ 13000rpm.

15- Discard flow through and tap top of collection tube dry.

16- Put spin column back into same collection tube and add 700ul of PE buffer.

17- Let sit for ~1min before

18- Centrifuge 1min @13000 rpm

19- Put EB buffer in the heat block

20- Discard flow through, tap dry collection tube

21- Put spin column back into same collection tube.

22- Using p10 remove all excess liquid from purple o ring inside spin column - do not touch silica filter with pipette tip.

23- Centrifuge 1min @ top speed.

24- Prepare fresh 1.5ml tube with lid removed.

25- Place spin column into new 1.5ml tube and discard old collection tube

26- Add pre-heated EB buffer (22.5ul) directly to the filter without touching it with your tip, i.e get tip close to it so the liquid goes directly onto it and none gets stuck above the purple o-ring.

27- Let sit for 1min

28- Centrifuge 1min @13000 rpm

29- <u>Transfer flow through into strip tube in preparation for next step.</u>

30- Bring out barcoded adapters

31- Prepare Ligation reaction

Add 1ul of P7 adapter to cleaned DNA in PCR tube	
Add 1ul of the correct P5 barcoded adapter to each PCR tube	
Prepare Ligation Mastermix	
	ul
10xT4 Ligase Buffer	4
PEG-4000 (50% solution)	4
H20	9
T4 DNA ligase (1000ceu/uL = 5 u/uL)	1
Total volume	18
Add 18ul of MM to each PCR tube	
Pipette mix gently + flash spin	
Incubate on thermal cycler 22°C	60min

32- After LIGASE reaction repeat step 9 to 31 – use all the DNA template

Minelute cleanup (Enzymatic Reaction Cleanup kit)		
200ul PB Buffer	60s	13000rpm
750ul Buffer PE	60s	13000rpm
	60s	top speed

33- Use Ice cube to stop ligase reaction before adding templates (add strip tubes one by one)

34- Prepare Bst fill in reaction

6) Bst fill-in reaction final volume 40ul

	ul
10x Thermopol-Buffer	4
25 mM each (100mM total) dNTPs	1
H2O	13.5
Bst DNA Polymerase (8 U/ul)	1.5
Total volume	20

Add 20ul mastermix to each tube of DNA

Incubate on thermal cycler	
Preheat lid 95°C	30min
Heatkill Bst	10min

35- Amplify the library using IS7 and IS8 primers

Library Amplification	
HiFi PCRs	
dH20	13.25
10x Gold Buffer	2.5
50mM MgSO4	1.25
25 mM each (100mM total) dNTPs	0.25
IS7_short_amp.P5	1.25
IS8_short_amp.P7	1.25
HiFi Taq Polymerase	0.25
LIBRARY DNA	5
Final volume	25
aliquot 20ul into PCR tubes	

D. Laboratory protocol for the SSL method

Day 1:	
Prepare your own wash buffers, 1% Tween-20 and 2% Tween-20	
Heat denaturation and dephosphorylation	
Prepare mastermix:	
	μl
10x T4 RNA ligation buffer	8
2% Tween 20	2
FastAP (1U/μl; ThermoFisher)	1
H20	14.6
Volume of MMx to add to DNA	25.6

DNA extract	20
Total volume	45.6
flick mix and spin down	
Incubate on thermocycler: DENAT	37ºC
	95ºC
Place immediately in ice block - take to thermocycler	
Ligation of first adapter	
Prepare mastermix:	
	μl
50% PEG-8000 (NEB)	32
100mM ATP (ThermoFisher)	0.4
CL78/Splinter (10/20μM)	1
T4 DNA Ligase (30U/μl; ThermoFisher)	1
volume of MMx to add to dentured DNA	34.4
Denatured DNA	45.6
Total volume	80
flick mix and spin down	
Incubate on thermocycler: LIG (for 1 Hr)	37ºC
1 mins	95ºC
∞	10ºC
Freeze at -20ºC until proceeding with next steps	
Day 2:	
Immobilization of ligation products on beads	
After each step, pellet beads using a magnetic stand and discard the supernatant	
Keep 1x Wash buffer + SDS heated @ 55C between washes	
For each hybridisation reaction, transfer 20μl of MyOne Streptavidin C1 magnetic beads per sample to a 1.5 ml tube (max. 5 reactions per tube)	
Wash 2 times with 500μl 1x Wash buffer + SDS (1M NaCl; 10 mM Tris-HCl pH8; 1 mM EDTA pH8; 0.05% Tween-20 and 0.5% SDS) at room temperature	
Resuspend beads in 250μl 1x Wash Buffer + SDS (multiplied by the number of reactions, e.g. 1ml for 4 reactions)	
Transfer 250μl bead solution to new 1.5ml lobind tube per sample.	
Thaw ligated DNA and incubate for 1 min at 95ºC and immediately transfer to ice block for 2-5 mins.	
Add ligation DNA to bead suspension, mix well and rotate at room temperature for 20 mins.	
Pellet beads and remove supernatant	**Keep, label - post bead ligation supernatant.**
Add 200μl 0.1x Wash buffer + SDS (0.1M NaCl; 10 mM Tris-HCl pH8; 1 mM EDTA pH8; 0.05% Tween-20 and 0.5% SDS)	

Vortex for 8 s to resuspend the beads, quick spin	
Pellet beads and remove supernatant	
Stringency wash for splinter removal	
Add 100µl stringency wash buffer (0.1x SSC; 0.1% SDS) to beads from previous step	
resuspend beads by vortexing, quick spin	
Incubate on thermoshaker for 3 mins at 45ºC with 1400rpm mixing for 3s every 30s	
Pellet beads and remove supernatant	
Add 200µl 0.1x Wash Buffer (0.1M NaCl; 10 mM Tris-HCl pH8; 1 mM EDTA pH8; 0.05% Tween-20)	
Resuspend beads by vortexing for 8s, quick spin	
Primer annealing and extension	
Prepare 'Fill-in' mastermix:	
	µl
H20	39.1
10x Klenow reaction buffer	5
25mM dNTP	0.4
1% Tween-20	2.5
100mM CL130	1
Total volume	48
Pellet beads and remove supernatant	
Add 48µl fill-in mix to beads and resuspend by vortexing, quick spin	
Incubate on heatblock for 2 mins at 65ºC	
Transfer immediately to ice block for 5 mins	
transfer to tube rack at room temperature	
Add 2µl Klenow fragment (10U/ul)	
Resuspend beads by vortexing for 3s	
Incubate for 5 mins at room temp with vortex mixing every 60s, then incubate on thermoshaker for 25 mins at 35ºC and 450rpm mixing, increase to 1400rpm every 5 mins for 2s	
Post-extension washes	
Pellet beads and remove supernatant	
Add 200µl 0.1x Wash Buffer + SDS, resuspend by vortexing for 8s	
Pellet beads and remove supernatant	
Add 100µl Stringency Buffer, resuspend by vortexing	
Incubate on thermoshaker for 3 mins at 45ºC with 3s mixing every 30s	
Pellet beads and remove supernatant	
Add 200µl 0.1x Wash Buffer, resuspend by vortexing for 8s	
Ligation of second adapter, library elution	
Prepare ligation mastermix:	

	µl
H20	73.5
10x T4 DNA ligase buffer	10
50% PEG-4000	10
100mM CL53/73	2
1% Tween-20	2.5
Total volume	98
Pellet beads and remove supernatant	
Add 98ul of ligation mastermix to each sample	
Add 2ul of T4 DNA Ligase (5U/µl) to each sample for total reaction volume of 100ul	
Resuspend beads and ligation mix by vortexing for 3s, quick spin	
Incubate in your hands and shake them like maraccas for 2s every 2 mins for 1 hour or alternatively on heat block for 1hr at 22ºC	
Pellet beads and remove supernatant	
Add 200µl 0.1x Wash buffer + SDS and resuspend by vortexing for 8s	
Pellet beads and remove supernatant	
Add 100µl Stringency wash buffer and resuspend by vortexing	
Incubate on thermoshaker for 3 mins at 45ºC with 3s mixing every 30s	
Pellet beads and remove supernatant	
Add 200µl 0.1x Wash buffer and resuspend by vortexing for 8s	
Elution of Library	
Pellet beads and remove supernatant	
Add 50µl EB + 0.05% Tween-20 (10mM Tris-HCl, pH8; 0.05% Tween-20) and resuspend by vortexing for 8s	
Transfer bead suspension to new 0.2ml PCR tube	
Incubate for 1 min at 95ºC	
<u>Immediately place tube in magnetic rack, pellet beads and transfer supernatant to new 1.5ml lobind tube</u>	
7) Library First Amplification	
Set this PCR up in the ancient lab, perform thermocycling in Darling Building	
HiFi PCRs	
	µl
dH20	13.25
10x Gold Buffer	2.5
50mM MgSO4	1.25
25 mM each (100mM total) dNTPs	0.25
IS4	1.25
GAII _10	1.25
HiFi Taq Polymerase	0.25
LIBRARY DNA	5
Final volume	25
aliquot 20ul into PCR tubes	

"The advent of agriculture" "The Worst Mistake in the History of the Human Race"

Jared Diamond, American geographer and historian

Chapter III

Global Ancient Dental Calculus Assessment Examining Ancient Oral Microbiota Responses to Agriculture

Statement of Authorship

Title of Paper	Using ancient DNA from ancient Near Eastern and African individuals to examine detailed oral microbiota responses to agriculture.
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Publication Details	In preparation for submission to *npj Biofilms and Microbiome*

Principal Author

Name of Principal Author (Candidate)	Muslihudeen Abdul-Aziz
Contribution to the Paper	Designed the experiment. Performed all the laboratory work, data analysis and interpretation of results. Wrote the manuscript.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.

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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate to include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Collected the samples and edited the manuscript.

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Signature	Date 21/11/18

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Global Ancient Dental Calculus Assessment Examining Ancient Oral Microbiota Responses to Agriculture

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Abstract

The human oral microbiome is intricately linked with human health and disease. Recent research suggests that the transition to Agriculture may have resulted in changes in the oral microbiome, and that these changes now underlie many metabolic diseases. However, limited research has been done in the oral cavity and was geographically and temporally limited, despite the diversity of agricultural types, developments and adoption processes. Here, we use ancient DNA from 269 dental calculus samples spanning 8,000 years to unveil the oral microbiota of 37 ancient hunter-gatherers and 232 ancient agriculturalists from around the world, including the first ancient oral microbiome data from Near Eastern individuals. We explore the compositional and functional changes in these ancient microbiomes and examine how the advent of agriculture and environmental differences contribute to microbiome differences and potentially impacted modern health. Taxonomically, we find that the hunter-gatherer samples were enriched in *Pseudoremibacter alactolyticus*, *Actinomyces israelii* and *Olsenella uli*, while agriculturalist samples were enriched in species such as *Peptostreptococcus bacterium oral taxon 113*, *Actinomyces cardiffensis* and *Streptococcus sp. DD04*. Functionally, we find an enrichment in microbial functions involved in the degradation of sugar in agriculturalists while in hunter-gatherers we find an enrichment in functions involved in the breakdown and utilization of collagen based proteins. Furthermore, we find oral microbiomes from both ancient hunter-gatherer and agriculturalist samples can be classified into groups dominated by specific bacterial species. For example, ancient hunter-gatherer microbiota was divided into three compositional types dominated by *Pseudoremibacter alactolyticus, Methanobrevibacter oralis* or *Streptococcus oralis*, while the ancient agriculturalist microbiota was divided into two groups dominated by either *M. oralis* or *S. oralis*. This suggests that while diet may drive the functional differences between ancient hunter-gatherer and agriculturalist microbiome, ancient taxonomic composition is likely dominated by other factors, including microbial ecology in the oral cavity, host genetics, oral hygiene, and/or environment. These results will be of great value to understanding how disease, changes in human culture, and adaptation to unique environments impact our microbiome, and will further enrich our understanding of human prehistory.

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Introduction

The human oral microbiota is an important aspect of human health and disease. The oral microbiota is a complex community that includes over 700 diverse bacterial species (Aas, Paster, Stokes, Olsen, & Dewhirst, 2005). These microbes are linked to the human immune and endocrine systems (E.R. Herrero et al. 2018), where they can communicate with the host (Cugini, Klepac-Ceraj, Rackaityte, Riggs, & Davey, 2013). A majority of these microbes are commensal symbionts, such as *Gemella* and *Granulicatella* (Aas et al., 2005), while others, such as *Porphyromonas gingivalis*, are pathobionts – microbes which normally exist as commensal symbionts but become pathogenic when microbial composition shifts to a state of imbalance (Cerf-Bensussan & Gaboriau-Routhiau, 2010; Hornef, 2015). This imbalance can then cause localized oral diseases, such as periodontal disease and caries, but can also contribute to systemic diseases, such as heart disease, cancer and rheumatoid arthritis (Jia et al., 2018).

A shift towards imbalance in oral microbiota composition may occur due to numerous factors, including salivary flow rate (Gomar-Vercher, Simón-Soro, Montiel-Company, Almerich-Silla, & Mira, 2018; Proctor et al., 2018), antibiotic/antimicrobial agents, the weakening of the immune system (Starr et al., 2018), changes in oral hygiene (Belstrøm et al., 2018), host genetics (Abdul-Aziz et al. 2016; Gomez et al. 2017), shared living environment (Shaw et al., 2017), diet (Giacaman, 2017), and lifestyle (Michaud et al., 2013). Diet and lifestyle have recently dominated the focus of oral microbiome research; a number of recent studies compared the differences in oral microbiota between modern hunter-gatherers, traditional farmers (agriculturalists) and western Industrialized populations (Clemente et al., 2015; Lassalle et al., 2017; Li et al., 2014; Nasidze, Li, Quinque, Tang, & Stoneking, 2009). Contemporary hunter-gatherer populations, such as the Yanomami of Venezuela (Clemente et al., 2015), Filipino hunter gatherers (Lassalle et al., 2017), and the Batwa of Uganda (Nasidze et al. 2011; Chapter IV), have higher microbial diversity than agricultural and Industrial populations. Similarly, these populations have an oral microbiota composition that is well balanced between pathobionts and beneficial commensal bacteria, promoting oral health (Herrero et al. 2016), while in contrast, agriculturalists and Industrialists have a decrease in the abundance of beneficial commensal bacteria

and an increase in pathobionts (Lassalle et al., 2017). This suggests that the advent of subsistence based on agriculture and subsequently Industrialization impacted many aspects of human health and disease.

Although differences in subsistence strategies between populations have begun to be characterized, it is important to note that the handful of contemporary hunter-gatherer populations that have been studied are not analogous to prehistorical foragers (Crittenden & Schnorr, 2017). In order to understand how these changes have impacted the modern microbiota, we need to examine individuals who are past ancestors of modern populations. A bold new tool in unveiling the evolutionary past of the human oral microbiota is the use of ancient DNA (aDNA) in paleomicrobiome research. Recently, calcified dental plaque (calculus) was identified as an unprecedented source of ancient oral microbial DNA. While several early studies confirmed the presence of aDNA in dental calculus ((De La Fuente, Flores, and Moraga 2013; Preus et al. 2011). Adler et al. (2013) were the first to use high throughput DNA sequencing techniques to reconstruct the oral microbiota. In this study, the oral microbiota shifted in response to Agriculture, which was linked to a skeletal increase in oral disease in a small subset of European individuals. Subsequent papers have further characterized the presence of oral pathobionts in ancient dental calculus (Warinner et al. 2014; Weyrich et al. 2017). More recently, Farrer et al. (2018) used dental calculus to identify associations between microbiota composition and oral and systemic health in medieval and post medieval British individuals.

While these latest advances have allowed us to unveil the past, our understanding or oral microbiota adaptation in the past remains limited, largely due to the previous sample sizes and geographical distribution. Thus far, most studies have been limited to European continent due to ease of access and improved preservation, which has led to gaps in places such as the Near East, Asia, and Africa, where poor preservation conditions prevail but where important human cultural shifts, such as the agricultural transition, began. Furthermore, the lack of wider geographical sampling has created a gap in the understanding of the diversity of ancient hunter-gather microbiomes around the world. We hypothesize that various hunter-gatherer populations have different oral microbiota compositions, largely due to the variety of environments and diets. There is also a gap in terms of our understanding of the diversity of ancient agriculturalists.

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While Agriculture was independently discovered in various locations, the main component of most domesticated crops was their high carbohydrate content. We hypothesize that this would have resulted in selection for bacterial taxa specific to this diet (Giacaman, 2017), thereby resulting in similar levels of diversity and lack of variation in the oral microbiome composition compared to hunter-gatherers. We posit that an agriculturalist diet with a limited number of plants and animals would have decreased microbial diversity and likely selected for bacterial taxa involved in the breakdown of carbohydrate rich food. In other words, agriculturalist populations would have similar levels of diversity compared to each other, and all agriculturalist populations would have less diversity than hunter-gatherers. A number of microorganisms involved in carbohydrate metabolism are also known oral pathogens (Kaidonis & Townsend, 2016), so we expect to observe an increase in pathogenic bacteria in agriculturalists compared to hunter-gatherers. Although perhaps not obvious, there is also a scarcity of research on the oral microbiome composition of ancient agriculturalists globally, as many modern studies use Industrialized or Modernized populations as proxies for agriculturalists. However, Industrialization and Modernization likely had its own unique impacts on the oral microbiome.

Here, we characterize and compare ancient hunter-gather and agriculturalist oral microbiomes on a global scale, including rarely examined ancient African and Asian (Including Near Eastern populations and new data from ancient Europe. Alongside previously published ancient dental calculus data, this is largest dental calculus study to date in sample size (n=270) and geographical distribution (n = 14 countries with 56 unique sites). Using this unique dataset, we provide a detailed assessment on how the subsistence based on agriculture may have impacted the taxonomic and functional composition of the oral microbiota in ancient populations.

Material and Methods

Ancient dental calculus sample collection

We obtained ethics approval for this study from the University of Adelaide Human Research Ethics Committee (H-2012-108). New ancient dental calculus samples (n =136) were collected from a wide geographical distribution, including

samples from Africa (n=7), the Near East (n=22), Europe (n=80), Asia (n=21), North America (n=1) and the Pacific (n=5), spanning 7,000 years of human pre-history (Fig. 1). Supragingival dental calculus deposits were dislodged from the surface of tooth samples using a sterile dental pick. Gentle pressure was applied in parallel to the tooth surface to avoid enamel damage as previously described (Weyrich, Dobney, & Cooper, 2015). Collected fragments were then stored in sterile sealed zip bags for transportation to the ancient DNA facility at the Australian Centre for Ancient DNA (ACAD), University of Adelaide, Australia. Sample metadata, including oral health status of each individual, defined based on an assessment of skeletal remains for signs of oral and other diseases was also recorded at this stage (Table S1A).
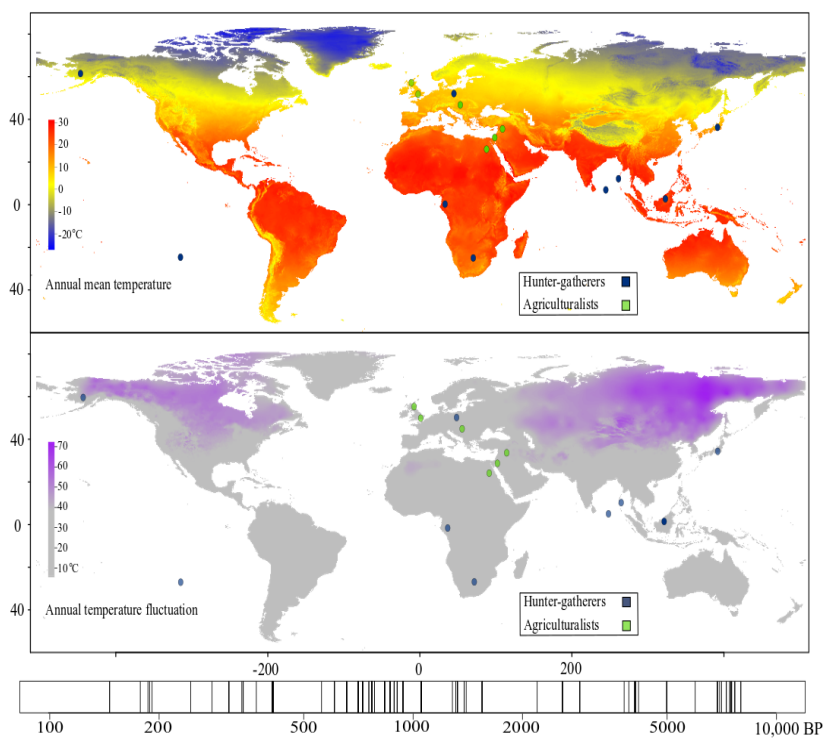
Figure 1. Location of 269 samples used in the analysis according to their global variation in mean temperature, temperature fluctuation, subsistence strategy and timeline of sample age (years BP). The sparsity of sites with annual mean temperature >20°C, reflects known preservation and sampling bias towards cooler climates.

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Ancient DNA facility and low-endogenous DNA considerations

We performed all sample processing and laboratory procedures prior to PCR amplification at the specialized ancient DNA facility at the University of Adelaide. The facility was designed to allow ancient DNA research to be performed in a low contamination environment by using positive air pressure in the general facility, as well as routine cleaning with a 3% sodium hypochlorite solution and treatment with ultraviolet light each night. To further limit the introduction of modern contaminant DNA, all experiments were performed within UV-treated, still-air hoods located in isolated, still-air rooms. All personnel accessed the facility using a dedicated single access room and wore disposable full body suits, gloves, and face masks.

DNA extraction, library preparation and sequencing

To minimize environmental contamination, we first decontaminated the dental calculus samples using UV irradiation for 15 minutes on each side, followed by soaking in two ml of 5% sodium hypochlorite for three minutes, rinsing in 90% ethanol for a minute, and drying at room temperature for two minutes. Each sample was then immediately placed into sterile plastic tubes and crushed on the side of the tube with sterile tweezers. We then extracted DNA from each individual using an in-house silica-based DNA extraction method, as previously described (Brotherton et al., 2013) but with decreased buffer volumes (1.72 ml lysis buffer (1.6 ml EDTA; 100 µl SDS; 20 µl 20 mg ml^{-1} proteinase K) and 3 ml guanidine DNA-binding buffer) (Weyrich et al., 2017). At this stage, we included two negative controls that were processed in sequence before and after the samples respectively. As environmental controls, we also separately extracted and prepared libraries for soil samples obtained from directly adjacent to the skeleton at the archaeological sites where dental calculus samples were obtained, where available (n=2; Hungary and Niger).

Extracted DNA from each sample was amplified using 16S rRNA primers (Caporaso et al., 2012) to verify the extraction of microbial DNA, assess the presence of inhibitors, and roughly assess the preservation state of each individual sample. Only DNA from samples that successfully amplified the 16S rRNA gene

as visualized on a 2.5% agarose gel using electrophoresis were used to construct shotgun metagenomic libraries (Fig. S1.).

Shotgun metagenomic libraries were then generated from the successfully extracted dental calculus samples, as previously described (Meyer & Kircher, 2010), which included using unique combinations of 7 bp forward and reverse barcodes. We used 13 cycles of PCR for the first amplification with P5/P7 barcoded adapters, followed by an additional 13 cycles for the addition of a GAII-index and sequencing primers. The constructed libraries were then purified using Ampure XP (Beckman Coulter, USA), quantified using an Agilent TapeStation (Agilent Technologies, USA), and pooled at equimolar concentrations. Samples with low DNA yield (< 2 ng/µl) were omitted from sequencing. We then sequenced the remaining libraries on the Illumina NextSeq platform (Illumina, USA) using the 2 x 150 bp configuration.

Previously published ancient dental calculus data used for comparative analysis

In addition, raw DNA sequences from previously publicly available datasets (Farrer et al. 2018; Weyrich et al. 2017) of ancient dental calculus samples from Africa (n=3) and Europe (n= 130) were downloaded as FASTQ files and processed using the same pipeline as sequenced data (Table S1A).

Bioinformatic analysis of taxonomic composition

We used Illumina's bcl2fastq software to convert the sequenced data into FASTQ file format. Data was then trimmed, demultiplexed, and merged using AdapterRemoval v2 (Schubert, Lindgreen, & Orlando, 2016) based on the unique P5/P7 barcoded adapters. Only merged reads were used to limit the addition of long DNA fragments that likely represent modern DNA contamination. Taxonomic composition was generated from sequenced data using MEGAN Alignment Tool (MALT) v 0.3.8 (Herbig et al., 2016). MALTn aligned DNA reads from samples against an in-house database created using 47,696 archaeal and bacterial genome assemblies from the NCBI Assembly database (Eisenhofer and Weyrich (In preparation 2018)). The resulting alignment based blast-text files were then converted in RMA files using the blast2rma script included with the program

MEGAN v 6.11.1 (Huson et al., 2016) with the following Last Common Ancestor (LCA) parameters: weighted-LCA=80% , minimum bitscore=42, minimum E-value=0.01, minimum support percent=0.1. The resulting RMA files were then analysed in MEGAN for downstream analyses.

We then explored the impact of modern DNA contamination within our sequenced data sets, which has been shown to influence ancient microbiota studies (Eisenhofer, Cooper, & Weyrich, 2017; Kennedy et al., 2014; Korlević et al., 2014; Salter et al., 2014). We compared biological samples versus laboratory controls using a Bray Curtis dissimilarity in a Principal Coordinates Analysis (PCoA) (Warinner et al. 2014; Weyrich et al. 2017) to assess if contamination was driving the signal without our unfiltered biological samples.

Samples were assessed for ancient DNA authenticity by two methods (1) comparison to extraction blank controls and (2) by estimation of cytosine deamination using MapDamage (Jónsson, Ginolhac, Schubert, Johnson, & Orlando, 2013). (1) In MEGAN, we used subtractive filtering to remove species found in the extraction blank controls from ancient dental calculus samples, as previously described (Weyrich, et al., 2017). Any samples with fewer than 100,000 sequences post-filtering were removed from further analysis. (2) MapDamage was used to control for cytosine deamination by using as reference a bacterial species, *Anaerolineaceae oral taxon 439*, that was found to be widely abundant in the ancient dental calculus samples. Samples that failed these two authenticity step or had less than 100,000 reads were also omitted at this stage, as it is unlikely that an accurate microbiota assemblage can be obtained with fewer sequences (Hillmann et al., 2018). Unfiltered data is provided in the Supplementary Material.

Subsequent statistical analysis was then performed using Calypso v. 8.68 (Zakrzewski et al., 2017). Filtered, species-level taxonomic composition data was exported from MEGAN as a BIOM file and then converted into Calypso V3 files using the Calypso converter. We then removed rare taxa (less than 0.01% relative abundance across all samples) and performed total sum normalisation (TSS) combined with square root transformation (Hellinger transformation) on the dataset. TSS normalises count data by dividing feature read counts by the total number of reads in each sample thereby converting raw feature counts to relative

abundance. Calypso was then used to perform alpha diversity and beta diversity analysis. Taxonomic discrimination between groups was performed on the dataset using LefSe (Segata et al. 2011). ANOSIM (Buttigieg & Ramette, 2014; CLARKE, 1993) was used within Calypso to test for statistical significance in composition between groups. In Calypso, the unsupervised grouping of samples with similar community composition into clusters is achieved by hierarchical clustering. Core microbiome analysis was performed by defining the core microbiome as taxa found in 60% of samples in a defined group. Network analysis was generated within Calypso by first computing associations between all taxa using the Spearman's rank correlation. The resulting pairwise correlations were then converted into dissimilarities and used to ordinate nodes in a two dimensional plot by PCoA. We grouped sites into country of origin for simplicity. For comparative alpha diversity analysis between sample sites, sites with less than two samples were not included within the comparison.

Bioinformatic analysis of functional composition

Although we used the more accurate MALTn (nucleotide to nucleotide alignment) for taxonomic analysis, it is not yet able to perform functional analysis. Therefore, we a functional composition for each sample using DIAMOND v 0.9.13 (Buchfink, Xie, & Huson, 2014). DIAMOND used protein based alignment of sequenced data from samples against the NCBI nr (5th December 2017) database. The resulting alignment based DAA files were then converted into RMA files using the meganizer software tool included with the program MEGAN v 6.11.1 (Huson et al., 2016) with the following Last Common Ancestor (LCA) parameters: weighted-LCA=80% , minimum bitscore=42, minimum E-value=0.01, minimum support percent=0.01. Samples were then uploaded into MEGAN for further analysis.

Samples were again assessed for ancient DNA authenticity by comparison to extraction blank controls using the DIAMOND outputs. We took a conservative approach and used subtractive filtering to remove functions found in the extraction blank controls from ancient dental calculus samples. As damage profiles cannot yet be calculated on functional profiles without reference based mapping, this stage

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was not performed. Samples with fewer than 100,000 reads were again omitted at this stage.

We then explored both taxonomic and functional information present within the data set. We verified that taxonomic profiles reflected the results identified earlier using MALTn. Next, we explored the functional profiles relating to diet, so we extracted amino acid and carbohydrate functions at level 3 from the SEED database and exported it out of MEGAN as STAMP format files. Subsequent statistical analysis and data visualisation was performed using STAMP v. 2.1.3 (Parks, Tyson, Hugenholtz, & Beiko, 2014). Differential abundance of functions between groups was tested using a Kruskal-Wallis and Welch's T-test with Storey's FDR correction in STAMP.

Results

A robust and authentic oral microbiota composition was obtained from ancient Near Eastern and African dental calculus samples.

In this study, we extracted aDNA from dental calculus samples from the Near East, Africa, and Asia (Table S1A). As much of these areas are hot and dry and known for poor DNA preservation, we developed an assessment scheme to monitor successful DNA extractions and library preparations of poorly preserved dental calculus specimens. To assess the successful extraction of aDNA and assess the presence of inhibitors, we amplified the V4 region of the 16S ribosomal RNA gene. This was successful for 62% of extracted samples from which shotgun libraries were prepared. Following library preparation, we then selected only those that had adequate DNA concentration (> 2 ng/µl) using Agilent TapeStation. About 84% of our samples had adequate amounts of DNA and were sequenced. Following sequencing, 90% of samples were found to have adequate number of sequenced reads (>100,000 sequences). Our success rate from samples from harsh preservation environments in Africa and the Near East was 30% (Fig. S1). Overall, we were able to obtain a 47% success rate in reconstructing robust oral microbiota composition from ancient calculus samples irrespective of geographical region.

Total sequenced data analysed included previously published data was 824,313,024 reads with an average of 3,041,745 (± 825,672) reads per ancient

dental calculus sample with short average fragment lengths (average size 82 bp), as expected for ancient DNA (Table S1B). An average of 56.8% (±10.5%) of reads were taxonomically assigned using MALT, as previously observed for ancient dental calculus samples (Farrer et al. 2018) (Table S1B).

The levels of microbial diversity and microbiota composition in ancient dental calculus samples was significantly different from controls (ANOVA p < 0.05) (EBCs and associated soil samples) (Fig. S2). A majority of ancient dental calculus samples (n=270) clustered with previously published ancient dental calculus samples and were similar to them in taxonomic composition. However, one sample clustered with controls and was composed of known common laboratory and environmental taxa (Cornet et al. 2018; Goffau et al. 2018; Salter et al. 2014). This sample was removed from subsequent analysis.

A conservative approach was then taken to remove microorganisms observed in our EBCs using subtractive filtering. After removing any species present in laboratory controls, we retained an average of 97.78% of reads per sample remained, highlighting good sample preservation. Following this step, all samples were then subsampled to a minimum of 104,270 reads (Table S1B) for further diversity analyses. To our knowledge, this is the most diverse dataset of dental calculus samples to be published to date.

We used *Anaerolineaceae bacterium oral taxon 439*, an abundant taxon present with at 1,000 sequences per sample in all of our ancient dental calculus samples for DNA damage analysis using MapDamage 2.0 (Jónsson et al., 2013). Sequencing mapping to *Anaerolineaceae bacterium oral taxon 439* were characterized by short fragment sizes and deamination at the terminal ends, as expected for aDNA and distinct from modern calculus samples (Fig. S3). As previous research has shown that there are differences in microbial composition of dental calculus samples from various tooth types (Farrer et al. 2018), we examined the impact of tooth type, as well as other potential confounders to the dataset, such as tooth surface, sex, individual ages at death, and location of calculus on the gingival margin (e.g., in relationship to the cementoenamel junction), on microbial composition of the samples. Microbial composition was not significantly differentiated between samples of different sexes (ANOSIM p > 0.05) and

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individual ages (ANOSIM p > 0.05) (Fig. S4). However, we did find differences between various tooth type (ANOSIM p < 0.05), tooth surfaces (ANOSIM p < 0.05) and location of calculus on the gingival margin (LCGM) in relationship to the cementoenamel junction (ANOSIM p < 0.05) (Fig. S5). However, these explain very little variation in our dataset (R^2= 6-18%), likely due to the cross-population nature of our study design (Farrer et al. 2018). Although subsequent results were done using all tooth types, tooth surfaces and LCGM, the following results were similar to results obtained by only using molars with supragingival plaque on the lingual surface, which formed the majority of the samples in this dataset (Fig. S12).

Significant differences were observed in the oral microbiome composition of ancient hunter-gatherers from different global locations.

We first characterized the composition of the oral microbiota of ancient hunter-gatherers from six locations in our dataset to understand oral microbial diversity within global ancient hunter-gather populations (Fig. 1). The core ancient hunter-gatherer microbiota consisted of 10 shared oral microbial taxa, including *Actinomyces sp. oral taxon 414, Anaerolineaceae bacterium oral taxon 439, Bacteroidetes oral taxon 274*, as well as potentially pathogenic taxa, such as *Tannerella forsythia and Treponema denticola* (Table S2A). While overall microbial diversity measured using Shannon's diversity index was not found to be significantly different between distinct ancient hunter-gatherer populations (ANOVA p > 0.05) (Fig. 2A), the number of bacterial taxa measured as microbial richness was significantly different between groups (ANOVA p < 0.05) (Fig. 2B). Indeed, significantly different oral microbiota composition was observed between hunter-gatherer populations from different locations (PCoA of Bray Curtis distances) with 40% of the variation explained by geographic location (Adonis R^2 = 0.416; p < 0.05). Hunter-gatherer samples from South Africa had the highest diversity of microbial taxa, while hunter-gather samples from the Easter Island, Chile had the fewest number of microbial taxa. South African samples were dominated by *Actinomyces georgiae*, while the Easter Island samples were dominated by *Pseudoremibacter alactolyticus* and *Tannerella forsythia*.

When the taxonomic composition of the hunter-gatherer samples was quantitatively visualized using a hierarchical clustered taxa bar plot, we observed

that the three groups were present in samples regardless of geographical distribution (Fig. 2A&C). These three groups were dominated by three different bacterial genera: *Pseudoremibacter*, *Methanobrevibacter* and *Streptococcus.*
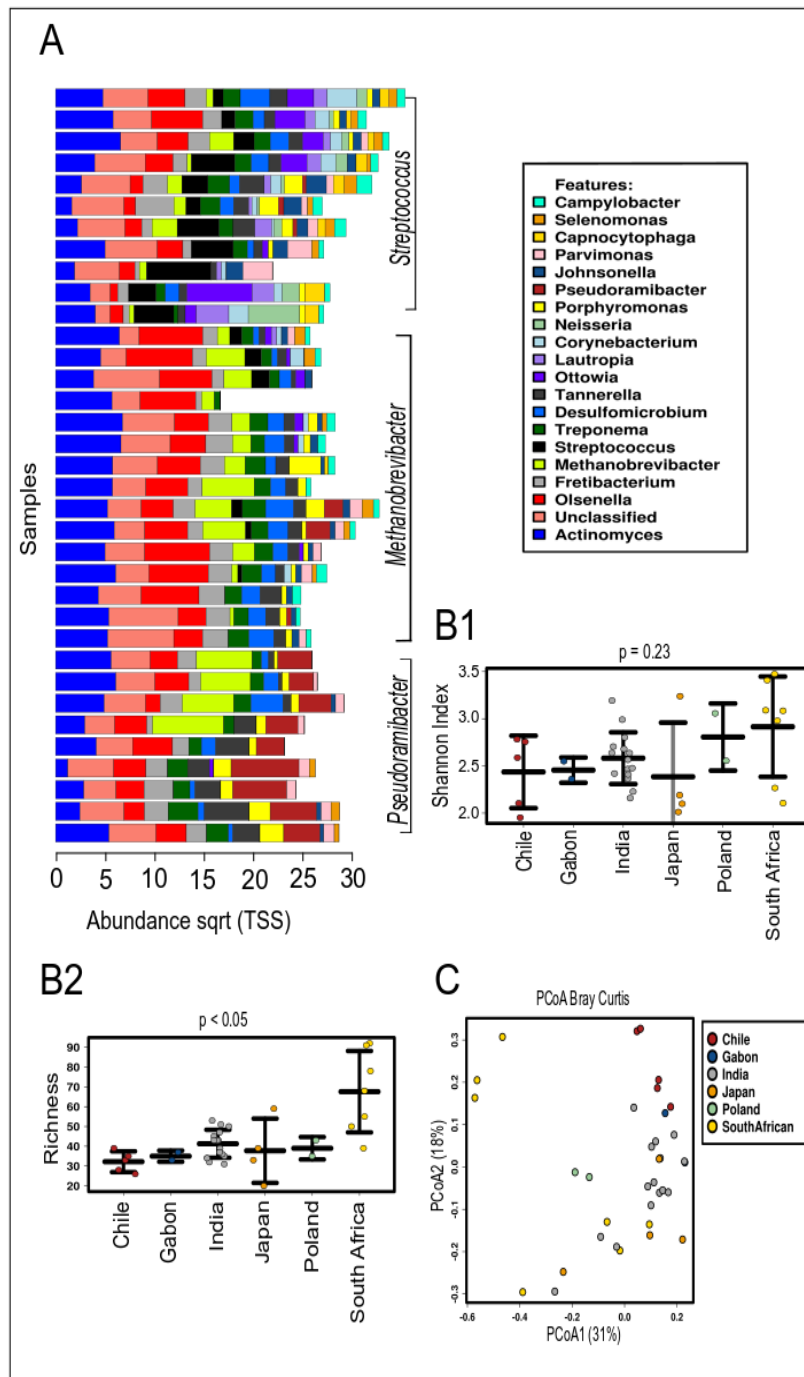
Figure 2. A. Taxa bar plot of top 20 genera in the oral microbiota of hunter-gatherers (HG) from six sampled locations. Rarefied at 104,000 sequences per sample. The hunter-gatherer oral microbiota was divided into three groups by composition,

driven by *Pseudoremibacter*, *Methanobrevibacter* and *Streptococcus*. B1. Shannon diversity index metric for the oral microbiota of Hunter-gatherers from six sampled locations. B2. Microbial richness metric for the oral microbiota of Hunter-gatherers from six sampled locations. Overall microbial diversity measured using Shannon's diversity index was not found to be significantly different between various hunter-gatherer populations (p > 0.05). However, the number of bacterial taxa, measured as microbial richness, was significantly different between groups (p < 0.05). Hunter-gatherer samples from South Africa were found to have the highest number of microbial taxa while hunter-gather samples from the Easter Islands, Chile had the lowest. C. PCoA plot based on Bray Curtis distances of oral microbial samples of hunter-gatherers from six sampled locations. Rarefied at 104,000 sequences per sample. Significantly different oral microbiome composition was observed between HG populations from various locations with 40-50% of the variation explained by geographic location (ANOSIM p < 0.05).

We then explored the microbial composition that defined these three groups using a network analysis for identifying co-occurring bacteria. This revealed that bacterial taxa in the *Methanobrevibacter* group included species such as *Olsenella uli* and *Peptostreptococceae bacterium oral taxon 113* that occurred together significantly more often in this group compared to the *Streptococcus* group. The group dominated by *Streptococcus* had co-occurring taxa that included *Lautropia mirabilis*, *Ottowia sp. oral taxon 894*, *Leptotrichia sp. oral taxon 212*, and *Corynebacterium matruchotti*. The third distinct group, dominated by *Pseudoremibacter*, formed a third group that fell between the first two groups (Fig. 2) and co-occurred with *Slackia exigua, Porphyromonas gingivalis and Treponema maltophilum* at the species level (Fig. S6). Whilst the *Methanobrevibacter* and *Streptococcus* groups were previously observed (Farrer et al. 2016), this was the first time the *Pseudoremibacter* group has been described.

We also explored levels of oral microbial pathogens within the six hunter-gatherer populations. We found significantly different abundances in opportunistic oral pathogens, such as *Streptococcus oralis*, *Porphyromonas gingivalis* and *Tannerella forsythia*, across various populations (ANOVA p < 0.05) (Fig. S7). Hunter-gatherer samples from Easter Island, Chile had the highest abundance of *Tannerella forsythia* and *Porphyromonas gingivalis*, while South African samples had the highest abundance of *Streptococcus oralis*.

We explored the functional diversity between the six hunter-gatherer populations but found no significant functional differences between locations, which suggests that the hunter-gatherer microbiota was functionally conserved

(Kruskal-Wallis, Storey's FDR corrected p-value > 0.05). Functions such as N-Acetyl-Galactosamine and Galactosamine Utilization, Maltose and Maltodextrin Utilization and Beta-Glucoside Metabolism were found in all hunter-gatherer groups (Kruskal-Wallis, Storey's FDR corrected p-value < 0.05).

Oral microbiota in agricultural communities varies globally prior to Industrialization

We explored the oral microbiota composition of ancient agriculturalists from six locations. We began by examining the core oral microbiota in agriculturalists, which consisted of 15 shared microbial taxa, including pathogens, such as *Treponema denticola* and *Tannerella forsythia* as observed with hunter-gatherers, and taxa that were specific to agriculturalists, including *Fretibacterium fastidiosum* and *Desulfomicrobium orale* (Table S2B). We then explored alpha diversity between the different sample locations. We found that both microbial diversity measured using Shannon's diversity index and number of microbial taxa measured as microbial richness significantly varied between different agricultural populations (ANOVA p < 0.05) (Fig. 3B). Contrary to hunter-gatherers, we observed that microbial composition in agriculturalists was not differentiated by location using Bray Curtis dissimilarity visualized on a PCoA plot (ANOSIM p > 0.05). However, we found that agriculturalist samples were divided into two groups based on their composition, rather than the three found in hunter-gatherers. This was further analysed using a hierarchical clustered taxa bar plot, where we observed that the first group was dominated by *Methanobrevibacter* and the second was dominated by *Streptococcus* (Fig. 3A&C).
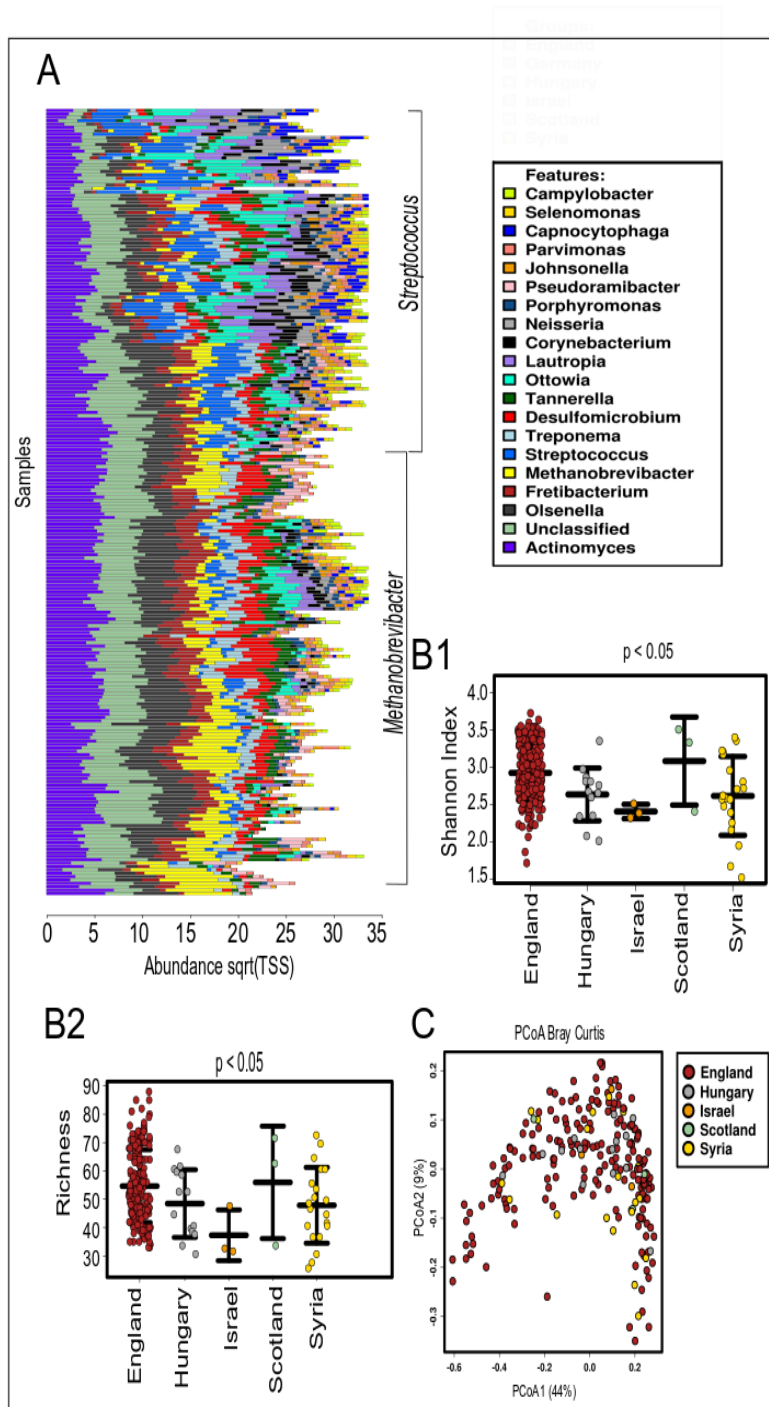
Figure 3. A. Taxa bar plot of top 20 genera in the oral microbiota of agriculturalists from six sampled locations. Rarefied at 100,000 sequences per sample. Oral microbiota in agriculturalists is divided into two groups by composition, driven by *Methanobrevibacter* and *Streptococcus*. B1. Shannon diversity index metric for the oral microbiota of agriculturalists from six sampled locations B2. Microbial richness metric for the agriculturalists from six sampled locations. Both microbial diversity measured using Shannon's diversity index

and number of microbial taxa, measured as, microbial richness significantly varied between different agricultural populations (ANOVA p > 0.05). C. PCoA plot based on Bray Curtis distances of oral microbial samples of agriculturalists from six sampled locations. Rarefied at 104,000 sequences per sample. Oral microbiome composition was not significantly different between agriculturalists populations from various locations (ANOSIM p > 0.05).

These two groups were also previously observed by Farrer et al. (2018), and that data set is included in this analysis, potentially biasing our results. However, we completed the same beta-diversity analysis without the Farrer et al.'s (2018) data, and we see the same trend without its inclusion (Fig. S15). The two groups we observe were similar to the two identified in hunter-gatherers. For example, the *Methanobrevibacter* group was defined by the presence of taxa such as *Methanobrevibacter oralis*, *Olsnella sp. oral taxon 807*, *Actinomyces sp. oral taxon 414*, *Eggerthia catenaformis*, *Filifactor alocis Fretibacterium fastidiosum*, *Desulfobulbus propionicus*, while the *Streptococcus* group was defined by the presences of *Lautropia mirabilis*, *Neisseria elongata*, *Ottowia sp. oral taxon 894*, *Capnocytophaga granulosa*, *Aggregatibacter naeslundii*, *Leptotrichia sp. oral taxon 212*, *Candidatus Saccharibacetria oral taxon TM7x* and *Corynebacterium matruchotii*. The microbial composition of the two groups was then further explored using a network analysis that identified co-occurring bacteria. The *Methanobrevibacter* group observed in the agricultural samples was similar to that observed in hunter-gathers, with taxa such as *Anaerolineaceae sp. oral taxon 439*, *Olsnella sp. oral taxon 807* and *Peptostreptococceae bacterium oral taxon 113* significantly co-occurring within the *Methanobrevibacter* group. The *Streptococcus* group contains unique clusters that include species such as *Lautropia mirabilis, Ottowia sp. Oral taxon 894*, *Leptotrichia sp. Oral taxon 212* and *Corynebacterium matruchotii* (p < 0.05) (Fig. S8).

We explored levels of oral microbial pathogens between the agricultural populations, finding significant differences in the abundance of predicted pathogenic bacteria such as *Treponema denticola* and *Porphyromonas gingivalis* (ANOVA p < 0.05). We find that the abundance of predicted pathogenic taxa was highest in samples from England and Scotland while lowest in samples from Israel. Both the Hungarian and the Syrian samples fell between these two groups. (Fig. S9). It is important to note here that the English and Scottish samples were mostly

from the medieval and postmedieval age and were relatively younger than the Hungarian and Syrian samples.

In order to assess if functional differences were present in the microbiome of different agricultural populations, we explored their functional repertoire. However, we found no significant functional differences between them (Kruskal-Wallis, Storey's FDR corrected p-value > 0.05). The agriculturalist oral microbiome shared functions such as Starch degradation in Plants, Lactose & Lactate utilization, Sucrose utilization and, Galactose degradation in plants amongst all groups (Kruskal-Wallis, Storey's FDR corrected p-value < 0.05).

Significant taxonomic and functional differences exist between agriculturalists and hunter-gatherers

Following a separate analysis of the two ancient groups, we then compared the oral microbiota of both global populations of ancient hunter-gatherers and agriculturalists to explore the impacts of Agriculture on the oral microbiota further.

Taxonomically, we found that the core microbiota between hunter-gatherers and agriculturalists consisted of eleven shared genera (Table S2C) amongst which are *Methanobrevibacter*, *Tannerella*, *Camplyobacter*, *Desulfomicrobium*, *Filifactor*, *Porphyromonas*, *Johnsonella*, *Olsenella*, *Treponema*, *Actinomyce*s and *Fretibacterium.* In addition, it is important to note that while *Streptococcus* was found in 80% of agriculturalists it was only found in 50% of hunter-gatherers.

Next, we examined the total diversity between the hunter-gatherers and agriculturalists. We found that the agriculturalist oral microbiota had significantly higher overall microbial diversity (Shannon index) and number of bacterial taxa (microbial richness) than that of hunter-gatherers ($p < 0.05$) (Fig. 4B). This is in contrast to most modern comparisons between hunter-gatherers and agriculturalists (Lassalle et al., 2017; Nasidize et al., 2009). Higher diversity and microbial richness in agriculturalists was characterized by higher prevalence of low abundant genera such as *Selemonas*, *Filifactor,* and *Leptotrichia*. However, when we removed samples from medieval and post-medieval time periods (All agriculturalists) from the analysis, we find no significance between hunter-gatherers and agriculturalists ($p > 0.05$) (Fig. S14).

We then compared the oral microbial composition between the two groups using Bray Curtis similarity visualized on a PCoA plot, while we found no visible clusters between the two groups, differences between agriculturalists and hunter-gatherers were significant (ANOSIM p < 0.05) (Fig. 4C). Comparatively, the oral microbiota of hunter-gatherers was enriched with microbial species such as *Pseudoremibacter alactolyticus*, *Actinomyces israelii* and *Olsenella uli*, while the agriculturalist oral microbiota was enriched with species such as *Peptostrepotcoccus bacterium oral taxon 113*, *Actinomyces cardiffensis* and *Streptococcus sp. DD04*. (Fig. 5 & Fig. S13).

Furthermore, compositionally, we found that there were two distinct clusters dominated by either *Streptococcus* or *Methanobrevibacter* that spread across the divide in subsistence strategy between the two. Conversely, the *Pseudoremibacter* group initially observed in the hunter-gatherer samples was subsumed by the *Methanobrevibacter* group which had the same taxa co-occurring within it as described earlier (p < 0.05) (Fig. 4A). Once again, we used network analysis to identify co-occurring bacteria in the combined dataset of hunter-gatherers and agriculturalists; this analysis revealed the same bacterial taxa in the *Methanobrevibacter* group, such as *Methanobrevibacter*, *Olsnella* and *Peptostreptococceae*.
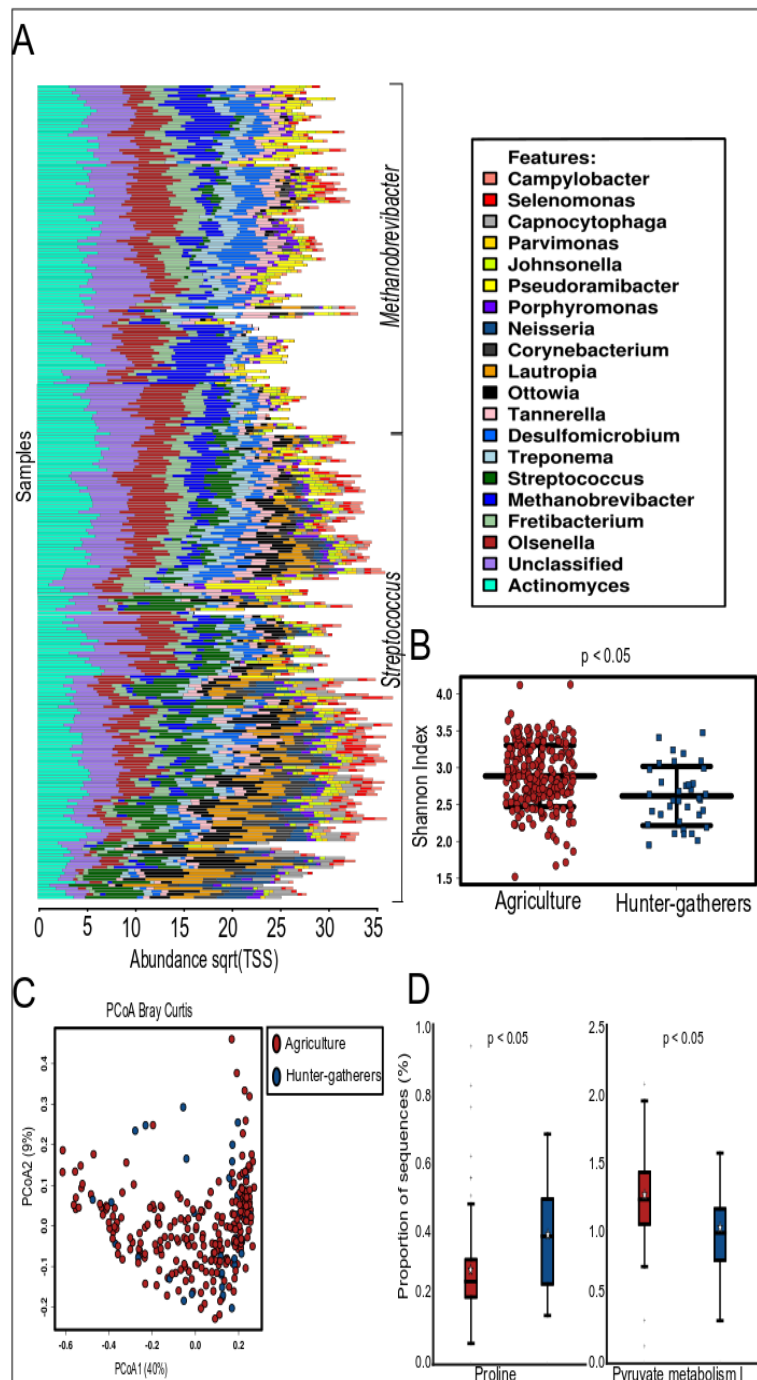
Figure 4. A. Taxa bar plot of top 20 genera in the oral microbiota of hunter-gatherers and agriculturalist. Rarefied at 104,000 sequences per sample. Oral microbiota in hunter-gatherers and agriculturalists was divided into two groups by composition, driven by *Methanobrevibacter* and *Streptococcus*. B1. Shannon diversity index metric for the oral microbiota of hunter-gatherers and agriculturalists. B2. Microbial richness metric for the oral microbiota of hunter-gatherers and agriculturalists. Agriculturalists had higher diversity and microbial richness compared to hunter-gatherers (ANOVA $p > 0.05$). C. PCoA plot based on Bray Curtis distances of oral microbial samples of hunter-gatherers and

agriculturalists. Rarefied at 104,000 sequences per sample. Significant differences were observed between agriculturalists and hunter-gatherers (ANOSIM p < 0.05). D. A comparison of amino acid and carbohydrate microbial functions between hunter-gatherers and agriculturalists using Kruskal-Wallis test and corrected with Storey's FDR. Agriculturalists were significantly enriched in metabolic functions such as Pyruvate metabolism I and II. Meanwhile, hunter-gatherers were significantly enriched in metabolic function Proline, 4-hydroxyproline uptake and utilization (p-value < 0.05).

Similarly, the *Streptococcus* group contained significantly co-occurred taxa that were observed earlier, such as *Lautropia* and *Leptotrichia* (Fig. 6). In order to understand factors driving the differences in composition observed between the *Streptococcus* group and *Methanobrevibacter* group, we tested if diet type, individual age, sample age, sex, tooth type, tooth surface, disease status and gingival site of calculus correlated with either group. However, none of these factors explained the two groups. These taxonomic results were replicated using the DIAMOND aligned data (Fig. S11).

We next explored significant functional differences between the two groups. Agriculturalists were significantly enriched in metabolic functions related to the use of plant based carbon sources for energy, such as pyruvate metabolism I and II (Muegge et al., 2011), compared to hunter-gatherers. Meanwhile, hunter-gatherer populations were significantly enriched in 4-Hydroxyproline uptake and utilization, which is related to the utilization of amino acids from a meat based diet (Wu et al., 2011) (Kruskal-Wallis, Storey's FDR corrected p-value < 0.05) (Fig. 4D). To ensure the increase in amino acid metabolism was not due to proteolytic activity as a result of disease, we explored 4-Hydroxyproline
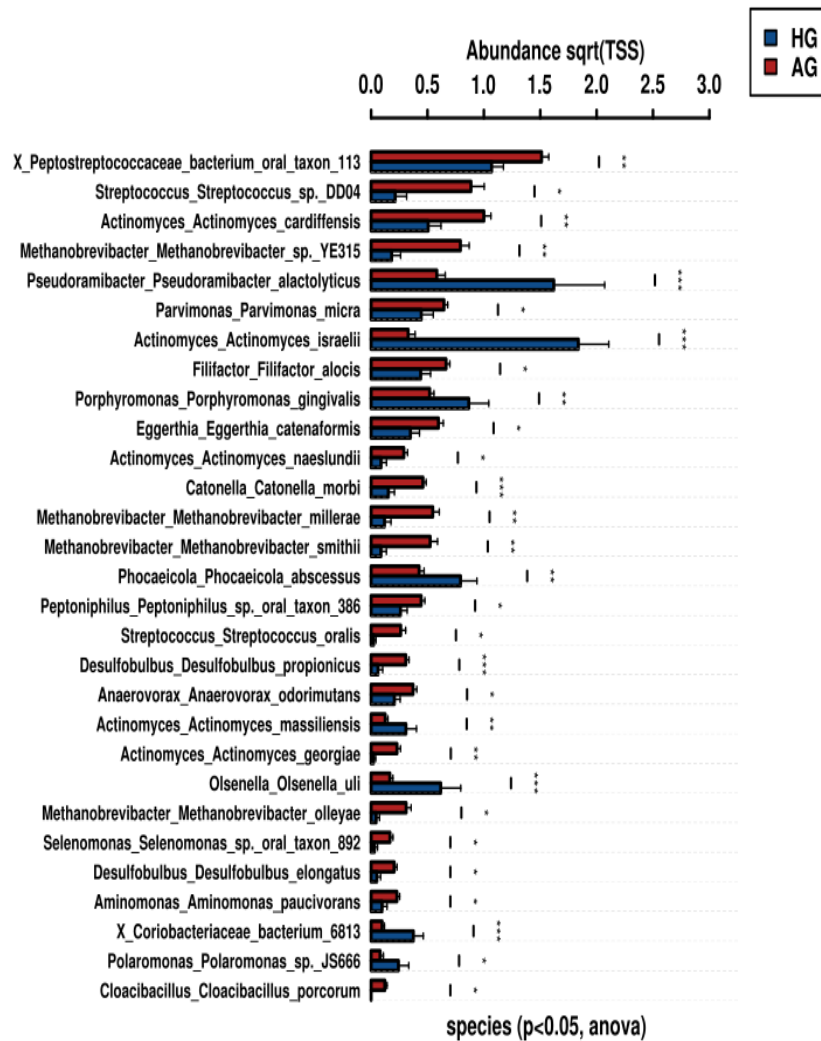
Figure 5. Microbial species with significant differences in oral microbiota composition between ancient hunter-gatherers (HG) and agriculturalist (AG) samples (ANOVA p < 0.05). Taxa such as *Pseudoremibacter alactolyticus*, *Actinomyces israelii* and *Olsenella uli* were enriched in the hunter-gatherer samples while species such as *Peptostrepotcoccus bacterium oral taxon 113*, *Actinomyces cardiffensis* and *Streptococcus sp. DD04* were enriched in the agricultural samples.

levels between all diseased and all healthy individuals in our dataset and found no significance between the two groups (Kruskal-Wallis, Storey's FDR corrected p-value > 0.05) (Fig. S10), which may suggest that 4-Hydroxyproline degradation in these samples represents dietary meat degradation rather than proteolytic activity due to disease.

We next explored the functional profiles of the two taxonomic grouping we identified in all samples (*i.e.* the taxonomic groups dominated by either *Methanobrevibacter* or *Streptococcus* species). We found that

Methanobrevibacter dominated group was significantly enriched in functions related to amino acid and protein degradation, such as valine degradation and methanogenesis (Kruskal-Wallis, Storey's FDR corrected p-value < 0.05) (Fig. 7). In contrast, the *Streptococcus* dominated group was significantly enriched in sugar based metabolic functions, such as sucrose utilization. Notably, the *Streptococcus* group was also significantly enriched in functions linked with milk-based metabolism, including lactose and lactate utilization (Kruskal-Wallis, Storey's FDR corrected p-value < 0.05). These functions were previously observed by Farrer et al. (2018) (Fig. 7) in ancient Britain, again suggesting that oral microbial functions may reflect past diets. However, these results suggest that the oral microbiome may reflect dietary differences globally across different cultural groups.

Figure 6. Network analysis of co-occurring taxa within the hunter-gatherer and agriculturalist oral microbiota. Two groups were identified, one driven by *Methanobrevibacter* and the other *Streptococcus*. Within the *Methanobrevibacter* group, taxa such as *Methanobrevibacter*, *Anaerolineaceae*, *Olsnella* and *Peptostreptococceae* occurred significant more often together and clustered away from the *Streptococcus*

group which significantly co-occurred with taxa such as *Lautropia, Fusobacterium* and *Leptotrichia*.
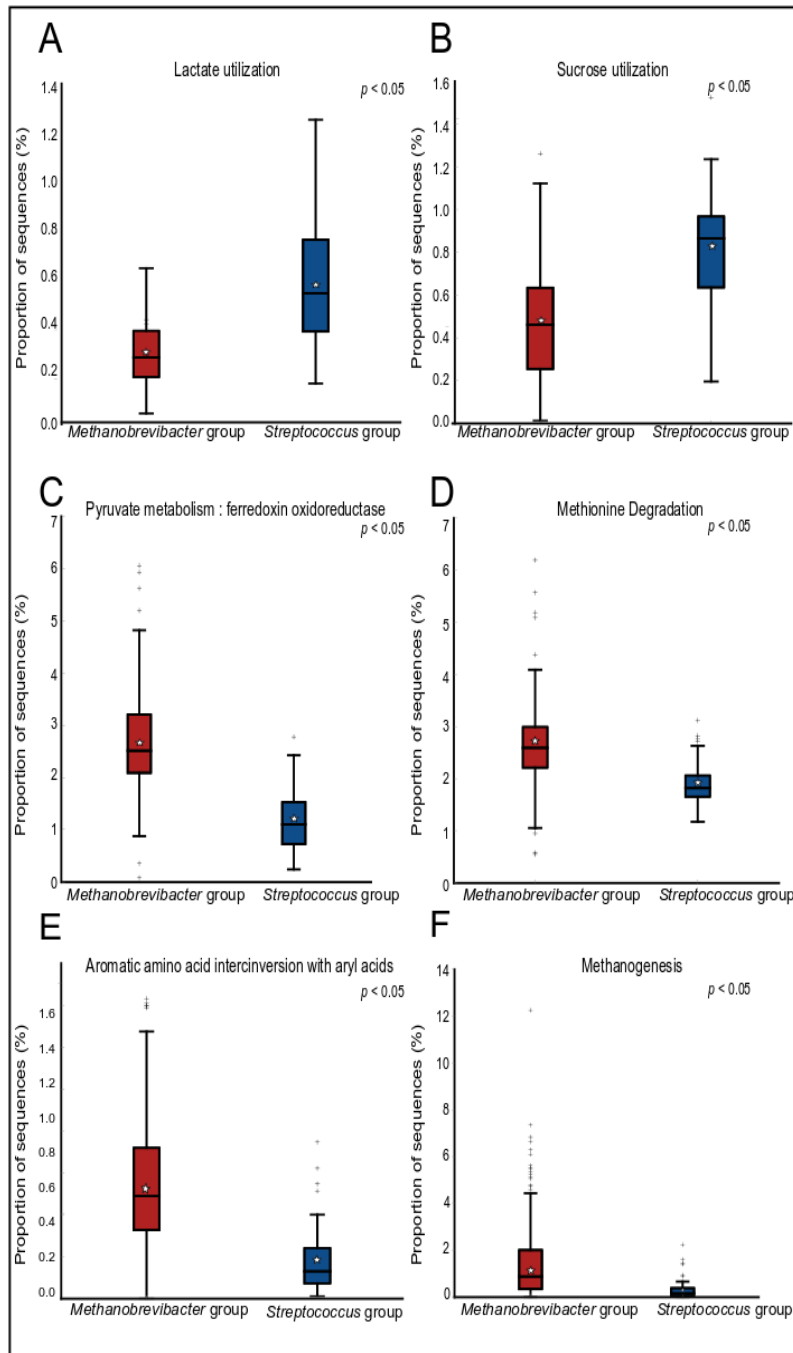
Figure 7. A comparison of amino acid and carbohydrate microbial functions between the *Methanobrevibacter* and *Streptococcus* groups using Kruskal-Wallis and corrected with Storey's FDR. The *Methanobrevibacter* group was significantly enriched in functions related to amino acid and protein degradation such as Valine degradation and Methanogenesis. While, the *Streptococcus* group was significantly enriched in Sugar based metabolic functions such as Sucrose utilization as well as being significantly enriched with milk based metabolic functions such as Lactose and Lactate utilization ($p < 0.05$).

Discussion

Here, we have reconstructed a robust and authentic oral microbiota composition of ancient hunter-gatherer and agriculturalist individuals. Using updated contaminant assessment measures, we observed distinct ecological differences in the oral microbiota, with oral microbiota composition divided into microbiota types driven by specific bacteria around which a microbial community aggregates. We also find microbial functional differences between agriculturalists and hunter-gatherers, with functions related to the breakdown of amino acids enriched in hunter-gatherers, while the agriculturalist oral microbiome was enriched in microbial functions related to uptake and utilization of starch and the breakdown of sugar.

There has been very little research in paleomicrobiology from subtropical and tropical environments, such as the Near East and Africa. This has been due to the poor preservation of ancient DNA in those environments and the increased potential for contamination due to poor preservation (Hofreiter et al., 2015; Kistler, Ware, Smith, Collins, & Allaby, 2017; Nieves-Colón et al., 2018; Wade, 2015). Therefore, controlling for contamination of crucial importance if we are to access samples from those sites. Furthermore, contamination has been an area of recent debate in paleomicrobiome research (de Goffau et al., 2018; Eisenhofer, Weyrich, Eisenhofer, & Weyrich, 2018; Salter et al., 2014; Warinner et al., 2017). To ensure we obtained a robust oral microbiota signal, crucial steps were taken to reduce both environmental and laboratory contamination. We used subtractive filtering to remove taxa found in extraction blank controls (negative controls) to ensure that the microbiome composition we reconstructed was from the oral cavity. In addition, we also collected soil samples from archaeological sites to monitor and control for potential environmental contamination. Post-sequencing, we applied a conservative approach by filtering out microbial taxa found in laboratory controls from ancient samples, as well as rare taxa (0.01% of total abundance). This was done as background contamination has been shown to impact the integrity of the microbial composition (Eisenhofer et al., 2018), and rare taxa present at abundances < 0.01% of the total may be the result of sequencing errors (Dickie, 2010).We also used improved databases and more accurate alignment algorithms such as MALTn, instead of MALTx, and improve LCA parameters within MEGAN to obtain more

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precise assignments of microbial taxa and reduce the assignment of spurious reads, thereby further reducing false positive microbial assignments within the data (Eisenhofer and Weyrich (In preparation 2018)). These methods and techniques were used to ensure an accurate reconstruction of the ancient oral microbiome composition from 269 dental calculus samples with very low risks of contamination and sequencing errors.

Notably, we found that the level of oral microbial diversity in agricultural samples was higher than hunter-gatherer populations. This is contrary to previous observations where modern hunter-gatherer groups have consistently been shown to have higher microbial diversity compared non hunter-gatherers, agriculturalists (Clemente et al., 2015; Lassalle et al., 2017; Nasidze et al., 2011). However, this may also be due to technical issues in the way samples are processed and analysed. Furthermore, it is important to note that modern agriculturalists have been previously used compare against hunter-gatherers in modern research, and in many cases, these agriculturalists have also been Industrialized to varying extents. This Industrialisation potentially through homogenisation of food supply, pesticide use and antibiotics has been shown to lower microbial diversity and increase pathogenic load (Clemente et al. 2015; Cordain et al. 2005; Gillings, Paulsen, and Tetu 2015, Abdul-Aziz et al (In preparation)). While, ancient agricultural samples used in this study would not have been influenced Industrialization, they could have maintained their oral microbiota as they transitioned to farming. Other potential explanations could be the advent of monoculture based agriculture and the large scale processing of food.

We found that the core ancient hunter-gatherer microbiota was similar to that observed in modern hunter-gatherers. These species conserved between both types of dietary strategies may represent an ancestral oral microbiota that has existed in the oral cavity (Clemente et al., 2015; Lassalle et al., 2017). We also observed that the agriculturalist and hunter-gatherer oral microbiota fell into different taxonomic groupings, when analysed individually (i.e. only hunter-gatherers or only agriculturalists.) For example, the hunter-gatherer microbiota was divided in to three groups, each dominated by either *Pseudoremibacter*, *Methanobrevibacter* and *Streptococcus*. While the *Methanobrevibacter* and *Streptococcus* groups were also observed in the agriculturalists, the

Pseudoremibacter group was not as prominent in agriculturalists and was found to collapse within the *Methanobrevibacter* group. The absence of the *Pseudoremibacter* group in agriculturalist samples, although observed in the hunter-gatherer samples may be as the result of the introduction of rare taxa through Agriculture that replace the niche inhabited by this group thereby reducing its influence or may point towards selective pressure from the other two groups due to cultural and dietary change. This additional taxonomic group in hunter-gatherers may also be the result of a number of different factors such as the ecology of the formation of calculus in the oral cavity, changing oral hygiene practices, host genetics as well as a host of yet unknown environmental factors.

Within the remaining two taxonomic groupings or compositional types, individuals segregated across the dataset irrespective of subsistence strategy or geography. Similar oral 'groups' have been found previously. For example, a similar *Streptococcus* group was described in a study that compared the saliva of modern Italian omnivore, ovo-lacto-vegetarian and vegan individuals (Hansen et al., 2018). They described the presence of three "salivary types" that divided their dataset irrespective of diet. In addition to the *Streptococcus* group, they also found two other groups, one driven by *Neisseria –Fusobacterium* and another by *Prevotella*. However, we did not find evidence of these other two groups in our ancient samples. Another recent study by Farrer et al. (2018) also explored the impact of diet on the oral microbiota of the ancient British population through time and discovered that their oral microbiota was divided into three different groups, *Streptococcus*, *Methanobrevibacter* and a modern group. The data from the Farrer et al. (2016) was also used in this study. Based on the consistence of these two findings on single populations and our own findings within wider and more diverse human populations over 7000-year span, it appears that *Streptococcus* and *Methanobrevibacter* groups are two distinct ecological communities that form the biofilm in dental plaque across global populations.

We propose that these two communities represent distinct ecological communities forming two extremes of a gradient, similar to the community recently described by Welch et al. (2016). The bacterial species that we find within each of these groups have been found to co-exist and aggregate with one another. Within the *Methanobrevibacter* group, *Methanobrevibacter* presence correlated with that of *Anaerolineaceae*. These two taxa have been found to syntrophically cooperate

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with one another (Liang et al., 2015). Functionally, the *Methanobrevibacter* group was found to be enriched in functions related to amino acid degradation which may suggest affinity with a meat based diet. Although it was present in both agriculturalist and hunter-gatherer samples. The *Methanobrevibacter* was more prevalent in hunter-gather and older samples in the dataset, which we cautiously suggest may point towards this group as being more ancient than the *Streptococcus* group. The *Streptococcus* group was found with other genera, such as *Neisseria*, *Leptotrichia*, *Capnocytophaga* and *Actinomyces* with which it has been found to coexist as biofilms forming a hedgehog structure (Welch et al. 2016).

Even though, our hunter-gatherer and agriculturalist samples did not form separate clusters based on their microbial composition, we did find differences in the abundance of specific microbial taxa between the two groups. For example, we found taxa associated with periodontal disease and dental abscess such as *Porphyromonas gingivalis* and *Olsnella uli* (enriched in hunter-gatherers), as well as *Catonella morbi* and *Eggerthia catenaformis* (enriched in agriculturalists) that indicates that periodontal disease may have been present in both cultural groups irrespective of diet (Adler et al., 2013). The large number of pathogenic taxa that we find in our results is also symptomatic of an over-representative bias towards pathogenic taxa in the NCBI and other microbial genomic databases. In addition, the oral microbiota of agriculturalists was enriched in two species of *Streptococcus* bacteria which have been associated with an increase in the intake of a diet rich in starch (Adler et al., 2013; Giacaman, 2017; Lassalle et al., 2017). This may be as a result of increased amounts of carbohydrate rich foods compared with hunter-gatherer diets. These taxonomic results reflect the findings from our functional analysis. The functional differences we found were also observed between typical animal-derived and typical plant-derived diets within the historic British population studied by Farrer et al., (2018). However, previous research that examined the salivary microbiota of vegans and non-vegans found that diet had no significant impact on taxonomic composition but they found distinct functions/metabolomes between the two groups (De Filippis et al., 2014). However, recent research has showed that the oral microbiome of individuals living a western lifestyle are similar in composition and are characterised by low microbial diversity (Clemente et al., 2015; Lassalle et al., 2017) (Chapter IV). These factors would have significant impact on their results. Furthermore, it is important to note that the study by De

Filippis et al., (2014) was preformed using saliva samples from contemporary populations within a homogenous European environment in Italy compared to our ancient data that covers diverse global locations and a heterogeneous mix of different populations.

We also tested a variety of metadata factors, including sex, oral geography, etc., and found no correlations with sample metadata and hunter-gatherers or agriculturalists. Surprisingly, the disease status of samples did not correlate with taxonomic compositions in agriculturalists or hunter-gatherers, either of the two microbiota groups observed in ancient samples. Based on the functional data, that diet may play a role in the differences between hunter-gathers and agriculturalists, and the differences in taxonomic groupings. Other factors that may drive the partitioning we observed into the two groups, include cooking practices (Gillings et al., 2015; Perry, Kistler, Kelaita, & Sams, 2015; Schnorr, Sankaranarayanan, Lewis, & Warinner, 2016), hygiene practices (Belstrøm et al., 2018) and host genetics (Gomez et al. 2017). However, the results from this study show that the influence of disease is not conclusive though it is important to note the paucity of disease information available for ancient samples. Due to the nature of the samples obtained for research in paleomicrobiology, there is a sampling bias towards diseased individuals with large deposits of dental calculus. Furthermore, as we are yet to understand the post-mortem processes that occur in the oral cavity that may result in changes to composition of the microbial community, the possibility of taphonomic bias could also impact some of our results. Finally, there are many challenges in collecting accurate metadata on samples especially when it concerns donated samples as well as the difficulty in assessing state of disease and other pathological factors due to absence of uniform standards which may result in metadata bias that might affect our conclusions.

The similarities between hunter-gatherers and agriculturalists may be due to poor definitions of past archaeological sites or cultures. It is difficult to define a past hunter-gatherer in the same way as they are characterized today. Our definition in this study is based on archaeological assessment of excavation sites where no evidence of agricultural practices has been found, but there is certainly not a single, clearly defined and agreed upon definition of what constitutes a hunter-gatherer. Modern hunter-gatherer populations are commonly defined as groups where a

substantial amount of their diet is obtained from wild natural sources. Different hunter-gatherer groups have different subsistence strategies depending on the environment and season (Bernal, Novellino, Gonzalez, & Ivan Perez, 2007; Dubois, Girard, Lapointe, & Shapiro, 2017; Speth, 1987). Many hunter-gatherer groups, such as the historical Swaiyano people of Papua New Guinea (Guddemi, 1992), also practiced short term horticulture in addition to hunting and gathering. Furthermore, modern ethnographic analysis of 229 modern hunter-gatherer groups has revealed that hunter-gatherer diets are substantially varied by environment (Ströhle and Hahn 2011). As hunter-gatherer diets depend on their environment, the wide variety of diets may explain differences observed in the level of bacterial diversity and composition we observed between sample locations. It was notable to see an increase in opportunistic pathogens such as *Streptococcus* species, associated with a diet rich, in carbohydrates in tropical South African hunter-gatherers. This is similar to what was observed in the ancient Moroccan hunter-gathers who were shown to rely on highly cariogenic wild plants, such as acorns and pine nuts for sustenance (Humphrey et al., 2014) and further underlies the diversity of the hunter-gatherer diet. Another factor that might explain our findings is change in dental hygiene; hygiene differences between the two groups may play a role in differences observed between hunter-gatherer groups due to differences in the methods used for dental hygiene or comparative absence of dental hygiene practices in one population versus another (Schnorr et al., 2016). Additionally, host genetics has recently been shown to influence oral microbiota composition by modulating the host immune system (Gensollen, Iyer, Kasper, & Blumberg, 2016; Gomez et al., 2017). The interaction between host genetics and oral microbiome is another factor that that might drive the differences we find. These results further open up interesting avenues of research to explore how host genetics, diet, environment and lifestyle interact to influence the human oral microbiome.

In this study using ancient dental calculus samples from 269 individuals sourced from diverse regions of the world spanning a period of more than 7000 years, we explored the compositional and functional differences between ancient hunter-gatherers and agriculturalists in order to examine how the transition towards agriculture had impacted the human oral microbiome and subsequently modern health. We find that significant taxonomical and functional differences exist

between the two cultural groups. Furthermore, we also discover the presence of two ecological communities dominated by *Streptococcus* and *Methanobrevibacter* irrespective of geographical location, culture based on diet or disease status of individual samples. These findings further expand our understanding of the prehistoric oral microbiome and how cultural shifts in the past may have influenced its structure and composition. However, further research involving larger sample sizes and from more diverse geographical locations not covered in this study such as South America will be required to confirm that these findings are not limited to specific geographic locations. Towards this aim, improved metadata collection and new sample processing techniques will be needed to improve access to aDNA from poorly preserved tropical and subtropical environments. To understand how these shifts have impacted the oral microbiome at the community level, we will also need to improve our current models of microbial ecology to include a more detailed framework of how various microbial taxa are organized and function in the oral cavity especially in biofilms. With these steps, we will obtain a clearer understanding of how changes in human diet, culture and lifestyle have impacted the human oral microbiome through time, which could potentially open up new avenues for healthier dietary practices in line with maintaining the diversity of human oral microbiome and the potential development of new therapies to stem the increasing tide of oral disease and metabolic diseases associated with the disruption of microbiome composition in populations around the world.

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References

1. Aas, J. A., Paster, B. J., Stokes, L. N., Olsen, I., & Dewhirst, F. E. (2005). Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology*, *43*(11), 5721–32. http://doi.org/10.1128/JCM.43.11.5721-5732.2005
2. Abdul-Aziz, M. A. M. A.-R., Cooper, A., & Weyrich, L. S. L. S. (2016). Exploring relationships between host genome and microbiome: new insights from genome-wide association studies. *Frontiers in Microbiology*, *7*(OCT), 1611. http://doi.org/10.3389/fmicb.2016.01611
3. Adler, C. J., Dobney, K., Weyrich, L. S., Kaidonis, J., Walker, A. W., Haak, W., … collected samples, J. (2013). Sequencing ancient calcified dental plaque shows changes in oral microbiota with dietary shifts of the Neolithic and Industrial revolutions Europe PMC Funders Group. *Nat Genet*, *45*(4), 450–455. http://doi.org/10.1038/ng.2536
4. Belstrøm, D., Sembler-Møller, M. L., Grande, M. A., Kirkby, N., Cotton, S. L., Paster, B. J., … Holmstrup, P. (2018). Impact of Oral Hygiene Discontinuation on Supragingival and Salivary Microbiomes. *JDR Clinical & Translational Research*, *3*(1), 57–64. http://doi.org/10.1177/2380084417723625
5. Bernal, V., Novellino, P., Gonzalez, P. N., & Ivan Perez, S. (2007). Role of Wild Plant Foods Among Late Holocene Hunter-Gatherers From Central and North Patagonia (South America): An Approach From Dental Evidence. *Am J Phys Anthro-Pol*, *133*, 1047–1059. http://doi.org/10.1002/ajpa.20638
6. Brotherton, P., Haak, W., Templeton, J., Brandt, G., Soubrier, J., Jane Adler, C., … Ziegle, J. S. (2013). Neolithic mitochondrial haplogroup H genomes and the genetic origins of Europeans. *Nature Communications*, *4*, 1764. http://doi.org/10.1038/ncomms2656
7. Buchfink, B., Xie, C., & Huson, D. H. (2014). Fast and sensitive protein alignment using DIAMOND. *Nature Methods*, *12*(1), 59–60. http://doi.org/10.1038/nmeth.3176
8. Buttigieg, P. L., & Ramette, A. (2014). A guide to statistical analysis in microbial ecology: a community-focused, living review of multivariate data analyses. *FEMS Microbiology Ecology*, *90*(3), 543–550. http://doi.org/10.1111/1574-6941.12437
9. Caporaso, J. G., Lauber, C. L., Walters, W. a, Berg-Lyons, D., Huntley, J., Fierer, N., … Knight, R. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME Journal*, *6*(8), 1621–1624. http://doi.org/10.1038/ismej.2012.8
10. Cerf-Bensussan, N., & Gaboriau-Routhiau, V. (2010). The immune system and the gut microbiota: friends or foes? *Nature Reviews Immunology*, *10*(10), 735–744. http://doi.org/10.1038/nri2850
11. CLARKE, K. R. (1993). Non-parametric multivariate analyses of changes in community structure. *Austral Ecology*, *18*(1), 117–143. http://doi.org/10.1111/j.1442-9993.1993.tb00438.x
12. Clemente, J. C., Pehrsson, E. C., Blaser, M. J., Sandhu, K., Gao, Z., Wang, B., … Dominguez-Bello, M. G. (2015). The microbiome of uncontacted Amerindians. *Science Advances*, *1*(3), e1500183–e1500183.

http://doi.org/10.1126/sciadv.1500183

13. Cordain, L., Eaton, S. B., Sebastian, A., Mann, N., Lindeberg, S., Watkins, B. A., … Brand-Miller, J. (2005). Origins and evolution of the Western diet: health implications for the 21st century. *The American Journal of Clinical Nutrition*. http://doi.org/10.1016/s0920-5861(96)00158-7

14. Cornet, L., Meunier, L., Van Vlierberghe, M., Léonard, R. R., Durieu, B., Lara, Y., … Baurain, D. (2018). Consensus assessment of the contamination level of publicly available cyanobacterial genomes. *PloS One*, *13*(7), e0200323. http://doi.org/10.1371/journal.pone.0200323

15. Crittenden, A. N., & Schnorr, S. L. (2017). Current views on hunter-gatherer nutrition and the evolution of the human diet. *American Journal of Physical Anthropology*, *162*(S63), e23148. http://doi.org/10.1002/ajpa.23148

16. Cugini, C., Klepac-Ceraj, V., Rackaityte, E., Riggs, J. E., & Davey, M. E. (2013). *Porphyromonas gingivalis* : keeping the pathos out of the biont. *Journal of Oral Microbiology*, *5*(1), 19804. http://doi.org/10.3402/jom.v5i0.19804

17. De Filippis, F., Vannini, L., La Storia, A., Laghi, L., Piombino, P., Stellato, G., … Ercolini, D. (2014). The Same Microbiota and a Potentially Discriminant Metabolome in the Saliva of Omnivore, Ovo-Lacto-Vegetarian and Vegan Individuals. *PLoS ONE*, *9*(11), e112373. http://doi.org/10.1371/journal.pone.0112373

18. de Goffau, M. C., Lager, S., Salter, S. J., Wagner, J., Kronbichler, A., Charnock-Jones, D. S., … Parkhill, J. (2018). Recognizing the reagent microbiome. *Nature Microbiology*, *3*(8), 851–853. http://doi.org/10.1038/s41564-018-0202-y

19. De La Fuente, C., Flores, S., & Moraga, M. (2013). DNA from Human Ancient Bacteria: A Novel Source of Genetic Evidence from Archaeological Dental Calculus. *Archaeometry*, *55*(4), 767–778. http://doi.org/10.1111/j.1475-4754.2012.00707.x

20. Dickie, I. A. (2010). Insidious effects of sequencing errors on perceived diversity in molecular surveys. *New Phytologist*, *188*(4), 916–918. http://doi.org/10.1111/j.1469-8137.2010.03473.x

21. Dubois, G., Girard, C., Lapointe, F.-J., & Shapiro, B. J. (2017). The Inuit gut microbiome is dynamic over time and shaped by traditional foods, *5*. http://doi.org/10.1186/s40168-017-0370-7

22. Eisenhofer, R., Cooper, A., & Weyrich, L. S. (2017). Reply to Santiago-Rodriguez et al.: proper authentication of ancient DNA is essential. *FEMS Microbiology Ecology*, *93*(5). http://doi.org/10.1093/femsec/fix042

23. Eisenhofer, R., Weyrich, L., Eisenhofer, R., & Weyrich, L. S. (2018). Proper Authentication of Ancient DNA Is Still Essential. *Genes*, *9*(3), 122. http://doi.org/10.3390/genes9030122

24. Eisenhofer R, W. L. (n.d.). Assessing alignment-based taxonomic classification of ancient microbial DNA.

25. Farrer, A. (2016). Ancient DNA studies of dental calculus. The University of Adelaide.

26. Farrer, A. G., Bekvalac, J., Redfern, R., Gully, N., Dobney, K., Cooper, A., & Weyrich, L. S. (2018). Biological and cultural drivers of oral microbiota in Medieval and Post-Medieval London, UK. *BioRxiv*, 343889.

http://doi.org/10.1101/343889

27. Gensollen, T., Iyer, S. S., Kasper, D. L., & Blumberg, R. S. (2016). How colonization by microbiota in early life shapes the immune system. *Science*, *352*(6285). http://doi.org/10.1126/science.aad9378

28. Giacaman, R. (2017). Sugars and beyond. The role of sugars and the other nutrients and their potential impact on caries. *Oral Diseases*. http://doi.org/10.1111/odi.12778

29. Gillings, M. R., Paulsen, I. T., & Tetu, S. G. (2015). Ecology and Evolution of the Human Microbiota: Fire, Farming and Antibiotics. *Genes*, *6*(3), 841–57. http://doi.org/10.3390/genes6030841

30. Gomar-Vercher, S., Simón-Soro, A., Montiel-Company, J. M., Almerich-Silla, J. M., & Mira, A. (2018). Stimulated and unstimulated saliva samples have significantly different bacterial profiles. *PLOS ONE*, *13*(6), e0198021. http://doi.org/10.1371/journal.pone.0198021

31. Gomez, A., Espinoza, J. L., Harkins, D. M., Leong, P., Saffery, R., Bockmann, M., … Nelson, K. E. (2017a). Host Genetic Control of the Oral Microbiome in Health and Disease. *Cell Host & Microbe*, *22*(3), 269–278.e3. http://doi.org/10.1016/j.chom.2017.08.013

32. Gomez, A., Espinoza, J. L., Harkins, D. M., Leong, P., Saffery, R., Bockmann, M., … Nelson, K. E. (2017b). Host Genetic Control of the Oral Microbiome in Health and Disease. *Cell Host & Microbe*, *22*(3), 269–278.e3. http://doi.org/10.1016/J.CHOM.2017.08.013

33. Guddemi, P. (1992). When Horticulturalists Are like Hunter-Gatherers: The Sawiyano of Papua New Guinea. *Ethnology*, *31*(4), 303. http://doi.org/10.2307/3773422

34. Hansen, T. H., Kern, T., Bak, E. G., Kashani, A., Allin, K. H., Nielsen, T., … Pedersen, O. (2018). Impact of a vegan diet on the human salivary microbiota. *Scientific Reports*, *8*(1), 5847. http://doi.org/10.1038/s41598-018-24207-3

35. Herbig, A., Maixner, F., Bos, K. I., Zink, A., Krause, J., & Huson, D. H. (2016). *MALT: Fast alignment and analysis of metagenomic DNA sequence data applied to the Tyrolean Iceman. bioRxiv*. Cold Spring Harbor Labs Journals. Retrieved from http://biorxiv.org/content/early/2016/04/27/050559.abstract

36. Herrero, E. R., Fernandes, S., Verspecht, T., Ugarte-Berzal, E., Boon, N., Proost, P., … Teughels, W. (2018). Dysbiotic Biofilms Deregulate the Periodontal Inflammatory Response. *Journal of Dental Research*, 002203451775267. http://doi.org/10.1177/0022034517752675

37. Herrero, E. R., Slomka, V., Boon, N., Bernaerts, K., Hernandez-Sanabria, E., Quirynen, M., & Teughels, W. (2016). Dysbiosis by neutralizing commensal mediated inhibition of pathobionts. *Scientific Reports*, *6*(1), 38179. http://doi.org/10.1038/srep38179

38. Hillmann, B., Al-Ghalith, G. A., Shields-Cutler, R., Zhu, Q., Gohl, D., Beckman, K. B., … Knights, D. (2018). Evaluating the information content of shallow shotgun metagenomics. *BioRxiv*, 320986. http://doi.org/10.1101/320986

39. Hofreiter, M., Paijmans, J. L. A., Goodchild, H., Speller, C. F., Barlow, A., Fortes, G. G., … Collins, M. J. (2015). The future of ancient DNA: Technical advances and conceptual shifts. *BioEssays*, *37*(3), 284–293. http://doi.org/10.1002/bies.201400160

40. Hornef, M. (2015). Pathogens, Commensal Symbionts, and Pathobionts: Discovery and Functional Effects on the Host. *ILAR Journal*, *56*(2), 159–162. http://doi.org/10.1093/ilar/ilv007

41. Humphrey, L. T., De Groote, I., Morales, J., Barton, N., Collcutt, S., Bronk Ramsey, C., & Bouzouggar, A. (2014). Earliest evidence for caries and exploitation of starchy plant foods in Pleistocene hunter-gatherers from Morocco. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(3), 954–9. http://doi.org/10.1073/pnas.1318176111

42. Huson, D. H., Beier, S., Flade, I., Górska, A., El-Hadidi, M., Mitra, S., … Tappu, R. (2016). MEGAN Community Edition - Interactive Exploration and Analysis of Large-Scale Microbiome Sequencing Data. *PLoS Computational Biology*, *12*(6), e1004957. http://doi.org/10.1371/journal.pcbi.1004957

43. Jia, G., Zhi, A., Lai, P. F. H., Wang, G., Xia, Y., Xiong, Z., … Ai, L. (2018). The oral microbiota – a mechanistic role for systemic diseases. *BDJ*, *224*(6), 447–455. http://doi.org/10.1038/sj.bdj.2018.217

44. Jónsson, H., Ginolhac, A., Schubert, M., Johnson, P. L. F. F., & Orlando, L. (2013). MapDamage2.0: Fast approximate Bayesian estimates of ancient DNA damage parameters. *Bioinformatics*, *29*(13), 1682–4. http://doi.org/10.1093/bioinformatics/btt193

45. Kaidonis, J., & Townsend, G. (2016). The "sialo–microbial–dental complex" in oral health and disease. *Annals of Anatomy*, *203*, 85–89. http://doi.org/10.1016/j.aanat.2015.02.002

46. Kennedy, N. A., Walker, A. W. A., Berry, S. H., Duncan, S. H. S., Farquarson, F. M., Louis, P., … Holtrop, G. (2014). The Impact of Different DNA Extraction Kits and Laboratories upon the Assessment of Human Gut Microbiota Composition by 16S rRNA Gene Sequencing. *PLOS ONE*, *9*(2), 207214. http://doi.org/10.1371/journal.pone.0088982

47. Kistler, L., Ware, R., Smith, O., Collins, M., & Allaby, R. G. (2017). A new model for ancient DNA decay based on paleogenomic meta-analysis. *Nucleic Acids Research*, *45*(11), 6310–6320. http://doi.org/10.1093/nar/gkx361

48. Korlević, P., Gerber, T., Gansauge, M.-T. M.-T., Hajdinjak, M., Nagel, S., Aximu-Petri, A., & Meyer, M. (2014). Reducing microbial and human contamination in dna extractions from ancient bones and teeth. *BioTechniques*, *59*(2), 87–93. http://doi.org/10.2144/000114320

49. Lassalle, F., Spagnoletti, M., Fumagalli, M., Shaw, L., Dyble, M., Walker, C., … Balloux, F. (2017). Oral microbiomes from hunter-gatherers and traditional farmers reveal shifts in commensal balance and pathogen load linked to diet. *Molecular Ecology*, *27*(1), 182–195. http://doi.org/10.1111/mec.14435

50. Li, J., Quinque, D., Horz, H.-P., Li, M., Rzhetskaya, M., Raff, J. A., … Stoneking, M. (2014). Comparative analysis of the human saliva microbiome from different climate zones: Alaska, Germany, and Africa. *BMC Microbiology*, *14*(1), 316. http://doi.org/10.1186/s12866-014-0316-1

51. Liang, B., Wang, L.-Y., Mbadinga, S. M., Liu, J.-F., Yang, S.-Z., Gu, J.-D., & Mu, B.-Z. (2015). Anaerolineaceae and Methanosaeta turned to be the dominant microorganisms in alkanes-dependent methanogenic culture after long-term of incubation. *AMB Express*, *5*(1), 117.

146

http://doi.org/10.1186/s13568-015-0117-4

52. Mark Welch, J. L., Rossetti, B. J., Rieken, C. W., Dewhirst, F. E., & Borisy, G. G. (2016). Biogeography of a human oral microbiome at the micron scale. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(6), E791-800. http://doi.org/10.1073/pnas.1522149113

53. Meyer, M., & Kircher, M. (2010). Illumina sequencing library preparation for highly multiplexed target capture and sequencing. *Cold Spring Harbor Protocols*, *2010*(6), pdb.prot5448. http://doi.org/10.1101/pdb.prot5448

54. Michaud, D. S., Izard, J., Rubin, Z., Johansson, I., Weiderpass, E., Tjønneland, A., … Bueno-de-Mesquita, H. B. (2013). Lifestyle, dietary factors, and antibody levels to oral bacteria in cancer-free participants of a European cohort study. *Cancer Causes & Control : CCC*, *24*(11), 1901–9. http://doi.org/10.1007/s10552-013-0265-2

55. Muegge, B. D., Kuczynski, J., Knights, D., Clemente, J. C., Gonzalez, A., Fontana, L., … Gordon, J. I. (2011). Diet drives convergence in gut microbiome functions across mammalian phylogeny and within humans. *Science (New York, N.Y.)*, *332*(6032), 970–4. http://doi.org/10.1126/science.1198719

56. Nasidze, I., Li, J., Quinque, D., Tang, K., & Stoneking, M. (2009). Global diversity in the human salivary microbiome. *Genome Research*, *19*(4), 636–43. http://doi.org/10.1101/gr.084616.108

57. Nasidze, I., Li, J., Schroeder, R., Creasey, J. L., Li, M., & Stoneking, M. (2011). High Diversity of the Saliva Microbiome in Batwa Pygmies. *PLoS ONE*, *6*(8), e23352. http://doi.org/10.1371/journal.pone.0023352

58. Nieves-Colón, M. A., Ozga, A. T., Pestle, W. J., Cucina, A., Tiesler, V., Stanton, T. W., & Stone, A. C. (2018). Comparison of two ancient DNA extraction protocols for skeletal remains from tropical environments. *American Journal of Physical Anthropology*, *166*(4), 824–836. http://doi.org/10.1002/ajpa.23472

59. Parks, D. H., Tyson, G. W., Hugenholtz, P., & Beiko, R. G. (2014). STAMP: statistical analysis of taxonomic and functional profiles. *Bioinformatics (Oxford, England)*, *30*(21), 3123–4. http://doi.org/10.1093/bioinformatics/btu494

60. Perry, G. H., Kistler, L., Kelaita, M. A., & Sams, A. J. (2015). Insights into hominin phenotypic and dietary evolution from ancient DNA sequence data. *Journal of Human Evolution*, *79*, 55–63. http://doi.org/10.1016/j.jhevol.2014.10.018

61. Proctor, D. M., Fukuyama, J. A., Loomer, P. M., Armitage, G. C., Lee, S. A., Davis, N. M., … Relman, D. A. (2018). A spatial gradient of bacterial diversity in the human oral cavity shaped by salivary flow. *Nature Communications*, *9*(1), 681. http://doi.org/10.1038/s41467-018-02900-1

62. Salter, S. S. J., Cox, M. J., Turek, E. M., Calus, S. T., Cookson, W. O., Moffatt, M. F., … Pop, M. (2014). Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biology*, *12*(1), 87. http://doi.org/10.1186/s12915-014-0087-z

63. Schnorr, S. L., Sankaranarayanan, K., Lewis, C. M., & Warinner, C. (2016). Insights into human evolution from ancient and contemporary microbiome studies. *Current Opinion in Genetics & Development*, *41*, 14–26. http://doi.org/10.1016/j.gde.2016.07.003

64. Schubert, M., Lindgreen, S., & Orlando, L. (2016). AdapterRemoval v2: rapid adapter trimming, identification, and read merging. *BMC Research Notes*, *9*(1), 88. http://doi.org/10.1186/s13104-016-1900-2

65. Shaw, L., Ribeiro, A. L. R., Levine, A. P., Pontikos, N., Balloux, F., Segal, A. W., … Smith, A. M. (2017). The Human Salivary Microbiome Is Shaped by Shared Environment Rather than Genetics: Evidence from a Large Family of Closely Related Individuals. *MBio*, *8*(5), e01237-17. http://doi.org/10.1128/mBio.01237-17

66. Speth, J. D. (1987). Early hominid subsistence strategies in seasonal habitats. *Journal of Archaeological Science*, *14*(1), 13–29. http://doi.org/10.1016/S0305-4403(87)80003-1

67. Starr, J. R., Huang, Y., Lee, K. H., Murphy, C. M., Moscicki, A.-B., Shiboski, C. H., … Paster, B. J. (2018). Oral microbiota in youth with perinatally acquired HIV infection. *Microbiome*, *6*(1), 100. http://doi.org/10.1186/s40168-018-0484-6

68. Wade, L. (2015). Breaking a tropical taboo. *Science*. http://doi.org/10.1126/science.349.6246.370

69. Warinner, C., Herbig, A., Mann, A., Fellows Yates, J. A., Weiß, C. L., Burbano, H. A., … Krause, J. (2017). A Robust Framework for Microbial Archaeology. *Annual Review of Genomics and Human Genetics*, *18*(1), 321–356. http://doi.org/10.1146/annurev-genom-091416-035526

70. Warinner, C., Matias Rodrigues, J. F., Vyas, R., Trachsel, C., Shved, N., Grossmann, J., … Cappellini, E. (2014). Pathogens and host immunity in the ancient human oral cavity. *Nature Publishing Group*, *46*. http://doi.org/10.1038/ng.2906

71. Warinner, C., Rodrigues, J. F. M., Vyas, R., Trachsel, C., Shved, N., Grossmann, J., … Cappellini, E. (2014). Pathogens and host immunity in the ancient human oral cavity. *Nature Genetics*, *46*(4), 336–44. http://doi.org/10.1038/ng.2906

72. Weyrich, L. S., Dobney, K., & Cooper, A. (2015). Ancient DNA analysis of dental calculus. *Journal of Human Evolution*, *79*, 119–124. http://doi.org/10.1016/j.jhevol.2014.06.018

73. Weyrich, L. S., Duchene, S., Soubrier, J., Arriola, L., Llamas, B., Breen, J., … Cooper, A. (2017). Neanderthal behaviour, diet, and disease inferred from ancient DNA in dental calculus. *Nature Publishing Group*, *544*. http://doi.org/10.1038/nature21674

74. Wu, G., Bazer, F. W., Burghardt, R. C., Johnson, G. A., Kim, S. W., Knabe, D. A., … Spencer, T. E. (2011). Proline and hydroxyproline metabolism: implications for animal and human nutrition. *Amino Acids*, *40*(4), 1053–63. http://doi.org/10.1007/s00726-010-0715-z

75. Wu, G. D., Compher, C., Chen, E. Z., Smith, S. A., Shah, R. D., Bittinger, K., … Lewis, J. D. (2016). Comparative metabolomics in vegans and omnivores reveal constraints on diet-dependent gut microbiota metabolite production. *Gut*, *65*(1), 63–72. http://doi.org/10.1136/gutjnl-2014-308209

76. Zakrzewski, M., Proietti, C., Ellis, J. J., Hasan, S., Brion, M.-J., Berger, B., & Krause, L. (2017). Calypso: a user-friendly web-server for mining and visualizing microbiome-environment interactions. *Bioinformatics (Oxford, England)*, *33*(5), 782–783. http://doi.org/10.1093/bioinformatics/btw725

Supplementary Material
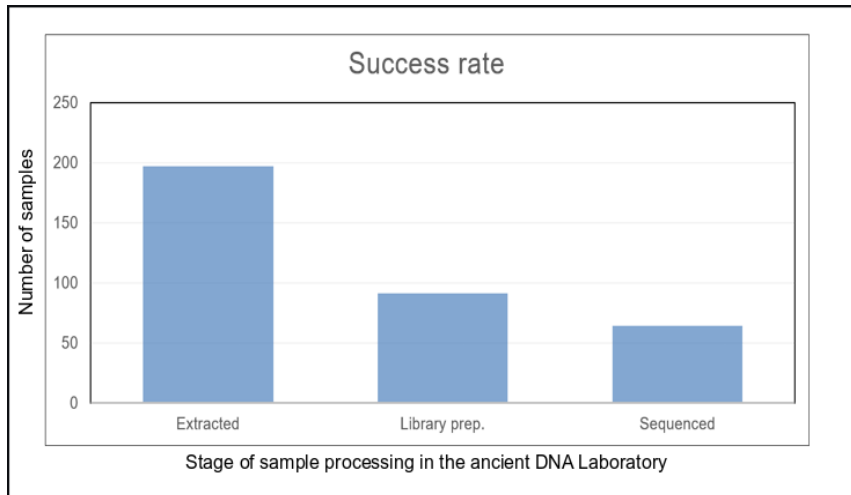
Figures

Figure S1. Success rate of samples from harsh preservation environments in Africa and the Near East at different stages of the laboratory process. Our success rate from samples from harsh preservation environments in Africa and the Near East was 30%.
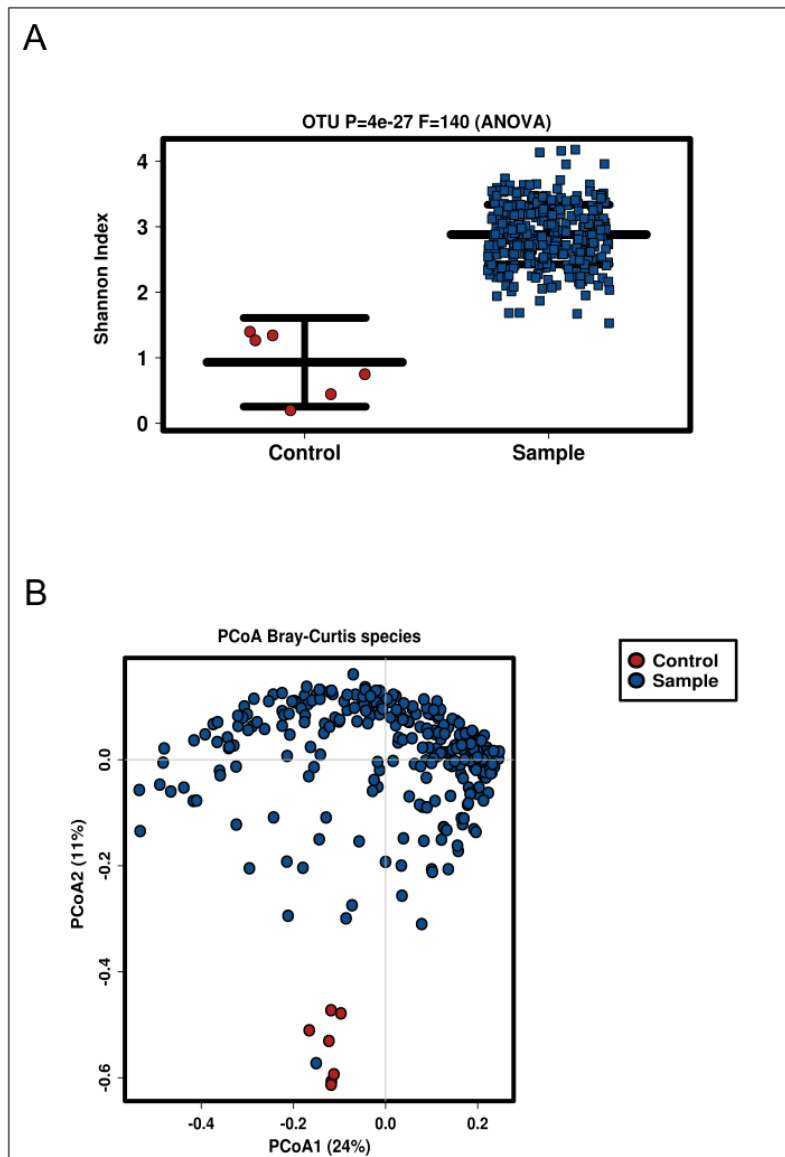
Figure S2. A. Comparison of microbial diversity between samples and controls computed using Shannon diversity index. B. Comparison of microbial composition between samples and controls based PCoA plot of on Bray-Curtis similarity. The levels of microbial diversity and microbiota composition in ancient dental calculus samples were significantly different from controls (ANOVA & ANOSIM p < 0.05).
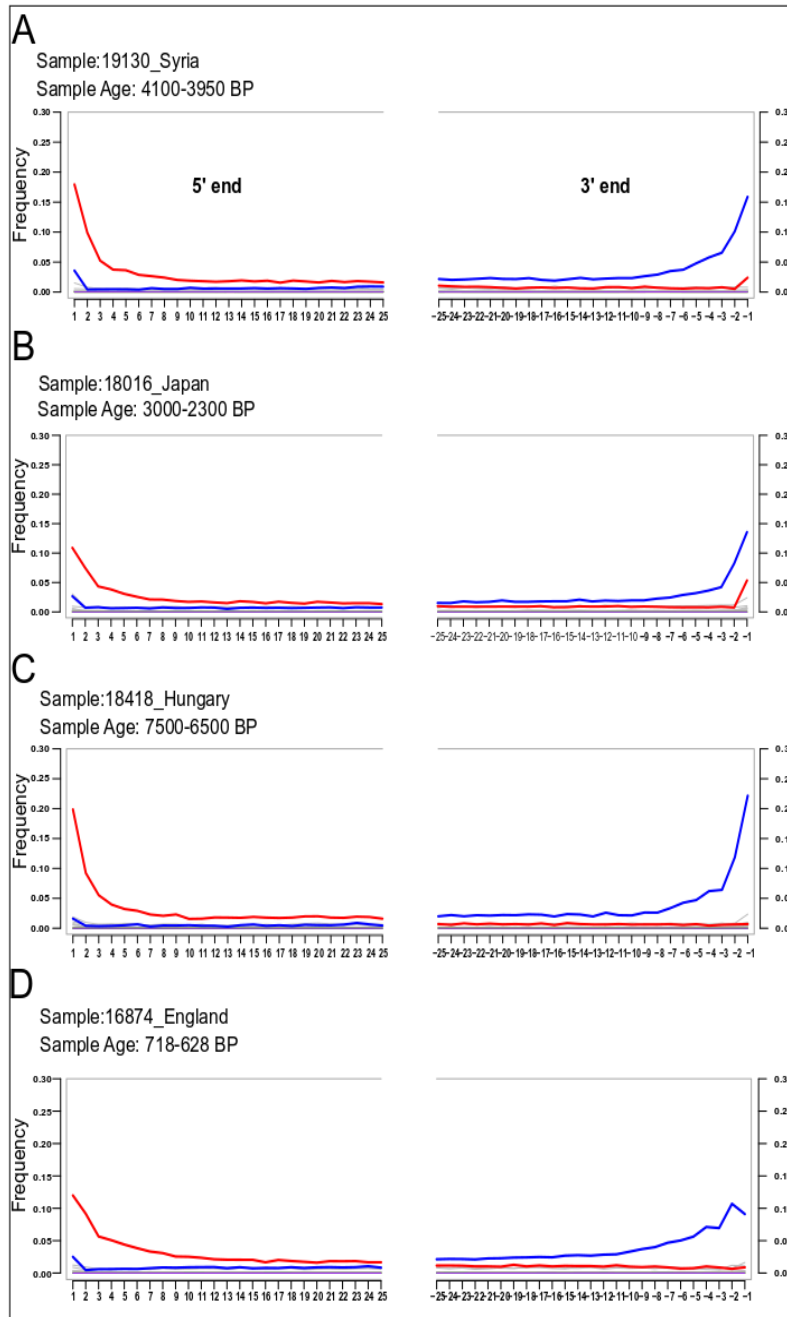
Figure S3. Map Damage plots based on Ancient DNA Analysis using *Anaerolineaceae bacterium oral taxon 439* in a range of samples from various time periods. In comparison to modern calculus samples, ancient dental calculus samples are characterized by short fragment sizes and deamination at the terminal ends, representative of aDNA (A-D). Map Damage plots provide information on age and preservation state of the sample.
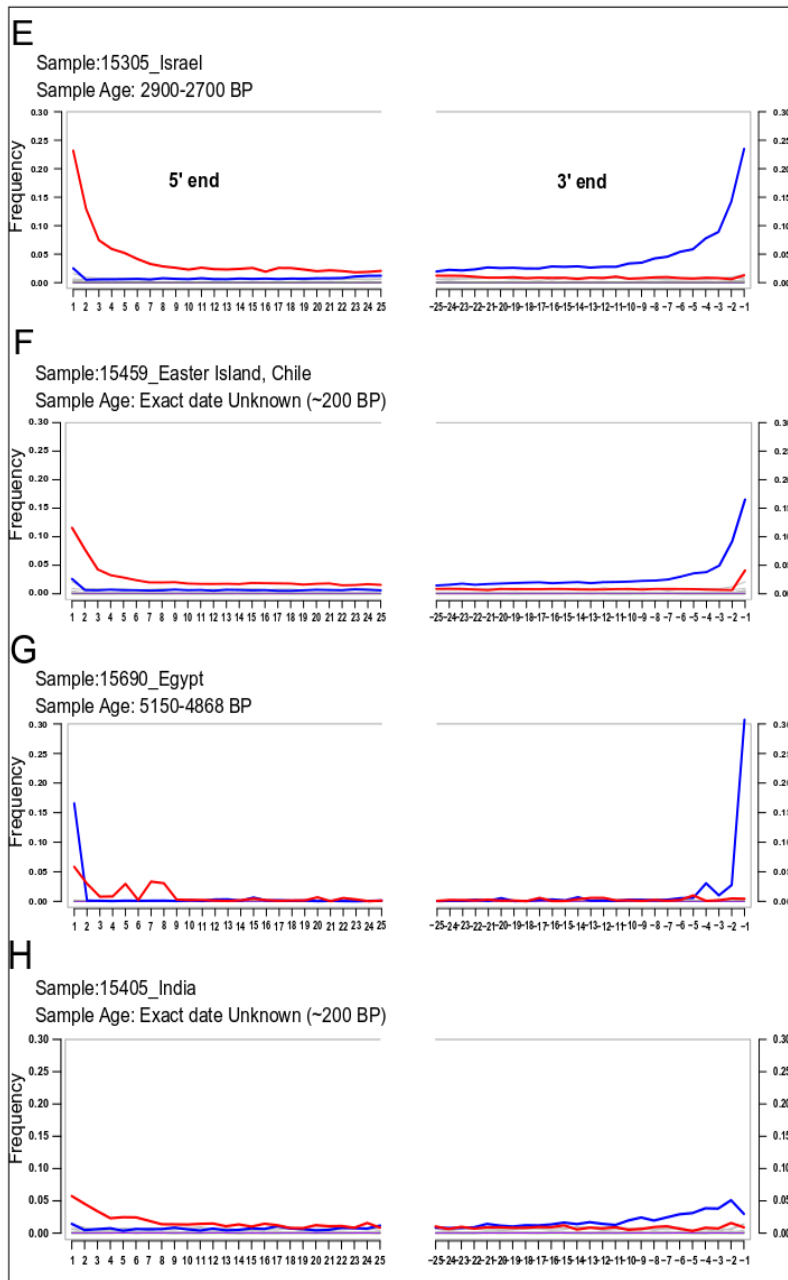
Figure S3. Map Damage plots based on Ancient DNA Analysis using *Anaerolineaceae bacterium oral taxon 439* in a range of samples from various time periods. In comparison to modern calculus samples, ancient dental calculus samples are characterized by short fragment sizes and deamination at the terminal ends, representative of aDNA (E-H). Map Damage plots provide information on age and preservation state of the sample.
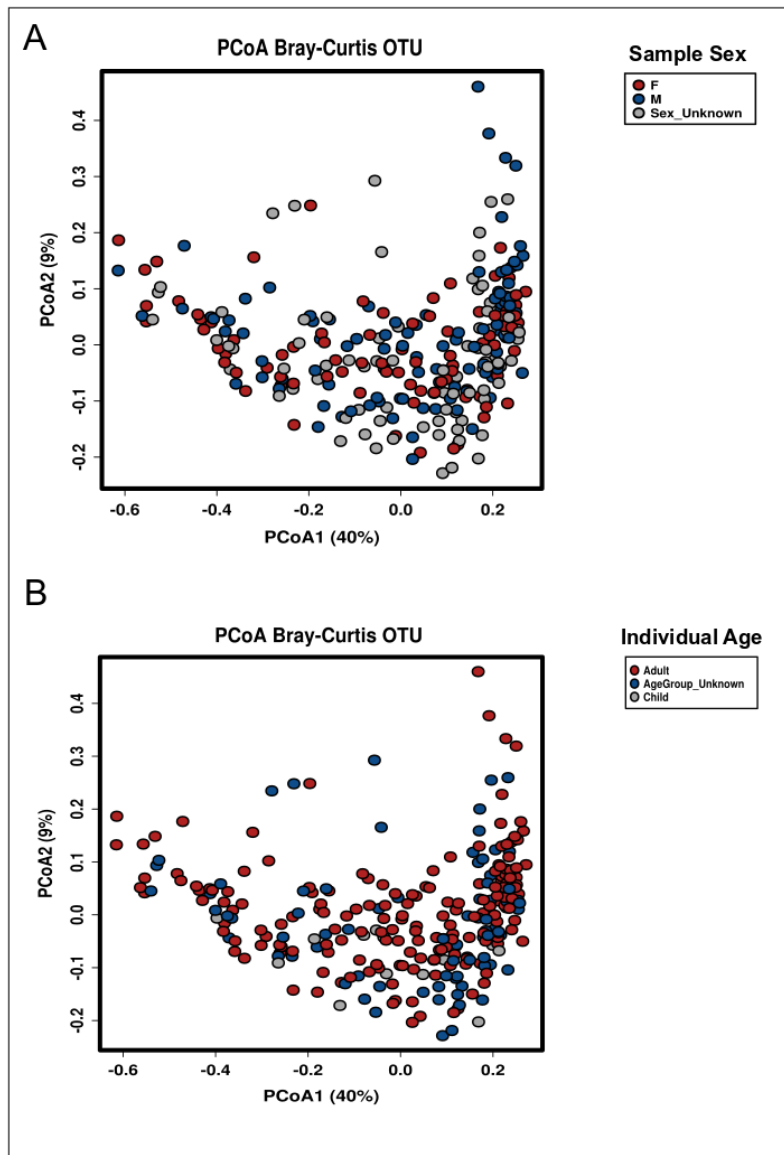
Figure S4. Impact of potential confounders on the analysis. Beta diversity, based on PCoA plot of Bray-Curtis similarities between microbial composition and sample characteristics: A. Sex and B. Individual Age. No significant clusters were observed (ANOSIM p > 0.05). Samples lacking information on Sex and Individual Age were removed to calculate statistical significance but left in these PCoA plots.
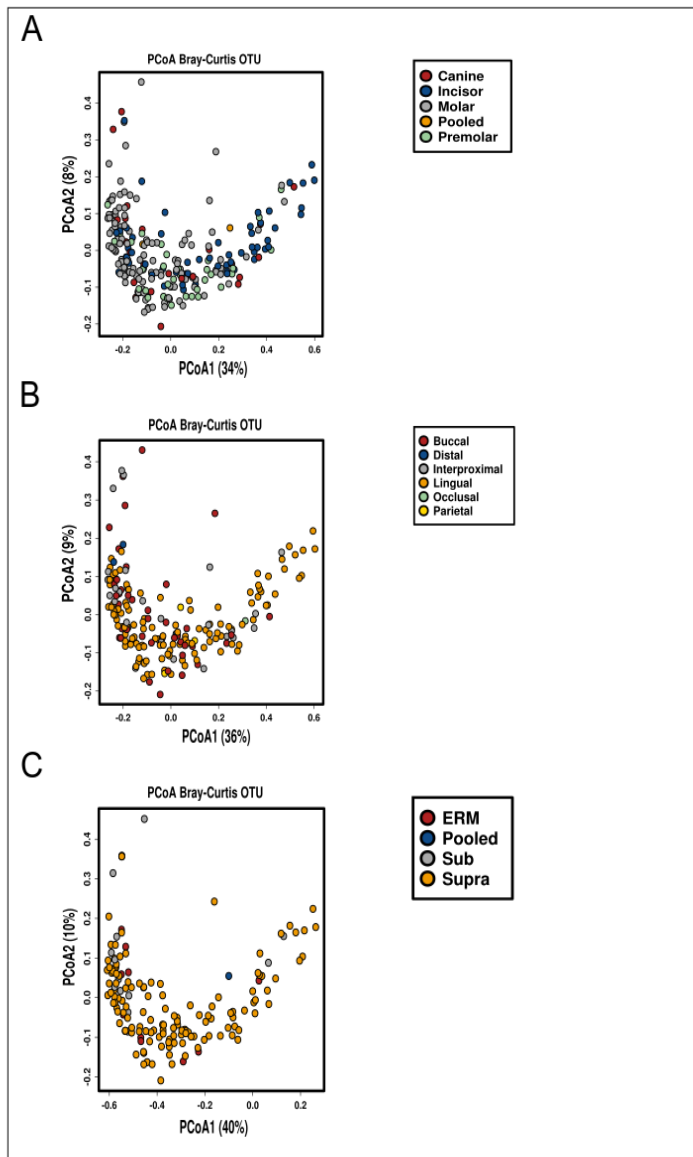
Figure S5. Impact of potential confounders on the analysis. Beta diversity, based on PCoA plot of Bray-Curtis similarities between microbial composition and sample characteristics: A. Tooth Type, B. Tooth Surface, C. Location of dental calculus on gingival margin (Supragingival/Subgingival) (LCGM). Significant clusters were observed (ANOSIM $p < 0.05$). Samples lacking information on Tooth Type, Tooth Surface and LCGM were removed from this analysis.

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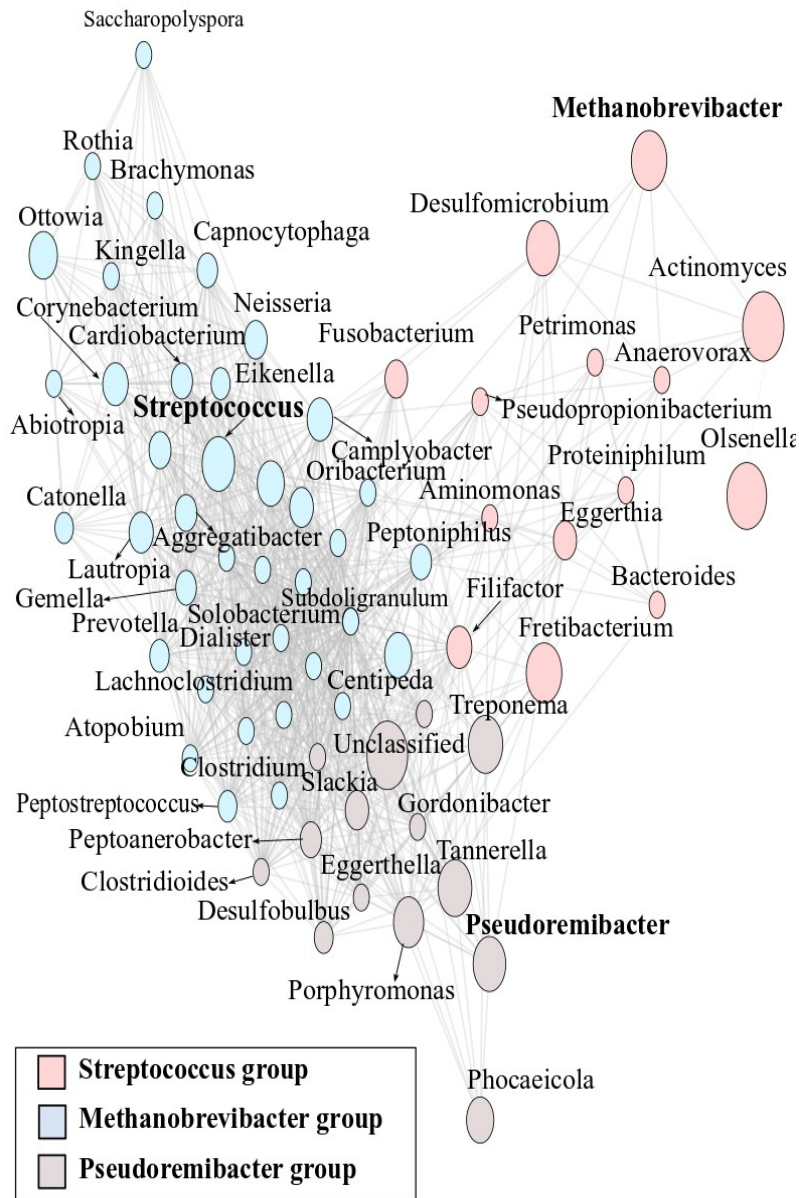
Figure S6. Network analysis of co-occurring taxa within the hunter-gatherer oral microbiota. Bacterial taxa in the *Methanobrevibacter* group such as *Anaerolineaceae*, *Olsnella* and *Peptostreptococceae* occurred significant more often together and clustered away from the *Streptococcus* group which significantly co-occurred with taxa such as *Lautropia*, *Ottowia*, *Leptotrichia* and *Corynebacterium* (Spearman's rank correlation p < 0.05). The third distinct group, the *Pseudoremibacter* group formed in between these first two groups and co-occurred with *Slackia, Porphyromonas and Treponema*.
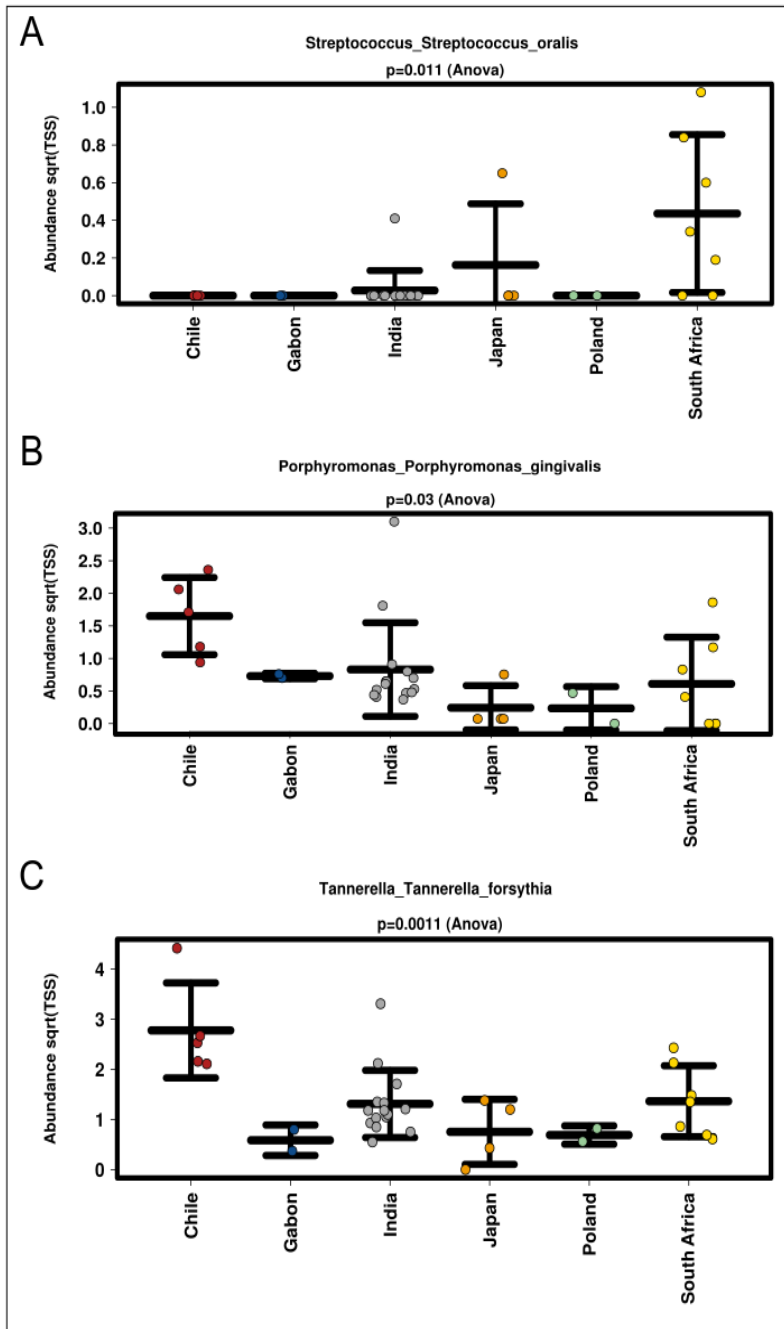
Figure S7. Predicted oral pathogens in the oral microbiota of hunter-gatherers from six sampled locations. A. *Streptococcus Oralis*, B. *Porphyromonas Gingivalis* C. *Tannerella forsythia*.
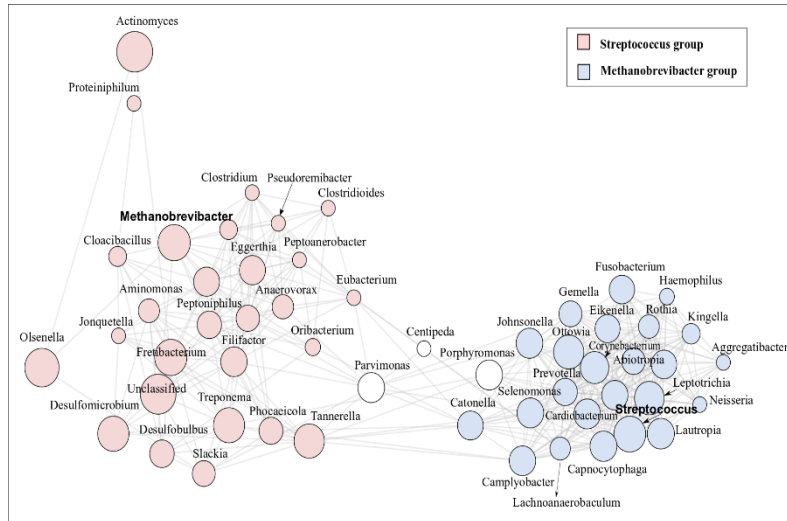
Figure S8. Network analysis of co-occurring taxa within the agriculturalist oral microbiota. *Methanobrevibacter* group observed in the agricultural samples was similar to that observed in hunter-gathers, with taxa such as *Anaerolineaceae*, *Olsnella* and *Peptostreptococceae* occurring within the group with *Methanobrevibacter* significantly more often together. The *Streptococcus* group clusters away from the first group and significantly co-occurred with taxa such as *Lautropia, Ottowia*, *Leptotrichia* and *Corynebacterium* (Spearman's rank correlation p < 0.05). Notably, we find that *Fusobacterium* co-occurred equally between the two groups.
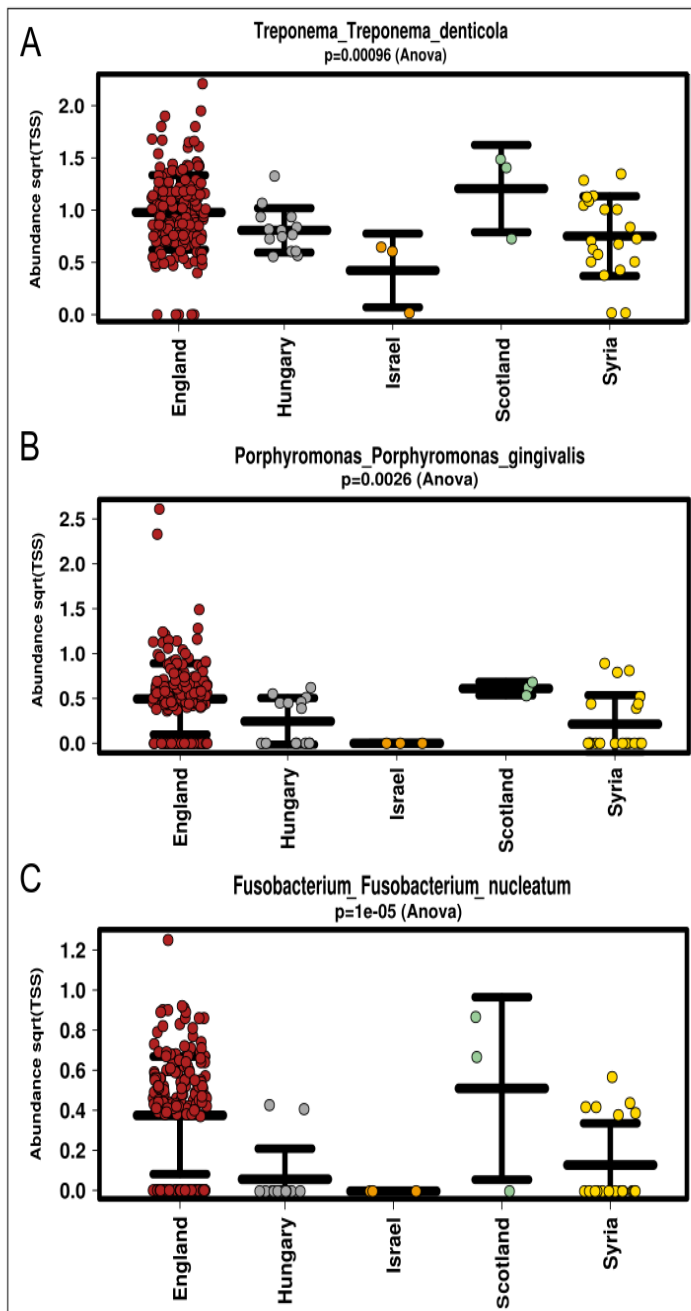
Figure S9. Predicted oral pathogens in the oral microbiota of agriculturalists from five sampled locations. A. *Treponema denticola* B. *Porphyromonas Gingivalis* C *Fusobacterium nucleatum*.
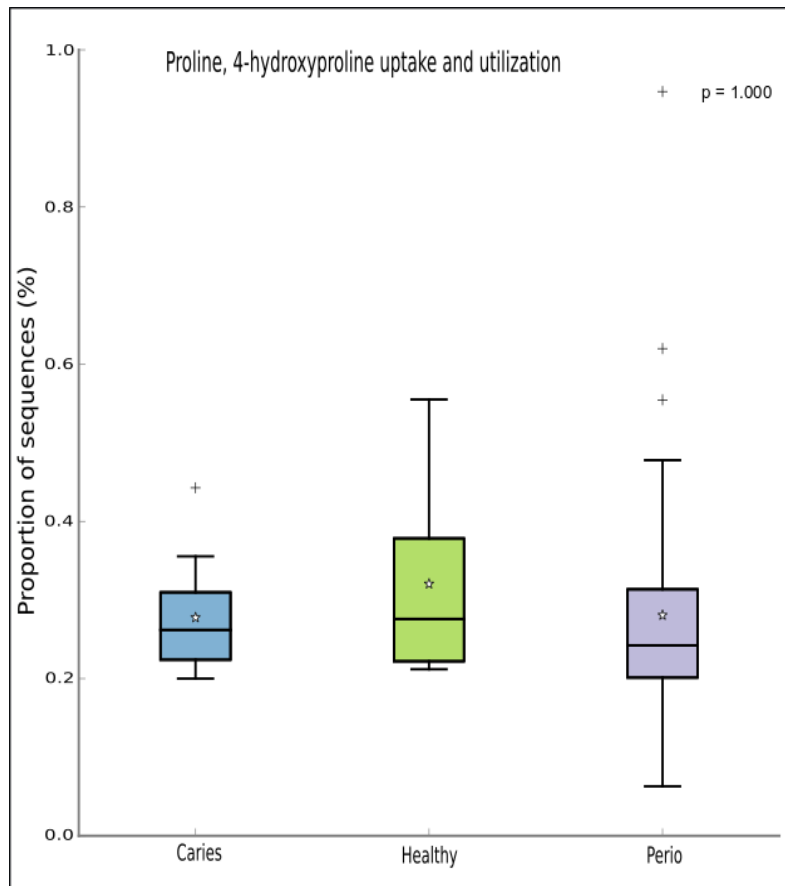
Figure S10. Comparison of the Proline levels between diseased and healthy individuals. Proline was more prevalent in healthy individuals (Kruskal-Wallis, Storey's FDR corrected p-value > 0.05).
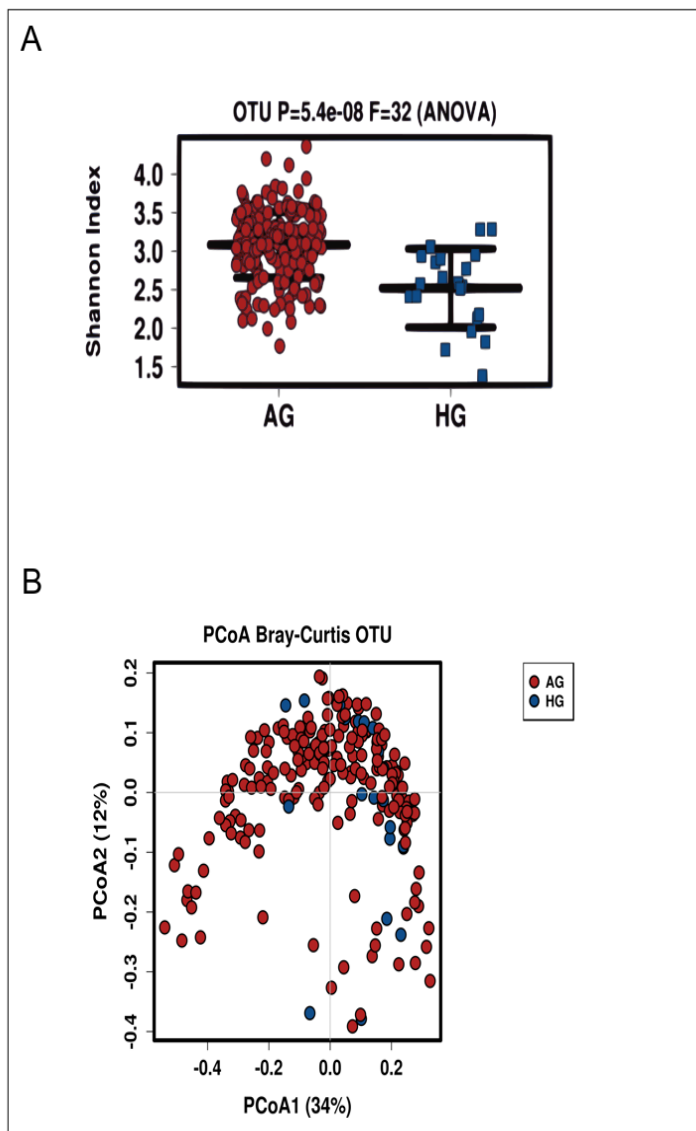
Figure S11. Confirmation that results obtained from using taxonomic data using DIAMOND is similar to that from MEGAN. A. Alpha diversity using Shannon diversity index between hunter-gatherers and agriculturalists. B. PCoA of Bray Curtis distances between the agricultural and hunter-gatherer oral microbiota. Taxonomic results from DIAMOND is similar to that of MEGAN/MALT.

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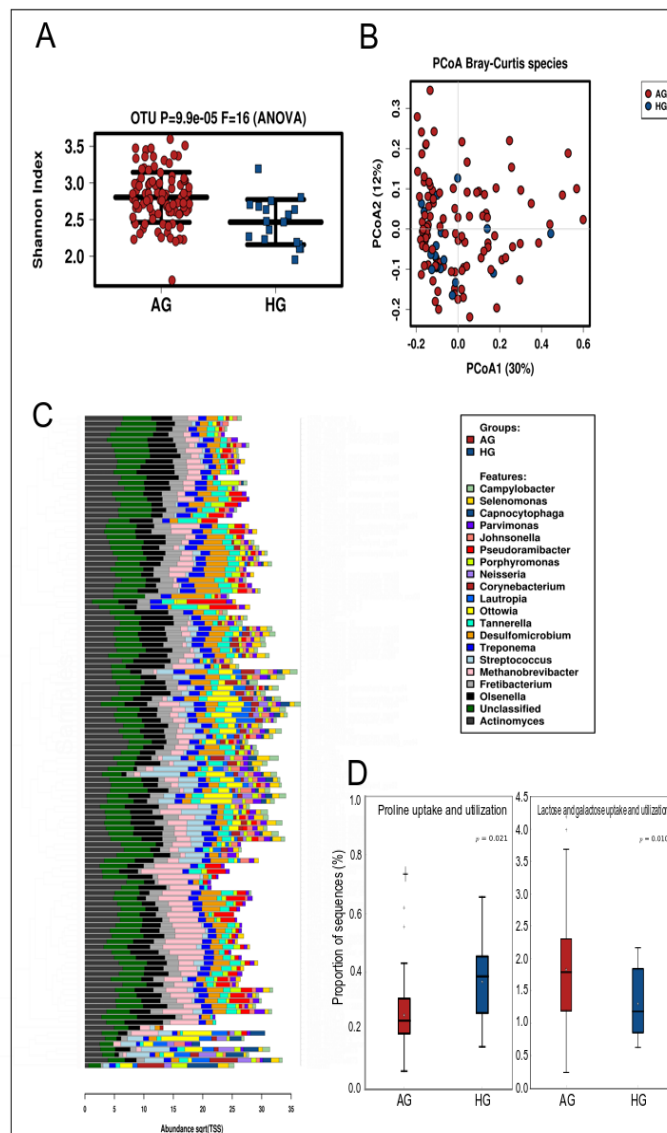
Figure S12. Analysis of microbial composition between ancient hunter-gatherers and agriculturalists using only molars (tooth type), lingual (tooth surface), supragingival plaque (LCGM). A. Alpha diversity between agricultural and hunter-gatherer oral microbiota. B. PCoA plot of Bray Curtis distances between agricultural and hunter-gatherer oral microbiota. C. Taxonomic bar plot between hunter-gatherer and agriculturalist oral microbiota. D. Functional analysis between hunter-gatherer and agricultural microbiota. Taxonomic and functional results using only one tooth type, tooth surface and LCGM is similar to results obtained combining tooth types, tooth surface and LCGM (ANOVA; $p < 0.05$)
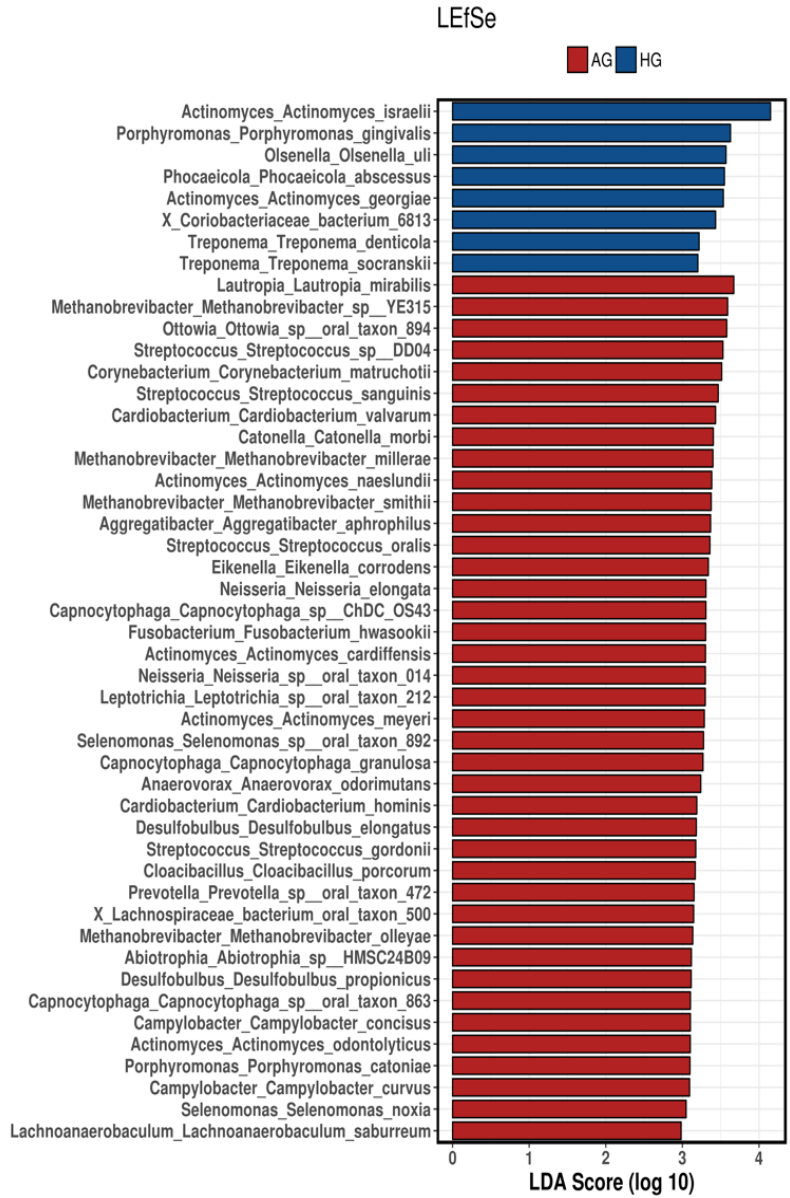
Figure S13. Top 50 most discriminative bacteria species in the oral microbiota of ancient hunter-gatherers and agriculturalists determined by LEfSe analysis.
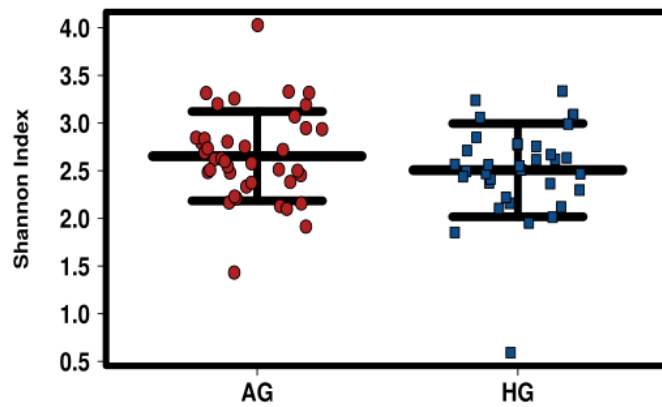
Figure S14. Shannon diversity index metric for the oral microbiota of hunter-gatherers and agriculturalists. Here we removed samples from England that were from the medieval and post-medieval age. We find no significance differences in the microbial diversity between hunter-gatherers and agriculturalist populations once these English samples are removed (ANOVA p < 0.05).
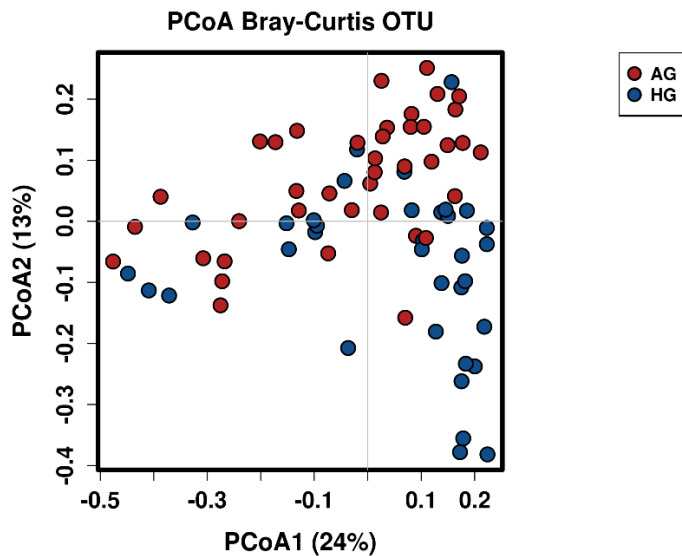
Figure S15. Confirmation of results obtained when Farrer et al (2018)'s data was excluded from the analysis is similar to Fig 4. PCoA of Bray Curtis distances between the agricultural and hunter-gatherer oral microbiota. Taxonomic results using MEGAN/MALT.

Tables

Table S1A. Metadata table of samples used in this study.

Sample Name	Group_composition	Country	ToothType	ToothSurface	LCGM	SampleAge_YBP	Sex	Age	Diseased_state	Culture	DataSource
12014_x10_Dudka_PolishHG1	NAN	Poland	NA	NA	NA	7550	NA	NA	NA	HG	Weyrich et al. 2017
12017_X10_Dudka_PolishHG2	Methano_group	Poland	NA	NA	NA	7550	NA	NA	NA	HG	Weyrich et al. 2017
12018_Syria_1	Methano_group	Syria	NA	NA	NA	1400-1000	F	20_30	NA	AG	NewData
12023_Syria_4	Methano_group	Syria	NA	NA	NA	150-50	M	40_60	NA	AG	NewData
12024_Syria_5	Strepto_group	Syria	NA	NA	NA	150-50	NA	NA	Perio	AG	NewData
12027_Syria_8	Methano_group	Syria	NA	NA	NA	1300-200	F	NA	Healthy	AG	NewData
13204_AfrPP2	NAN	SouthAfrica	NA	NA	NA	570	NA	NA	NA	HG	Weyrich et al. 2017
13208_AfrSF2	NAN	SouthAfrica	NA	NA	NA	3835	NA	NA	NA	HG	Weyrich et al. 2017
13213_AfrPP1	NAN	SouthAfrica	NA	NA	NA	740	NA	NA	NA	HG	Weyrich et al. 2017
15305p_Lachish	Methano_group	Israel	Incisor	Buccal	Supra	2900-2700	NA	NA	Perio	AG	NewData
15307p_Lachish	Methano_group	Israel	Molar	Buccal	NA	2900-2700	NA	NA	Perio	AG	NewData
15309p_Lachish	Methano_group	Israel	Incisor	Buccal	NA	2900-2700	NA	NA	Perio	AG	NewData
15340_SouthAfrica_1	Methano_group	SouthAfrica	Molar	Lingual	NA	NA	M	Adult	NA	HG	NewData
15342_SouthAfrica_3	Methano_group	SouthAfrica	Molar	Distal	Supra	NA	NA	Adult	NA	HG	NewData
15343_X10_SouthAfr_HG	Strepto_group	SouthAfrica	Premolar	Lingual	Sub	NA	M	Adult	NA	HG	NewData
15344_SouthAfrica_5	Strepto_group	SouthAfrica	Premolar	Buccal	Supra	NA	M	12_15	NA	HG	NewData
15354_Gabon_1	Methano_group	Gabon	Molar	Lingual	Sub	NA	NA	NA	NA	HG	NewData
15356_Gabon_2	Methano_group	Gabon	Molar	Lingual	Supra	NA	F	NA	NA	HG	NewData
15394_Nicobar_Islands_1	Methano_group	India	Molar	Buccal	ERM	NA	NA	NA	NA	HG	NewData
15395_Nicobar_Islands_2	Methano_group	India	Molar	Lingual	ERM	NA	NA	NA	NA	HG	NewData
15396_Andaman_4	Strepto_group	India	Molar	Lingual	ERM	NA	NA	NA	NA	HG	NewData
15397_Andaman_5	Methano_group	India	Premolar	Buccal	Sub	NA	NA	NA	NA	HG	NewData
15398_Andaman_6	Methano_group	India	Molar	Buccal	Sub	NA	NA	NA	Perio_Caries	HG	NewData
15400_Andaman_7	Methano_group	India	Molar	Lingual	ERM	NA	NA	NA	NA	HG	NewData
15401_Andaman_8	Methano_group	India	Molar	Buccal	ERM	NA	NA	NA	NA	HG	NewData
15402_Andaman_9	Methano_group	India	Molar	Distal	Supra	NA	NA	NA	Caries	HG	NewData
15403_Andaman_10	Methano_group	India	Molar	Lingual	Supra	NA	NA	NA	NA	HG	NewData
15404_Andaman_11	Methano_group	India	Premolar	Buccal	ERM	NA	NA	NA	Perio_Caries	HG	NewData
15405_Andaman_12	Methano_group	India	Premolar	Lingual	ERM	NA	NA	NA	Perio	HG	NewData
15406_Andaman_1	Methano_group	India	Molar	Buccal	ERM	5017-4317	NA	NA	NA	HG	NewData
15407_Andaman_13	Methano_group	India	Molar	Lingual	Sub	NA	NA	NA	Perio	HG	NewData
15408_Andaman_14	Methano_group	India	Incisor	Lingual	Sub	NA	NA	NA	Perio	HG	NewData
15410_Andaman_16	Methano_group	India	Molar	Lingual	NA	NA	NA	NA	NA	HG	NewData
15411_Borneo_1	Methano_group	Indonesia	Molar	Buccal	Sub	NA	NA	NA	NA	HG	NewData
15455_Easter_Island_1	Methano_group	Chile	Canine	Buccal	ERM	NA	NA	NA	Perio	HG	NewData
15457_Easter_Island	NAN	Chile	Molar	Buccal	NA	NA	NA	NA	NA	HG	NewData
15458_Easter_Island_3	Methano_group	Chile	Premolar	Buccal	Sub	NA	NA	NA	NA	HG	NewData
15459_Easter_Island_4	Cluster_wEBC	Cluster_wEl	Cluster_wE	Cluster_wEBC	Cluste	Cluster_wEBC	Clust	Cluster_	NA	Cluster_	NewData
15460_Easter_Island_5	Methano_group	Chile	Molar	Buccal	Sub	NA	NA	NA	NA	HG	NewData
15461_Easter_Island_6	Methano_group	Chile	Molar	Distal	ERM	NA	NA	NA	NA	HG	NewData
15690_AncientEgypt2	Methano_group	Egypt	Molar	Buccal	Supra	5150-4686	NA	NA	Perio	AG	NewData
16811_Medieval_SpitalSquare	Strepto_group	England	Incisor	Interproximal	Supra	738-783	F	36-45	NA	AG	Farrer et al. 2018
16812_Medieval_SpitalSquare	Strepto_group	England	Incisor	Lingual	Supra	738-783	F	18-25	NA	AG	Farrer et al. 2018
16813_Medieval_SpitalSquare	Strepto_group	England	Incisor	Interproximal	Supra	738-783	M	18-25	NA	AG	Farrer et al. 2018
16814_Medieval_SpitalSquare	Methano_group	England	Incisor	Buccal	Supra	738-783	F	>46	NA	AG	Farrer et al. 2018
16815_Medieval_SpitalSquare	Methano_group	England	Molar	Interproximal	Supra	738-783	F	26-35	NA	AG	Farrer et al. 2018
16816_Medieval_SpitalSquare	Methano_group	England	Incisor	Buccal	Supra	738-783	M	18-25	NA	AG	Farrer et al. 2018
16818_Medieval_SpitalSquare	Methano_group	England	Molar	Lingual	Supra	738-783	M	36-45	NA	AG	Farrer et al. 2018
16819_Medieval_SpitalSquare	Methano_group	England	Premolar	Interproximal	Supra	738-783	F	26-35	NA	AG	Farrer et al. 2018
16820_Medieval_SpitalSquare	Methano_group	England	Molar	Lingual	Supra	738-783	M	26-35	NA	AG	Farrer et al. 2018
16821_Medieval_SpitalSquare	Strepto_group	England	Incisor	Lingual	Supra	738-783	M	Adult	NA	AG	Farrer et al. 2018
16823_Medieval_SpitalSquare	Methano_group	England	Premolar	Interproximal	Supra	738-783	M	36-45	NA	AG	Farrer et al. 2018
16825_Medieval_SpitalSquare	Methano_group	England	Molar	Lingual	Supra	738-783	M	36-45	NA	AG	Farrer et al. 2018
16826_Medieval_SpitalSquare	Methano_group	England	Molar	Lingual	Supra	738-783	F	36-45	NA	AG	Farrer et al. 2018
16828_Medieval_SpitalSquare	Methano_group	England	Molar	Lingual	Supra	738-783	F	18-25	NA	AG	Farrer et al. 2018
16829_Medieval_SpitalSquare	Methano_group	England	Incisor	Buccal	Supra	738-783	M	36-45	Healthy	AG	Farrer et al. 2018
16830_Medieval_SpitalSquare	Strepto_group	England	Premolar	Buccal	Supra	738-783	F	36-45	NA	AG	Farrer et al. 2018
16832_Medieval_SpitalSquare	Strepto_group	England	Incisor	Buccal	Supra	738-783	F	26-35	NA	AG	Farrer et al. 2018
16833_Medieval_SpitalSquare	Methano_group	England	Molar	Lingual	Supra	738-783	M	36-45	Healthy	AG	Farrer et al. 2018
16834_Medieval_MertonPriory	Strepto_group	England	Canine	Lingual	Supra	796-718	M	26-35	Healthy	AG	Farrer et al. 2018
16835_Medieval_MertonPriory	Strepto_group	England	Incisor	Lingual	Supra	718-628	F	36-45	Perio_Caries	AG	Farrer et al. 2018
16836_Medieval_MertonPriory	Strepto_group	England	Canine	Lingual	Supra	796-718	F	36-45	Perio_Caries	AG	Farrer et al. 2018
16837_Medieval_MertonPriory	Methano_group	England	Molar	Lingual	Supra	901-796	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16838_Medieval_MertonPriory	Strepto_group	England	Incisor	Lingual	Supra	796-718	M	36-45	Perio	AG	Farrer et al. 2018
16839_Medieval_MertonPriory	Strepto_group	England	Premolar	Lingual	Supra	901-796	F	36-45	Perio	AG	Farrer et al. 2018
16840_Medieval_MertonPriory	Strepto_group	England	Canine	Lingual	Supra	901-718	M	26-35	Perio	AG	Farrer et al. 2018
16841_Medieval_MertonPriory	Methano_group	England	Incisor	Buccal	Supra	796-718	M	18-25	Caries	AG	Farrer et al. 2018
16842_Medieval_MertonPriory	Strepto_group	England	Canine	Lingual	Supra	901-796	F	36-45	Perio	AG	Farrer et al. 2018
16843_Medieval_MertonPriory	Methano_group	England	Molar	Lingual	Supra	796-718	F	26-35	Caries	AG	Farrer et al. 2018
16844_Medieval_MertonPriory	Strepto_group	England	Incisor	Interproximal	Supra	718-628	M	18-25	Perio_Caries	AG	Farrer et al. 2018
16845_Medieval_MertonPriory	Methano_group	England	Incisor	Lingual	Supra	901-796	F	36-45	Perio_Caries	AG	Farrer et al. 2018
16846_Medieval_MertonPriory	Methano_group	England	Premolar	Lingual	Supra	901-796	M	36-45	Caries	AG	Farrer et al. 2018
16847_Medieval_MertonPriory	Strepto_group	England	Incisor	Lingual	Supra	718-628	F	36-45	Perio	AG	Farrer et al. 2018
16848_Medieval_MertonPriory	Methano_group	England	Canine	Buccal	Supra	718-628	F	36-45	Perio_Caries	AG	Farrer et al. 2018

16849_Medieval_MertonPriory	Strepto_group	England	Premolar	Lingual	Supra	952-478	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16850_Medieval_MertonPriory	Methano_group	England	Molar	Interproximal	Supra	952-478	M	36-45	Healthy	AG	Farrer et al. 2018
16851_Medieval_MertonPriory	Methano_group	England	Premolar	Buccal	Supra	952-478	M	>46	Perio_Caries	AG	Farrer et al. 2018
16852_Medieval_MertonPriory	Methano_group	England	Premolar	Lingual	Supra	952-478	F	36-45	Perio	AG	Farrer et al. 2018
16853_Medieval_MertonPriory	Methano_group	England	Molar	Lingual	Supra	952-478	F	36-45	Perio	AG	Farrer et al. 2018
16854_Medieval_MertonPriory	Methano_group	England	Molar	Lingual	Supra	952-478	F	26-35	Perio	AG	Farrer et al. 2018
16855_Medieval_MertonPriory	Methano_group	England	Premolar	Lingual	Supra	952-478	F	>46	Perio	AG	Farrer et al. 2018
16856_Medieval_MertonPriory	Methano_group	England	Premolar	Lingual	Supra	952-478	F	>46	Perio	AG	Farrer et al. 2018
16857_Medieval_MertonPriory	Strepto_group	England	Canine	Lingual	Supra	952-478	M	26-35	Perio	AG	Farrer et al. 2018
16858_Medieval_MertonPriory	Methano_group	England	Incisor	Buccal	Supra	718-628	M	>46	Perio_Caries	AG	Farrer et al. 2018
16860_Medieval_MertonPriory	Strepto_group	England	Incisor	Lingual	Supra	901-796	M	26-35	Perio_Caries	AG	Farrer et al. 2018
16861_Medieval_MertonPriory	Methano_group	England	Molar	Lingual	Supra	796-718	F	36-45	Perio_Caries	AG	Farrer et al. 2018
16862_Medieval_MertonPriory	Strepto_group	England	Premolar	Lingual	Supra	796-718	F	36-45	Perio_Caries	AG	Farrer et al. 2018
16863_Medieval_MertonPriory	Methano_group	England	Canine	Interproximal	Supra	901-480	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16864_Medieval_MertonPriory	Strepto_group	England	Molar	Buccal	Supra	901-480	F	36-45	Perio_Caries	AG	Farrer et al. 2018
16865_Medieval_MertonPriory	Strepto_group	England	Molar	Interproximal	Supra	796-718	M	>46	Perio_Caries	AG	Farrer et al. 2018
16867_Medieval_MertonPriory	Methano_group	England	Molar	Buccal	Supra	718-628	F	36-45	Perio	AG	Farrer et al. 2018
16869_Medieval_MertonPriory	Strepto_group	England	Premolar	Lingual	Supra	901-480	F	36-45	Perio_Caries	AG	Farrer et al. 2018
16871_Medieval_MertonPriory	Methano_group	England	Premolar	Lingual	Supra	718-628	M	36-45	Perio	AG	Farrer et al. 2018
16872_Medieval_MertonPriory	Methano_group	England	Premolar	Lingual	Supra	901-480	M	>46	Perio_Caries	AG	Farrer et al. 2018
16873_Medieval_MertonPriory	Strepto_group	England	Canine	Lingual	Supra	796-718	F	36-45	Caries	AG	Farrer et al. 2018
16874_Medieval_MertonPriory	Methano_group	England	Molar	Lingual	Supra	718-628	M	>46	Perio_Caries	AG	Farrer et al. 2018
16876_Medieval_MertonPriory	Methano_group	England	Molar	Lingual	Supra	796-718	M	26-35	Healthy	AG	Farrer et al. 2018
16877_Medieval_MertonPriory	Methano_group	England	Incisor	Lingual	Supra	718-628	F	36-45	Healthy	AG	Farrer et al. 2018
16878_Medieval_MertonPriory	Methano_group	England	Incisor	Lingual	Supra	718-628	M	26-35	Perio_Caries	AG	Farrer et al. 2018
16879_Medieval_MertonPriory	Methano_group	England	Incisor	Lingual	Supra	796-718	M	26-35	Perio	AG	Farrer et al. 2018
16880_Medieval_MertonPriory	Methano_group	England	Molar	Lingual	Supra	718-628	M	18-25	Perio_Caries	AG	Farrer et al. 2018
16881_Medieval_MertonPriory	Methano_group	England	Molar	Lingual	Supra	901-480	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16883_Medieval_MertonPriory	Strepto_group	England	Incisor	Lingual	Supra	901-480	F	36-45	Perio_Caries	AG	Farrer et al. 2018
16884_Medieval_MertonPriory	Methano_group	England	Molar	Lingual	Supra	718-628	F	>46	Perio	AG	Farrer et al. 2018
16885_Medieval_MertonPriory	Methano_group	England	Molar	Lingual	Supra	796-718	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16886_Medieval_MertonPriory	Strepto_group	England	Canine	Lingual	Supra	796-718	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16887_Medieval_MertonPriory	Strepto_group	England	Premolar	Lingual	Supra	901-480	F	>46	Caries	AG	Farrer et al. 2018
16888_Medieval_MertonPriory	Methano_group	England	Incisor	Lingual	Supra	718-628	F	18-25	Perio_Caries	AG	Farrer et al. 2018
16889_Medieval_MertonPriory	Methano_group	England	Molar	Lingual	Supra	901-480	F	36-45	Perio_Caries	AG	Farrer et al. 2018
16890_Medieval_MertonPriory	Methano_group	England	Molar	Lingual	Supra	796-718	F	36-45	Perio_Caries	AG	Farrer et al. 2018
16891_Medieval_MertonPriory	Methano_group	England	Molar	Lingual	Supra	718-628	F	26-35	Perio	AG	Farrer et al. 2018
16892_PostMedieval_CrossBones	Methano_group	England	Molar	Lingual	Supra	420-165	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16893_PostMedieval_CrossBones	Methano_group	England	Molar	Buccal	Supra	420-165	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16894_PostMedieval_CrossBones	Methano_group	England	Canine	Interproximal	Sub	420-165	M	>46	Healthy	AG	Farrer et al. 2018
16896_PostMedieval_CrossBones	Methano_group	England	Premolar	Interproximal	Sub	420-165	F	>46	Perio_Caries	AG	Farrer et al. 2018
16897_PostMedieval_CrossBones	Strepto_group	England	Incisor	Lingual	Supra	420-165	F	26-35	Perio	AG	Farrer et al. 2018
16898_PostMedieval_CrossBones	Methano_group	England	Canine	Buccal	Supra	420-165	M	18-25	Perio	AG	Farrer et al. 2018
16899_PostMedieval_CrossBones	Strepto_group	England	Molar	Buccal	Supra	420-165	F	36-45	Perio_Caries	AG	Farrer et al. 2018
16900_PostMedieval_CrossBones	Methano_group	England	Incisor	Interproximal	Supra	420-165	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16901_PostMedieval_CrossBones	Methano_group	England	Incisor	Lingual	Supra	420-165	F	18-25	Perio_Caries	AG	Farrer et al. 2018
16903_PostMedieval_StBenetShe	Methano_group	England	Molar	Lingual	Sub	345	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16905_PostMedieval_StBenetShe	Strepto_group	England	Molar	Buccal	Sub	348-165	F	26-35	Perio_Caries	AG	Farrer et al. 2018
16906_PostMedieval_StBenetShe	Strepto_group	England	Incisor	Interproximal	Supra	348-165	F	36-45	Perio	AG	Farrer et al. 2018
16907_PostMedieval_StBenetShe	Methano_group	England	Molar	NA	Supra	193	M	>46	Perio	AG	Farrer et al. 2018
16911_PostMedieval_StBenetShe	Methano_group	England	Molar	Lingual	Supra	348-165	F	36-45	Caries	AG	Farrer et al. 2018
16913_Medieval_StBenetShereho	Methano_group	England	Canine	Lingual	Supra	738-352	M	36-45	Perio	AG	Farrer et al. 2018
16914_Medieval_StBenetShereho	Methano_group	England	Molar	NA	Supra	768-518	M	36-45	Perio	AG	Farrer et al. 2018
16915_Medieval_StBenetShereho	Methano_group	England	Molar	Buccal	Supra	768-518	M	36-45	Perio	AG	Farrer et al. 2018
16916_Medieval_StBenetShereho	Methano_group	England	Premolar	Lingual	Supra	738-352	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16917_Medieval_StBenetShereho	Methano_group	England	Molar	Interproximal	Supra	738-352	F	36-45	Perio_Caries	AG	Farrer et al. 2018
16918_Medieval_StBenetShereho	Strepto_group	England	Incisor	Lingual	Supra	738-352	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16919_PostMedieval_ChelseaOld	Methano_group	England	Molar	Lingual	Supra	318-168	M	26-35	Perio_Caries	AG	Farrer et al. 2018
16920_PostMedieval_ChelseaOld	Strepto_group	England	Incisor	Lingual	Supra	286	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16921_PostMedieval_ChelseaOld	Methano_group	England	Canine	Lingual	Supra	318-168	F	26-35	Perio_Caries	AG	Farrer et al. 2018
16922_PostMedieval_ChelseaOld	Strepto_group	England	Incisor	Interproximal	Supra	191	M	>46	Perio_Caries	AG	Farrer et al. 2018
16923_PostMedieval_ChelseaOld	Methano_group	England	Molar	Lingual	Supra	318-168	F	26-35	Perio_Caries	AG	Farrer et al. 2018
16925_PostMedieval_ChelseaOld	Strepto_group	England	Canine	Interproximal	Supra	182	M	>46	Perio	AG	Farrer et al. 2018
16926_PostMedieval_ChelseaOld	Methano_group	England	Premolar	Lingual	Supra	318-168	F	36-45	Perio_Caries	AG	Farrer et al. 2018
16927_PostMedieval_ChelseaOld	Methano_group	England	Premolar	Lingual	Supra	196	F	>46	Perio_Caries	AG	Farrer et al. 2018
16930_Medieval_StMaryGraces	Methano_group	England	Molar	Lingual	Supra	618-480	F	18-25	Perio	AG	Farrer et al. 2018
16931_Medieval_StMaryGraces	Methano_group	England	Molar	Lingual	Supra	668-480	M	36-45	Perio	AG	Farrer et al. 2018
16933_Medieval_StMaryGraces	Methano_group	England	Molar	Interproximal	Supra	618-480	M	36-45	Perio	AG	Farrer et al. 2018
16935_Medieval_MertonPriory	Methano_group	England	Molar	Lingual	Supra	718-628	F	36-45	Perio_Caries	AG	Farrer et al. 2018
16936_Medieval_MertonPriory	Methano_group	England	Incisor	Buccal	Supra	718-628	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16937_Medieval_StMaryGraces	Methano_group	England	Molar	Lingual	Supra	618-480	F	26-35	Perio_Caries	AG	Farrer et al. 2018
16938_Medieval_StMaryGraces	Methano_group	England	Molar	Lingual	Supra	618-480	F	26-35	Perio	AG	Farrer et al. 2018
16939_Medieval_StMaryGraces	Methano_group	England	Molar	Interproximal	Supra	618-480	M	>46	Perio	AG	Farrer et al. 2018
16940_Medieval_StMaryGraces	Methano_group	England	Molar	Interproximal	Supra	668-480	M	18-25	Perio	AG	Farrer et al. 2018
16941_Medieval_StMaryGraces	Methano_group	England	Incisor	Buccal	Supra	618-480	M	26-35	Perio_Caries	AG	Farrer et al. 2018
16942_Medieval_StMaryGraces	Strepto_group	England	Incisor	Interproximal	Supra	618-480	F	36-45	Caries	AG	Farrer et al. 2018
16944_Medieval_StMaryGraces	Methano_group	England	Molar	Lingual	Supra	668-480	M	26-35	Perio_Caries	AG	Farrer et al. 2018

Sample	Group	Country	Tooth	Surface	Supra	Date	Sex	Age	Health	Context	Source
16948_Medieval_StMaryGraces	Methano_group	England	Molar	Lingual	Supra	668-480	M	26-35	Caries	AG	Farrer et al. 2018
16949_Medieval_StMaryGraces	Methano_group	England	Incisor	Buccal	Supra	618-480	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16950_Medieval_StMaryGraces	Methano_group	England	Molar	Interproximal	Supra	668-480	F	26-35	Caries	AG	Farrer et al. 2018
16952_Medieval_GuildhallYard	Methano_group	England	Molar	Lingual	Supra	878-788	F	>46	Perio	AG	Farrer et al. 2018
16953_Medieval_GuildhallYard	Strepto_group	England	Incisor	Lingual	Supra	878-788	M	18-25	Caries	AG	Farrer et al. 2018
16955_Medieval_GuildhallYard	Methano_group	England	Molar	Lingual	Supra	878-788	F	>46	Perio	AG	Farrer et al. 2018
16956_Medieval_GuildhallYard	Methano_group	England	Premolar	Lingual	Supra	878-788	F	26-35	Perio	AG	Farrer et al. 2018
16957_Medieval_GuildhallYard	Methano_group	England	Premolar	Buccal	Supra	878-788	M	36-45	Perio	AG	Farrer et al. 2018
16959_Medieval_GuildhallYard	Strepto_group	England	Incisor	Lingual	Supra	878-788	F	26-35	Perio_Caries	AG	Farrer et al. 2018
16961_PostMedieval_StBrides	Strepto_group	England	Incisor	Lingual	Supra	952-478	NA	NA	Caries	AG	Farrer et al. 2018
16962_Medieval_BermondseyAbb	Methano_group	England	Molar	Buccal	Supra	952-478	M	18-25	Perio_Caries	AG	Farrer et al. 2018
16963_Medieval_BermondseyAbb	Strepto_group	England	Incisor	Lingual	Supra	952-478	M	26-35	Perio_Caries	AG	Farrer et al. 2018
16966_Medieval_BermondseyAbb	Strepto_group	England	Premolar	Lingual	Supra	952-478	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16967_Medieval_BermondseyAbb	Methano_group	England	Premolar	Lingual	Supra	952-478	M	18-25	Perio	AG	Farrer et al. 2018
16968_Medieval_BermondseyAbb	Methano_group	England	Molar	Lingual	Supra	952-478	M	36-45	Perio_Caries	AG	Farrer et al. 2018
16969_Medieval_BermondseyAbb	Methano_group	England	Molar	Lingual	Supra	952-478	M	18-25	Perio_Caries	AG	Farrer et al. 2018
16970_Medieval_BermondseyAbb	Methano_group	England	Molar	Buccal	Supra	952-478	M	>46	Perio	AG	Farrer et al. 2018
16971_Medieval_BermondseyAbb	Methano_group	England	Premolar	Lingual	Supra	952-478	M	36-45	Perio	AG	Farrer et al. 2018
18015_Japan_Jomon_1	Methano_group	Japan	Premolar	Buccal	NA	3000-2300	M	NA	NA	HG	NewData
18016_Japan_Jomon_2	Methano_group	Japan	Incisor	Buccal	NA	3000-2300	M	NA	NA	HG	NewData
18018_Japan_Jomon_4	Methano_group	Japan	Molar	Buccal	NA	3000-2300	M	NA	NA	HG	NewData
18019_Japan_Jomon_5	Methano_group	Japan	Premolar	Lingual	NA	3000-2300	M	NA	NA	HG	NewData
18402_X10_Hungary9	Methano_group	Hungary	Premolar	NA	NA	8000-7500	NA	NA	NA	AG	NewData
18407_Hungary1	Methano_group	Hungary	NA	NA	NA	8200-6500	NA	NA	NA	AG	NewData
18408_Hungary2	Methano_group	Hungary	NA	NA	NA	8200-6500	NA	NA	NA	AG	NewData
18413_Hungary10	Strepto_group	Hungary	Molar	NA	NA	7450-7350	NA	NA	NA	AG	NewData
18414_Hungary3	Methano_group	Hungary	Premolar	NA	NA	7300-7000	NA	NA	NA	AG	NewData
18416_Hungary4	Methano_group	Hungary	Molar	NA	NA	7500-7000	F	20_30	NA	AG	NewData
18418_Hungary5	Methano_group	Hungary	NA	NA	NA	7500-6500	NA	NA	NA	AG	NewData
18421_Hungary11	Methano_group	Hungary	Canine	NA	NA	6900-6400	M	40_60	NA	AG	NewData
18422_Hungary6	Methano_group	Hungary	NA	NA	NA	6900-6400	NA	NA	NA	AG	NewData
18427_Hungary7	Methano_group	Hungary	Canine	NA	NA	6900-6400	F	18_25	NA	AG	NewData
18428p_Hungary12	Methano_group	Hungary	NA	NA	NA	7000-5340	NA	NA	NA	AG	NewData
18442_Hungary8	Strepto_group	Hungary	Canine	NA	NA	7300-6900	F	7	NA	AG	NewData
18447p_Hungary14	Methano_group	Hungary	NA	NA	NA	6900-6760	NA	NA	NA	AG	NewData
18448p_Hungary13	Methano_group	Hungary	NA	NA	NA	7085-6990	NA	NA	NA	AG	NewData
18501_Pictish2	Methano_group	Scotland	Molar	NA	NA	NA	NA	NA	NA	AG	NewData
18502_Pictish3	NAN	Scotland	NA	NA	NA	NA	NA	NA	NA	AG	NewData
18503_Pictish3	Methano_group	Scotland	Molar	Lingual	NA	NA	NA	NA	NA	AG	NewData
19130_Syria_6	Methano_group	Syria	Incisor	NA	NA	4100-3950	NA	NA	Healthy	AG	NewData
19132_Syria_7	Methano_group	Syria	Incisor	NA	NA	3950-2700	NA	NA	NA	AG	NewData
19137_Syria_9	Strepto_group	Syria	Incisor	NA	NA	1400-1000	NA	1_12	NA	AG	NewData
19141_Syria_10	Methano_group	Syria	Canine	NA	NA	1400-1000	NA	NA	NA	AG	NewData
19150_Syria_12	Methano_group	Syria	Molar	NA	NA	2600-2300	F	40_50	NA	AG	NewData
19155_Syria_14	Methano_group	Syria	Molar	NA	NA	2600-2300	M	45	NA	AG	NewData
19157_Syria_15	Methano_group	Syria	Molar	NA	NA	2600-2300	M	50	NA	AG	NewData
19158_Syria_16	Methano_group	Syria	Molar	NA	NA	4100-3800	F	>18	NA	AG	NewData
19159_Syria_17	Strepto_group	Syria	Molar	NA	NA	2900-1800	NA	NA	NA	AG	NewData
19161_Syria_18	Methano_group	Syria	Molar	NA	NA	4200-4100	NA	>18	NA	AG	NewData
19163_Syria_19	Methano_group	Syria	Molar	NA	NA	250-50	NA	>18	NA	AG	NewData
19164_Syria_20	Strepto_group	Syria	Incisor	NA	NA	2600-2300	NA	NA	NA	AG	NewData
19166_Syria_21	Methano_group	Syria	Molar	NA	NA	2600-2300	F	40_45	NA	AG	NewData
19167_X10_Syria	Methano_group	Syria	Canine	NA	NA	6000-5300	F	30_35	NA	AG	NewData
19171_Syria_23	Methano_group	Syria	Incisor	NA	NA	4100-3950	NA	NA	NA	AG	NewData
19172_Syria_24	Strepto_group	Syria	Incisor	NA	NA	4100-3950	NA	NA	NA	AG	NewData
19358_Alaska	Strepto_group	USA	Incisor	Lingual	Supra	378	NA	NA	NA	HG	NewData
BreedonOnTheHill12855	Methano_group	England	Molar	Lingual	NA	1342	NA	NA	Perio	AG	Farrer et al. 2018
BreedonOnTheHill12857	Strepto_group	England	NA	NA	NA	1342	NA	NA	Perio	AG	Farrer et al. 2018
BreedonOnTheHill12858	Methano_group	England	NA	NA	NA	1342	NA	NA	Perio	AG	Farrer et al. 2018
BreedonOnTheHill12861	Methano_group	England	NA	NA	NA	1342	NA	NA	Perio_Caries	AG	Farrer et al. 2018
BreedonOnTheHill12864	Methano_group	England	NA	NA	NA	1342	NA	NA	Perio	AG	NewData
Hinxton16487	Methano_group	England	Molar	Lingual	NA	NA	F	40_50	Healthy	AG	NewData
Hinxton16488	Methano_group	England	Canine	Interproximal	NA	NA	M	>18	Perio	AG	NewData
Hinxton16489	Methano_group	England	Molar	Interproximal	NA	2217	M	Middle	Perio_Caries	AG	NewData
Hinxton16490	Strepto_group	England	Molar	Interproximal	NA	1418-1118	F	Young.M	Perio	AG	NewData
Hinxton16491	Strepto_group	England	Incisor	Interproximal	NA	NA	F	Middle	Perio	AG	NewData
Hinxton16492	Methano_group	England	Incisor	U	NA	NA	NA	Child.15	Perio	AG	NewData
Hinxton16493	Methano_group	England	Canine	Interproximal	NA	NA	M	Adult	Perio	AG	NewData
Hinxton16494	Methano_group	England	Molar	Interproximal	NA	NA	M	Middle	Perio_Caries	AG	NewData
Hinxton16495	Methano_group	England	Canine	Lingual	NA	NA	F	Young.M	Perio	AG	NewData
IsleOfLewis15931	Methano_group	England	Molar	Lingual	NA	NA	M	NA	NA	AG	NewData
IsleOfPamona15920	Strepto_group	England	Molar	Lingual	NA	NA	NA	NA	NA	AG	NewData

Sample Name	Group	Country				Date	Sex	Age	Perio	AG	Dataset
Jewbury8815	Strepto_group	England	NA	NA	NA	848-728	M	40-50	NA	AG	NewData
Jewbury8822	Strepto_group	England	NA	NA	NA	848-728	F	30-40	NA	AG	NewData
Jewbury8830	Strepto_group	England	NA	NA	NA	848-728	F	40	NA	AG	NewData
Jewbury8835	Methano_group	England	NA	NA	NA	848-728	M	40-50	NA	AG	NewData
Jewbury8838	Strepto_group	England	NA	NA	NA	848-728	M	50	NA	AG	NewData
Jewbury8839	Methano_group	England	NA	NA	Supra	848-728	F	20-30	NA	AG	NewData
Jewbury8860	Methano_group	England	NA	NA	NA	848-728	M	20-30	NA	AG	NewData
Jewbury8866	Methano_group	England	NA	NA	NA	848-728	F	Adult	NA	AG	NewData
Kirkhill15923	Methano_group	England	Molar	NA	NA	NA	NA	NA	NA	AG	NewData
Kirkhill15924	Methano_group	England	Molar	NA	NA	NA	NA	NA	NA	AG	NewData
Linton16499	Methano_group	England	Canine	U	NA	1328-1118	M	YoungA	NA	AG	NewData
Linton16529	NAN	England	Incisor	Lingual	NA	1328-1118	M	Adolesc	Perio	AG	NewData
NewarkBay8887	Strepto_group	England	Molar	Lingual	NA	NA	NA	NA	NA	AG	NewData
NewarkBay8888	Methano_group	England	Canine	Lingual	NA	NA	NA	NA	NA	AG	NewData
Oakington15806	Methano_group	England	Molar	NA	NA	1568-1418	F	Adult	NA	AG	NewData
Oakington15807	Methano_group	England	Molar	NA	NA	1568-1418	F	U	NA	AG	NewData
Oakington15809	Methano_group	England	Molar	NA	NA	1568-1418	F	U	NA	AG	NewData
Oakington15810	Methano_group	England	PreMolar	NA	NA	1568-1418	F	Over45	NA	AG	NewData
Oakington15811	Methano_group	England	Molar	NA	NA	1568-1418	M	25-30	NA	AG	NewData
Oakington16496	Methano_group	England	Molar	NA	NA	1568-1418	F	Middle	NA	AG	NewData
Oakington16500	Methano_group	England	Molar	NA	NA	1568-1418	NA	Child	NA	AG	NewData
Oakington16540	Methano_group	England	Molar	NA	NA	1568-1418	NA	Child	NA	AG	NewData
Raunds8333	Methano_group	England	Incisor	Buccal	NA	1068-868	M	35-45	NA	AG	NewData
Raunds8338	Strepto_group	England	Incisor	Lingual	NA	1068-868	M	35-45	NA	AG	NewData
Raunds8346	Methano_group	England	Incisor	Lingual	NA	1068-868	M	35-45	NA	AG	NewData
Raunds8347	Methano_group	England	Premolar	Buccal	NA	1068-868	M	>45	NA	AG	NewData
Raunds8348	Methano_group	England	NA	Lingual	NA	1068-868	NA	NA	NA	AG	NewData
Raunds8867	Methano_group	England	Molar	Lingual	NA	1068-868	F	17-25	NA	AG	NewData
Raunds8869	Methano_group	England	Molar	Lingual	NA	1068-868	M	17-25	NA	AG	NewData
Raunds8872	Methano_group	England	Molar	Lingual	NA	1068-868	F	25-35	NA	AG	NewData
SkaeBrae15919	Methano_group	England	Molar	NA	NA	NA	M	Adolesc	NA	AG	NewData
StBrides12849	Methano_group	England	NA	NA	NA	418-318	NA	NA	NA	AG	NewData
StBrides12850	Methano_group	England	NA	NA	NA	418-318	NA	NA	NA	AG	NewData
StBrides12866	Methano_group	England	Molar	Parietal	NA	418-318	NA	NA	NA	AG	NewData
StBrides12867	Methano_group	England	Molar	Parietal	NA	418-318	NA	NA	NA	AG	NewData
StBrides12870	Strepto_group	England	Molar	Lingual	NA	418-318	NA	NA	Perio	AG	NewData
StHelensOnWalls8873	Strepto_group	England	Molar	Occlusal	NA	918-468	F	>60	NA	AG	NewData
StHelensOnWalls8875	Methano_group	England	Molar	Lingual	NA	918-468	F	NA	NA	AG	NewData
StHelensOnWallsy8874	Methano_group	England	Molar	Lingual	NA	918-468	F	20-25	NA	AG	NewData
StNiniansIsle15927	Methano_group	England	Molar	U	NA	NA	F	NA	NA	AG	NewData
StNiniansIsle15928	Strepto_group	England	Molar	Parietal	NA	NA	M	10_18	NA	AG	NewData
StNiniansIsle15929	Strepto_group	England	Molar	U	NA	NA	NA	NA	NA	AG	NewData
Yorkshire8890	Methano_group	England	Molar	Lingual	NA	4117-1218	NA	NA	NA	AG	NewData
Yorkshire8892	Strepto_group	England	Molar	Lingual	NA	4117-1218	NA	NA	NA	AG	NewData
Yorkshire8893	Strepto_group	England	Incisor	Lingual	NA	4117-1218	NA	NA	NA	AG	NewData
Yorkshire8895	Methano_group	England	Premolar	Lingual	NA	4117-1218	NA	NA	NA	AG	NewData
15302_LachishSoil	Env. Control	Env. Contro	Env. Contro	Env. Control	Env. C	Env. Control	Env. (	Env. Col	Env. Control	Env. Con	NewData
18433_HungarySoil	Env. Control	Env. Contro	Env. Contro	Env. Control	Env. C	Env. Control	Env. (	Env. Col	Env. Control	Env. Con	NewData
18523_EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	NewData
18699_EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	NewData
19077_EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	NewData
19107p_EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	NewData
19128_EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	NewData
19361_EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	EBC	NewData

Table S1B. Sequencing data of samples used in this study.

Sample Name	Prefilter Assigned reads	Normalised reads	PostFilter Reads	%reads Post filteration at Species Level
12014_x10_Dudka_PolishHG1	1523383	104276	86305	82.76592888
12017_X10_Dudka_PolishHG2	1427845	104274	96875	92.90427144
12018_Syria_1	582559	104268	103430	99.19630184
12023_Syria_4	985344	104278	103086	98.85690174
12024_Syria_5	1137793	104278	103142	98.91060435
12027_Syria_8	5147428	104273	103458	99.21839786
13204_AfrPP2	118575	104558	103849	99.32190746
13208_AfrSF2	135710	104553	103914	99.38882672
13213_AfrPP1	125187	104621	104041	99.44561799
15305p_Lachish	333904	104275	96299	92.35099497
15307p_Lachish	2813707	104272	103103	98.87889366
15309p_Lachish	249700	104379	100643	96.42073597
15340_SouthAfrica_1	465660	104269	101023	96.88689831
15342_SouthAfrica_3	529859	104279	102785	98.56730502
15343_X10_SouthAfr_HG	20889796	104270	103352	99.11959336
15344_SouthAfrica_5	1861756	104276	102202	98.0110476
15354_Gabon_1	463647	104275	104164	99.89355071
15356_Gabon_2	470150	104282	104282	100

15394_Nicobar_Islands_1	861316	104387	103955	99.58615536
15395_Nicobar_Islands_2	845337	104274	103809	99.5540595
15396_Andaman_4	830936	104282	102715	98.49734374
15397_Andaman_5	669527	104270	103869	99.6154215
15398_Andaman_6	720931	104276	104141	99.87053589
15400_Andaman_7	617780	104273	97600	93.60045266
15401_Andaman_8	794494	104274	103117	98.89042331
15402_Andaman_9	612299	104273	104273	100
15403_Andaman_10	642644	104271	103060	98.83860325
15404_Andaman_11	1084776	104269	103841	99.58952325
15405_Andaman_12	794239	104278	104111	99.83985117
15406_Andaman_1	1016327	104276	104276	100
15407_Andaman_13	594996	104274	103040	98.8165794
15408_Andaman_14	737531	104269	103230	99.00353892
15410_Andaman_16	117161	104308	87624	84.00506193
15411_Borneo_1	109407	104308	87624	84.00506193
15455_Easter_Island_1	357082	104270	94225	90.36635657
15457_Easter_Island	1093283	104274	104013	99.74969791
15458_Easter_Island_3	508011	104273	103950	99.69023621
15459_Easter_Island_4	Cluster_wEBC	Cluster_wEBC	Cluster_wEBC	Cluster_wEBC	
15460_Easter_Island_5	742042	104279	103590	99.33927253
15461_Easter_Island_6	499407	104277	103830	99.57133404
15690_AncientEgypt2	1020443	104275	84227	80.77391513
16811_Medieval_SpitalSquare	1487819	104270	101728	97.5620984
16812_Medieval_SpitalSquare	1706517	104271	104056	99.79380652
16813_Medieval_SpitalSquare	5400499	104279	102838	98.61813021
16814_Medieval_SpitalSquare	1387042	104276	101653	97.48456021
16815_Medieval_SpitalSquare	1134116	104280	103784	99.5243575
16816_Medieval_SpitalSquare	1080278	104271	100845	96.71433093
16818_Medieval_SpitalSquare	2187093	104267	93198	89.38398535
16819_Medieval_SpitalSquare	440249	104278	99067	95.00278103
16820_Medieval_SpitalSquare	1152766	104275	102007	97.82498202
16821_Medieval_SpitalSquare	1984948	104274	102520	98.31789324
16823_Medieval_SpitalSquare	1057985	104275	101524	97.36178374
16825_Medieval_SpitalSquare	1196106	104279	99774	95.67985884
16826_Medieval_SpitalSquare	1302260	104274	101391	97.23516888
16828_Medieval_SpitalSquare	938924	104282	97680	93.66908958
16829_Medieval_SpitalSquare	3695922	104275	101339	97.18436826
16830_Medieval_SpitalSquare	1519114	104273	99552	95.47246171
16832_Medieval_SpitalSquare	2497687	104274	103771	99.51761705
16833_Medieval_SpitalSquare	1019194	104276	103494	99.25006713
16834_Medieval_MertonPriory	1634565	104271	104145	99.87916103
16835_Medieval_MertonPriory	1284180	104273	103942	99.68256404
16836_Medieval_MertonPriory	813433	104273	103966	99.70558054
16837_Medieval_MertonPriory	610451	104279	102973	98.7475906
16838_Medieval_MertonPriory	2760124	104270	104112	99.84847032
16839_Medieval_MertonPriory	923297	104276	91876	88.10848134
16840_Medieval_MertonPriory	1079477	104279	103601	99.34982115
16841_Medieval_MertonPriory	629363	104272	103797	99.54446064
16842_Medieval_MertonPriory	3019662	104278	103685	99.4313278
16843_Medieval_MertonPriory	2472892	104279	101838	97.65916436
16844_Medieval_MertonPriory	1236946	104277	104096	99.82642385
16845_Medieval_MertonPriory	1020835	104273	103788	99.5348748
16846_Medieval_MertonPriory	561308	104281	101498	97.33124922
16847_Medieval_MertonPriory	4875150	104274	103829	99.57323973
16848_Medieval_MertonPriory	906284	104274	101606	97.44135643
16849_Medieval_MertonPriory	1082289	104272	102746	98.53651987
16850_Medieval_MertonPriory	1349071	104272	98954	94.89987724
16851_Medieval_MertonPriory	1126658	104276	101719	97.54785377

16852_Medieval_MertonPriory	4713937	104277	103893	99.63175005
16853_Medieval_MertonPriory	3732346	104273	102398	98.20183557
16854_Medieval_MertonPriory	1399867	104278	103921	99.65764591
16855_Medieval_MertonPriory	883175	104278	103629	99.37762519
16856_Medieval_MertonPriory	938214	104266	103016	98.80114323
16857_Medieval_MertonPriory	5664420	104270	104045	99.78421406
16858_Medieval_MertonPriory	1320461	104278	102257	98.06191143
16860_Medieval_MertonPriory	1168675	104273	102141	97.95536716
16861_Medieval_MertonPriory	1317730	104279	103486	99.23954008
16862_Medieval_MertonPriory	1187342	104270	100468	96.35369713
16863_Medieval_MertonPriory	641678	104269	103661	99.41689284
16864_Medieval_MertonPriory	1033799	104268	103714	99.46867687
16865_Medieval_MertonPriory	671783	104276	104148	99.87724884
16867_Medieval_MertonPriory	979345	104270	102328	98.13752757
16869_Medieval_MertonPriory	1193633	104270	103344	99.11192097
16871_Medieval_MertonPriory	1067897	104281	103783	99.52244417
16872_Medieval_MertonPriory	859913	104273	103867	99.61063746
16873_Medieval_MertonPriory	1582722	104275	100538	96.41620714
16874_Medieval_MertonPriory	1103758	104272	103876	99.62022403
16876_Medieval_MertonPriory	934593	104275	103459	99.21745385
16877_Medieval_MertonPriory	955417	104276	103347	99.10909509
16878_Medieval_MertonPriory	1067208	104275	101278	97.1258691
16879_Medieval_MertonPriory	1013038	104272	103717	99.46773822
16880_Medieval_MertonPriory	868080	104273	102427	98.22964718
16881_Medieval_MertonPriory	716832	104272	99430	95.35637563
16883_Medieval_MertonPriory	813715	104278	104132	99.85998964
16884_Medieval_MertonPriory	1445054	104275	101825	97.65044354
16885_Medieval_MertonPriory	934093	104275	103249	99.01606329
16886_Medieval_MertonPriory	1302924	104272	101200	97.05385914
16887_Medieval_MertonPriory	1898939	104274	100681	96.55427048
16888_Medieval_MertonPriory	1236067	104275	104162	99.8916327
16889_Medieval_MertonPriory	743055	104276	102684	98.47328244
16890_Medieval_MertonPriory	577926	104279	100414	96.29359699
16891_Medieval_MertonPriory	598554	104264	103973	99.72090079
16892_PostMedieval_CrossBones	550120	104268	102177	97.99459086
16893_PostMedieval_CrossBones	1255355	104278	104278	100
16894_PostMedieval_CrossBones	1009299	104269	104269	100
16896_PostMedieval_CrossBones	260857	104272	103953	99.69406936
16897_PostMedieval_CrossBones	2365043	104273	102820	98.60654244
16898_PostMedieval_CrossBones	628283	104271	104086	99.82257771
16899_PostMedieval_CrossBones	1318959	104273	103362	99.12633184
16900_PostMedieval_CrossBones	705711	104272	103768	99.51664876
16901_PostMedieval_CrossBones	1197679	104275	101986	97.80484296
16903_PostMedieval_StBenetSher	737261	104275	103075	98.84919684
16905_PostMedieval_StBenetSher	3064223	104271	103967	99.70845201
16906_PostMedieval_StBenetSher	1635764	104280	100233	96.11910242
16907_PostMedieval_StBenetSher	588834	104280	104128	99.85423859
16911_PostMedieval_StBenetSher	2136767	104273	103913	99.65475243
16913_Medieval_StBenetShereho	1083686	104277	101650	97.48074839
16914_Medieval_StBenetShereho	2918133	104277	99865	95.76896152
16915_Medieval_StBenetShereho	2178990	104271	103799	99.54733339
16916_Medieval_StBenetShereho	1744057	104271	102786	98.57582645
16917_Medieval_StBenetShereho	719316	104273	103367	99.13112695
16918_Medieval_StBenetShereho	4742448	104281	103550	99.29900941
16919_PostMedieval_ChelseaOld(	1045460	104273	104113	99.84655663
16920_PostMedieval_ChelseaOld(	2119448	104275	103668	99.4178854
16921_PostMedieval_ChelseaOld(	1538047	104270	103864	99.61062626
16922_PostMedieval_ChelseaOld(	575906	104273	103886	99.62885886
16923_PostMedieval_ChelseaOld(	1630479	104277	104277	100
16925_PostMedieval_ChelseaOld(	897123	104283	104053	99.77944631
16926_PostMedieval_ChelseaOld(	1586589	104273	103805	99.55117816
16927_PostMedieval_ChelseaOld(	1133315	104283	103378	99.13216919
16930_Medieval_StMaryGraces	1858033	104272	104133	99.8666948
16931_Medieval_StMaryGraces	854966	104271	103978	99.71900145

16933_Medieval_StMaryGraces	1150491	104269	103939	99.68351092
16935_Medieval_MertonPriory	2618973	104278	103011	98.78497861
16936_Medieval_MertonPriory	1427444	104271	99414	95.34194551
16937_Medieval_StMaryGraces	1625210	104284	101527	97.35625791
16938_Medieval_StMaryGraces	855734	104275	101712	97.54207624
16939_Medieval_StMaryGraces	8586322	104275	104275	100
16940_Medieval_StMaryGraces	750202	104267	102572	98.37436581
16941_Medieval_StMaryGraces	679292	104276	103405	99.16471671
16942_Medieval_StMaryGraces	2717834	104279	102300	98.10220658
16944_Medieval_StMaryGraces	753279	104279	104279	100
16948_Medieval_StMaryGraces	686416	104276	103736	99.48214354
16949_Medieval_StMaryGraces	531909	104277	103732	99.47735359
16950_Medieval_StMaryGraces	929670	104282	104282	100
16952_Medieval_GuildhallYard	1604682	104277	103769	99.512836
16953_Medieval_GuildhallYard	935865	104274	104059	99.79381246
16955_Medieval_GuildhallYard	1448760	104279	97325	93.33135147
16956_Medieval_GuildhallYard	1755205	104278	102963	98.73894781
16957_Medieval_GuildhallYard	1787478	104274	103740	99.48788768
16959_Medieval_GuildhallYard	963629	104277	103669	99.41693758
16961_PostMedieval_StBrides	1188253	104275	103879	99.62023496
16962_Medieval_BermondseyAbb	891462	104277	103220	98.98635365
16963_Medieval_BermondseyAbb	1509564	104279	103498	99.25104767
16966_Medieval_BermondseyAbb	1404289	104273	102817	98.60366538
16967_Medieval_BermondseyAbb	1458652	104271	104049	99.78709325
16968_Medieval_BermondseyAbb	1329091	104274	102520	98.31789324
16969_Medieval_BermondseyAbb	2221613	104274	103889	99.63078044
16970_Medieval_BermondseyAbb	591502	104264	101213	97.07377427
16971_Medieval_BermondseyAbb	1972905	104277	102952	98.72934588
18015_Japan_Jomon_1	328742	104270	97983	93.9704613
18016_Japan_Jomon_2	627388	104274	101116	96.97144063
18018_Japan_Jomon_4	488214	104274	103955	99.69407522
18019_Japan_Jomon_5	571077	104281	92253	88.46577996
18402_X10_Hungary9	20895324	104279	102968	98.74279577
18407_Hungary1	1816918	104276	103178	98.9470252
18408_Hungary2	1207328	104266	76682	73.54458788
18413_Hungary10	609457	104278	101646	97.47597768
18414_Hungary3	671994	104275	98294	94.26420523
18416_Hungary4	935794	104270	103100	98.87791311
18418_Hungary5	961063	104271	103449	99.21166959
18421_Hungary11	588279	104270	101935	97.76062146
18422_Hungary6	1141170	104281	87243	83.66145319
18427_Hungary7	497419	104275	104275	100
18428p_Hungary12	1046166	104277	100529	96.40572705
18442_Hungary8	217459	104277	103178	98.94607632
18447p_Hungary14	584926	104275	102090	97.90457924
18448p_Hungary13	1136711	104276	104276	100
18501_Pictish2	868387	104277	98627	94.58173902
18502_Pictish3	1161821	104270	101114	96.97324254
18503_Pictish3	231342	104280	102135	97.94303797
19130_Syria_6	125364	104269	101322	97.1736566
19132_Syria_7	2136928	104278	104278	100
19137_Syria_9	1117506	104282	103365	99.12065361
19141_Syria_10	618766	104274	88545	84.91570286
19150_Syria_12	875195	104277	99885	95.7881412
19155_Syria_14	1391944	104279	103097	98.86650236
19157_Syria_15	754330	104272	99390	95.31801442
19158_Syria_16	943700	104271	101769	97.60048336
19159_Syria_17	576308	104277	103249	99.0141642
19161_Syria_18	2012063	104277	101987	97.80392608
19163_Syria_19	3012758	104274	102502	98.30063103
19164_Syria_20	3110654	104271	103711	99.46293792
19166_Syria_21	15793306	104272	103470	99.23085776
19167_X10_Syria	829112	104271	101781	97.61199183
19171_Syria_23	1100683	104278	88536	84.9038148
19172_Syria_24	457617	104273	102879	98.66312468
19358_Alaska	2347033	104271	103945	99.68735315
BreedonOnTheHill12855	1976822	104274	94950	91.05817366
BreedonOnTheHill12857	2273424	104276	103749	99.49461046
BreedonOnTheHill12858	2068196	104272	101339	97.18716434

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BreedonOnTheHill12861	1833489	104272	100335	96.22429799
BreedonOnTheHill12864	2274378	104277	103908	99.64613481
Hinxton16487	1841231	104273	103931	99.67201481
Hinxton16488	2770129	104277	94621	90.74004814
Hinxton16489	1807736	104275	104160	99.8897147
Hinxton16490	2952456	104276	104276	100
Hinxton16491	2189747	104279	103547	99.298037
Hinxton16492	2636460	104280	103624	99.37092443
Hinxton16493	1856468	104273	103272	99.04001995
Hinxton16494	1809283	104273	104030	99.76695789
Hinxton16495	543767	104275	102482	98.28050827
IsleOfLewis15931	2668052	104272	102241	98.05220961
IsleOfPamona15920	1636951	104282	103005	98.77543584
Jewbury8815	1996703	104278	104025	99.75737931
Jewbury8822	1423946	104275	104024	99.75929034
Jewbury8830	2517024	104276	103324	99.08703824
Jewbury8835	1120657	104277	103512	99.26637705
Jewbury8838	1506441	104277	104277	100
Jewbury8839	143207	104278	103756	99.49941503
Jewbury8860	460145	104276	103964	99.70079405
Jewbury8866	2778836	104274	104274	100
Kirkhill15923	753893	104272	102136	97.95151143
Kirkhill15924	2149493	104282	101940	97.75416659
Linton16499	2639678	104277	103311	99.07362122
Linton16529	23754732	104279	103965	99.69888472
NewarkBay8887	2548100	104272	104103	99.83792389
NewarkBay8888	1518977	104265	103358	99.13010118
Oakington15806	104275	104275	95357	91.44761448
Oakington15807	2270935	104271	104108	99.84367657
Oakington15809	2429095	104276	104118	99.84847904
Oakington15810	3011911	104273	100688	96.5619096
Oakington15811	1200257	104276	103486	99.24239518
Oakington16496	932200	104281	103967	99.6988905
Oakington16500	2384174	104275	99539	95.45816351
Oakington16540	2179432	104274	103759	99.50610891
Raunds8333	2420842	104275	102186	97.99664349
Raunds8338	2678674	104274	101898	97.72138788
Raunds8346	2716168	104272	100921	96.7862897
Raunds8347	1826826	104273	98806	94.75703202
Raunds8348	525633	104264	103437	99.20682115
Raunds8867	1473333	104268	102639	98.43767983
Raunds8869	3172933	104275	103546	99.30088708
Raunds8872	2138194	104277	101528	97.36375231
SkaeBrae15919	361349	104281	98679	94.62797633
StBrides12849	1732308	104274	103704	99.45336325
StBrides12850	2809034	104275	101887	97.7099017
StBrides12866	2306303	104281	101580	97.40988291
StBrides12867	3405399	104273	101532	97.37132335
StBrides12870	2691017	104274	103426	99.18675796
StHelensOnWalls8873	3500992	104282	102699	98.48200073
StHelensOnWalls8875	2321078	104268	100608	96.48981471
StHelensOnWallsy8874	2157030	104280	103735	99.47736862
StNiniansIsle15927	2806207	104277	103713	99.45913289
StNiniansIsle15928	2041895	104278	101547	97.38103915
StNiniansIsle15929	1619477	104270	101726	97.5601803
Yorkshire8890	811347	104278	103706	99.45146627
Yorkshire8892	726251	104267	91396	87.65573
Yorkshire8893	3199831	104268	102068	97.89005256
Yorkshire8895	2217317	104278	93895	90.04296208
15302_LachishSoil	Env. Control	Env. Control	Env. Control	Env. Control
18433_HungarySoil	Env. Control	Env. Control	Env. Control	Env. Control
18523_EBC	EBC	EBC	EBC	EBC
18699_EBC	EBC	EBC	EBC	EBC
19077_EBC	EBC	EBC	EBC	EBC
19107p_EBC	EBC	EBC	EBC	EBC
19128_EBC	EBC	EBC	EBC	EBC
19361_EBC	EBC	EBC	EBC	EBC

Table S2A. Core hunter-gatherer microbiota

Taxa
Peptostreptococceae Bacterium oral taxon 113
Actinomyces sp. oral taxon 414
Desulfomicrobium orale
Fretibacterium fastidiosum
Treponema socranskii
Treponema denticola
Anaerolineaceae bacterium oral taxon 439
Candidatus Sacchribacteria oral taxon TM7x
Bacteroidetes oral taxon 274
Tannerella forsythia

Table S2B. Core agricultural microbiota

Taxa
Tannerella forsythia
Peptostreptococceae Bacterium oral taxon 113
Candidatus Sacchribacteria oral taxon TM7x
Anaerolineaceae bacterium oral taxon 439
Bacteroidetes oral taxon 274
Treponema maltophilum
Treponema denticola
Anaerolineaceae bacterium oral taxon 439
Eubacterium spahenum
Actinomyces cardiffensis
Actinomyces dentalis
Actinomyces oris
Actinomyces sp. oral taxon 414
Olsenella sp. oral taxon 807
Fretibacterium fastidiosum
Desulfomicrobium orale

Table S2C. Core microbiota of both hunter-gatherer and agriculturalists

Taxa
Methanobrevibacter oralis
Unclassified
Tannerella forsythia
Campylobacter gracilis
Desulfomicrobium orale
Filifactor alocis
Porphyromonas gingivalis
Fretibacterium fastidiosum
Johnsonella ignava
Olsenella sp. oral taxon 807
Treponema denticola
Actinomyces sp. oral taxon 414

"If we are to live together in peace, we must come to know each other better."

Lyndon B. Johnson, 36th President of the United States

Chapter IV

Industrialisation Dramatically Impacts Oral Microbiota on a Multi-Continental Scale

Statement of Authorship

Title of Paper	Industrialisation dramatically impacts oral microbiota on a multi-continental scale
Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished and Unsubmitted work written in manuscript style
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Principal Author

Name of Principal Author (Candidate)	Muslihudeen Abdul-Aziz		
Contribution to the Paper	Designed the experiment. Performed all the laboratory work, data analysis and interpretation of results. Wrote the manuscript.		
Overall percentage (%)	70%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	29/11/18

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

    i.   the candidate's stated contribution to the publication is accurate (as detailed above);

    ii.   permission is granted for the candidate to include the publication in the thesis; and

    iii.   the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Alan Cooper		
Contribution to the Paper	Provided funding. Assisted with the collection of samples. Discussed and edited the manuscript.		
Signature		Date	

Name of Co-Author	Laura S. Weyrich
Contribution to the Paper	Provided funding, Co-designed the experiments. Advised on data analysis and interpretation of results, and discussed and edited the manuscript.
Signature	Date 21/11/18

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Industrialisation Dramatically Impacts Oral Microbiota on a Multi-Continental Scale

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Abstract

The human microbiota plays a crucial role in our health and has been implicated in systemic disease. Recent research suggests that lifestyle changes, such as the transition to agriculture and Industrialisation, may have resulted in alterations to the oral microbiota. However, limited geographical and temporal sampling has hindered a more detailed understanding of these changes, and others have argued that oral microbiota may be more resistant to industrialized changes compared to the gut microbiota, due to deep co-evolutionary relationships between oral microbes and its metabolism of host proteins. Here, we compare microbiota from Batwa rainforest hunter-gatherers and neighbouring Bakiga subsistence farmers from southwest Uganda. Using 16S ribosomal RNA gene sequencing, we obtain a robust salivary oral microbiota signal but find no significant differences in microbial diversity or composition between the two Ugandan populations, which may reflect the Batwa's move from hunting and gathering to subsistence agriculture since 1991. We also compare these microbiota with published worldwide data from Tanzanians, Yanomami hunter-gatherers from Venezuela, and industrialized populations from the United States, Europe, and Australia. As previously observed, the diversity of oral microbiota in people living rural and traditional lifestyles was higher than that of industrialized populations. However, the Uganda individuals possessed the highest oral microbial diversity of any population yet studied, and oral microbiota from individuals living rural or traditional lifestyles was differentiated according to continent. Agricultural and industrialized populations were also found to have lower numbers of known pathogenic oral taxa compared to hunter-gatherers. These results suggest that both diet and the environment play a complex interactive role in the composition and structure of the oral microbiome.

Research highlights

- A robust salivary oral microbiota data was obtained from two populations, rainforest hunter-gatherers (Batwa) and traditional farmers (Bakiga) living in Uganda.

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- No significant differences were found between the Batwa and Bakiga oral microbiota, despite unique evolutionary histories and past differences in diet.

- When analysed with published worldwide data, Industrialisation was found to be the single most significant factor driving salivary oral microbiota differentiation.

- Oral microbiota from those with traditional lifestyles was stratified according to the geography.

- Potential oral pathogens were found to be higher in hunter-gatherer populations.

Introduction

The human oral microbiota consists of billions of bacterial cells that form biofilms on the different surfaces and crevices in the oral cavity, thriving in diverse communities (Mark Welch et al. 2016; Proctor et al. 2018; Sender, Fuchs, and Milo 2016). Similarly, the oral microbiome is defined as the totality of microbial genes and the surrounding environmental conditions in the oral cavity (Marchesi and Ravel 2015). The past two decades of research on the oral microbiota and its microbiome have shown that over 700 various species can exist in the mouth, consisting of diversity that spans the entire bacterial phylogenetic tree currently known to microbiologists (Aas et al. 2005; Krishnan, Chen, and Paster 2017). Amongst these species, ~200 bacterial species are typically found in the oral cavity of each individual. While some play critical roles in oral disease (*e.g. Streptococcus mutans, Porphyromonas gingivalis,* and *Pseudomonas aeruginosa* (Kitada, de Toledo, and Oho 2009)) others, such as Streptococcus *salivarius* and commensals such as *Gemella* and *Granulicatella* species, are associated with a healthy oral microbiota (Aas et al. 2005). The overall community composition of the oral microbiota is impacted and shaped by the flow of saliva (Proctor et al. 2018), diet (Adler et al. 2013; Arensburg 1996; Giacaman 2017; Weyrich et al. 2017), and by the host immune system (Gomez et al. 2017). Research has also explored and characterised differences in the oral microbiome between diseased and healthy individuals (Chen et al. 2018; Kitada, de Toledo, and Oho 2009; Li et al. 2014; Sampaio-Maia et al. 2016; Yang et al. 2012). This has revealed that the oral

microbiota shifts towards a state of compositional change causing disease during both oral (*e.g.* caries and periodontal disease) and systemic diseases, such as heart disease, arthritis, and cancer (Le Bars et al. 2017; Sampaio-Maia et al. 2016; Simpson and Thomas 2016; Zhang et al. 2015).

The oral microbiota in different populations may also be unique depending on the specific evolutionary history of those people. The co-evolutionary processes that drive evolutionary differences in oral microbiota are predicted to be diverse and likely involve specific human-microbe co-evolution (Blaser 2006), adaptation to past cultural transitions or revolutions (*e.g.* adoption of agriculture during the Neolithic Revolution), and the introduction of new bacterial taxa as humans encountered new environments or dietary food sources (Adler et al. 2013; Weyrich, Dobney, and Cooper 2015). These scenarios would have had immense impacts on oral microbial composition and subsequently on human health (Carding et al. 2015; Logan, Jacka, and Prescott 2016; McDonald et al. 2016). A recent approach tested this theory by examining short and damaged DNA fragments of oral bacterial taxa extracted from ancient calcified dental plaque. This unveiled significant shifts in oral microbiota that coincided with past cultural transitions (*i.e.* advent of agriculture and Industrialisation) (Adler et al. 2013; Warinner et al. 2014), suggesting that the evolutionary signals from a population may generate unique microbial signatures. In modern populations, evidence of these histories may still be present. One study explored these deeper co-evolutionary relationships by examining oral microbiota based on ethnicity, revealing ethnicity-associated differences in oral microbiota diversity (Mason et al. 2013). Another study explored African populations compared to Alaskan Native Americans and Germans, revealing African specific signals (Li et al., 2014). Together, these studies suggest that the oral microbiota in unique populations may retain signatures of their past evolutionary histories. However, it remains unclear as to how much these evolutionary signals of the past are maintained in populations today.

Rapid Industrialisation over the past two centuries, with the advent of processed food and medication such as antibiotics, has resulted in better health outcomes and increased lifespans in many populations (Steckel 1999; Szreter 2004). However, rapid Industrialisation has also been linked to increases in diseases of affluence, such as heart disease, obesity, and Type 2 Diabetes (Basch, Samuel,

and Ethan 2013; Tuohy and Del Rio 2014). Recently, research has identified strong links between these diseases and changes in the microbiota (Belkaid and Hand 2014; Parekh, Balart, and Johnson 2015), and further studies have suggested that these changes may originate from Industrialisation (Logan, Jacka, and Prescott 2016; Valle Gottlieb et al. 2017). While early microbiota research suggested that these changes were isolated to the gut, recent evidence suggests that similar Industrial changes occurred within the oral microbiota (Adler et al. 2013; Clemente et al. 2015). Much of this research was done by comparing the oral microbiota of hunter-gatherer groups, such as the Yanomami (Venezuela) (Clemente et al. 2015), the Batwa (Uganda) (Nasidze et al. 2011), Native Alaskans (United States) (Li et al. 2014), and those in semi-urban environments from Tanzania (Bisanz et al. 2015), Sierra Leone, and Democratic Republic of Congo (Nasidze et al. 2011), to those living a Western, industrialized lifestyle (Li et al. 2014; Nasidze et al. 2009). industrialized populations are generally found to have lower microbial diversity due to their homogenous diets and lifestyles, compared to non-industrialized populations, and were enriched in taxa linked to caries and periodontal disease (Clemente et al. 2015; Li etc. 2014; Nasidze et al. 2009, 2011). However, many of these studies had small sample sizes (< 30 individuals) and only compared a single hunter-gather community to that of a single industrialized population, raising questions to the broader, more general impacts of Industrialisation on different human populations.

Amongst the many factors that are predicted to alter the microbiota during Industrialisation, the diet has been proposed as one of the single most significant factors to influence the gut microbiota (David et al. 2014; De Filippo et al. 2010; Muegge et al. 2011). Similar research in the oral cavity has suggested that dietary differences may play a role in the mouth. A recent Danish study showed differences in oral microbial community structure and taxonomic composition between vegans and omnivores and (Hansen et al. 2018), but a similar study on Italians did not find dietary differences (De Filippis et al. 2014). However, research by Takeshita et al. (2015) compared larger scale dietary differences in different populations (Japanese and Koreans) and revealed that Koreans had lower oral microbial diversity than the Japanese, which was likely tied to a diet heavier in spicy, salty, and fermented food. Similarly, studies on Batwa hunter-gatherers in Uganda identified significant

differences in their oral microbiota compared to other African agriculturalists from Sierra Leone and the DRC, as well as Germans and Indigenous populations from Alaska (Li et al. 2014; Nasidze et al. 2011). However, all of these studies compared dietary differences across different human populations, where the impacts of the environment, evolutionary history, or general lifestyle on the oral microbiota cannot be controlled. A recent study by Lassalle et al. (2017) addressed a shared environment by comparing the oral microbiomes of three pairs of hunter-gatherers and their neighbouring traditional farmers in the Philippines. They reported differences in community structure and levels of diversity between the two lifestyles, concluding that dietary shifts have had an impact on the human oral microbiome. However, this has not been shown in other human populations, leaving remaining questions about the extent to which dietary differences likely contribute to changes in oral microbiota.

Here, we use a 16S rRNA amplicon based sequencing approach using the V4 region to understand factors that drive oral microbiota diversity of a pair of populations in the same environment, located in South-western Uganda: a hunter-gather group, the Indigenous Batwa pygmies (also known as the Twa) and Bantu-speaking farmers, the Bakiga. The Batwa were expelled from their rainforest home in 1991 during the creation of Bwindi Impenetrable National Park; they now live alongside the Bakiga in areas around the park in Kanungu district, Uganda, Central East Africa. The Batwa communities lost legal access to the rainforest and traditional food sources and they experienced negative shifts in health (Disko and Tugendhat 2014; Ohenjo et al. 2006). The Bakiga are the Batwa's Bantu-speaking agricultural neighbours who migrated into the region as part of the Bantu expansion between 2,000-3,000 years ago (Pakendorf, Bostoen, and De Filippo 2011; A. Plumptre et al. 2004). We compared these two populations and other oral microbiota data obtained from hunter-gatherers, agriculturalists, and industrialized populations to provide a global context on the impacts of Industrialisation on oral microbiota composition.

Material and Methods

Batwa and Bakiga dataset

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Ethics Approval

The saliva samples used in this study were collected with informed consent and approved by the Institutional Review Boards of Makerere University, Uganda (protocol 2009-137) and the University of Chicago, USA (16986A). Approval for this study was also obtained from the University of Adelaide, Australia (3808).

Sample Collection

The study consists of 96 saliva samples from two Ugandan populations: Indigenous Batwa (n=87) and Bakiga (n=9), randomly selected from a more extensive collection of saliva samples used in a population genetics study into the evolutionary history of the pygmy phenotype in African rainforest hunter-gatherers (Perry et al. 2014). Samples were collected over one field season in 2010 into Oragene collection tubes. Samples of adults from both groups were collected from settlements at eight different locations in Kanungu district, Uganda (Fig. 1; Table S1A): Buhoma, Byumba, Kebiremu, Kihembe, Kitariro, Mpungu, Mukono and Netko. The samples were collected from 55 females and 41 males.

Figure 1. Location of Bwindi Impenetrable National Park and Kanungu district, Uganda where Batwa and Bakiga samples were collected.

DNA extraction, library preparation and sequencing

Saliva was collected in Oragene DNA collection kits (DNA Genotek Inc., Canada) and stored at -80°C following shipment to the United States. A volume of 200 µL of saliva per sample was transferred into new tubes and shipped frozen to the University of Adelaide for further analysis. Cells were lysed from saliva in 2 mL tubes using glass beads vortexed with 470 µL EDTA (0.5M), 30 µL SDS (10%), and 20 µL proteinase K (20 mg/mL). DNA was then extracted from the samples using an in-house silica-based DNA extraction protocol previously described (Brotherton et al. 2013). To account for the use of small volumes of saliva, we modified the total volume of DNA binding buffer to the following: 1.6 mL lysis buffer (1.46 mL Guanidinium DNA binding buffer, 8 µL NaCl (5M), 20.80 µL Triton-X 100, 24.53 µL sterile water, 88.88 µL NaOAc (3M)). Two blank extraction controls were included for every extraction batch of twenty samples (n=10).

Extraction was followed by amplification using polymerase chain reactions (PCR) using specific barcoded primers from a stretch of the V4 region of the 16S ribosomal RNA (rRNA) encoding gene. This reaction consisted of a forward primer 515 F and a reverse primer 806 R (Caporaso et al. 2012); each primer consisted of the appropriate Illumina adapter, an 8-nucleotide index sequence i5 and i7, a 10-nucleotide pad sequence, a 2-nucleotide linker, and the gene-specific primer. All samples were amplified in triplicate including additional PCR negative controls. Each PCR reaction was done in triplicate and consisted of: 18.05 µL sterile water, 1 µL DNA extract, 0.25 µL of Hi-Fi Taq (ThermoFisher Scientific), 2.5 µL of 10X Hi-Fi Taq (ThermoFisher Scientific), 1 µL MgCl$_2$ (50 mM), 0.2 µL dNTPs (25 mM), and 1 µL each for the forward and reverse primers. The following PCR conditions were used: initial denaturing (95°C, 6 minutes); followed by 37 cycles of denaturation (95°C, 30 seconds), annealing (50°C, 30 seconds) and elongation (72°C, 90 seconds); and final extension (60°C, 10 minutes). Following amplification, the triplicate reactions were pooled, and the PCR products were visualised using electrophoresis on a 2.5% agarose gel. Quantification was then performed using Qubit 2.0 (Life Technologies). Samples were pooled at equimolar concentrations and purified using magnetic Ampure beads (Beckman Coulter). The

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pooled DNA library was then quantified using Tapestation 2200 (Agilent) and KAPA SYBR fast Universal master mix qPCR assay (Geneworks). DNA sequencing was performed using Illumina MiSeq 150 bp paired-end chemistry generating a total of 4,258,543 (range 11,412-114,994) paired-end reads. A mean of 41,964 reads was obtained per sample, while the mean for successfully sequenced blank extraction control and PCR blanks (EBC) (n=4) was 4,773 reads.

Data processing

Paired-end reads were demultiplexed using Illumina bcl2fastq software (v1.8.4) and merged using fastq-join (v.1.3.1) (Aronesty 2013). Merged paired-end reads were de-multiplexed and processed in Quantitative Insights Into Microbial Ecology v2 (QIIME2 2017.9) (Bolyen et al. 2018)(Navas-Molina et al. 2013) using Deblur (v.1.02) (Amir et al. 2017), which uses a sequence variant method for OTU picking to increase the accuracy and maximise the specificity of the 16S rRNA gene reads. Data was trimmed at 120 nt for processing in Deblur. The resultant sub Operational Taxonomic Units (sOTUs) or amplicon sequence variants (ASV) then underwent *de novo* multiple sequence alignment using MAFFT (QIIME2 – 2017.9). Taxonomy was assigned using a pre-trained naïve Bayes classifier (Bokulich et al. 2018) and the QIIME2 "q2-feature-classifier" plugin. The classifier was trained on the Silva 119 (Quast et al. 2013) 99% reference tree OTUs reference data set on the V4 region amplified by 515/806 primers. Environmental and lab contaminant taxa that were found to be differentially abundant between controls and samples were filtered out using QIIME2 "q2-feature-table filter feature" command. Alpha and beta diversity were computed using QIIME2 "q2-diversity" plugin with rarefaction set at 1,000 sequences per sample. (C. Lozupone et al. 2011; C. Lozupone and Knight 2005). Alpha diversity for single metadata categories was compared with the Kruskal-Wallis test. The unweighted UniFrac distance between samples was tested with a non-parametric PERMANOVA (Anderson 2001) and ANOSIM (CLARKE 1993). Differential abundance testing was performed using Analysis of Composition of Microbiomes (ANCOM) (Mandal et al. 2015), as implemented in QIIME2 "q2-composition" plugin. Taxonomic discriminants between groups were performed on the 100 most dominant OTUs using LEfSe (Segata et al. 2011). Core taxa comparison was processed using the QIIME2 "q2-feature-table core feature" command. Core microbiome was set to identify taxa

found in 90% of all samples in a group. Identified core taxa were then used to draw a Venn diagram using InteractiVenn (Heberle et al. 2015). Calypso was used to independently verify alpha and beta diversity results from QIIME2 and generate figures (Zakrzewski et al. 2017). Before analysis using Calypso, total sum normalisation (TSS) combined with square root transformation (Hellinger transformation) was performed on the filtered dataset. TSS normalises count data by dividing feature read counts by the total number of reads in each sample. The method converts raw feature counts to relative abundance. Scripts used in the data processing and analysis steps are provided in the supplementary document.

Worldwide analysis

Data selection and curation

To compare the oral microbiota composition of Batwa and Bakiga with those from other worldwide populations, we selected projects with published data from microbial study management platform QIITA (http://qiita.microbio.me). The selected projects were those with oral microbiome samples with data from the V4 16S rRNA gene region using the same primer sets (Fig. S8) (Table 1; Table S1B). The dataset including respective metadata was then curated for samples from individuals at a single time point, who were adults, and who had not undergone antibiotic or another study-specific treatment that may have changed the oral microbiome composition. We also uploaded our unprocessed, merged Batwa and Bakiga read into QIITA and processed them using the parameters described below. This total dataset (n=273) consisted of 162 females and 108 males. Sex information was not available for three samples.

Data analysis

The total dataset was pre-processed within QIITA using a QIIME2 implementation with the following parameters: data was trimmed to 100nt to match data already present within QIITA, and OTU picking was done using Deblur (v1.0.3) (Amir et al. 2017). The sOTUs obtained were then inserted into the Greengenes 13_8 (McDonald et al. 2012) 99% reference tree using SEPP (Mirarab, Nguyen, and Warnow 2011). The resultant dataset was then imported in QIIME2 (2018.2) on a local workstation for the worldwide analysis. Alpha and beta diversity

calculations were computed using QIIME2 "q2-diversity" plugin (C. A. Lozupone et al. 2007; C. Lozupone and Knight 2005) with rarefaction set at 1,000 sequences per sample. Alpha diversity for single metadata categories was compared with the Kruskal-Wallis test. The unweighted UniFrac distance between samples was tested with a non-parametric PERMANOVA (Anderson 2001) and ANOSIM (Clarke 1993). Differential abundance testing was performed using ANCOM (Mandal et al. 2015) using the "q2-composition" command. Core taxa comparison was processed with the QIIME2 "q2-feature-table core feature" command, as identified within 95% of all samples. Previously published a list of environmental and laboratory contaminants were filtered from the dataset (Salter et al. 2014). Calypso was used to independently verify alpha and beta diversity results from QIIME and generate figures (Zakrzewski et al. 2017). Before analysis using Calypso, total sum normalisation (TSS) combined with square root transformation (Hellinger transformation) was performed on the dataset. TSS normalises count data by dividing feature read counts by the total number of reads in each sample. The method converts raw feature counts to relative abundance. Scripts used in the data processing and analysis will be provided in the online version of the manuscript.

Table 1. List of datasets used in the worldwide analysis.

QIITA Project Name	Location of data on QIITA	No. of samples used	Citation
Tanzanian population	https://qiita.ucsd.edu/study/description/2024	15	(Bisanz et al. 2015)
American Gut Project - prep 1164 (industrialized)	https://qiita.ucsd.edu/study/description/10317	41	(McDonald et al. 2018)
The microbiome of the uncontacted Amerindians project (Yanomami)	https://qiita.ucsd.edu/study/description/10052	17	(Clemente et al. 2015)
Batwa/Bakiga project	https://qiita.ucsd.edu/study/description/11492	96	*This study*
Temporal variability of the microbiome project (industrialized)	https://qiita.ucsd.edu/study/description/2148	104	(Franzosa et al. 2015)

Results

A robust oral microbiota signal was obtained from Ugandan saliva samples

Contamination has been shown to be a significant issue in recent microbiota studies, especially those that have limited endogenous DNA (Salter et al. 2014; Weiss et al. 2014). As our samples were initially collected in 2010, we first sought to verify the presence of a robust oral microbial signal from our data against negative laboratory controls. Alpha and beta diversity of our controls were compared with those of our saliva samples from Batwa and Bakiga. Alpha diversity measured using Faith's Phylogenetic Distance (PD) revealed that biological samples had significantly higher bacterial diversity when compared to controls (Kruskal-Wallis, $p = 0.01$) (Fig. 2A). A beta diversity comparison of the microbial composition between samples and controls using unweighted UniFrac visualised with a PCoA plot revealed that controls clustered significantly away from all biological samples, explaining 21.92% and 10.03% of the variation on axis 1 and 2, respectively ($p < 0.05$, ANOSIM) (Fig. 2B). Using both ANCOM and LEfSe analysis to detect taxa that differed in abundance between the Ugandan samples and controls, we found that the controls were significantly dominated by common environmental and lab contaminants, including *Comamonas*, *Pseudomonas,* and *Acinetobacter,* while our samples were dominated by the oral bacterial genera *Prevotella*, *Rothia, Veillonella,* and *Neisseria* (Fig. 2C). We also compared the microbial composition obtained from the Ugandan samples with published oral microbiome data from the Human Microbiome Project. The top ten core bacterial genera in our dataset were genera that have been identified as oral microbial taxa in the Human Microbiome Project (Fig. 2D), reflecting the robustness of the oral microbiota signal obtained from Ugandan saliva samples.

No significant differences were found between the Batwa and Bakiga oral microbiota

Nasidize *et al.* (2011) reported higher oral microbiota diversity amongst the Batwa compared to other African agricultural populations from different locations, suggesting that the higher levels of diversity likely originated from the

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unique rainforest hunter-gatherer lifestyle of the Batwa. Therefore, we first explored differences in the microbiota between the Batwa and neighbouring agriculturalists, the Bakiga. Alpha diversity of the two subject groups measured with Faith's PD did not reveal significant differences in the abundance of bacterial diversity between the two groups (Kruskal-Wallis, p=0.056) (Fig. 3A), although the mean level of diversity was slightly higher in the Batwa. This trend is compatible with previous findings (Nasidize *et al.* 2011), however we may be underpowered to detect significance. Similar results were obtained using additional alpha diversity metrics, including Chao1, Shannon Index and Evenness (Fig. S1 A-H).
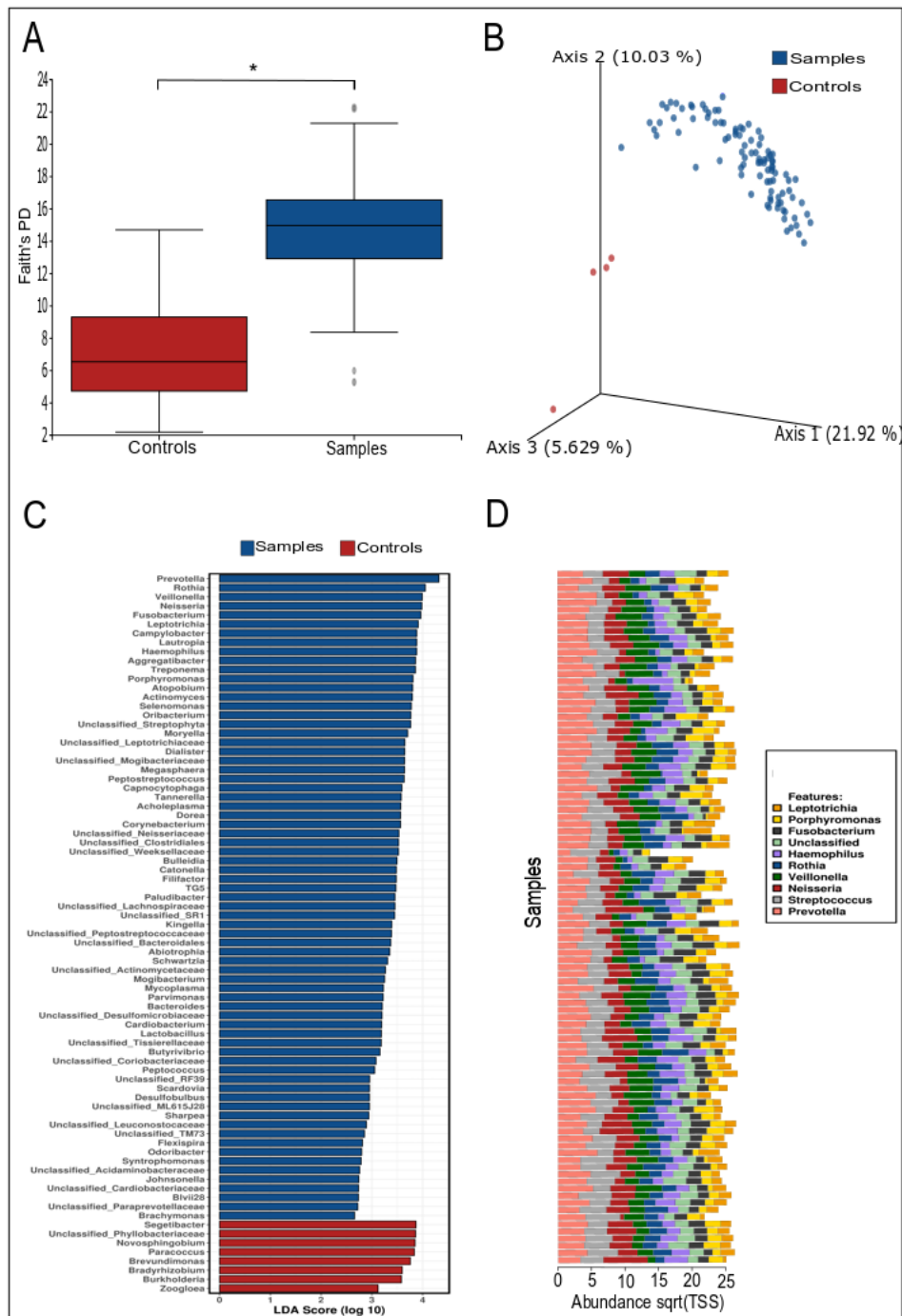
Figure 2. A. Faith's phylogenetic diversity (PD) metric for Batwa and Bakiga oral microbiota and laboratory controls. OTU tables were rarefied at 1000 sequences per sample. Biological salivary microbiota samples collectively had significantly more phylogenetically distinct taxa compared with controls (p < 0.05; Kruskal-Wallis). B. PCoA plot based on unweighted UniFrac distances calculated on OTU tables of oral samples was rarefied at 1000 sequences per sample (p < 0.05). C. Top discriminative genera between Ugandan samples and controls were determined by LEfSe analysis. D. The top 10 most abundant oral genera found in the oral microbiota from Ugandan individuals were compared.

Beta diversity analyses based on unweighted UniFrac distances revealed an absence of population-specific clusters, as no significant separation was observed between the oral microbiota of Batwa and Bakiga (p > 0.05) (Fig. 3B). This was also true for non-phylogenetic based distance metrics, including Bray-Curtis, Jaccard, and Binomial metrics (Fig. S2 A-F). We also explored the data for unique species delineating the two groups using ANCOM. However, no detectable significant differences were observed. These results were in direct contrast to what was previously reported by Nasidize, *et al.*, 2011, where the Batwa was compositionally different from other African groups. These results suggest that recent factors may have led to homogenization in the hunter-gatherer microbiota in the Batwa population or that shared environment has a larger impact on the microbiota of the Batwa and Bakiga than dietary differences.

Local geography contributes to within Ugandan microbiota differences

Next, we assessed differences within the Batwa and Bakiga populations to identify population-specific factors that may drive diversity within a population with traditional lifestyles. First, we explored the potential for sex-based differences, as observed in the gut microbiota of other African hunter-gatherer populations (Schnorr et al. 2014). No significant differences were observed in alpha diversity and beta diversity between the two sexes of Batwa individuals (p > 0.05, Kruskal-Wallis) (Fig. S6A and C). No significant differences were observed in alpha diversity and beta diversity between the two sexes of Batwa individuals (p > 0.05, Kruskal-Wallis) (Fig. S6A and C). We next assessed differences within the Batwa population amongst the eight settlement sites.

Alpha diversity analyses using Faith's PD revealed that samples collected from Kebiremu (n=11) had significantly higher bacterial diversity (p < 0.01, Kruskal-Wallis; Fig. S3A) than in other sites. This was driven by an increase in abundance of genera such as *Porphyromonas*, *Treponema*, *Acholeplasma*, *Selenomonas* and *Desulfomicrobium*. However, no significant clusters or separation was observed between any of the collection sites when beta diversity

was measured using unweighted UniFrac on a PCoA plot (p = > 0.05, Fig. S5B).
Overall, population-specific differences were limited to local geography.
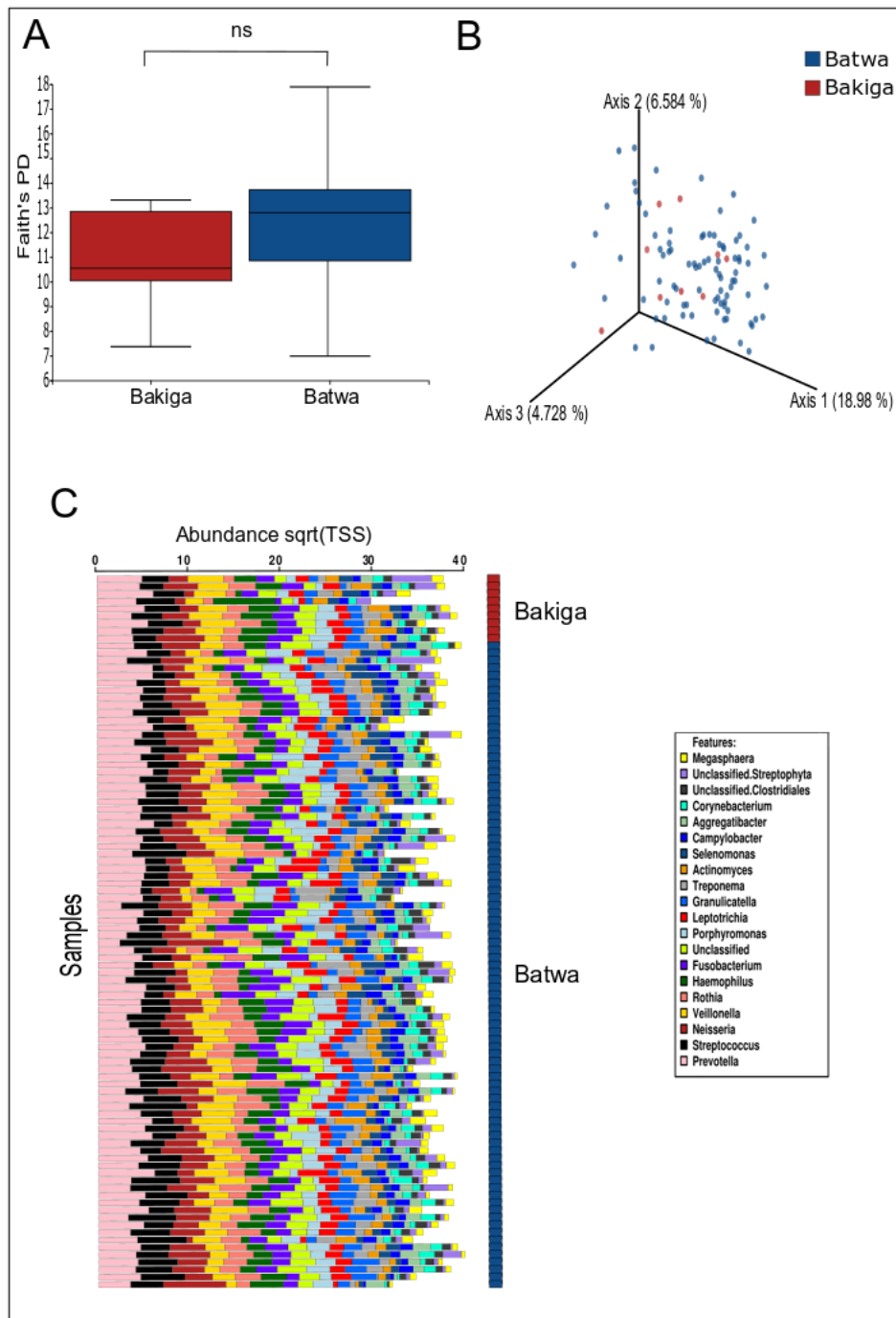
Figure 3. A. Faith's phylogenetic diversity (PD) of Batwa and Bakiga oral samples. OTU
tables were rarefied at 1000 sequences per sample (p > 0.05; Kruskal-Wallis). B. PCoA

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plot based on unweighted UniFrac distances calculated on OTU tables of Batwa and Bakiga oral samples was rarefied at 1000 sequences per sample (p > 0.05).
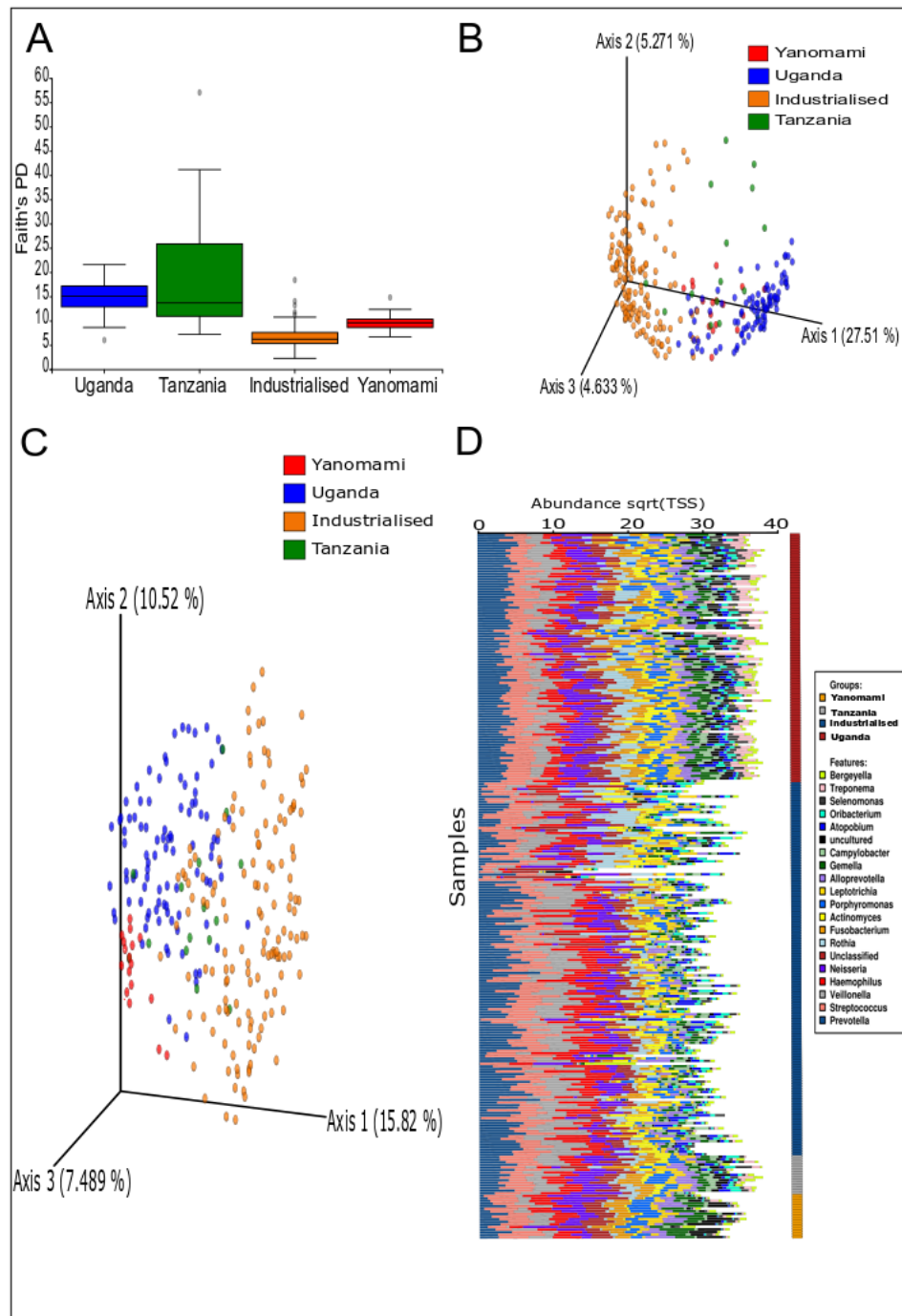
Figure 4. A. Faith's phylogenetic diversity (PD) is displayed for microbiota from Uganda, semi-urban Tanzanians (Bisanz et al. 2015), Yanomami of Venezuela (Clemente et al. 2015), and an Industrialised group (Urban Americans, Europeans and Australians (Flores et al. 2014; Mcdonald et al. 2018)). OTU tables were rarefied at 1000 sequences per sample (p < 0.05, Kruskal-Wallis) B. PCoA plot based on unweighted UniFrac distances was calculated on OTU tables of oral samples from the four population groups that were rarefied at 1000 sequences per

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sample (p < 0.05). C. Bray Curtis distances calculated on OTU tables of oral samples from the four population groups was rarefied at 1000 sequences per sample and plotted in a PCoA plot (p < 0.05).

Industrialisation is the single most significant factor driving global oral microbiota differences

To further explore the variation of the oral microbiome across populations, we performed a worldwide analysis using the Ugandan oral microbiome data in conjunction with publicly available V4 16S rRNA data from Tanzanians (Bisanz et al. 2015), Yanomami hunter-gatherers of Venezuela (Clemente et al. 2015), and urban industrialized group from the United States of America, Europe, and Australia (Flores et al. 2014; McDonald, Hyde, Debelius, Morton, Gonzalez, Gunderson, et al. 2018). Alpha diversity using Faith's PD (Kruskal-Wallis test, $p < 0.05$) indicated significant differences in the levels of bacterial diversity between all four groups. The Batwa/Bakiga group had the highest bacterial diversity, followed by Tanzanians, the Yanomami, and finally industrialized populations, which had the lowest overall bacterial diversity (Fig. 4A). This is unexpected, as the Yanomami are the only population within the data set that still maintains a traditional hunter-gatherer lifestyle and was previously reported to contain the highest oral bacterial diversity of any population studied to date (Clemente et al. 2015). This finding was further replicated using other alpha diversity metrics (Fig. S4). We also examined the beta diversity of the four population groups using unweighted UniFrac distances and Bray Curtis on a PCoA plot (Fig. 4B and C). This result indicated significant differences in the composition of the oral microbiota between the four population groups (ANOSIM (R=0.68, $p < 0.05$); PERMANOVA (F=35.22, $p < 0.05$)). Three distinct clusters were observed on axis 1, which explained 27% of the variation observed. The first cluster separates the Ugandan, Tanzanian, and Yanomami samples from industrialized populations, while the third axis separates Yanomami samples from the African samples. This result was also replicated with different beta diversity metrics (Fig. S6). Taxa such as *Streptococcu*s, *Fusobacterium* and *Actinomyces* were substantially higher in industrialized compared to the other three populations. Overall, we find significant differences between populations that correlated with the level of Industrialisation.

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Traditional lifestyles are differentiated according to the continent

We next explored differences among the non-Industrial populations. In a PCoA plot constructed from UniFrac distances, axes 2 and 3 separated the two African populations from the Yanomami population and explained 5.27% and 4.63% of the total variation respectively within the entire data set. On this axis, the Tanzanian samples fell between the Ugandan and industrialized samples, forming a gradient between the two populations (Fig. 4C; Fig S9 B and D). On axis 2, rural Ugandans were clustered away from semi-urbanised Tanzanians. We also examined the taxonomic composition of the worldwide data using ANCOM, which revealed higher levels of taxa such as *Gracilibacteria* and *Capnocytophaga* in Yanomami and taxa such as *Lactobacillus salivarius*, *Acholeplasma* species, and *Treponema amylovarum* in Ugandan samples. (Table S2). Overall, continental geography appears to differentiate the non-Industrial microbiota within this data set.

Predicted oral pathogens are proportionally lower in agriculturalist communities

We explored differences in the abundance of specific genera that contain oral pathogens between traditional and Industrial oral microbiota. Samples from non-agricultural communities, such as the Batwa and Yanomami, generally had a higher abundance of potential oral pathogens (Fig. 5B). Batwa had significantly enriched levels of opportunistic oral pathogens, including *Mycoplasma*, *Peptostreptococcus*, *Lautropia* and *Filifactor* as well as known oral pathogens *Streptococcus*, *Treponema* and *Porphyromonas*. However, we also found an increase in potentially beneficial taxa within the same populations, including *Ruminococcaceae*, *Bifidobacterium*, *Butryvibrio* and *Lactobacillus salivarius*. These taxa have been shown to play a role in ensuring a balanced, healthy oral microbial ecosystem (Burton et al. 2011; Haukioja 2010; Neville and O'Toole 2010; Picard et al. 2005). A Similar pattern of enrichment in both pathogens and beneficial taxa was observed in the Yanomami albeit with different bacterial taxa, such as *Alloprevotella*, *Gemella*, *Porphyromonas*, *Aggregatibacter* as pathogens and *Howardella* being beneficial (Fig. 5C). These beneficial taxa were either absent or found at very low levels in industrialized populations.

Core microbiota analysis in the oral microbiota

We also explored the core microbiota of all populations by looking at taxa shared amongst 60% of individuals within each group (Table S3A) and for 90% of all individuals in our dataset (Table S3B). Eight taxa were shared globally across different human populations and lifestyles; *Actinomyces, Neisseria*, *Rothia, Prevotella*, *Gemella, Streptococcus*, *Veillonella* and *Haemophilus* taxa. We next compared core microbiota of the Industrial populations to the core of the non-Industrial populations. Non-industrialised populations had a larger core microbiota (n=19) consisting of twelve taxa that were missing in the core microbiota of industrialized populations (n=11) (Table S3B) (Fig 5A). While pathogenic taxa such as *Streptococcus* and *Veillonella* were maintained in the core microbiome of industrialized populations, beneficial taxa such as *Ruminococcaceae* were absent (Table S3A). Within non-Industrial populations, the African core oral microbiota included *Ruminococcaceae*, *Stomatobaculum* and *Selenomonas* (Table S3A).
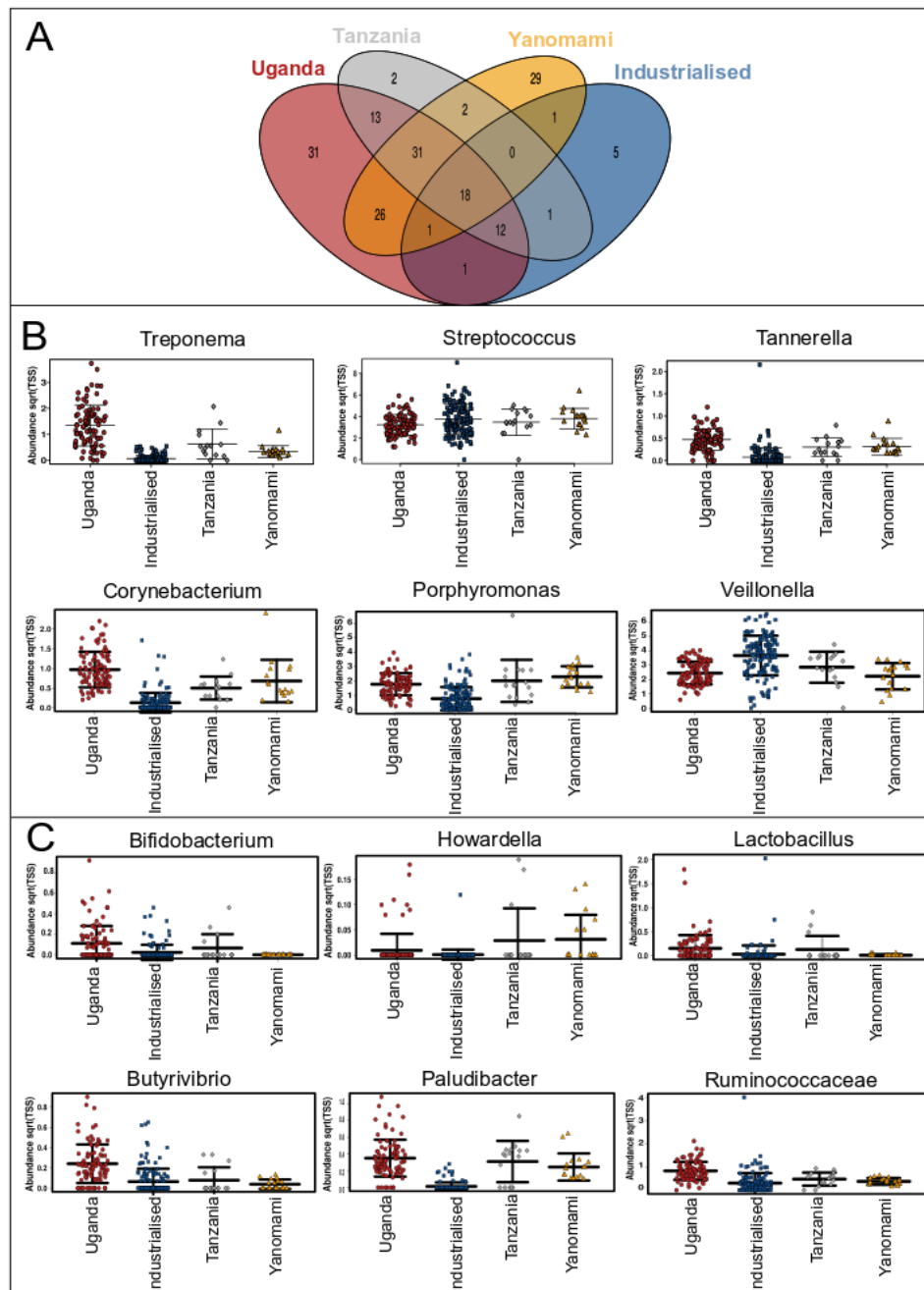
Figure 5. A. Venn diagram with unique and shared taxa between the industrialised and non-industrialised populations ($p < 0.05$, ANOVA). B. Predicted oral pathogens in the oral microbiota of worldwide populations adhering to different subsistence strategies. C. Abundance of beneficial bacteria in the oral microbiota of four worldwide populations adhering to different subsistence strategies and levels of urbanisation ($p < 0.05$, ANOVA).

Discussion

In this study, we explored the oral microbiome of the Batwa, a hunter-gather group, and the Bakiga, an agriculturalist group, living in the same rainforest environment in Uganda. We observed no significant differences in community structure or oral microbial diversity between the two populations, despite their different evolutionary histories and different diets. However, we find significant differences in community structure between these populations and other global population groups with diverse subsistence strategies. Using a larger sample size from the Batwa, we replicate previous reports of their high level of oral microbial diversity (Li et al. 2014; Nasidze et al. 2011). We find that the Batwa have the highest oral microbial diversity amongst the analysed populations from diverse global locations we analysed. This research adds to our growing understanding of the global diversity of the human oral microbiome and the impact that shifts in diet, environment, and genetics play in shaping oral health or disease.

Contrary to previous findings, we found few detectable differences between the Batwa and the Bakiga. Previous studies suggested that the Batwa lifestyle and its associated diet supported a significant increase in oral microbial diversity compared to those living an agrarian lifestyle (Nasidze et al. 2011). Although we lacked detailed dietary data in this study, contemporary and historical research has characterised the Batwa as an Indigenous African hunter-gatherer group that primarily source their food via hunting, fishing, and gathering of wild yams and honey in the African rainforest (Disko and Tugendhat 2014; A. Plumptre et al. 2004). In contrast, the Bakiga moved into the rainforest region with the Bantu expansion and have a strong cultural affinity for agriculture (Ohenjo et al. 2006). While these two different dietary strategies should have resulted in the unique oral microbiota, we instead observe a homogenization of microbiota between the two populations. Two distinct scenarios could explain these findings. First, the Batwa microbiota could have become more like the microbiota in Bakiga as the result of a shift towards a more agricultural diet when access to rainforest food sources was removed by law. The Batwa were displaced from their ancestral lands in 1991, as a result of the declaration of their forest habitation as a protected area, known as the Bwindi Impenetrable National Park, to protect the endangered mountain gorillas. This has limited Batwa access to the forest for traditional hunting and

gathering, resulting in their marginalisation and leading many Batwa to provide labour for the Bakiga in exchange for agricultural food products (Ohenjo et al. 2006).

While specific similarities in diet remain unclear, it is, therefore, possible that diet altered the microbiota in the Batwa. Second, the Bakiga migration to the edges of a highly diverse tropical environment could have resulted in microbiota alterations thereby significantly shifting their microbiota to one that is similar to that of Batwa; for example, the Bakiga may have changes in their diet, such as the consumption of wild game, or obtained similar microorganisms through the use of a shared common rainforest environment and water resources. Despite these similarities, alpha diversity was generally higher in the Batwa individuals, and several unique species such as *Rubrobacter*, *Streptococcus sobrinus*, *Eggerthia catenaformis*, *Treponema pectinovorum*, and *Leptotrichia sp. oral taxon 847* were observed. This may reflect their long-term co-evolutionary history and adaptation to an earlier hunter-gatherer lifestyle as has been observed in other animal species (Sanders 2015).

While diet and environment likely play key roles in the Batwa and Bakiga microbiota, additional factors may also contribute to the homogenization of oral microbiota between the two Ugandan populations, including host genetics (Abdul-Aziz et al. 2016; Gomez et al. 2017), oral hygiene practices (Belstrøm et al. 2018), and state of general health (Shaw et al. 2017). While a unique genetic history of the two populations could support differences in the oral microbiota, this is not what we observed. Recent genetic research has explored the genetic history of the Batwa and Bakiga populations and the potential for genetic exchanges between the two populations (Perry et al. 2014). Several reports have indicated that there has been limited genetic exchange between these populations, as intermarriages are frowned upon however, recent research shows that there are extensive levels of admixture between the two populations (Patin et al. 2014, 2017; Perry et al. 2014; Perry and Verdu 2017). These shared genetics in addition to other factors, such as hygiene or health status, may play key roles in the selection of similar microbiota in the two populations. While limited data exist on hygiene or health in these population, we also posit that general oral health status may not be an active driver in microbiota similarities, as the Batwa are not known to have poor health (Walker and Hewlett 1990), while the Bakiga suffer from poorer oral health (Agwu et al. 2015), however,

there is a lack of detail oral health assessments of the two populations. Nevertheless, based on our results and available information, we posit that a mixture of shared dietary and environmental factors likely selected for the similarities in oral microbial communities between these two populations.

While the Batwa and Bakiga shared similar oral microbiota, the two were distinct compared to other global populations and, along with other people living more traditional or rural lifestyles (*i.e.* Tanzanian and Yanomami), contained significantly different oral microbial community structure and diversity compared to industrialized populations. We find the highest level of bacterial diversity in the Batwa, which is possibly emblematic of their past hunter-gather lifestyle and diet, as well as of exposure to an incredibly bio-diverse tropical rainforest environment. The Bwindi impenetrable park surrounds many of the settlements from which our samples were collected. It is known to be amongst the most globally diverse areas in fauna and flora (Plumptre et al. 2007). This finding broadly gives credence to the hypothesis that Industrialisation leads to a reduction in bacterial diversity and a shift in microbial community structure. While agriculture has its impacts, it is likely possible that these changes in industrialized populations result in a state of imbalance that explains the recent increase in Industrial lifestyle associated oral and systemic diseases (Cordain et al. 2005; Marsh; Rosier et al. 2014).

However, the gradient of rural Tanzanians between remote Batwa and industrialized populations suggests that the Industrial shifts may take longer than previously thought or occur differently in specific populations. The Tanzanian samples represent a semi-urbanised African environment, as they were collected from Buswelu, Ilemela district in the Mwanza Region, which has the second highest rate of urbanisation in Tanzania (National Bureau of Standard 2013). Our findings suggest that the Tanzanian oral microbiome is in transition to an industrialized state, which potentially explains why Tanzanians overlaps with populations with heavily homogenised diets (Industrial), as well as those with higher dietary variation (hunting and gathering). In Tanzania, Industrialisation is characterised by a reduction in overall diversity of food, as well as an increased consumption of sugar, soft drinks, polished rice, and refined flour in place of traditional foods. Indeed, we find an American soft drink bottling company less than 5 km away from the sample collection site. While many factors contribute to the process of Industrialisation, the homogenisation of diet as a result of Industrialisation is likely

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an active driver in the observed decreased diversity, including a decrease in the beneficial bacteria that balance the proliferation of pathogenic bacteria. This may additionally underlie the increases in oral microbial diseases, as populations urbanise and societies industrialise (Skelly et al. 2018; Steckel 1999; Szreter 2004). An alternative hypothesis may be that the gradient observed in the Tanzanian samples is due to underlying unique evolutionary histories of various African populations before Industrial exposure. Following Industrialisation, this underlying composition may then result in different oral microbial transitions in different populations. All Industrial samples utilised in this paper are mostly from individuals of European ancestry, so examining African and South American populations as the urbanise and industrialise is critical to better understand this transition.

We also find differences in levels of bacterial diversity, community structure, and types of beneficial bacteria between the two indigenous hunter-gatherer populations, the Yanomami of Venezuela and the Batwa of Uganda. These differences could be explained by the diversity in environments, diets, or a multitude of other factors between them. However, we must also consider that these factors are cumulative in different evolutionary histories between the two populations. It is likely that the oral microbiota of the two populations are of distinct evolutionary histories and therefore would start at different diversities prior to Industrialization impacts. As the two populations begin to urbanise, different transitionary or even final stages in the oral microbiota may develop between them. This possibility implies that separate Indigenous populations may respond differently to Industrialisation, which could result in unique oral microbiota that supports oral and systemic diseases in unique ways. Further long-term research will be required to monitor how hunter-gather microbiota respond to Industrialisation in diverse contexts. We currently know very little about this process, the rate at which oral microbiota change, and how this may contribute to differences in oral and systemic health. This is critical research, as it has significant implications for the Indigenous health and the development of novel ways to treat the oral health issues in all human populations.

To accurately characterise the oral microbiota of the Batwa and Bakiga within a global context, we had to tackle and address some critical issues concerning meta-analyses of microbiota data sets: sample size, contamination, and

taxonomic resolution. First, recent microbiome research has shown that appropriate sample sizes and representative sampling is essential to accurately characterise the microbiome (Knight et al. 2018). To ensure this, we doubled the sample size of Batwa compared to the previous study that explored the Batwa oral microbiome (Nasidze et al. 2011). We also expanded the sampling locations of the Batwa in Uganda to seven sites to increase the geographic representation within the dataset. To obtain a global context, we performed a worldwide analysis incorporating another 170 samples using the microbial study management platform QIITA, resulting in the largest worldwide analysis of published oral 16S rRNA gene (V4) data to our knowledge. As research into microbiomes expands, meta-analyses of similar data sets from published studies will be crucial to our understanding of human microbiota from various geographical locations. In this study, we were limited to only available data from the V4 region to standardise the analysis and taxonomic identification. This limited our ability to compare our results with previously published data with different 16S rRNA variable (V) regions such as that of Nasidze et al. (2011). However, a recently published method, fragment insertion, allows for the analysis of 16S rRNA across various 16S variable regions and fragment lengths (Janssen et al. 2018).

Second, laboratory and environmental contamination can critically impact the levels of diversity and microbiome structure of meta-analyses (Salter et al. 2014). Here, we assessed contamination within the samples throughout the worldwide analysis and use sOTUs to identify better and track cross-contamination. In the future, additional methods in the future, such as decontam (Davis 2017), will continue to improve estimates of contamination in the worldwide analysis. Lastly, the use of 16S rRNA gene amplicon sequencing is limited in its taxonomic resolution and ability to accurately characterise bacterial function. The use of whole genome (shotgun) metagenomic analysis has the potential for higher taxonomic resolution and the identification of functionally relevant taxa, drastically improving our interpretation of meta-analyses. While the cost of shotgun sequencing is still prohibitive for many laboratories, recent research has shown that cheaper shallow shotgun sequencing would provide similar accuracy to deeper shotgun sequencing, significantly reducing the costs of use for this technology (Hillmann et al. 2018). While shotgun analysis of saliva samples can be complicated due to high levels of human DNA, some recent approaches have been reported to mitigate this challenge

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(Feechery 2013, Marota 2018). Continued improvements in these areas will allow for more robust meta-analyses and ensure we can obtain an accurate understanding of how cultural and lifestyle changes impact the human oral microbiome.

References

1. Aas, Jørn A et al. 2005. "Defining the Normal Bacterial Flora of the Oral Cavity." *Journal of clinical microbiology* 43(11): 5721–32. http://www.ncbi.nlm.nih.gov/pubmed/16272510 (August 19, 2015).
2. Abdul-Aziz, M.A. Muslihudeen Abdul-Razaq, Alan Cooper, and Laura S. L.S. Weyrich. 2016. "Exploring Relationships between Host Genome and Microbiome: New Insights from Genome-Wide Association Studies." *Frontiers in Microbiology* 7(OCT): 1611. http://journal.frontiersin.org/article/10.3389/fmicb.2016.01611/full (October 10, 2016).
3. Adler, Christina J et al. 2013. "Sequencing Ancient Calcified Dental Plaque Shows Changes in Oral Microbiota with Dietary Shifts of the Neolithic and Industrial Revolutions." *Nature genetics* 45(4): 450–5, 455e1. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3996550&tool=pmcentrez&rendertype=abstract.
4. Agwu, Ezera, John C. Ihongbe, Joseph O. Ezeonwumelu, and Moazzam M. Lodhi. 2015. "Baseline Burden and Antimicrobial Susceptibility of Pathogenic Bacteria Recovered from Oral Lesions of Patients with HIV/AIDS in South-Western Uganda." *Oral Science International* 12(2): 59–66. https://www-sciencedirect-com.proxy.library.adelaide.edu.au/science/article/pii/S134886431500018X (August 27, 2018).
5. Amir, Amnon et al. 2017. "Deblur Rapidly Resolves Single-Nucleotide Community Sequence Patterns" ed. Jack A. Gilbert. *mSystems* 2(2): e00191-16. http://msystems.asm.org/lookup/doi/10.1128/mSystems.00191-16 (February 19, 2018).
6. Anderson, Marti J. 2001. "A New Method for Non Parametric Multivariate Analysis of Variance." *Austral ecology* 26: 32–46. http://onlinelibrary.wiley.com/doi/10.1111/j.1442-9993.2001.01070.pp.x/full.
7. Arensburg, B. 1996. "Ancient Dental Calculus and Diet." *Human Evolution* 11(2): 139–45. http://link.springer.com/10.1007/BF02437397 (July 6, 2018).
8. Aronesty, Erik. 2013. "Comparison of Sequencing Utility Programs." *The Open Bioinformatics Journal* 7(1): 1–8. http://benthamopen.com/ABSTRACT/TOBIOIJ-7-1 (April 14, 2018).
9. Le Bars, Pierre et al. 2017. "The Oral Cavity Microbiota: Between Health, Oral Disease, and Cancers of the Aerodigestive Tract." *Canadian Journal of Microbiology* 63(6): 475–92. http://www.nrcresearchpress.com/doi/pdf/10.1139/cjm-2016-0603?src=recsys& (February 6, 2018).
10. Basch, Corey H., Lalitha Samuel, and Danna Ethan. 2013. "Obesity, Diabetes and Heart Disease: Effects of Globalization on Population Health, Preventive Efforts, and the Importance of Social Change." *International Journal of Health Promotion and Education* 51(4): 185–97. http://www.tandfonline.com/doi/abs/10.1080/14635240.2013.787732

(April 26, 2018).

11. Belkaid, Yasmine, and Timothy W Hand. 2014. "Role of the Microbiota in Immunity and Inflammation." *Cell* 157(1): 121–41. http://www.sciencedirect.com/science/article/pii/S0092867414003456 (July 15, 2014).

12. Belstrøm, D. et al. 2018. "Impact of Oral Hygiene Discontinuation on Supragingival and Salivary Microbiomes." *JDR Clinical & Translational Research* 3(1): 57–64. http://www.ncbi.nlm.nih.gov/pubmed/29662960 (April 22, 2018).

13. Bisanz, Jordan E et al. 2015. "Microbiota at Multiple Body Sites during Pregnancy in a Rural Tanzanian Population and Effects of Moringa-Supplemented Probiotic Yogurt" ed. H. L. Drake. *Applied and Environmental Microbiology* 81(15): 4965–75. http://www.ncbi.nlm.nih.gov/pubmed/25979893 (November 27, 2017).

14. Blaser, Martin J. 2006. "Who Are We? Indigenous Microbes and the Ecology of Human Diseases." *EMBO reports* 7(10): 956–60. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1618379&tool=pmcentrez&rendertype=abstract (October 7, 2015).

15. Bokulich, Nicholas A et al. 2018. "Optimizing Taxonomic Classification of Marker Gene Amplicon Sequences." https://peerj.com/preprints/3208/ (April 14, 2018).

16. Brotherton, Paul et al. 2013. "Neolithic Mitochondrial Haplogroup H Genomes and the Genetic Origins of Europeans." *Nature communications* 4: 1764. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3978205&tool=pmcentrez&rendertype=abstract (February 15, 2016).

17. Burton, J., P. Wescombe, P. Cadieux, and J. Tagg. 2011. "Beneficial Microbes for the Oral Cavity: Time to Harness the Oral Streptococci?" *Beneficial Microbes* 2(2): 93–101. http://www.wageningenacademic.com/doi/10.3920/BM2011.0002 (April 3, 2018).

18. Caporaso, J Gregory et al. 2012. "Ultra-High-Throughput Microbial Community Analysis on the Illumina HiSeq and MiSeq Platforms." *The ISME Journal* 6(8): 1621–24. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3400413&tool=pmcentrez&rendertype=abstract (July 9, 2014).

19. Carding, Simon et al. 2015. "Dysbiosis of the Gut Microbiota in Disease." *Microbial ecology in health and disease* 26: 26191. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4315779&tool=pmcentrez&rendertype=abstract (March 14, 2015).

20. Chen, Casey et al. 2018. "Oral Microbiota of Periodontal Health and Disease and Their Changes after Nonsurgical Periodontal Therapy." *The ISME Journal* 12(5): 1210–24. http://www.nature.com/articles/s41396-017-0037-1 (January 20, 2018).

21. CLARKE, K. R. 1993. "Non-parametric Multivariate Analyses of Changes in Community Structure." *Australian Journal of Ecology* 18(1): 117–43.

22. Clemente, J. C. et al. 2015. "The Microbiome of Uncontacted Amerindians." *Science Advances* 1(3): e1500183–e1500183. http://advances.sciencemag.org/cgi/doi/10.1126/sciadv.1500183 (April 18,

2015).

23. Cordain, Loren et al. 2005. "Origins and Evolution of the Western Diet: Health Implications for the 21st Century." *The American journal of clinical nutrition*.

24. David, Lawrence A. et al. 2014. "Diet Rapidly and Reproducibly Alters the Human Gut Microbiome." *Nature* 505(7484): 559–63. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3957428&tool =pmcentrez&rendertype=abstract (July 9, 2014).

25. Disko, Stefan, and Helen Tugendhat. 2014. *World Heritage Sites and Indigenous Peoples' Rights*. eds. Stefan Disko and Helen Tugendhat. Copenhagen: Transaction Publishers. https://www.iwgia.org/images/documents/popular-publications/world-heritage-sites-final-eb.pdf (July 2, 2018).

26. De Filippis, Francesca et al. 2014. "The Same Microbiota and a Potentially Discriminant Metabolome in the Saliva of Omnivore, Ovo-Lacto-Vegetarian and Vegan Individuals" ed. Gabriele Berg. *PLoS ONE* 9(11): e112373. http://dx.plos.org/10.1371/journal.pone.0112373 (June 8, 2018).

27. De Filippo, Carlotta et al. 2010. "Impact of Diet in Shaping Gut Microbiota Revealed by a Comparative Study in Children from Europe and Rural Africa." *Proceedings of the National Academy of Sciences of the United States of America* 107(33): 14691–96. http://www.scopus.com/inward/record.url?eid=2-s2.0-77957075815&partnerID=tZOtx3y1.

28. Flores, Gilberto E et al. 2014. "Temporal Variability Is a Personalized Feature of the Human Microbiome." *Genome Biology* 15(12). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4252997/pdf/13059_2014 _Article_531.pdf (November 27, 2017).

29. Franzosa, Eric A et al. 2015. "Identifying Personal Microbiomes Using Metagenomic Codes." *Proceedings of the National Academy of Sciences* 112(22).

30. Giacaman, RA. 2017. "Sugars and beyond. The Role of Sugars and the Other Nutrients and Their Potential Impact on Caries." *Oral Diseases*. http://doi.wiley.com/10.1111/odi.12778 (October 18, 2017).

31. Gomez, Andres et al. 2017. "Host Genetic Control of the Oral Microbiome in Health and Disease." *Cell Host & Microbe* 22(3): 269–278.e3. http://www.sciencedirect.com/science/article/pii/S1931312817303463 (September 18, 2017).

32. Hansen, Tue H. et al. 2018. "Impact of a Vegan Diet on the Human Salivary Microbiota." *Scientific Reports* 8(1): 5847. http://www.nature.com/articles/s41598-018-24207-3 (April 15, 2018).

33. Haukioja, Anna. 2010. "Probiotics and Oral Health." *European journal of dentistry* 4(3): 348–55. http://www.ncbi.nlm.nih.gov/pubmed/20613927 (July 6, 2018).

34. Heberle, Henry et al. 2015. "InteractiVenn: A Web-Based Tool for the Analysis of Sets through Venn Diagrams." *BMC Bioinformatics* 16(1): 169. http://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-015-0611-3 (October 8, 2018).

35. Hillmann, Benjamin et al. 2018. "Evaluating the Information Content of Shallow Shotgun Metagenomics." *bioRxiv*: 320986. https://www.biorxiv.org/content/biorxiv/early/2018/05/12/320986.full.pdf ?%3Fcollection= (May 15, 2018).

36. Janssen, Stefan et al. 2018. "Phylogenetic Placement of Exact Amplicon Sequences Improves Associations with Clinical Information" ed. Nicholas Chia. *mSystems* 3(3): e00021-18. http://msystems.asm.org/lookup/doi/10.1128/mSystems.00021-18 (June 5, 2018).

37. Kitada, K, A de Toledo, and T Oho. 2009. "Increase in Detectable Opportunistic Bacteria in the Oral Cavity of Orthodontic Patients." *International Journal of Dental Hygiene* 7(2): 121–25. http://www.ncbi.nlm.nih.gov/pubmed/19416094 (April 3, 2018).

38. Knight, Rob et al. 2018. "Best Practices for Analysing Microbiomes." *Nature Reviews Microbiology* 16(7): 1. http://www.nature.com.proxy.library.adelaide.edu.au/articles/s41579-018-0029-9.pdf (June 5, 2018).

39. Krishnan, K., T. Chen, and B J Paster. 2017. "A Practical Guide to the Oral Microbiome and Its Relation to Health and Disease." *Oral diseases* 23(3): 276–86. http://www.ncbi.nlm.nih.gov/pubmed/27219464 (October 23, 2017).

40. Lassalle, Florent et al. 2017. "Oral Microbiomes from Hunter-Gatherers and Traditional Farmers Reveal Shifts in Commensal Balance and Pathogen Load Linked to Diet." *Molecular Ecology* 27(1): 182–95. http://www.ncbi.nlm.nih.gov/pubmed/29165844 (November 27, 2017).

41. Li, Jing et al. 2014. "Comparative Analysis of the Human Saliva Microbiome from Different Climate Zones: Alaska, Germany, and Africa." *BMC Microbiology* 14(1): 316. http://www.scopus.com/inward/record.url?eid=2-s2.0-84924137629&partnerID=tZOtx3y1 (October 16, 2015).

42. Logan, Alan C, Felice N Jacka, and Susan L Prescott. 2016. "Immune-Microbiota Interactions: Dysbiosis as a Global Health Issue." *Current allergy and asthma reports* 16(2): 13. http://www.ncbi.nlm.nih.gov/pubmed/26768621 (January 18, 2016).

43. Lozupone, Catherine et al. 2011. "UniFrac: An Effective Distance Metric for Microbial Community Comparison." *ISME Journal* 5(2): 169–72.

44. Lozupone, Catherine A, Micah Hamady, Scott T Kelley, and Rob Knight. 2007. "Quantitative and Qualitative Beta Diversity Measures Lead to Different Insights into Factors That Structure Microbial Communities." *Applied and environmental microbiology* 73(5): 1576–85. http://www.ncbi.nlm.nih.gov/pubmed/17220268 (April 9, 2018).

45. Lozupone, Catherine, and Rob Knight. 2005. "UniFrac : A New Phylogenetic Method for Comparing Microbial Communities UniFrac : A New Phylogenetic Method for Comparing Microbial Communities." *Applied and environmental microbiology* 71(12): 8228–35.

46. Mandal, Siddhartha et al. 2015. "Analysis of Composition of Microbiomes: A Novel Method for Studying Microbial Composition." *Microbial Ecology in Health & Disease* 26: 27663. http://www.ncbi.nlm.nih.gov/pubmed/26028277 (June 1, 2017).

47. Marchesi, Julian R., and Jacques Ravel. 2015. "The Vocabulary of

Microbiome Research: A Proposal." *Microbiome* 3(1): 31. http://www.microbiomejournal.com/content/3/1/31 (July 31, 2015).

48. Mark Welch, Jessica L. et al. 2016. "Biogeography of a Human Oral Microbiome at the Micron Scale." *Proceedings of the National Academy of Sciences of the United States of America* 113(6): E791-800. http://www.ncbi.nlm.nih.gov/pubmed/26811460 (January 15, 2018).

49. Marsh, P. D. "Are Dental Diseases Examples of Ecological Catastrophes?" *Microbiology* 149(2): 279–94. http://www.microbiologyresearch.org/docserver/fulltext/micro/149/2/279.pdf?expires=1527554076&id=id&accname=guest&checksum=4EBB15118FAFA748758EECFA61BF04D3 (May 29, 2018).

50. Mason, Matthew R. et al. 2013. "Deep Sequencing Identifies Ethnicity-Specific Bacterial Signatures in the Oral Microbiome" ed. Indranil Biswas. *PLoS ONE* 8(10): e77287. http://dx.plos.org/10.1371/journal.pone.0077287.

51. McDonald, Daniel et al. 2012. "An Improved Greengenes Taxonomy with Explicit Ranks for Ecological and Evolutionary Analyses of Bacteria and Archaea." *The ISME journal* 6(3): 610–18. https://www.scienceopen.com/document/id/3aa95555-2522-488b-ae7b-06b115400a4e (July 9, 2014).

52. ———. 2016. "Extreme Dysbiosis of the Microbiome in Critical Illness." *mSphere* 1(4): e00199-16. http://msphere.asm.org/lookup/doi/10.1128/mSphere.00199-16.

53. McDonald, Daniel, Embriette R Hyde, Justine W Debelius, James T Morton, Antonio Gonzalez, Gail Ackermann, et al. 2018. "American Gut: An Open Platform for Citizen-Science Microbiome Research." *bioRxiv*: 277970. https://www.biorxiv.org/content/early/2018/03/07/277970 (April 7, 2018).

54. McDonald, Daniel, Embriette Hyde, Justine W. Debelius, James T. Morton, Antonio Gonzalez, Gail Ackermann, et al. 2018. "American Gut: An Open Platform for Citizen Science Microbiome Research" ed. Casey S. Greene. *mSystems* 3(3): e00031-18. http://msystems.asm.org/lookup/doi/10.1128/mSystems.00031-18 (June 5, 2018).

55. Mirarab, S., N. Nguyen, and T. Warnow. 2011. "SEPP: SATé-Enabled Phylogenetic Placement." In *Biocomputing 2012*, WORLD SCIENTIFIC, 247–58. http://www.worldscientific.com/doi/abs/10.1142/9789814366496_0024.

56. Muegge, Brian D. et al. 2011. "Diet Drives Convergence in Gut Microbiome Functions across Mammalian Phylogeny and within Humans." *Science (New York, N.Y.)* 332(6032): 970–74. http://www.ncbi.nlm.nih.gov/pubmed/21596990 (March 15, 2018).

57. Nasidze, Ivan et al. 2009. "Global Diversity in the Human Salivary Microbiome." *Genome research* 19(4): 636–43. http://genome.cshlp.org/content/19/4/636.short (November 16, 2015).

58. ———. 2011. "High Diversity of the Saliva Microbiome in Batwa Pygmies" ed. Francisco Rodríguez-Valera. *PLoS ONE* 6(8): e23352. http://dx.plos.org/10.1371/journal.pone.0023352 (August 30, 2017).

59. National Bureau of Standard. 2013. "2012 Population and Housing Census Population Distribution by Administrative Areas." *NBS ministry of*

finance.

60. Navas-Molina, José A. et al. 2013. "Advancing Our Understanding of the Human Microbiome Using QIIME." *Methods in Enzymology* 531: 371–444. http://linkinghub.elsevier.com/retrieve/pii/B9780124078635000198.

61. Neville, BA, and PW O'Toole. 2010. "Probiotic Properties of Lactobacillus Salivarius and Closely Related *Lactobacillus* Species." *Future Microbiology* 5(5): 759–74. http://www.futuremedicine.com/doi/10.2217/fmb.10.35 (April 24, 2018).

62. Ohenjo, Nyang'ori et al. 2006. "Health of Indigenous People in Africa." *Lancet (London, England).*

63. Pakendorf, Brigitte, Koen Bostoen, and Cesare De Filippo. 2011. "Molecular Perspectives on the Bantu Expansion: A Synthesis." *Language Dynamics and Change* 1(1): 50–88.

64. Parekh, Parth J, Luis A Balart, and David A Johnson. 2015. "The Influence of the Gut Microbiome on Obesity, Metabolic Syndrome and Gastrointestinal Disease." *Clinical and translational gastroenterology* 6: e91. http://dx.doi.org/10.1038/ctg.2015.16 (December 14, 2015).

65. Patin, Etienne et al. 2014. "The Impact of Agricultural Emergence on the Genetic History of African Rainforest Hunter-Gatherers and Agriculturalists." *Nature Communications* 5(1): 3163. http://www.nature.com/articles/ncomms4163 (July 3, 2018).

66. ———. 2017. "Dispersals and Genetic Adaptation of Bantu-Speaking Populations in Africa and North America." *Science (New York, N.Y.)* 356(6337): 543–46. http://www.ncbi.nlm.nih.gov/pubmed/28473590 (November 27, 2017).

67. Perry, George H., and Paul Verdu. 2017. "Genomic Perspectives on the History and Evolutionary Ecology of Tropical Rainforest Occupation by Humans." *Quaternary International* 448: 150–57. http://www.sciencedirect.com/science/article/pii/S104061821630115X (May 10, 2016).

68. Perry, George H et al. 2014. "Adaptive, Convergent Origins of the Pygmy Phenotype in African Rainforest Hunter-Gatherers." *Proceedings of the National Academy of Sciences of the United States of America* 111(35): E3596-603. http://www.pnas.org/content/111/35/E3596.full#sec-7 (May 4, 2016).

69. PICARD, C. et al. 2005. "Review Article: Bifidobacteria as Probiotic Agents - Physiological Effects and Clinical Benefits." *Alimentary Pharmacology and Therapeutics* 22(6): 495–512. http://doi.wiley.com/10.1111/j.1365-2036.2005.02615.x (June 9, 2018).

70. Plumptre, Andrew et al. 2004. 4 Albertine Rift Technical Reports Series *Socioeconomic Status of People in the Central Albertine Rift.*

71. Plumptre, Andrew J. et al. 2007. "The Biodiversity of the Albertine Rift." *Biological Conservation.*

72. Proctor, Diana M. et al. 2018. "A Spatial Gradient of Bacterial Diversity in the Human Oral Cavity Shaped by Salivary Flow." *Nature Communications* 9(1): 681. http://www.nature.com/articles/s41467-018-02900-1 (February 21, 2018).

73. Quast, Christian et al. 2013. "The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools." *Nucleic Acids Research* 41(D1): D590–96.

http://academic.oup.com/nar/article/41/D1/D590/1069277/The-SILVA-ribosomal-RNA-gene-database-project (April 14, 2018).

74. Rosier, Bob T., Marko De Jager, Egija Zaura, and Bastiaan P. Krom. 2014. "Historical and Contemporary Hypotheses on the Development of Oral Diseases: Are We There Yet?" *Frontiers in cellular and infection microbiology* 4: 92. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4100321&tool=pmcentrez&rendertype=abstract (May 22, 2016).

75. Salter, SJ Susannah J et al. 2014. "Reagent and Laboratory Contamination Can Critically Impact Sequence-Based Microbiome Analyses." *BMC Biology* 12(1): 87. http://www.biomedcentral.com/1741-7007/12/87 (October 10, 2016).

76. Sampaio-Maia, B. et al. 2016. "The Oral Microbiome in Health and Its Implication in Oral and Systemic Diseases." *Advances in applied microbiology* 97: 171–210. https://www.sciencedirect.com/science/article/pii/S0065216416301095?_rdoc=1&_fmt=high&_origin=gateway&_docanchor=&md5=b8429449ccfc9c30159a5f9aeaa92ffb&dgcid=raven_sd_recommender_email (December 8, 2016).

77. Schnorr, Stephanie L et al. 2014. "Gut Microbiome of the Hadza Hunter-Gatherers." *Nature communications* 5: 3654. http://www.nature.com/ncomms/2014/140415/ncomms4654/full/ncomms4654.html.

78. Segata, Nicola et al. 2011. "Metagenomic Biomarker Discovery and Explanation." *Genome Biology* 12(6): R60. http://genomebiology.biomedcentral.com/articles/10.1186/gb-2011-12-6-r60 (July 7, 2018).

79. Sender, Ron, Shai Fuchs, and Ron Milo. 2016. "Revised Estimates for the Number of Human and Bacteria Cells in the Body." *PLoS Biology* 14(8).

80. Shaw, Liam et al. 2017. "The Human Salivary Microbiome Is Shaped by Shared Environment Rather than Genetics: Evidence from a Large Family of Closely Related Individuals." *mBio* 8(5): e01237-17. http://www.ncbi.nlm.nih.gov/pubmed/28900019 (September 18, 2017).

81. Simpson, Kerri T., and John G. Thomas. 2016. "Oral Microbiome: Contributions to Local and Systemic Infections." *Current Oral Health Reports* 3(1): 45–55. http://link.springer.com/10.1007/s40496-016-0079-x (July 17, 2017).

82. Skelly, Emily, Kostas Kapellas, Alan Cooper, and Laura S. Weyrich. 2018. "Consequences of Colonialism: A Microbial Perspective to Contemporary Indigenous Health." *American Journal of Physical Anthropology*. http://www.ncbi.nlm.nih.gov/pubmed/30159882 (September 4, 2018).

83. Steckel, Richard. 1999. *Industrialization and Health in Historical Perspective*. Cambridge, MA. http://www.nber.org/papers/h0118.pdf (April 26, 2018).

84. Szreter, S. 2004. "Industrialization and Health." *British Medical Bulletin* 69(1): 75–86. https://academic.oup.com/bmb/article-lookup/doi/10.1093/bmb/ldh005 (April 26, 2018).

85. Takeshita, Toru et al. 2015. "Distinct Composition of the Oral Indigenous Microbiota in South Korean and Japanese Adults." *Scientific Reports* 4(1):

6990. http://www.nature.com/articles/srep06990 (June 4, 2018).

86. Tuohy, Kieran, and Daniele Del Rio. 2014. *Diet-Microbe Interactions in the Gut : Effects on Human Health and Disease*. Elsevier Science. https://books.google.com.au/books?id=MQR0AwAAQBAJ&pg=PA53&lpg=PA53&dq=diseases+of+affluence+microbiome&source=bl&ots=iw4ftdxRrn&sig=xFcZT6WptcTNOh1QOwQ3LVU0W6c&hl=en&sa=X&ved=0ahUKEwing7PA7tbaAhUBXLwKHaMoBTIQ6AEIdDAH#v=onepage&q=diseases%2520of%2520affluence%25 (April 26, 2018).

87. Valle Gottlieb, Maria Gabriela, Vera Elizabeth Closs, Vilma Maria Junges, and Carla Helena Augustin Schwanke. 2017. "Impact of Human Aging and Modern Lifestyle on Gut Microbiota." *Critical Reviews in Food Science and Nutrition*: 1–8. https://www.tandfonline.com/doi/full/10.1080/10408398.2016.1269054 (March 8, 2018).

88. Walker, Pl, and Bs Hewlett. 1990. "Dental Health Diet and Social Status among Central African Foragers and Farmers." *American Anthropologist* 92(2): 383–98. https://anthrosource-onlinelibrary-wiley-com.proxy.library.adelaide.edu.au/doi/pdf/10.1525/aa.1990.92.2.02a00080 (June 4, 2018).

89. Weiss, Sophie et al. 2014. "Tracking down the Sources of Experimental Contamination in Microbiome Studies." *Genome biology* 15(12): 564. http://genomebiology.com/2014/15/12/564 (May 11, 2017).

90. Weyrich, Laura S., Keith Dobney, and Alan Cooper. 2015. "Ancient DNA Analysis of Dental Calculus." *Journal of Human Evolution* 79: 119–24. https://ac-els-cdn-com.proxy.library.adelaide.edu.au/S0047248414002541/1-s2.0-S0047248414002541-main.pdf?_tid=3e735bda-9676-4977-b507-61c4a0c7c047&acdnat=1522637436_38a86a3a6b77342dcad9a3e7b605a431 (April 2, 2018).

91. Weyrich, Laura S et al. 2017. "Neanderthal Behaviour, Diet, and Disease Inferred from Ancient DNA in Dental Calculus." *Nature Publishing Group* 544. https://www.nature.com/nature/journal/v544/n7650/pdf/nature21674.pdf (November 1, 2017).

92. Yang, Fang et al. 2012. "Saliva Microbiomes Distinguish Caries-Active from Healthy Human Populations." *The ISME journal* 6(1): 1–10. http://www.nature.com.proxy.library.adelaide.edu.au/ismej/journal/v6/n1/full/ismej201171a.html (January 5, 2016).

93. Zakrzewski, Martha et al. 2017. "Calypso: A User-Friendly Web-Server for Mining and Visualizing Microbiome-Environment Interactions." *Bioinformatics (Oxford, England)* 33(5): 782–83. http://www.ncbi.nlm.nih.gov/pubmed/28025202 (July 5, 2018).

94. Zhang, Xuan et al. 2015. "The Oral and Gut Microbiomes Are Perturbed in Rheumatoid Arthritis and Partly Normalized after Treatment." *Nature medicine* (July): 1–13.

Supplementary Material
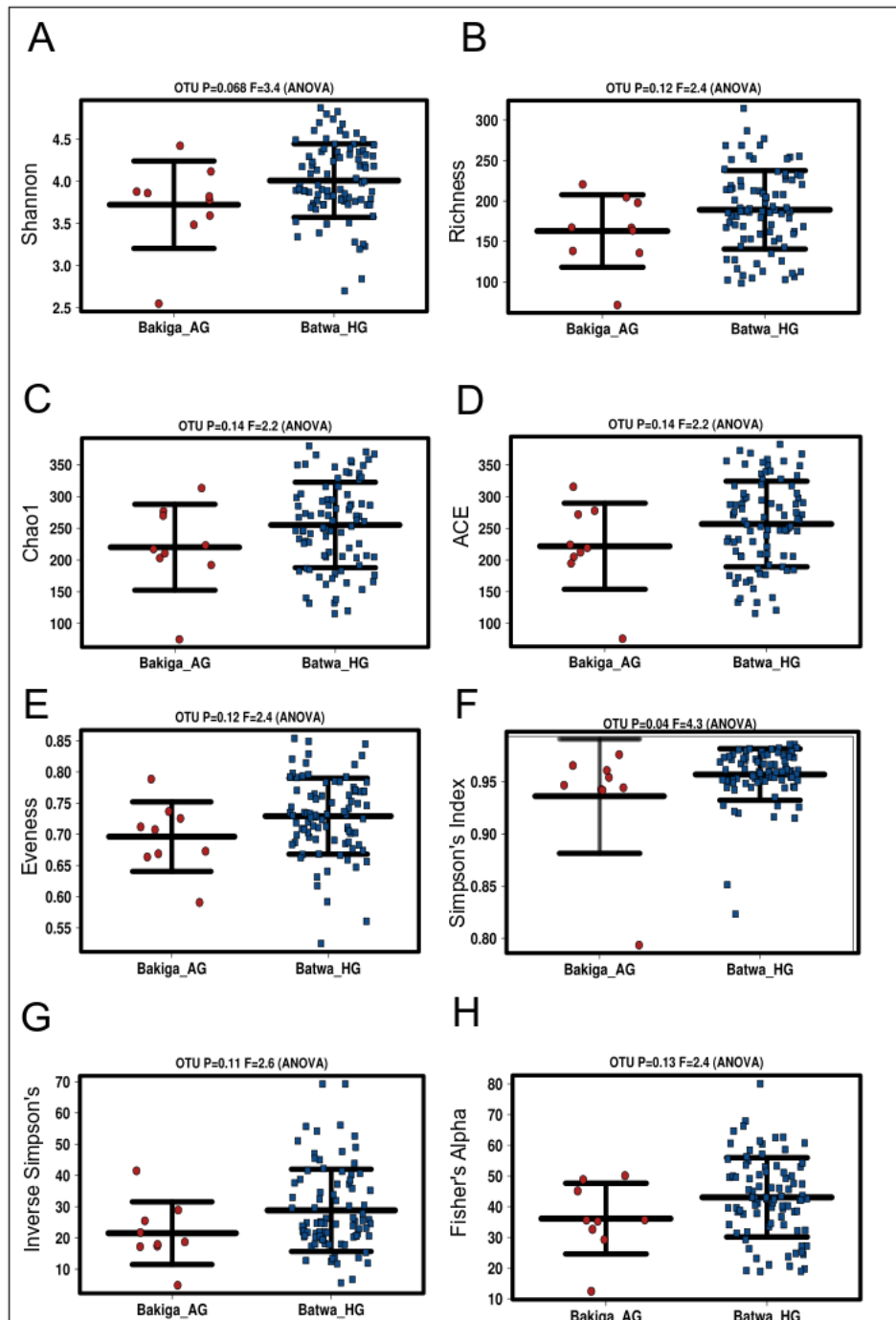
Figures

Figure S1. Alpha Diversity comparison between Batwa and Bakiga OTUs computed with A. Shannon index, B. Richness, C. Chao1, D. ACE, E. Evenness, F. Simpson's index, G. Inverse Simpson, H. Fisher's Alpha for Batwa and Bakiga

of Uganda. OTU tables rarefied at 1000 sequences per sample (p > 0.05, ANOVA).
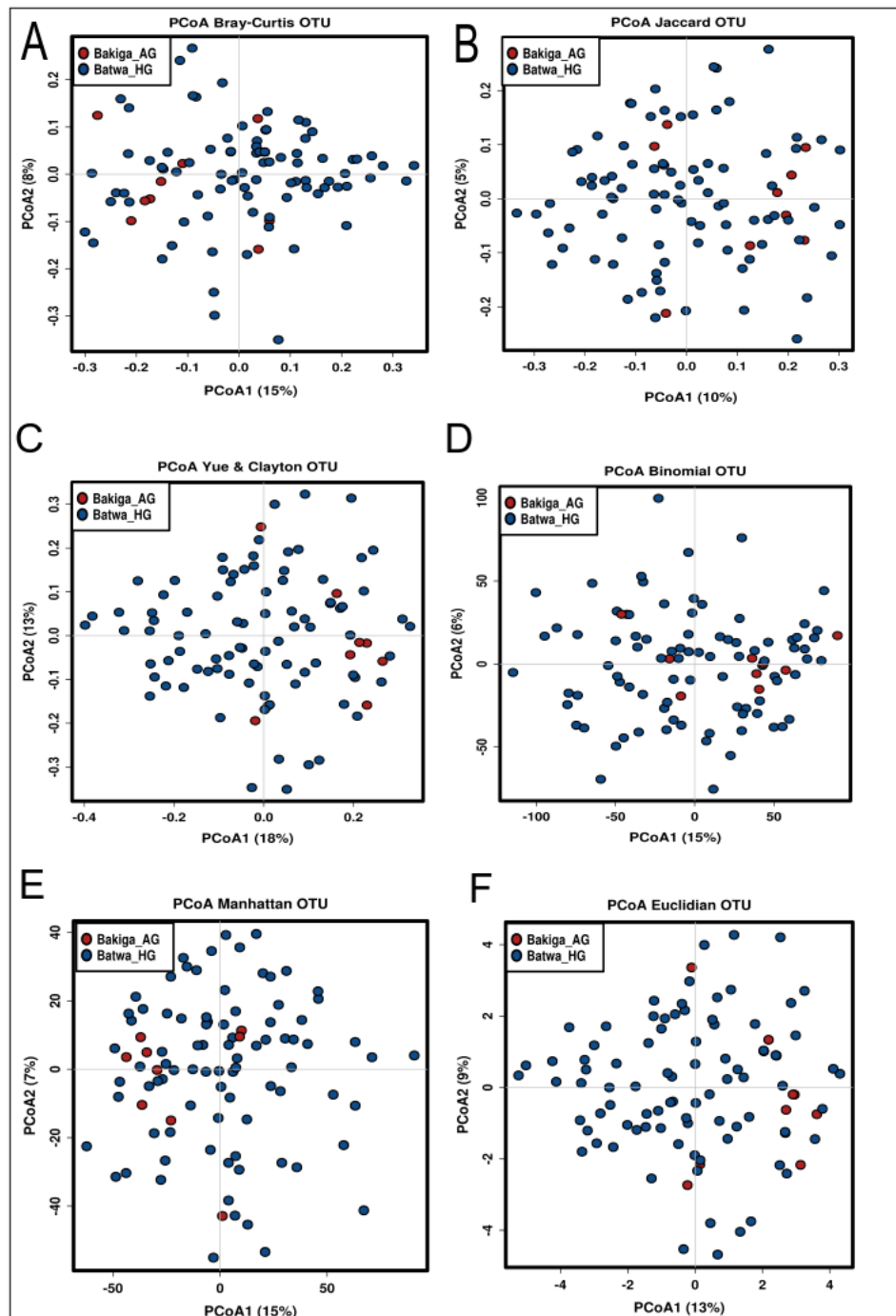
Figure S2. Beta diversity comparison between Batwa and Bakiga OTU, based on PCoA plot of: A. Bray-Curtis, B. Jaccard distance, C. Yue & Clayton, D. Binomial, E. Manhattan, F. Euclidian distance matrix. OTU tables rarefied at 1000 sequences per sample (p > 0.05, PERMANOVA).
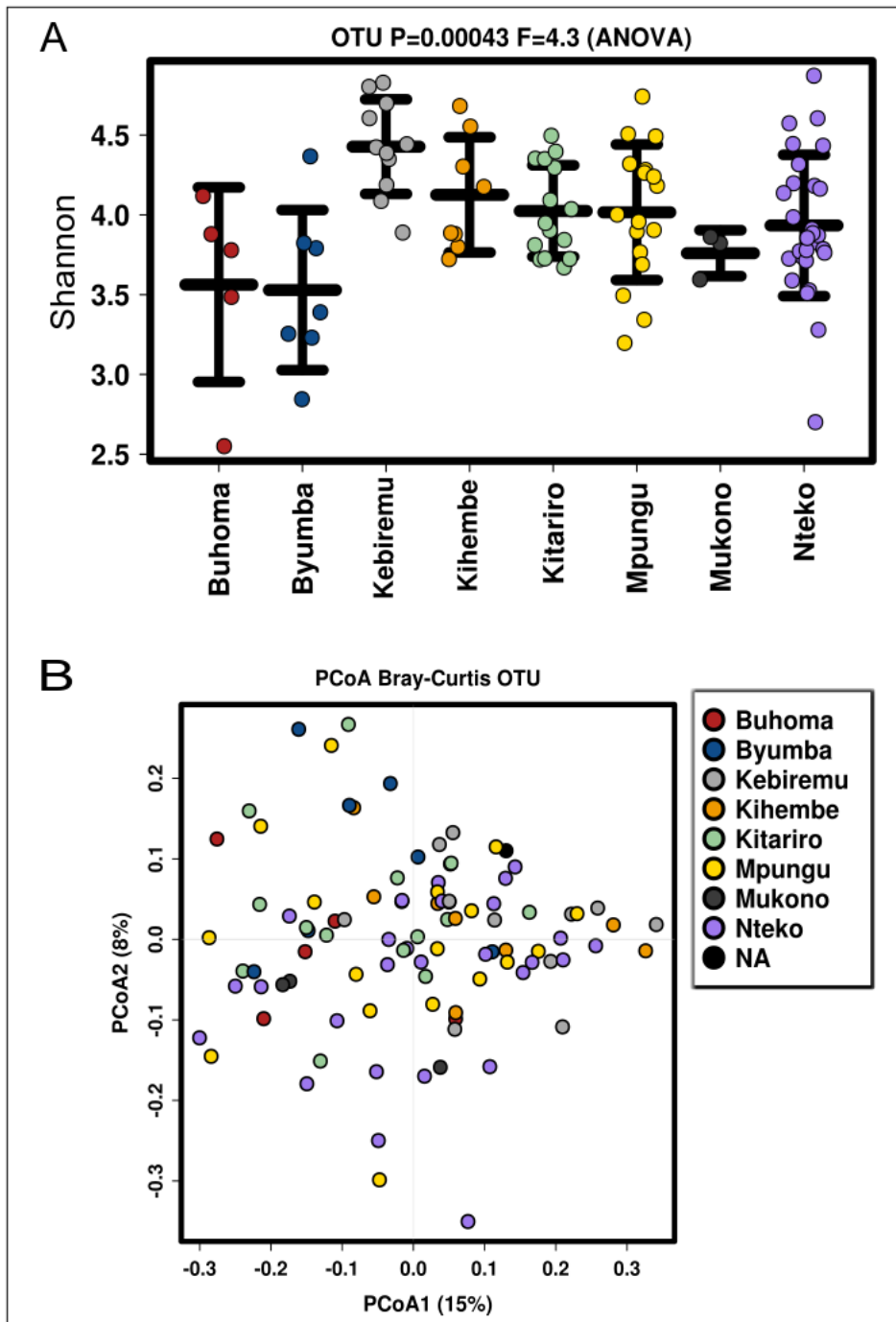
Figure S3. A. Alpha diversity comparison between Batwa and Bakiga OTUs computed with Shannon index for sampling locations for Batwa and Bakiga of Uganda. OTU tables rarefied at 1000 sequences per sample (p < 0.05, ANOVA). B. Beta diversity, based on PCoA of Bray-Curtis distance matrix (p > 0.05, ANOSIM).
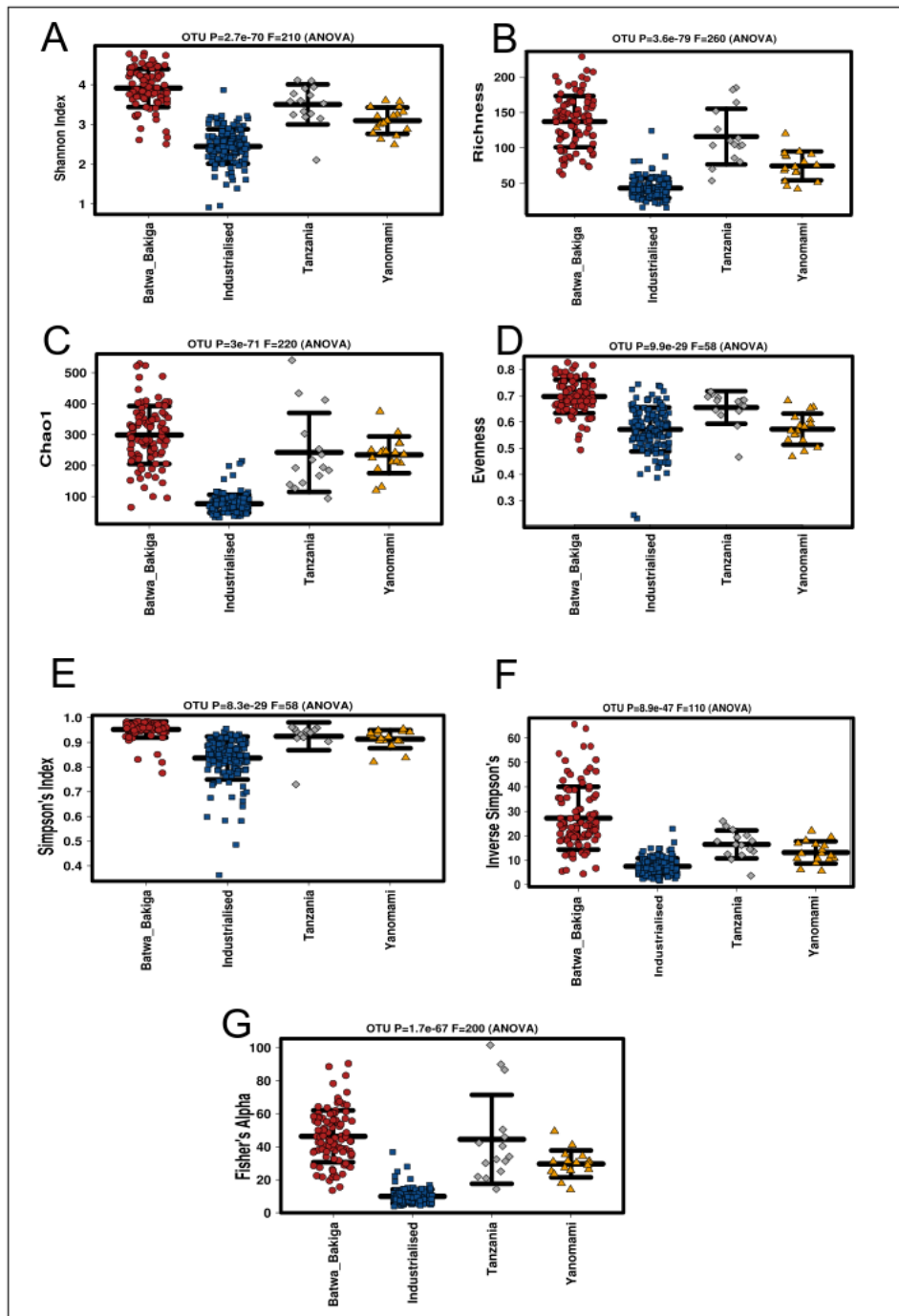
Figure S4. Alpha Diversity comparison between Batwa and Bakiga OTUs computed with A. Shannon index, B. Richness, C. Chao1, D. Evenness, E. Simpson's index, F. Inverse Simpson, G. Fisher's Alpha on worldwide data. OTU tables rarefied at 1000 sequences per sample. (p < 0.05, ANOVA).
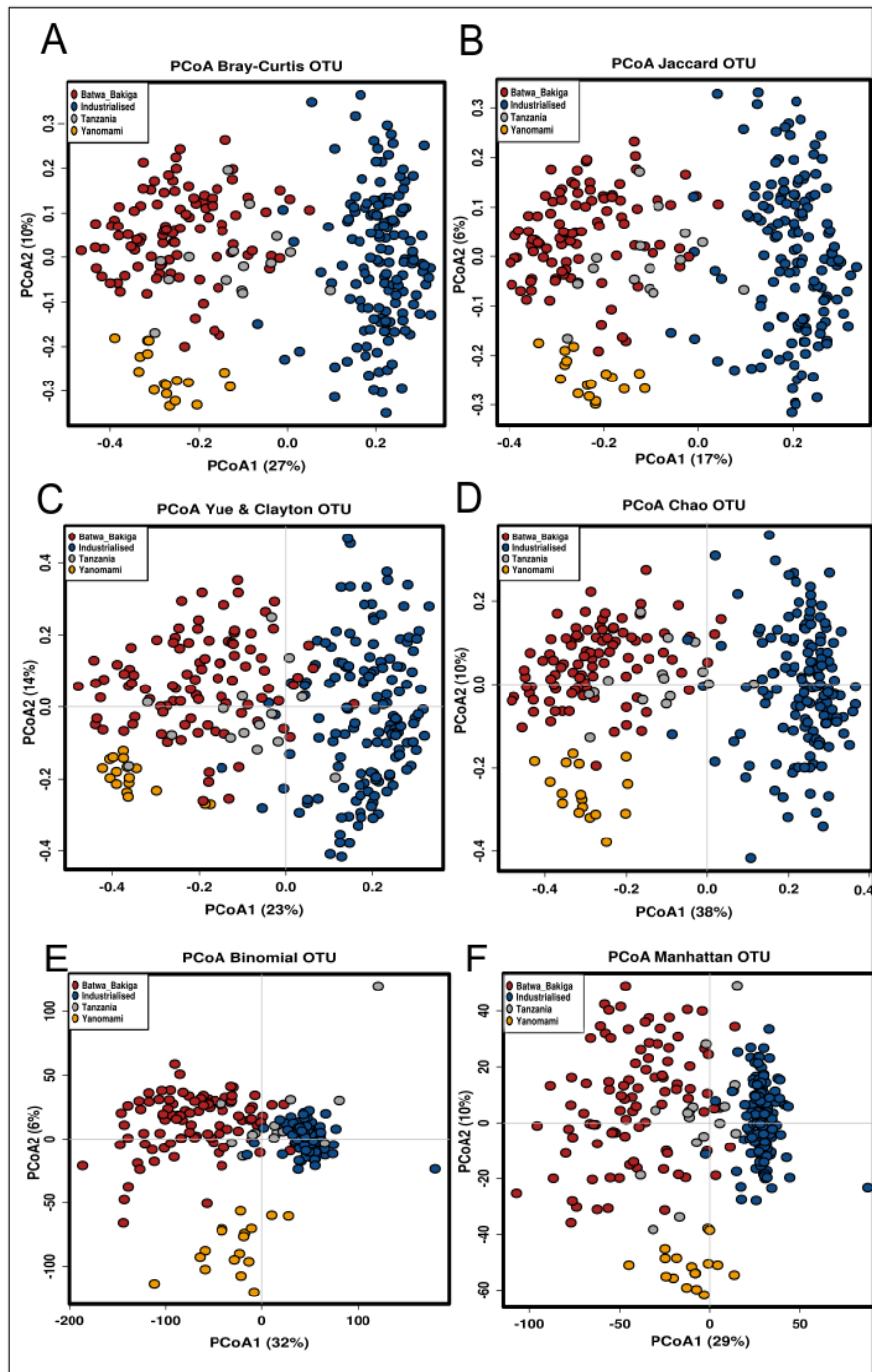
Figure S5. Beta diversity comparison between Batwa and Bakiga OTUs, based on PCoA plot of: A. Bray-Curtis, B. Jaccard distance, C. Yue & Clayton, D. Binomial, E. Manhattan distance matrix. OTU tables rarefied at 1000 sequences per sample (p < 0.05 ANOSIM).
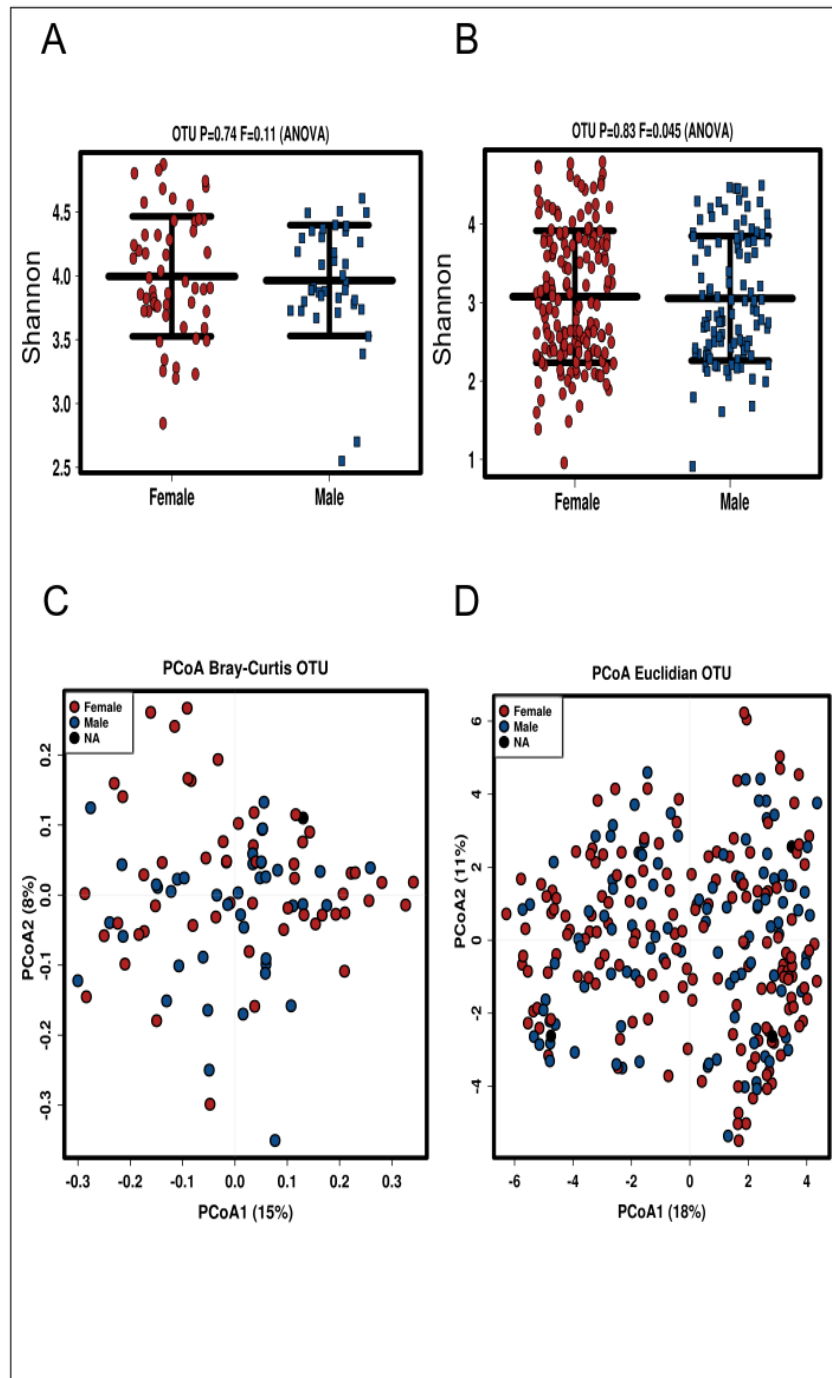
Figure S6. A. Alpha diversity comparison between Batwa and Bakiga OTUs computed with Shannon index. OTU tables rarefied at 1000 sequences per sample (p > 0.05, ANOVA). B. Beta diversity, based on PCoA plot of Bray-Curtis distance matrix. OTU tables rarefied at 1000 sequences per sample. C. Alpha diversity computed with Shannon index of sex for worldwide data. OTU tables rarefied at 1000 sequences per

sample. (p > 0.05, ANOVA). D. Beta diversity, based on PCoA plot of
Bray-Curtis distance matrix (p > 0.05, ANOVA).
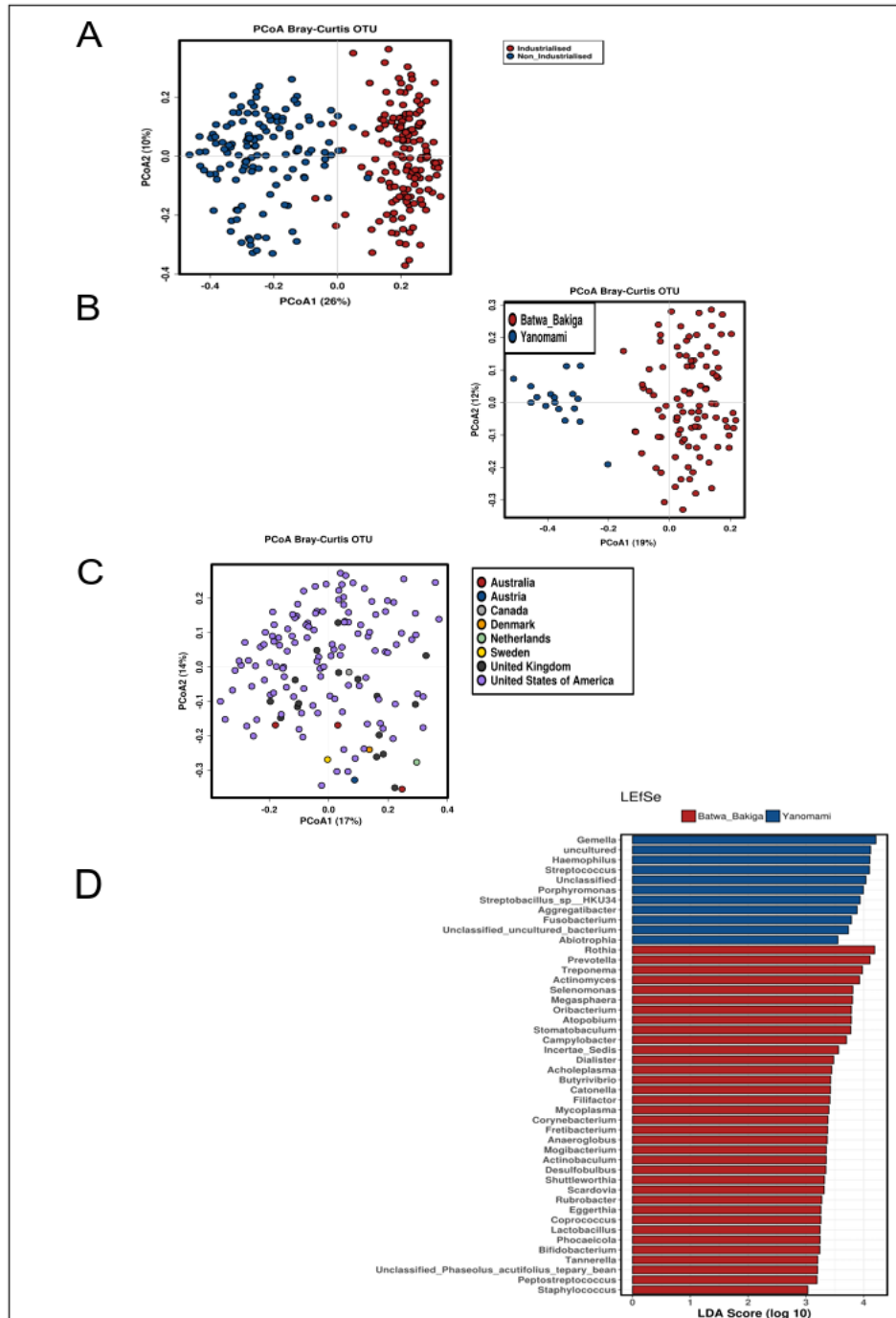
Figure S7. A. PCoA plot based on Bray Curtis distances calculated on OTU tables of
oral samples from industrialised and non-industrialised groups rarefied at 1000
sequences per sample (p < 0.05, ANOSIM). B. PCoA plot based on Bray Curtis
distances calculated on OTU tables of oral samples from Ugandan and Yanomami
groups rarefied at 1000 sequences per sample. Ugandan and Yanomami samples cluster

away from each other. C. PCoA plot based on Bray Curtis distances calculated on OTU tables of oral samples from countries comprising the industrialised group rarefied at 1000 sequences per sample. D. Top discriminative bacteria between the Ugandan and Yanomami were determined by LEfSe analysis.
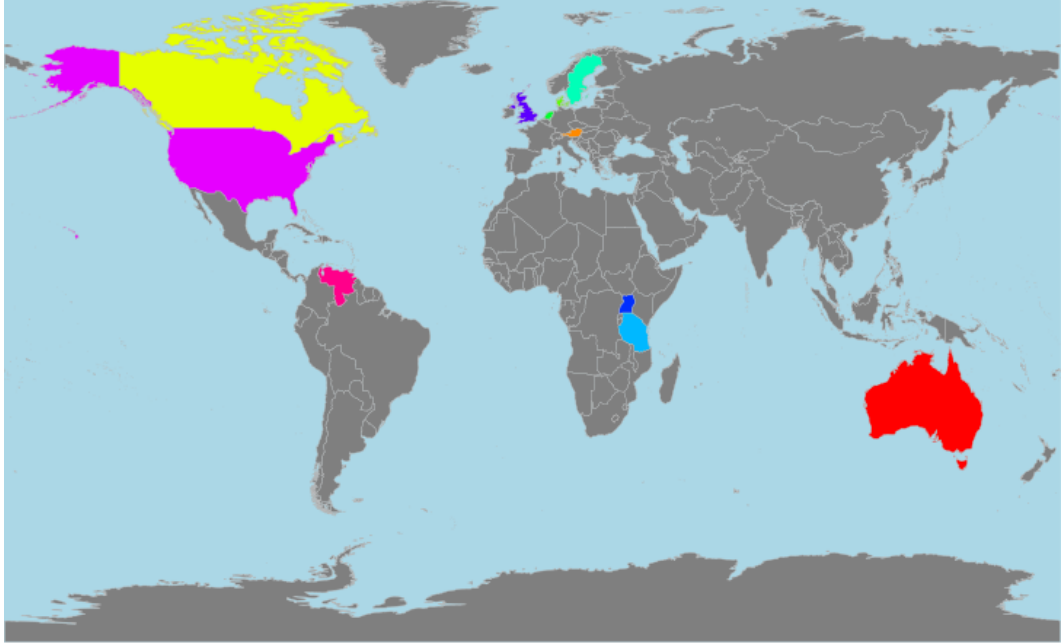
Figure S8. Location of oral microbiota samples used in the analysis of the four worldwide population groups

Tables

Table S1A. Metadata for the Batwa and Bakiga Ugandan samples.

ACAD_Number	Culture	CollectionSite	Sex
A19444	EBC	NA	NA
A19450	Batwa_HG	NA	NA
A19461	EBC	NA	NA
A19462	EBC	NA	NA
A19465	Batwa_HG	NA	NA
A19466	Batwa_HG	NA	NA
A19483	EBC	NA	NA
A19484	EBC	NA	NA
A19509	EBC	NA	NA
A19555	PCR	NA	NA
A19510	EBC	NA	NA
A19531	EBC	NA	NA
A19836	PCR	NA	NA
A19532	EBC	NA	NA
A19552	EBC	NA	NA
A19557	PCR	NA	NA
A19475	Bakiga_AG	Buhoma	Male
A19512	Batwa_HG	Kitariro	Male
A19463	Bakiga_AG	Buhoma	Male
A19528	Batwa_HG	Kihembe	Male
A19464	Batwa_HG	Kebiremu	Male
A19522	Bakiga_AG	Buhoma	Female
A19545	Batwa_HG	Kebiremu	Male
A19451	Bakiga_AG	Buhoma	Male
A19451	Bakiga_AG	Buhoma	Male
A19446	Batwa_HG	Kitariro	Male
A19487	Batwa_HG	Kitariro	Male
A19453	Batwa_HG	Mpungu	Male
A19508	Bakiga_AG	Mukono	Female
A19473	Batwa_HG	Nteko	Female
A19488	Batwa_HG	Kitariro	Male
A19505	Batwa_HG	Nteko	Male
A19456	Batwa_HG	Kihembe	Female
A19478	Batwa_HG	Kitariro	Male
A19501	Batwa_HG	Mpungu	Female
A19542	Batwa_HG	Nteko	Female
A19486	Batwa_HG	Kitariro	Female
A19551	Batwa_HG	Nteko	Male
A19448	Batwa_HG	Nteko	Female
A19447	Batwa_HG	Byumba	Male
A19534	Batwa_HG	Mpungu	Male
A19511	Batwa_HG	Kitariro	Male

A19513	Batwa_HG	Nteko	Male
A19543	Batwa_HG	Mpungu	Male
A19547	Batwa_HG	Kebiremu	Male
A19467	Bakiga_AG	Buhoma	Female
A19497	Batwa_HG	Byumba	Female
A19459	Batwa_HG	Nteko	Male
A19518	Batwa_HG	Byumba	Male
A19455	Bakiga_AG	Mukono	Female
A19477	Batwa_HG	Kihembe	Male
A19502	Batwa_HG	Mpungu	Female
A19504	Batwa_HG	Mpungu	Male
A19536	Batwa_HG	Kitariro	Male
A19540	Batwa_HG	Nteko	Female
A19449	Batwa_HG	Kebiremu	Female
A19458	Batwa_HG	Byumba	Female
A19485	Batwa_HG	Kitariro	Male
A19496	Batwa_HG	Nteko	Male
A19530	Batwa_HG	Nteko	Male
A19538	Batwa_HG	Kitariro	Female
A19514	Batwa_HG	Nteko	Female
A19460	Batwa_HG	Mpungu	Female
A19492	Batwa_HG	Kihembe	Male
A19520	Batwa_HG	Kihembe	Male
A19521	Batwa_HG	Nteko	Female
A19524	Batwa_HG	Nteko	Male
A19526	Batwa_HG	Kitariro	Male
A19529	Batwa_HG	Nteko	Male
A19533	Bakiga_AG	Mukono	Female
A19546	Batwa_HG	Mpungu	Female
A19523	Batwa_HG	Mpungu	Female
A19506	Batwa_HG	Nteko	Male
A19507	Batwa_HG	Byumba	Female
A19515	Batwa_HG	Nteko	Female
A19469	Batwa_HG	Mpungu	Female
A19503	Batwa_HG	Kitariro	Male
A19549	Batwa_HG	Nteko	Female
A19468	Batwa_HG	Kebiremu	Male
A19544	Batwa_HG	Nteko	Female
A19471	Batwa_HG	Mpungu	Female
A19472	Batwa_HG	Nteko	Female
A19454	Batwa_HG	Mpungu	Female
A19474	Batwa_HG	Mpungu	Female
A19482	Batwa_HG	Byumba	Female
A19490	Batwa_HG	Kihembe	Female

A19539	Batwa_HG	Nteko	Female
A19452	Batwa_HG	Nteko	Female
A19493	Batwa_HG	Kebiremu	Male
A19470	Batwa_HG	Nteko	Male
A19519	Batwa_HG	Mpungu	Female
A19527	Batwa_HG	Mpungu	Female
A19535	Batwa_HG	Nteko	Female
A19537	Batwa_HG	Kitariro	Female
A19481	Batwa_HG	Mpungu	Female
A19494	Batwa_HG	Kebiremu	Female
A19445	Batwa_HG	Kitariro	Female
A19516	Batwa_HG	Byumba	Female
A19491	Batwa_HG	Kihembe	Female
A19489	Batwa_HG	Kitariro	Female
A19500	Batwa_HG	Kebiremu	Female
A19498	Batwa_HG	Kebiremu	Female
A19476	Batwa_HG	Kihembe	Female
A19517	Batwa_HG	Nteko	Female
A19550	Batwa_HG	Nteko	Female
A19480	Batwa_HG	Kitariro	Male
A19499	Batwa_HG	Mpungu	Female
A19457	Bakiga_AG	Buhoma	Male
A19479	Batwa_HG	Kebiremu	Female
A19525	Batwa_HG	Nteko	Male
A19548	Batwa_HG	Nteko	Female
A19495	Batwa_HG	Nteko	Female
A19541	Batwa_HG	Nteko	Female

Table S1B. Metadata for the four population groups included in this study

Sample ID	country	culture	qiita_principal_inv	qiita_study_alias	qiita_study_id	gender
10317_20485	Sweden	Industrialised	Rob Knight	AGP	10317	male
10317_22373	Australia	Industrialised	Rob Knight	AGP	10317	female
10317_22374	Australia	Industrialised	Rob Knight	AGP	10317	female
10317_23669	Australia	Industrialised	Rob Knight	AGP	10317	male
10317_28573	Netherlands	Industrialised	Rob Knight	AGP	10317	female
10317_31067	Canada	Industrialised	Rob Knight	AGP	10317	male
10317_31838	USA	Industrialised	Rob Knight	AGP	10317	male
10317_33392	UK	Industrialised	Rob Knight	AGP	10317	female
10317_33558	UK	Industrialised	Rob Knight	AGP	10317	male
10317_3356	UK	Industrialised	Rob Knight	AGP	10317	male
10317_33567	UK	Industrialised	Rob Knight	AGP	10317	female
10317_33568	UK	Industrialised	Rob Knight	AGP	10317	female
10317_33585	UK	Industrialised	Rob Knight	AGP	10317	female
10317_33592	UK	Industrialised	Rob Knight	AGP	10317	female
10317_33593	UK	Industrialised	Rob Knight	AGP	10317	male
10317_36883	USA	Industrialised	Rob Knight	AGP	10317	female
10317_37945	UK	Industrialised	Rob Knight	AGP	10317	female
10317_382	UK	Industrialised	Rob Knight	AGP	10317	male
10317_38219	UK	Industrialised	Rob Knight	AGP	10317	male
10317_38223	Austria	Industrialised	Rob Knight	AGP	10317	male
10317_38445	USA	Industrialised	Rob Knight	AGP	10317	male
10317_41639	UK	Industrialised	Rob Knight	AGP	10317	male
10317_41668	UK	Industrialised	Rob Knight	AGP	10317	female
10317_41762	UK	Industrialised	Rob Knight	AGP	10317	male
10317_42637	UK	Industrialised	Rob Knight	AGP	10317	female
10317_42662	UK	Industrialised	Rob Knight	AGP	10317	male
10317_42693	Denmark	Industrialised	Rob Knight	AGP	10317	female
10317_43206	USA	Industrialised	Rob Knight	AGP	10317	male
10317_44325	USA	Industrialised	Rob Knight	AGP	10317	male
10317_45002	USA	Industrialised	Rob Knight	AGP	10317	female
10317_4501	USA	Industrialised	Rob Knight	AGP	10317	male
10317_46088	USA	Industrialised	Rob Knight	AGP	10317	female
10317_46126	USA	Industrialised	Rob Knight	AGP	10317	male
10317_46221	USA	Industrialised	Rob Knight	AGP	10317	male
10317_4778	USA	Industrialised	Rob Knight	AGP	10317	female
10317_47831	USA	Industrialised	Rob Knight	AGP	10317	female
10317_47842	USA	Industrialised	Rob Knight	AGP	10317	female
10317_48955	USA	Industrialised	Rob Knight	AGP	10317	male
10317_49028	USA	Industrialised	Rob Knight	AGP	10317	female
10317_49731	USA	Industrialised	Rob Knight	AGP	10317	male
10317_49827	USA	Industrialised	Rob Knight	AGP	10317	male
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10052.MG.O1	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	female
10052.MG.O1	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	female
10052.MG.O1	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	female
10052.MG.O1	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	female
10052.MG.O1	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	male

10052.MG.O2	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	female
10052.MG.O2	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	female
10052.MG.O4	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	male
10052.MG.O4	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	male
10052.MG.O5	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	male
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10052.MG.O5	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	male
10052.MG.O5	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	NA
10052.MG.O6	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	male
10052.MG.O7	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	male
10052.MG.O8	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	male
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11492.A19512	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	male
11492.A19513	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	male
11492.A19514	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19515	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19516	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19517	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19518	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	male
11492.A19519	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19520	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	male
11492.A19521	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19522	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19523	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19524	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	male
11492.A19525	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	male
11492.A19526	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	male
11492.A19527	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19528	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	male
11492.A19529	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	male
11492.A19530	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	male
11492.A19533	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19534	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	male
11492.A19535	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19536	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	male
11492.A19537	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19538	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19539	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19540	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19541	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female
11492.A19542	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicro	11492	female

11492.A19543	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicr	11492	male
11492.A19544	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicr	11492	female
11492.A19545	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicr	11492	male
11492.A19546	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicr	11492	female
11492.A19547	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicr	11492	male
11492.A19548	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicr	11492	female
11492.A19549	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicr	11492	female
11492.A19550	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicr	11492	female
11492.A19551	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicr	11492	male
2024.final.MN	Tanzania	Tanzania	Gregor Reid	TZ_probiotic_pregn	2024	female
2024.final.MN	Tanzania	Tanzania	Gregor Reid	TZ_probiotic_pregn	2024	female
2024.final.MN	Tanzania	Tanzania	Gregor Reid	TZ_probiotic_pregn	2024	female
2024.final.MN	Tanzania	Tanzania	Gregor Reid	TZ_probiotic_pregn	2024	female
2024.final.MN	Tanzania	Tanzania	Gregor Reid	TZ_probiotic_pregn	2024	female
2024.final.MN	Tanzania	Tanzania	Gregor Reid	TZ_probiotic_pregn	2024	female
2024.final.MN	Tanzania	Tanzania	Gregor Reid	TZ_probiotic_pregn	2024	female
2024.final.MN	Tanzania	Tanzania	Gregor Reid	TZ_probiotic_pregn	2024	female
2024.final.MN	Tanzania	Tanzania	Gregor Reid	TZ_probiotic_pregn	2024	female
2024.final.MN	Tanzania	Tanzania	Gregor Reid	TZ_probiotic_pregn	2024	female
2024.final.MN	Tanzania	Tanzania	Gregor Reid	TZ_probiotic_pregn	2024	female
2024.final.MN	Tanzania	Tanzania	Gregor Reid	TZ_probiotic_pregn	2024	female
2024.final.MN	Tanzania	Tanzania	Gregor Reid	TZ_probiotic_pregn	2024	female
2024.final.MN	Tanzania	Tanzania	Gregor Reid	TZ_probiotic_pregn	2024	female
2024.final.MN	Tanzania	Tanzania	Gregor Reid	TZ_probiotic_pregn	2024	female
2148.T20057	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20060	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20062	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20066	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20074	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20079	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20080	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20084	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	NA
2148.T20087	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20092	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20203	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20291	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20452	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20454	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20465	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20468	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20474	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20487	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20494	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20602	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female

2148.T20612	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20614	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20616	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20619	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20621	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20623	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20624	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20632	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20634	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20636	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20640	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20644	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20646	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20649	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20651	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20653	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20656	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20658	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20659	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20660	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20662	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20669	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20670	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20672	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20674	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20685	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20686	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20688	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20700	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20732	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20901	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20902	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20903	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20906	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20907	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20910	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20912	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20917	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20919	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20923	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20924	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20933	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20938	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20939	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20946	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20951	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20959	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female

2148.T20961	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20963	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20965	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20969	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20976	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20977	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20979	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20993	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20996	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20998	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T21007	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T21010	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T21023	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T21025	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T21030	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T21093	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T21102	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T21112	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	NA
2148.T21121	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T21124	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T21133	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T21137	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T21146	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T21148	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T21153	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T21154	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T21157	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T21159	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T21164	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T21187 A	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T21200	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T21228	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T21413	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T21417	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T21429	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
10052.MG.O1	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	male
10052.MG.O1	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	female
10052.MG.O1	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	male
10052.MG.O2	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	male
10052.MG.O2	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	female
10052.MG.O2	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	female
10052.MG.O2	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	female
10052.MG.O2	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	male
10052.MG.O3	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	male
10052.MG.O9	Venezuela_Br	Yanomami	Maria-Gloria Domir	Yanomani 2008	10052	female

11492.A19450	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicr	11492	NA
11492.A19465	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicr	11492	NA
11492.A19466	Uganda	Batwa_Bakiga	Laura Weyrich	UgandanSalivaMicr	11492	NA
2148.T20473	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20608	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20617	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20631	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20641	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20648	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20661	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20664	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20667	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20692	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20915	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20918	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	male
2148.T20942	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	female
2148.T20978	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	NA
2148.T21060	USA	Industrialised	Noah Fierer	Flores_tongue_EBI	2148	NA

Table S2. Analysis of composition (ANCOM) between the four population groups included in this study (mean abundance values).

Selected Taxa (ANCOM)	Batwa_Bakiga	Industrialised	Tanzania	Yanomami
Abiotrophia	0.11	0.022	0.33	0.39
Acholeplasma	0.33	0	0.003	0.0042
Actinobacillus	0.26	0.002	0.18	0.057
Actinobaculum	0.072	0.0013	0.029	0.0042
Actinomyces	1.8	3.28	1.57	0.13
Aeromonas	0.00036	0.18	0	0
Aggregatibacter	1.27	0.028	1.23	3.15
Alloprevotella	3.56	1.35	3.44	5.37
Anaeroglobus	0.06	0.00078	0.08	0.0018
Atopobium	0.68	1.14	0.62	0.02
Bacteroidales_genomosp._P4_oral_clone_MB2_G17	0.11	0	0.0017	0.027
Bacteroides	0.023	0.2	0.033	0.0052
Bergeyella	0.54	0.66	0.56	0.51
Bifidobacterium	0.041	0.0068	0.022	0
Butyrivibrio	0.094	0.021	0.023	0.0036
Capnocytophaga	0.59	0.17	0.49	0.88
Cardiobacterium	0.059	0.0023	0.036	0.063
Catonella	0.38	0.067	0.21	0.082
Chryseobacterium	0.002	0.35	0.0071	0
Coprococcus	0.038	0.0033	0.0097	0.0023
Corynebacterium	0.97	0.066	0.29	0.61
Desulfobulbus	0.033	0	0	0.00016
Dialister	0.33	0.022	0.14	0.058
Eggerthia	0.039	0.00016	0	0.00034

Filifactor	0.28	0.00094	0.11	0.043
Finegoldia	0.043	0.0014	0	0.014
Fretibacterium	0.078	0.00022	0.0075	0.0038
Fusobacterium	4.74	1.49	5.81	5.31
Gemella	2.57	0.88	1.53	7.62
Haemophilus	5.73	12.75	8.58	9.04
Incertae_Sedis	0.56	0.18	0.26	0.14
Johnsonella	0.057	0.024	0.025	0.022
Kingella	0.07	0.0054	0.11	0.054
Lachnoanaerobaculum	0.17	0.4	0.31	0.14
Lactobacillus	0.099	0.036	0.098	0.00038
Leptotrichia	3.05	1.67	2.82	2.09
Megasphaera	0.55	0.66	0.97	0
Mycoplasma	0.13	0.0056	0.065	0.007
Neisseria	9.56	8.08	14.42	12.58
Oribacterium	0.7	0.75	0.94	0.031
Paludibacter	0.17	0.0024	0.16	0.085
Parvimonas	0.13	0.096	0.21	0.15
Peptococcus	0.026	0.0038	0.021	0.015
Peptostreptococcus	0.4	0.17	0.027	0.16
Phocaeicola	0.043	0.000073	0.019	0.0056
Porphyromonas	3.78	1.27	3.59	5.77
Prevotella	16.13	17.17	12.04	6.12
RC9_gut_group	0.15	0.14	0.053	0.034
Rothia	5.63	6.55	2.48	0.69
Rubrobacter	0.059	0	0	0
Scardovia	0.047	0.0006	0.02	0.00021
Selenomonas	1.71	0.17	0.88	0.31
Shuttleworthia	0.036	0.0011	0.044	0
Solobacterium	0.12	0.22	0.24	0.081
Sphingobacterium	0.00026	0.16	0	0
Staphylococcus	0.013	0.049	0	0
Stomatobaculum	0.64	0.39	0.24	0.016
Streptobacillus_sp._HKU34	0.0069	0	0.0039	1.06
Streptococcus	11.55	16.23	14.41	15.35
Tannerella	0.28	0.052	0.14	0.13
Treponema	2.44	0.021	0.69	0.16
Unclassified	6.47	5.96	6.06	9.04
Unclassified.Phaseolus_acutifolius_tepary_bean	0.14	0.013	0.003	0
Unclassified.uncultured_bacterium	0.29	0.34	0.93	0.77
uncultured	1.84	0.28	1.15	5.5
uncultured_bacterium	0.13	0.000039	0.026	0.028
Veillonella	6.63	15.38	9.91	5.75
Weissella	0.035	0.0018	0	0.012

Table S3A. Study wide and population specific core microbiomes for the four population groups included in this study

A. Core microbiome was defined as taxa present shared between 60% of all samples within a group.

Core_Global_genus	Industrialised	African	South American (Yanoma	Non-Industrialised
Actinomyces	Actinomyces	Actinomyces	Abiotrophia	Actinomyces
Atopobium	Atopobium	Alloprevotella	Actinomyces	Alloprevotella
Carnobacteriaceae	Carnobacteriaceae	Atopobium	Alloprevotella	Bergeyella
Gemella	Gemella	Bergeyella	Bergeyella	Capnocytophaga
Haemophilus	Haemophilus	Campylobacter	Campylobacter	Corynebacterium
Leptotrichia	Neisseria	Capnocytophaga	Candidate division SR1	Fusobacterium
Prevotella	Oribacterium	Corynebacterium	Capnocytophaga	Gemella
Rothia	Prevotella	Fusobacterium	Catonella	Haemophilus
Streptococcus	Rothia	Gemella	Corynebacterium	Lachnoanaerobaculum
	Streptococcus	Haemophilus	Filifactor	Lactobacillales
	Veillonella	Lachnoanaerobaculum	Fusobacterium	Leptotrichia
		Lactobacillales	Gemella	Neisseria
		Leptotrichia	Haemophilus	Porphyromonas
		Megasphaera	Lachnoanaerobaculum	Prevotella
		Neisseria	Lactobacillales	Rothia
		Oribacterium	Lautropia	Ruminococcaceae
		Porphyromonas	Leptotrichia	Streptococcus
		Prevotella	Leptotrichiaceae	Treponema
		Rothia	Neisseria	Veillonella
		Ruminococcaceae	Paludibacter	Campylobacter
		Selenomonas	Peptostreptococcaceae	
		Stomatobaculum	Peptostreptococcus	
		Streptococcus	Porphyromonas	
		Treponema	Prevotella	
		Veillonella	Rothia	
			Ruminococcaceae	
			Solobacterium	
			Streptococcus	
			Tannerella	
			Treponema	
			Veillonella	

Table S3B. Study wide and population specific core microbiomes for the four population groups included in this study.

B. Core microbiome defined as taxa present shared between 90% of all samples with all populations.

Taxa
p__Proteobacteria__g__Neisseria_otu5147
p__Proteobacteria__g__Haemophilus_otu825
p__Proteobacteria__g__Campylobacter_otu8975
p__Fusobacteria__g__Leptotrichia__s__uncultured_bacterium_otu6162
p__Fusobacteria__g__Fusobacterium__s__uncultured_bacterium_otu3611
p__Firmicutes__g__Veillonella_otu8018
p__Firmicutes__g__uncultured__s__unidentified_otu5542
p__Firmicutes__g__Streptococcus_otu3405
p__Firmicutes__g__Solobacterium_otu5518
p__Firmicutes__g__Oribacterium__s__uncultured_bacterium_otu3223
p__Firmicutes__g__Mogibacterium__s__uncultured_bacterium_otu6994
p__Firmicutes__g__Lachnoanaerobaculum__s__uncultured_bacterium_otu1971
p__Firmicutes__g__Gemella_otu6453
p__Firmicutes__f__Carnobacteriaceae_otu3140
p__Bacteroidetes__g__Porphyromonas__s__uncultured_bacterium_otu8733
p__Bacteroidetes__g__Bergeyella__s__uncultured_bacterium_otu4214
p__Actinobacteria__g__Rothia__s__uncultured_bacterium_otu183
p__Actinobacteria__g__Actinomyces_otu1530

"Despite our monumental achievements in philosophy, technology and the arts, to bacteria, humans are no more than an organic mass to be utilized for growth and reproduction."

E.V. Sokurenko et al. (1999) Trends in Microbiology

Discussion

Discussion

The multitude of microbes that live symbiotically within the human body constitute the human microbiota and the totality of their genomes and the environment constitutes the human microbiome (Marchesi and Ravel 2015). As we continue to discover how much the microbiome is involved in many of the functions of the human body, including its abilities to maintain health and cause disease, the nascent field of microbiome research holds great promise to help explain the mechanisms behind a number of diseases and provide potential therapeutic targets. To reach that stage, research will need to characterise the diversity of the microbiome at a global level and understand how the microbiome has evolved into the microbial communities we observe in different human populations today. Furthermore, little is known about the factors that influence the microbiome and how those factors might have changed in the past and led to the imbalanced state of the microbiome that is observed in industrialised populations today.

Paleomicrobiome and modern microbiome research allow us to expand our current limited knowledge by enabling us to explore the microbiome of ancient and modern populations respectively, thereby shedding light on the how the human microbiome evolved through time and how the microbiome is composed today. In this thesis, I shed light on one aspect of the factors that influence the human microbiome, i.e. cultural change through diet. Human beings have undergone three major cultural changes in our evolutionary history: the advent of fire, the Agricultural transition and the Industrial revolution (Cordain et al. 2005; Gillings, Paulsen, and Tetu 2015). I posit that all three of these cultural changes have led to dietary changes by altering the type of nutrients taken into the body, thereby impacting our microbial communities. By characterising and comparing the oral microbiome of ancient hunter-gathers from pre-Agriculture to ancient early farmers globally, I explore for the first time how the transition from hunting and gathering to an agrarian culture might have changed the oral microbiome at a global level. In addition, I also compare the human oral microbiome of modern populations from diverse global regions with differing levels of Agriculture and Industrialisation,

including those in cultural transition to industrialisation, to explore how these recent cultural shifts are changing the microbiome of modern populations.

In this discussion Chapter, I begin the first section with a Chapter by Chapter synthesis of the main findings of this thesis and elucidate the contribution and significance of each research project to microbiome research. In the second section, I explore the broader contributions of this thesis to larger themes within the field of both paleomicrobiome and modern microbiome research, as well as limitations and potential future research approaches within these themes. I then conclude with some words of caution for the field of microbiome research moving forward and close with remarks on the overall significance and the potential of microbiome research.

Overall summary and significance

This thesis aimed to explore the impact of human cultural changes during the Agricultural transition on the human oral microbiome based on the hypothesis that these cultural changes have altered the human oral microbiome from an ancestral state resulting in the imbalance seen in the oral microbiome of industrialised populations today. In addition, it presents an analysis of the impact of both common and recently developed aDNA laboratory techniques in recovering microbiota from dental calculus. The data presented in this thesis provide unique insights into the association between the human oral microbiome and cultural changes through diet, including data from yet unstudied ancient and modern human populations. Therefore, this thesis adds to the tapestry of knowledge on the global diversity of the human oral microbiome.

In Chapter I, I introduce the microbiome in the human oral cavity and gut and briefly discuss the factors that influence its composition. I examine the influence of the human genome on the microbiome and how the interaction between the two "omes" contributes to human health and disease. I also review current research including the advent of Microbiome – Genome Wide Association Studies (GWAS), which I argue has a lot of potential but is currently limited by small sample sizes, absence of replication, and insufficient confirmatory and mechanistic explanations of significant associations that have been found. This review Chapter also suggests that combining the study of genomes and microbiomes of ancient

populations is needed to expand our understanding of long-term interactions between the host genome and microbiome. This research is also needed to unveil the co-evolutionary history of humans and their microorganisms, which will unearth insights into how the human microbiome co-evolved with the human genome to impact human health.

Chapter II compared two widely used and recently published ancient DNA (aDNA) extraction and library preparation methods on dental calculus samples of varying preservation states. I find that microbial composition reconstructed from aDNA extracted from well preserved dental calculus is not influenced by the methods used, but the composition in poorly preserved samples was markedly improved with the use of the single stranded library preparation method, which allowed access to ultrashort endogenous oral microbiota DNA fragments following subtractive filtering. This research is the first time the impact of aDNA extraction and library preparation methods is studied in the context of the reconstruction of ancient microbiomes from dental calculus. By exploring the impact of these methods on poorly preserved samples from difficult preservation environments, such as the Sahara Desert in Niger, it can help propel the field forward by presenting the best methods to access to aDNA from dental calculus samples from these environments, allowing for reconstruction of the microbiomes of samples from similar poorly preserved environments of Africa, the Near East, South East Asia, and other environments, where there has been a sparsity of paleomicrobiome studies so far.

Chapter III applies research findings from Chapter II to examine for the first time oral microbiota of ancient hunter gatherers and early farmers from diverse global populations. I use aDNA data extracted from 270 dental calculus samples spanning 7,000 years of human evolutionary history to unveil the oral microbiota of ancient individuals, including oral microbiomes from Near Eastern, Asian and African individuals. I explored the compositional and functional changes in these ancient microbiota and examined how the Agricultural transition impacted modern health. I find relative differences in abundance of specific bacterial taxa as well as differences in functional composition between ancient hunter-gatherer and agriculturalist samples. I also find both ancient hunter-gatherer and agriculturalist samples divided, irrespective of geography and disease state, into groups of different compositional types dominated by specific bacterial taxa which appear to

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form ecological communities that drive differences between samples. This suggests that, while diet may drive the functional differences between ancient hunter-gatherer and agriculturalist microbiome, taxonomic composition may be driven by other factors including microbial ecology in the oral cavity, host genetics, oral hygiene and host environment. This research significantly expands the diversity of oral microbiome data from ancient populations and brings to the fore questions regarding the different levels at which various factors influence the microbiome.

In Chapter IV, I return to the present with insights from the past, for a study on the microbiomes of contemporary modern populations. I compared the oral microbiota of two African populations living in the Central African rainforest in Uganda. The first population were until recently hunter-gatherers, known as the Batwa, while the second, their neighbours known as the Bakiga are traditional subsistence farmers. By sequencing the 16S ribosomal RNA gene from their saliva samples, I was able to reconstruct the oral microbiota of the Bakiga population for the first time and compared it to previously studied Batwa neighbours. However, I found no significant differences in microbial diversity or composition between the two Ugandan populations. Conversely, on comparison of this Ugandan microbiome data with published worldwide data from Tanzanians, Yanomami hunter-gatherers from Venezuela, and Industrialised populations from the United States, Europe, and Australia, I confirm findings, previously seen in the gut microbiome that the diversity of oral microbiota of people living rural and traditional lifestyles is higher compared to Industrial populations. Furthermore, unique to this study, I find that the Ugandan individuals possessed the highest oral microbial diversity of any rural or traditional human population studied to date. The oral microbiota from individuals living rural or traditional lifestyles was also differentiated according to continent, as the Ugandan hunter-gatherer oral microbiota was significantly different from the Yanomami in both diversity and community structure despite similarities in their lifestyle. Furthermore, a gradient in microbiome composition and diversity was found between the traditional Ugandans, semi-urban Tanzanians and industrialised western populations, moving towards lower diversity with increased level of Industrialisation. This research Chapter illustrates the impact of cultural changes such as the transition to agriculture and more recently Industrialisation has on the human oral microbiome as more populations industrialise globally resulting in an increase in diseases associated with an

imbalanced microbiome composition. This research also punctuates the need to better understand microbiome changes in respect to the recent changes caused by Industrialisation in order to develop potential therapeutics and techniques to reverse these changes.

In summary, the various Chapters of this thesis combine to shed light on the interaction between diet, environment, and human genetics on the oral microbiome of diverse global modern and ancient human populations. This research focus required a multidisciplinary approach necessitating an understanding of ecology, anthropology, archaeology, molecular biology, in addition to the relatively new fields of aDNA and microbiome research. In the following section, I go into detail on my contributions to broader overlapping themes within this research focus and also discuss current limitations and potential future research approaches.

Broader contributions, limitations and future approaches

Working with Ancient DNA: "Beware, Here be dragons!"

The ability to extract and sequence aDNA from fossilised material is a powerful tool that has allowed paleomicrobiome research to reconstruct the microbiomes of ancient human populations, thus allowing us to understand how the microbiome has changed through time. However, both the fields of aDNA and paleomicrobiome research are relatively new and there are a number of pitfalls to be avoided when it comes to working with ancient DNA, especially those that paleomicrobiome research field are grappling with. This section explores the contributions of this thesis in dealing with those pitfalls, such as lab methodology/protocol, contamination, sample preservation, sequencing costs, and the ever growing need for database space and computational power.

From dental calculus to ancient microbial DNA: Choosing a laboratory method

The choice of method used for aDNA extraction and library preparation from dental calculus has primarily been based on research on the use of aDNA in paleogenomics (Campos et al. 2012; Damgaard et al. 2015). Although these methods have been quite successful in obtaining aDNA from dental calculus samples from Neanderthals (Weyrich et al. 2017) and ancient Europeans (Warinner et al. 2015; Warinner et al. 2014), they have not been systematically assessed for

their impact on microbiome composition. In order to fill this gap in research, in Chapter II, I performed an in-depth assessment of the impact of recently published extraction and library preparation methods on oral microbiome composition reconstructed from dental calculus samples using both well preserved and poorly preserved samples. I demonstrated that widely used extraction and library preparation methods in the field (Meyer and Kircher 2010; Rohland and Hofreiter 2007) (Dabney et al. 2013; Gansauge et al. 2017) did not significantly impact the microbiome composition reconstructed from well preserved dental calculus samples. This result represents an important confirmation that currently used methods have not introduced biases into published reconstructed microbiome data that explore well preserved samples (Warinner et al. 2014; Weyrich et al. 2017). However, poorly preserved samples were significantly impacted by library preparation methods. I find that the single stranded library (SSL) preparation method significantly improved the reconstruction of microbiomes from poorly preserved samples. This finding will allow for the reconstruction of microbiomes from poorly preserved samples that have so far been unsuccessful.

Furthermore, research has recently shown that lab reagents can introduce contaminants during sample processing in the laboratory (Glassing et al. 2016; Salter et al. 2014); thus, it was crucial to assess the level of contamination that a given method introduces into the extraction and library preparation process. Hence, in Chapter II, I also performed an assessment of the impact of contamination on the use of recently published extraction and library preparation methods on oral microbiome composition reconstructed from dental calculus samples using both well preserved and poorly preserved samples. The choice of laboratory method had an impact on the level of contamination, with methods such as the SSL method, involving more reagents and processing steps increasing the level of contamination as a result of the increased risk of contaminant DNA from the laboratory environment entering sample tubes during extraction and library preparation steps. Therefore, a reduction in the number of reagents and steps in the currently used protocols may significantly improve the robustness of the reconstructed ancient microbiome from dental calculus samples. Notably, I find that the SSL method coupled with a subtractive filtering step significantly improved the microbial composition reconstructed from poorly preserved samples, allowing access to a robust oral microbiome composition in poorly preserved samples free of

contamination. Overall, these are significant advancements for paleomicrobiome research, as it allows access to oral microbiomes from ancient populations in locations where environmental conditions do not support good DNA preservation, such as Africa, the Near East and South East Asia. This advancement will expand our understanding of the oral microbiome of populations beyond well preserved environments of the European continent. Furthermore, my results showing that the SSL preparation method allows access to aDNA in poorly preserved dental calculus samples adds to the body of work done comparing the impact of the SSL method on poorly preserved bone and tooth samples. These findings have broader implications for the wider field of aDNA as it opens up the potential to obtain genomes from ancient human and hominin samples from poor preservation environments such as Sub Saharan Africa and tropical South East Asia.

Addressing contamination

Ever since the advent of the field of aDNA, contamination has been a topic of immense interest and discussion (Hagelberg, Hofreiter, and Keyser 2015; Stagg 2010; Willerslev and Cooper 2005), resulting in a number of controversies (Hagelberg, Hofreiter, and Keyser 2015; Pääbo 1985). However, a number of standards (Cooper and Poinar 2000) were subsequently established that resulted in reducing contamination, thereby improving the quality and reliability of ancient genomic data obtained. The field of paleomicrobiome research has benefited from this experience from its onset, resulting in robust laboratory procedures that reduced contamination. However, paleomicrobiome research is still grappling with unique sources of contamination, such as establishing if microbial DNA found in samples are endogenous or exogenous, as microorganisms are ubiquitous in all environments. To achieve that, in Chapter II, III and IV, I used a method initially employed in paleomicrobiome research at the Australian Centre of Ancient DNA (ACAD) called subtractive filtering (Weyrich et al. 2017). Subtractive filtering is the use of negative controls (extraction blank controls (EBCs)) that accompany samples through the laboratory processing steps to track the level of contamination from the lab environment or from the reagents used in the laboratory. These controls are also sequenced alongside the samples. Post sequencing, microbial taxa found within extraction blank controls are then used to filter out microbial taxa found in the samples. This method ensures exogenous microbial DNA from the

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laboratory environment that inadvertently gets sequenced with the endogenous microbial DNA does not confound the analysis and reconstruction of ancient oral microbiome from dental calculus. However, the exogenous microbial DNA could have also contaminated dental calculus sample prior to extraction, *e.g.* where the remains were buried (Pilli et al. 2013). In Chapters II and III, I assessed soil samples there were directly adjacent to the skeleton where dental calculus was sampled. These soil samples then underwent the same laboratory processing as dental calculus samples and were also sequenced. Sequencing soil samples then enabled me to use subtractive filtering to filter out environmental microbial DNA from the microbial composition of the dental calculus samples. This is a significant contribution to paleomicrobiome research, as there has been discussion around the possibility of microbial DNA contaminating dental calculus samples from the environment following a recent publication on obtaining aDNA from sediments (Slon et al. 2017; Willerslev et al. 2003). This method, while being conservative, ensures the reliability of paleomicrobiome data by removing false positives. However, it is not perfect, and there is still the possibility of contaminant taxa inadvertently being included in the analysis. Future approaches could involve the use of more effective bioinformatics approaches to flag common environmental and lab contaminants, which will need to be assessed for each lab specifically and then systematically filtered out at the data processing stage. Similar methods such as those developed for amplicon sequencing such as DECONTAM (Davis et al. 2017) that use statistical classification to identify contaminants could be further developed for the needs of paleomicrobiome research. The accurate characterisation and control of microbial contamination is a challenge not unique to paleomicrobiome research. For example, some of the contaminant taxa such as the genus *Acinetobacter* that we find in aDNA laboratories are also those that space agencies like NASA find in their clean rooms (Mogul et al. 2018). Therefore, these findings have broader implications for research fields where microbial contamination is an issue such as in space research (including astrobiology) and medical research in low biomass body sites like the human eye (Ozkan et al. 2017).

Screening before sequencing

The last two decades of advances in sequencing technologies have reduced the cost of DNA sequencing (Wetterstrand 2016). However, whole genome

sequencing costs including the costs of sample extraction and library preparation procedures for paleomicrobiome research, as we begin to sequencing larger numbers of samples, are still quite expensive for many research laboratories. Recent research showing that the cheaper amplicon sequencing approach to studying the oral microbiome of ancient populations introduces biases (Ziesemer et al. 2015) has led to a movement towards more expensive whole genome sequencing. In Chapter II and III, I attempted to extract aDNA from more than 250 dental calculus samples from the Near East and Africa with a success rate of 30%. Sequencing all of these samples, many of which are poorly preserved, would have resulted in a large number of failed sequencing runs resulting in an exorbitant loss in sequencing costs. Therefore, I used a screening method involving the amplification of the 16S ribosomal RNA gene using a universal primer and visualisation using gel electrophoresis to assess if ancient microbial DNA was successfully extracted from a sample. This screening method led to a reduction in sequencing costs that would have been lost, thereby allowing for the efficient use of sequencing funds for further samples. However, this approach also has its limitations as its lack of sensitivity for very low amounts of aDNA and there can be challenges with PCR amplification such as the presence of PCR inhibitors, that may introduce false positives into the assessment. However, these can be mitigated by using highly sensitive electrophoresis gels and by the purification of aDNA respectively. Another approach that could be used to assess the successful extraction of aDNA from dental calculus samples, although more expensive, is the use of highly sensitive DNA analysers such as the Agilent 2200 TapeStation system. Nonetheless, any approach that allows for the efficient screening of dental calculus samples will be crucial as the field begins to explore access to aDNA from samples from more diverse global locations that poorly preserve DNA. Furthermore, this screening method can also be used in the wider aDNA field of paleogenomics with the use universal primers for the organism being studied.

The human microbiome and cultural shifts through time

Recent research has showed that cultural shifts through diet has the ability to change both the gut microbiome and oral microbiome (Adler et al. 2013; David et al. 2014; Holmes et al. 2017). Furthermore, research on the gut microbiomes of populations in different parts of the world with different subsistence patterns

(Segata 2015) and between heavy meat eaters versus plant eaters match the microbiome profiles observed between carnivorous and herbivorous mammals respectively (David et al. 2014; Muegge et al. 2011). However, little is known on the impact of cultural shifts that have changed diet, such as Agriculture and the Industrial revolution on oral microbiome composition. In the following sections, I discuss the broader significance of my findings, limitations and future research approaches with regards to the impact of the transition to Agriculture and the Industrial revolution in modern populations.

The Agricultural transition and the oral microbiome

For most of human evolutionary history, we have led a nomadic hunter-gatherer lifestyle. Therefore, it has been hypothesised that our microbiome has evolved for specifically this type of diet (Hancock et al. 2010; Moeller et al. 2014; Muegge et al. 2011) and that changes in diet following the advent of fire, the Agricultural transition, and the Industrial revolution have led to a shift in the composition of our microbiome, and in some cases towards imbalance resulting in disease (Gillings, Paulsen, and Tetu 2015). In Chapter III, I explore the oral microbiome of hunter-gatherer population from diverse global populations and find that there is a high level of diversity between the different groups. As hunter-gatherer diets depend on their environment (Ströhle and Hahn 2011), the variety of possible diets between the different groups we sampled may explain differences observed in the level of bacterial diversity that we observed between different groups. It was notable to see an increase in bacteria such as *Streptococcus* species, associated with a diet rich in carbohydrates in South African hunter-gatherers. This is similar to recent research showing that ancient Moroccan hunter-gathers (Humphrey et al. 2014) had a starch-rich diet, which further suggests that ancient hunter-gatherers were not a monolithic dietary group. Although hunter-gatherers have been broadly defined as populations that obtain their diet from hunting of wild game and the gathering of wild plants (Crittenden and Schnorr 2017; Pontzer et al. 2012), it is difficult to define a hunter-gatherer group today due to the diversity in their diets and is even more difficult for ancient samples (Bernal et al. 2007; Guddemi 1992). However, the definition I've used in Chapter III is based on the archaeological assessment of excavation sites where no known evidence of agricultural practices was found. Notably my findings in Chapter IV that the

Ugandan, Batwa hunter-gatherer microbiome had no significant differences to that of their Bakiga, agriculturalists neighbours suggests that there are other factors besides diet that play an important role in the composition of the oral microbiome. In the case of the two Ugandan populations, the highly diverse rainforest environment that they both inhabit appears to have played an important role in homogenizing the two populations. However, other factors such as host genetics (Abdul-Aziz et al. 2016; Gomez et al. 2017), and, oral hygiene practices (Belstrøm et al. 2018) may also have a role in the structuring the composition of the human oral microbiome. It is important to note that this study is not exhaustive. Although I was able to expand the diversity and number of hunter-gatherer samples in Chapter III, data presented in this thesis is still far from capturing the full diversity of the global hunter-gatherer microbiome. Future research will require increases in sample size and diversity of hunter-gatherers to confirm these findings. These findings also have broader implications for anthropological research. In Chapter III, I also find that Agriculture has shifted the oral microbiome composition towards an enrichment in the abundance of *Streptococcus* bacteria which have been associated with an increase in the intake of a carbohydrate rich diet (Adler et al. 2013; Giacaman 2017; Lassalle et al. 2017). Furthermore, I also found significant functional differences between hunter-gatherers and agriculturalists that suggest that the advent of Agriculture likely led to enrichment in functions relating to the breakdown of sugars, thereby changing the functional repertoire of the human microbiome towards one capable of processing the changing diet and lifestyle. While recent research by Warinner et al. (2015) has shown the presence of milk based proteins in dental calculus, in Chapter III, I show that microbial functions related to lactose digestion were enriched in agriculturalist individuals. The presence of both milk proteins and functions related to the degradation of milk sugars in dental calculus further leads credence to changes in human culture, lifestyle and diet and their potential influence on the diversity, composition and function of the human oral microbiome. However, further research is needed to expand sample sizes and diversity of ancient samples from both hunter-gatherers and agriculturalists. Furthermore, in light of my findings regarding differences in functions between hunter-gatherers and agriculturalists, proteomic (Hendy et al. 2018) and metabolomics (Velsko et al. 2017) approaches on these ancient samples will shed further light on the changes that occurred in the microbiome as a result of

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the transition to Agriculture. In addition, my findings on functional differences between hunter-gatherers and agriculturalists mirrors what has been found in the human genome, where functions such as lactose tolerance were likely selected for in the human genome during the transition to Agriculture (Allentoft et al. 2015; Jones and Brown 2000; Larsen 1995) suggesting potential co-evolutionary processes (Abdul-Aziz, Cooper, and Weyrich 2016; Sullam et al. 2012) occurring between the genome and microbiome during this period of human cultural change. Future research integrating ancient genomic research and paleomicrobiome research would be well placed to unveil these co-evolutionary processes and their underlying mechanisms, the findings of which will have broader implications on our understanding of human evolution.

Enterotypes of the human oral microbiome?

Dental calculus is the calcified form of dental plaque (Akcalı and Lang 2018), which is itself a biofilm of various microbial taxa (Marsh 2004). This biofilm has been described as an ecological community (Welch et al. 2016; Utter, Welch, and Borisy 2016) with the presence of keystone species, primary and secondary colonizers (Kolenbrander and London 1993; Rosier et al. 2014). In Chapter III, I find that the oral microbiome of both ancient hunter-gatherers and agriculturalists consisted of distinct microbial compositions irrespective of their geographic location, cultural lifestyle and diet. These compositional groups driven primarily by either *Streptococcus* and *Methanobrevibacter* form what appears to be ecological communities within each group. For example, *Lautropia mirabilis* and *Ottowia sp. oral taxon 894* in the *Streptococcus* group have been shown to co-exist (Welch et al. 2016). Welch et al. (2016) showed that the plaque biofilm consists of complex microbial consortia formed by micro - environmental factors such as saliva flow, nutrient availability and oxygen levels. I posit that these composition types are the two extremes of continuous gradients of ecological communities that form different complex microbial consortia in the dental plaque of ancient populations before they calcified into dental calculus. This finding suggests that there may be different oral microbial composition types similar to enterotypes found in the gut microbiome (Arumugam et al. 2011). It is important to note here that the concept of enterotypes was debated in the microbiome research community

as their presence was found to be strongly influenced by methodology (Knights et al. 2014; Koren et al. 2013), however it has since been discovered that microbial composition does form continuous gradients that may be biologically significant (Knights et al. 2014; Yong 2012). Further research is required to confirm these findings and to unearth mechanistic explanations on factors that may drive the presence of one compositional type over another and possible transitionary states between them. Although, I did not find significant correlations between the compositional types and disease, potentially due to the paucity of metadata from ancient samples, further research is also required on the ecological interactions within members of the two compositional types and to test for any correlation with disease in other datasets. Characterising the ecological communities that comprise the human oral microbiome in ancient populations and how cultural transitions and other factors such as environment, host genome and disease has influenced them will allows us to better predict how future dietary changes might impact the human oral microbiome.

Hunter-gatherers and microbial diversity

In Chapter III, I find that the ancient hunter-gatherer microbiome is significantly less diverse compared to the ancient agriculturalist microbiome. This is contrary to previous observations where modern hunter-gatherer groups have consistently been shown to have higher microbial diversity compared agriculturalists (Clemente et al. 2015; Lassalle et al. 2017; Nasidze et al. 2011). However, it is important to note that modern agriculturalists that are compared with hunter-gatherers in research on contemporary populations have in many cases been industrialised to varying extents. In Chapter IV, I show that Industrialisation leads to lower microbial diversity and increases in pathogenic load. However, ancient agricultural samples used in the ancient study would not have been influenced industrialization, they would have maintained their oral microbiota as they transitioned to farming thereby maintaining a higher level of diversity. Although, we ensured we obtained a robust oral microbiome and controlled for contamination and potential sequencing errors and biases, the possibility remains that this reduction in the diversity of hunter-gathers may be due to sequencing artifacts within the aDNA dataset. However, I have tried to mitigate these biases by removing rare microbial taxa at 0.01% of abundance that may be the result of

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sequencing artifacts and also with the use of stringent alignment parameters in MALT. Ultimately, understanding how the hunter-gatherer microbiome was composed allows us to put both modern and historical oral microbiomes in context, thereby giving us a better baseline to characterize and define what constitutes a healthy oral microbiome.

Industrialization and the missing microbes

In Chapter IV of this thesis, I show that as populations industrialize there is a decrease in microbial diversity, specifically a decrease in beneficial taxa and an increase in opportunistic pathogenic taxa known as pathobionts, microbes that exist normally as commensal symbionts within the microbiome but become pathogenic when the composition shifts to imbalance (Hornef 2015). Pathobionts are associated with numerous diseases, such as periodontal disease (Jiao, Hasegawa, and Inohara 2014), Type II Diabetes (Tilg and Moschen 2015), and IBD (Kostic, Xavier, and Gevers 2014), strongly suggesting that changes in diversity and composition may be a driving factor that led the oral microbiome towards a state of imbalance and disease (Herrero et al. 2018). A few studies have explored restoration of these missing beneficial taxa such as *Gemella*, *Granulicatella*, and *Bifidobacterium*, but there has been limited success in using them as probiotics (Coqueiro et al. 2018; Jabr 2017; Mimi Pham et al. 2008). Researchers are exploring cultural immersion (Ruggles et al. 2018) and the use of biobanks (Ma et al. 2017); however, future research will have to find ways to ethically acquire these taxa from an ever dwindling numbers of populations living a non-industrialized lifestyle (Chuong et al. 2017; Rhodes 2016) and also find ways to ensure these beneficial microbial taxa are incorporated into the microbiomes of industrialized populations and are not just transient like some current probiotic based therapies (Ghouri et al. 2014; Mimi Pham 2008).

In Chapter IV, I also compared the microbiome of two Ugandan populations living side by side but with different diets. Although my initial expectation was to find significant differences between the two populations on account of their diets. My results showed that their microbiomes were surprisingly quite similar. It appeared that the diverse tropical rainforest environment in which both populations inhabited might have had a larger impact on their oral microbiomes compared to their diet. However, it might also be possible that both groups share unknown

similarities in diet such as the hunting of the same wild game from the forest or shared water sources that could have played a role in the homogenization of their oral microbiomes. These results significantly underline the complex interaction between diet and environment in shaping the human oral microbiome (Shaw et al. 2017). Future research with similar approaches in other parts of the world, such as those explored by Lassalle et al. (2017) in the Philippines, could control for environment between two groups. These approaches will be required to further elucidate the interactions between environment and diet on microbial composition. Furthermore, the use of 16S rRNA gene amplicon sequencing limited my analysis to only taxonomic resolution and lacked the ability to accurately characterize bacterial function. A future approach using whole genome (shotgun) metagenomic analysis has the potential for higher taxonomic resolution and the identification of functionally relevant taxa between the two Ugandan populations and within the wider global metadata set.

Expanding global diversity in microbiome research

In Chapter IV, I show that traditional lifestyles differentiated according to continent with different populations diverging in the oral microbiome composition based on their environment. Recent research has also showed that there are differences among the microbiomes of populations globally depending on their ethnic origin (Deschasaux et al. 2018; Mason et al. 2013; Zhou et al. 2018). These findings suggest that the microbiome may have had different evolutionary histories at different locations globally. Research projects such as Chapter III and Chapter IV of this thesis, where diverse populations are characterized and compared for the first time, are crucial in understanding the global diversity of the human oral microbiome. However, small sample sizes previously used in microbiome research have the potential to introduce biases and limit our understanding of this complete picture of human microbiome diversity. By forming long term collaborations with anthropologists, medical practitioners, archaeologists and museum curators, future research approaches will have increased access to samples to explore the ancient oral microbiome and compare it with the modern oral microbiome which will go beyond current siloed research and allow researchers to obtain a more complete picture of human oral microbiome diversity. However, there are a number of challenges in performing meta-analyses of ancient and modern oral microbiome

data. The impact of taphonomic bias on ancient microbiome composition is yet to be well studied but is crucial to address before comparing ancient samples with modern samples so as to ensure that the presence/absence or abundance of a specific microbial taxa is due to biologically significant factors rather than taphonomic bias. In addition, contamination will need be effectively addresses for modern microbiome studies. This ability to combine both datasets for an accurate analysis will allow us to identify microbial taxa and functions that might be present in ancient oral microbiomes but missing in modern oral microbiomes and vice versa. This study is the first comparison of hunter-gatherers and agriculturalists on a global level and lays the ground work for further research to include samples from more diverse global regions such as South America and Australia to confirm these finds on an even larger global scale.

Conclusion

Dental calculus preserves the oral microbiome and allows researchers to characterize changes in the microbiome before, during, and after cultural changes. The analysis of the dental calculus of 269 individuals from diverse global locations has enabled the first study of changes in the microbiome as a result of cultural shifts on a global level. These findings will allow us to predict how future changes in diet will influence our microbiome and impact our health. The findings of this thesis will also further expand our understanding of the evolution of the modern oral microbiota in response to changes in diet and environment. As the medical community moves towards microbial based therapies for various metabolic and infectious diseases, these findings and future research approaches have the potential to guide medical manipulations of the microbiota to prevent and cure disease. However, continuous improvement in lab processing methods, mitigation of the risk of contamination, accurate data analysis, and cautious interpretation of results will be essential.

The recent growth in publications on the influence of the culture through diet on the microbiome and on host genome microbiome interactions confirms that it is time for an in-depth exploration of these influences. To obtain a complete picture of these influences, diverse samples, more effective laboratory techniques, and novel metagenomic analysis tools will need to be developed. However, this is

beyond the capacity of many individual research laboratories and will require collaborations between archaeologists, microbiologists, statisticians and bioinformaticians. These collaborations have the potential to spur new initiatives and will enable us to harness the microbiome to prevent and cure disease which will be crucial as more countries develop and millions of people transition into the industrialised lifestyle and diet in the coming decades.

References

1. Abdul-Aziz, M.A. Muslihudeen Abdul-Razaq, Alan Cooper, and Laura S. L.S. Weyrich. 2016. "Exploring Relationships between Host Genome and Microbiome: New Insights from Genome-Wide Association Studies." *Frontiers in Microbiology* 7(OCT): 1611. http://journal.frontiersin.org/article/10.3389/fmicb.2016.01611/full (October 10, 2016).

2. Adler, Christina J, Keith Dobney, Laura S Weyrich, John Kaidonis, Alan W Walker, Wolfgang Haak, Corey J a Bradshaw, Grant Townsend, Arkadiusz Sołtysiak, Kurt W Alt, Julian Parkhill, and Alan Cooper. 2013. "Sequencing Ancient Calcified Dental Plaque Shows Changes in Oral Microbiota with Dietary Shifts of the Neolithic and Industrial Revolutions." *Nature genetics* 45(4): 450–5, 455e1. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3996550&tool=pmcentrez&rendertype=abstract.

3. Adler, Christina J, Keith Dobney, Laura S Weyrich, John Kaidonis, Alan W Walker, Wolfgang Haak, Corey JA Bradshaw, Grant Townsend, Arkadiusz Sołtysiak, Kurt W Alt, Julian Parkhill, Alan Cooper, et al. 2013. "Sequencing Ancient Calcified Dental Plaque Shows Changes in Oral Microbiota with Dietary Shifts of the Neolithic and Industrial Revolutions Europe PMC Funders Group." *Nat Genet* 45(4): 450–55. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3996550/pdf/emss-57665.pdf (September 3, 2018).

4. Akcalı, Aliye, and Niklaus P. Lang. 2018. "Dental Calculus: The Calcified Biofilm and Its Role in Disease Development." *Periodontology 2000* 76(1): 109–15. http://doi.wiley.com/10.1111/prd.12151 (January 7, 2018).

5. Allentoft, Morten E. et al. 2015. "Population Genomics of Bronze Age Eurasia." *Nature* 522(7555): 167–72. http://dx.doi.org/10.1038/nature14507 (June 10, 2015).

6. Arumugam, Manimozhiyan et al. 2011. "Enterotypes of the Human Gut Microbiome." *Nature* 473(7346): 174–80. http://www.ncbi.nlm.nih.gov/pubmed/21508958 (October 30, 2018).

7. Belstrøm, D. et al. 2018. "Impact of Oral Hygiene Discontinuation on Supragingival and Salivary Microbiomes." *JDR Clinical & Translational Research* 3(1): 57–64. http://www.ncbi.nlm.nih.gov/pubmed/29662960 (April 22, 2018).

8. Bernal, Valeria, Paula Novellino, Paula N Gonzalez, and S Ivan Perez. 2007. "Role of Wild Plant Foods Among Late Holocene Hunter-Gatherers From Central and North Patagonia (South America): An Approach From Dental Evidence." *Am J Phys Anthro-pol* 133: 1047–59. https://onlinelibrary-wiley-com.proxy.library.adelaide.edu.au/doi/pdf/10.1002/ajpa.20638 (June 17, 2018).

9. Bolyen, Evan et al. 2018. "QIIME 2: Reproducible, Interactive, Scalable, and Extensible Microbiome Data Science." *PeerJ Preprint*. https://peerj.com/preprints/27295/ (April 24, 2019).

10. Campos, Paula F. et al. 2012. "DNA in Ancient Bone - Where Is It Located and How Should We Extract It?" *Annals of Anatomy* 194(1): 7–

16. http://dx.doi.org/10.1016/j.aanat.2011.07.003.

11. Chuong, Kim H. et al. 2017. "Navigating Social and Ethical Challenges of Biobanking for Human Microbiome Research." *BMC Medical Ethics* 18(1): 1. http://bmcmedethics.biomedcentral.com/articles/10.1186/s12910-016-0160-y (October 30, 2018).

12. Clemente, J. C. et al. 2015. "The Microbiome of Uncontacted Amerindians." *Science Advances* 1(3): e1500183–e1500183. http://advances.sciencemag.org/cgi/doi/10.1126/sciadv.1500183 (April 18, 2015).

13. Cooper, a, and H N Poinar. 2000. "Ancient DNA: Do It Right or Not at All." *Science (New York, N.Y.)* 289(5482): 1139.

14. Coqueiro, Audrey Yule et al. 2018. "Probiotic Supplementation in Dental Caries: Is It Possible to Replace Conventional Treatment?" *Nutrire* 43(1): 6. https://nutrirejournal.biomedcentral.com/articles/10.1186/s41110-018-0064-3 (October 30, 2018).

15. Cordain, Loren et al. 2005. "Origins and Evolution of the Western Diet: Health Implications for the 21st Century." *The American journal of clinical nutrition*.

16. Crittenden, Alyssa N., and Stephanie L. Schnorr. 2017. "Current Views on Hunter-Gatherer Nutrition and the Evolution of the Human Diet." *American Journal of Physical Anthropology* 162(S63): e23148. http://doi.wiley.com/10.1002/ajpa.23148 (July 28, 2017).

17. Dabney, J. et al. 2013. "Complete Mitochondrial Genome Sequence of a Middle Pleistocene Cave Bear Reconstructed from Ultrashort DNA Fragments." *Proceedings of the National Academy of Sciences* 110(39): 15758–63. http://www.pnas.org/content/110/39/15758.abstract (January 10, 2016).

18. Damgaard, Peter B et al. 2015. "Improving Access to Endogenous DNA in Ancient Bones and Teeth." *Scientific Reports* 5.

19. David, Lawrence A. et al. 2014a. "Diet Rapidly and Reproducibly Alters the Human Gut Microbiome." *Nature* 505(7484): 559–63. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3957428&tool=pmcentrez&rendertype=abstract (July 9, 2014).

20. ———. 2014b. "Diet Rapidly and Reproducibly Alters the Human Gut Microbiome." *Nature* 505(7484): 559–63. http://www.nature.com/articles/nature12820 (October 13, 2018).

21. Deschasaux, Mélanie et al. 2018. "Depicting the Composition of Gut Microbiota in a Population with Varied Ethnic Origins but Shared Geography." *Nature Medicine*: 1. http://www.nature.com/articles/s41591-018-0160-1 (September 4, 2018).

22. Gansauge, Marie-Theres et al. 2017. "Single-Stranded DNA Library Preparation from Highly Degraded DNA Using T4 DNA Ligase." *Nucleic Acids Research* 45(10): 79. https://academic.oup.com/nar/article-abstract/45/10/e79/2948436 (July 25, 2018).

23. Ghouri, Yezaz A et al. 2014. "Systematic Review of Randomized Controlled Trials of Probiotics, Prebiotics, and Synbiotics in Inflammatory Bowel Disease." *Clinical and experimental gastroenterology* 7: 473–87. http://www.ncbi.nlm.nih.gov/pubmed/25525379 (April 24, 2018).

24. Giacaman, RA. 2017. "Sugars and beyond. The Role of Sugars and the

Other Nutrients and Their Potential Impact on Caries." *Oral Diseases*. http://doi.wiley.com/10.1111/odi.12778 (October 18, 2017).

25. Gillings, Michael R, Ian T Paulsen, and Sasha G Tetu. 2015. "Ecology and Evolution of the Human Microbiota: Fire, Farming and Antibiotics." *Genes* 6(3): 841–57. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4584332&tool=pmcentrez&rendertype=abstract (September 17, 2015).

26. Glassing, Angela et al. 2016. "Inherent Bacterial DNA Contamination of Extraction and Sequencing Reagents May Affect Interpretation of Microbiota in Low Bacterial Biomass Samples." *Gut pathogens* 8(1): 24. http://gutpathogens.biomedcentral.com/articles/10.1186/s13099-016-0103-7 (October 20, 2016).

27. Gomez, Andres et al. 2017. "Host Genetic Control of the Oral Microbiome in Health and Disease." *Cell Host & Microbe* 22(3): 269–278.e3. http://www.sciencedirect.com/science/article/pii/S1931312817303463 (September 18, 2017).

28. Guddemi, Phillip. 1992. "When Horticulturalists Are like Hunter-Gatherers: The Sawiyano of Papua New Guinea." *Ethnology* 31(4): 303. https://www.jstor.org/stable/3773422?origin=crossref (September 8, 2018).

29. Hagelberg, Erika, Michael Hofreiter, and Christine Keyser. 2015. "Ancient DNA: The First Three Decades." *Philosophical Transactions of the Royal Society B: Biological Sciences*.

30. Hancock, Angela M et al. 2010. "Colloquium Paper: Human Adaptations to Diet, Subsistence, and Ecoregion Are Due to Subtle Shifts in Allele Frequency." *Proceedings of the National Academy of Sciences of the United States of America* 107 Suppl(Supplement_2): 8924–30. http://www.pnas.org/cgi/content/long/107/Supplement_2/8924 (August 26, 2015).

31. Hendy, Jessica et al. 2018. "Proteomic Evidence of Dietary Sources in Ancient Dental Calculus." *Proceedings of the Royal Society B: Biological Sciences* 285(1883): 20180977. http://rspb.royalsocietypublishing.org/lookup/doi/10.1098/rspb.2018.0977 (July 25, 2018).

32. Herrero, E.R. et al. 2018. "Dysbiotic Biofilms Deregulate the Periodontal Inflammatory Response." *Journal of Dental Research*: 002203451775267. http://journals.sagepub.com/doi/10.1177/0022034517752675 (February 11, 2018).

33. Holmes, Andrew J. et al. 2017. "Diet-Microbiome Interactions in Health Are Controlled by Intestinal Nitrogen Source Constraints." *Cell Metabolism* 25(1): 140–51. http://www.ncbi.nlm.nih.gov/pubmed/27889387 (July 21, 2018).

34. Hornef, Mathias. 2015. "Pathogens, Commensal Symbionts, and Pathobionts: Discovery and Functional Effects on the Host." *ILAR Journal* 56(2): 159–62. https://academic.oup.com/ilarjournal/article-lookup/doi/10.1093/ilar/ilv007 (September 3, 2018).

35. Humphrey, Louise T et al. 2014. "Earliest Evidence for Caries and Exploitation of Starchy Plant Foods in Pleistocene Hunter-Gatherers from Morocco." *Proceedings of the National Academy of Sciences of the United*

States of America 111(3): 954–59.
http://www.ncbi.nlm.nih.gov/pubmed/24395774 (June 17, 2018).

36. Jabr, Ferris. 2017. "Probiotics Are No Panacea." *Scientific American* 317(1): 26–27.
http://www.nature.com/doifinder/10.1038/scientificamerican0717-26 (October 30, 2018).

37. Jiao, Y., M. Hasegawa, and N. Inohara. 2014. "The Role of Oral Pathobionts in Dysbiosis during Periodontitis Development." *Journal of Dental Research*.

38. Jones, Martin, and Terry Brown. 2000. "Agricultural Origins: The Evidence of Modern and Ancient DNA." *The Holocene* 10(6): 769–76.
http://hol.sagepub.com/content/10/6/769.abstract.

39. Knights, Dan et al. 2014. "Rethinking Enterotypes." *Cell host & microbe* 16(4): 433–37. http://www.ncbi.nlm.nih.gov/pubmed/25299329 (July 14, 2018).

40. Kolenbrander, P. E., and J. London. 1993. "Adhere Today, Here Tomorrow: Oral Bacterial Adherence." *Journal of Bacteriology*.

41. Koren, Omry et al. 2013. "A Guide to Enterotypes across the Human Body: Meta-Analysis of Microbial Community Structures in Human Microbiome Datasets" ed. Jonathan A. Eisen. *PLoS Computational Biology* 9(1): e1002863. http://dx.plos.org/10.1371/journal.pcbi.1002863 (November 23, 2018).

42. Kostic, Aleksandar D., Ramnik J. Xavier, and Dirk Gevers. 2014. "The Microbiome in Inflammatory Bowel Disease: Current Status and the Future Ahead." *Gastroenterology*.

43. Lassalle, Florent et al. 2017. "Oral Microbiomes from Hunter-Gatherers and Traditional Farmers Reveal Shifts in Commensal Balance and Pathogen Load Linked to Diet." *Molecular Ecology* 27(1): 182–95.
http://www.ncbi.nlm.nih.gov/pubmed/29165844 (November 27, 2017).

44. Ma, Yonghui, Hua Chen, Ruipeng Lei, and Jianlin Ren. 2017. "Biobanking for Human Microbiome Research: Promise, Risks, and Ethics." *Asian Bioethics Review* 9(4): 311–24.
https://link.springer.com/article/10.1007/s41649-017-0033-9 (October 30, 2018).

45. Marchesi, Julian R., and Jacques Ravel. 2015. "The Vocabulary of Microbiome Research: A Proposal." *Microbiome* 3(1): 31.
http://www.microbiomejournal.com/content/3/1/31 (July 31, 2015).

46. Mark Welch, Jessica L. et al. 2016. "Biogeography of a Human Oral Microbiome at the Micron Scale." *Proceedings of the National Academy of Sciences of the United States of America* 113(6): E791-800.
http://www.ncbi.nlm.nih.gov/pubmed/26811460 (January 15, 2018).

47. Marsh, P. D. 2004. "Dental Plaque as a Microbial Biofilm." *Caries Research* 38(3): 204–11.

48. Mason, Matthew R. et al. 2013. "Deep Sequencing Identifies Ethnicity-Specific Bacterial Signatures in the Oral Microbiome" ed. Indranil Biswas. *PLoS ONE* 8(10): e77287.
http://dx.plos.org/10.1371/journal.pone.0077287.

49. Meyer, Matthias, and Martin Kircher. 2010. "Illumina Sequencing Library Preparation for Highly Multiplexed Target Capture and Sequencing." *Cold Spring Harbor Protocols*.

50. Mimi Pham, Daniel A Lemberg and Andrew S Day. 2008. *Probiotics: Sorting the Evidence from the Myths*. https://www.mja.com.au/system/files/issues/188_05_030308/pha10499_f m.pdf (October 30, 2018).

51. Moeller, A. H. et al. 2014. "Rapid Changes in the Gut Microbiome during Human Evolution." *Proceedings of the National Academy of Sciences* 111(46): 16431–35. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4246287&tool =pmcentrez&rendertype=abstract.

52. Muegge, Brian D. et al. 2011. "Diet Drives Convergence in Gut Microbiome Functions across Mammalian Phylogeny and within Humans." *Science (New York, N.Y.)* 332(6032): 970–74. http://www.ncbi.nlm.nih.gov/pubmed/21596990 (March 15, 2018).

53. Nasidze, Ivan et al. 2011. "High Diversity of the Saliva Microbiome in Batwa Pygmies" ed. Francisco Rodríguez-Valera. *PLoS ONE* 6(8): e23352. http://dx.plos.org/10.1371/journal.pone.0023352 (August 30, 2017).

54. Ozkan, Jerome et al. 2017. "Temporal Stability and Composition of the Ocular Surface Microbiome." *Scientific Reports* 7(1): 9880. http://www.nature.com/articles/s41598-017-10494-9 (June 4, 2018).

55. Pääbo, Svante. 1985. "Molecular Cloning of Ancient Egyptian Mummy DNA." *Nature* 314(6012): 644–45. http://www.nature.com/articles/314644a0 (October 29, 2018).

56. Pilli, Elena et al. 2013. "Monitoring DNA Contamination in Handled vs. Directly Excavated Ancient Human Skeletal Remains" ed. Carles Lalueza-Fox. *PLoS ONE* 8(1): e52524. http://dx.plos.org/10.1371/journal.pone.0052524 (October 29, 2018).

57. Pontzer, Herman et al. 2012. "Hunter-Gatherer Energetics and Human Obesity" ed. Farid F. Chehab. *PLoS ONE* 7(7): e40503. http://dx.plos.org/10.1371/journal.pone.0040503 (November 27, 2017).

58. Rhodes, Rosamond. 2016. "Ethical Issues in Microbiome Research and Medicine." *BMC Medicine* 14(1).

59. Rohland, Nadin, and Michael Hofreiter. 2007. "Ancient DNA Extraction from Bones and Teeth." https://www-nature-com.proxy.library.adelaide.edu.au/articles/nprot.2007.247.pdf (September 24, 2018).

60. Rosier, Bob T., Marko De Jager, Egija Zaura, and Bastiaan P. Krom. 2014. "Historical and Contemporary Hypotheses on the Development of Oral Diseases: Are We There Yet?" *Frontiers in cellular and infection microbiology* 4: 92. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4100321&tool =pmcentrez&rendertype=abstract (May 22, 2016).

61. Ruggles, Kelly V et al. 2018. "Changes in the Gut Microbiota of Urban Subjects during an Immersion in the Traditional Diet and Lifestyle of a Rainforest Village." *mSphere* 3(4): e00193-18. http://www.ncbi.nlm.nih.gov/pubmed/30158281 (September 2, 2018).

62. Salter, SJ Susannah J et al. 2014. "Reagent and Laboratory Contamination Can Critically Impact Sequence-Based Microbiome Analyses." *BMC Biology* 12(1): 87. http://www.biomedcentral.com/1741-7007/12/87 (October 10, 2016).

63. Sanders, Jon G. 2015. "Disentangling the Coevolutionary Histories of Animal Gut Microbiomes." https://dash.harvard.edu/handle/1/17463127 (April 24, 2019).

64. Segata, Nicola. 2015. "Gut Microbiome: Westernization and the Disappearance of Intestinal Diversity." *Current Biology* 25(14).

65. Shaw, Liam et al. 2017. "The Human Salivary Microbiome Is Shaped by Shared Environment Rather than Genetics: Evidence from a Large Family of Closely Related Individuals." *mBio* 8(5): e01237-17. http://www.ncbi.nlm.nih.gov/pubmed/28900019 (September 18, 2017).

66. Slon, Viviane et al. 2017. "Neandertal and Denisovan DNA from Pleistocene Sediments." *Science (New York, N.Y.)* 356(6338): 605–8. http://www.ncbi.nlm.nih.gov/pubmed/28450384 (October 29, 2018).

67. Sokurenko, E V et al. 1999. "Pathoadaptive Mutations: Gene Loss and Variation in Bacterial Pathogens." *Trends in microbiology* 7(5): 191–95. http://www.ncbi.nlm.nih.gov/pubmed/10354593 (October 27, 2018).

68. Spencer Larsen, C. 1995. "Biological Changes in Human Populations with Agriculture." *Annual Review of Anthropology* 24(1): 185–213. http://anthro.annualreviews.org/cgi/doi/10.1146/annurev.anthro.24.1.185.

69. Stagg, Kelda. 2010. (Internal Report) "DNA Degradation in Teeth and Bones."

70. Ströhle, Alexander, and Andreas Hahn. 2011. "Diets of Modern Hunter-Gatherers Vary Substantially in Their Carbohydrate Content Depending on Ecoenvironments: Results from an Ethnographic Analysis." *Nutrition Research* 31(6): 429–35. http://www.ncbi.nlm.nih.gov/pubmed/21745624 (September 8, 2018).

71. Sullam, Karen E. et al. 2012. "Environmental and Ecological Factors That Shape the Gut Bacterial Communities of Fish: A Meta-Analysis." *Molecular Ecology* 21(13): 3363–78.

72. Tilg, Herbert, and Alexander R. Moschen. 2015. "Food, Immunity, and the Microbiome." *Gastroenterology* 148(6): 1107–19. https://www-sciencedirect-com.proxy.library.adelaide.edu.au/science/article/pii/S0016508515000128 (October 13, 2018).

73. Utter, Daniel R., Jessica L. Mark Welch, and Gary G. Borisy. 2016. "Individuality, Stability, and Variability of the Plaque Microbiome." *Frontiers in Microbiology*.

74. Velsko, Irina M et al. 2017. "The Dental Calculus Metabolome in Modern and Historic Samples." *Metabolomics : Official journal of the Metabolomic Society* 13(11): 134. http://www.ncbi.nlm.nih.gov/pubmed/29046620 (September 24, 2018).

75. Warinner, C. et al. 2015. "Direct Evidence of Milk Consumption from Ancient Human Dental Calculus." *Scientific Reports* 4(1): 7104. http://www.nature.com/articles/srep07104.

76. Warinner, C et al. "Direct Evidence of Milk Consumption from Ancient Human Dental Calculus." www.nature.com/scientificreports (September 3, 2018).

77. Warinner, Christina et al. 2014. "Pathogens and Host Immunity in the Ancient Human Oral Cavity." *Nature genetics* 46(4): 336–44. http://dx.doi.org/10.1038/ng.2906.

78. Wetterstrand, K A. 2016. "DNA Sequencing Costs: Data from the NHGRI

Large-Scale Genome Sequencing Program." *Dostępny w Internecie: www.genome.gov/sequencingcostsdata*.

79. Weyrich, Laura S et al. 2017. "Neanderthal Behaviour, Diet, and Disease Inferred from Ancient DNA in Dental Calculus." *Nature Publishing Group* 544. https://www.nature.com/nature/journal/v544/n7650/pdf/nature21674.pdf (November 1, 2017).

80. Willerslev, Eske et al. 2003. "Diverse Plant and Animal Genetic Records from Holocene and Pleistocene Sediments." *Science (New York, N.Y.)* 300(5620): 791–95. http://www.ncbi.nlm.nih.gov/pubmed/12702808 (October 29, 2018).

81. Willerslev, Eske, and Alan Cooper. 2005. "Ancient DNA." *Proceedings. Biological sciences / The Royal Society* 272(1558): 3–16. http://www.ncbi.nlm.nih.gov/pubmed/15875564 (June 15, 2017).

82. Yong, Ed. 2012. "Gut Microbial 'enterotypes' Become Less Clear-Cut." *Nature*. http://www.nature.com/doifinder/10.1038/nature.2012.10276 (November 23, 2018).

83. Zhou, Youlian et al. 2018. "Gut Microbiota Offers Universal Biomarkers across Ethnicity in Inflammatory Bowel Disease Diagnosis and Infliximab Response Prediction." *mSystems*.

84. Ziesemer, Kirsten A et al. 2015. "Intrinsic Challenges in Ancient Microbiome Reconstruction Using 16S RRNA Gene Amplification." *Scientific reports* 5: 16498. http://www.nature.com/srep/2015/151113/srep16498/full/srep16498.html (February 8, 2016).

"Difficulties are just things to overcome, after all."

Ernest Shackleton, Irish polar explorer who led three British expeditions
to the Antarctic

Curriculum Vitae

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Research Assistant (Intern)
July 2011 – Sept. 2011
Microbial Immunology Research Lab, **Penang, Malaysia**

PUBLICATIONS

Felix M. Key, **Muslihudeen A. Abdul-Aziz**, Roger Mundry, Benjamin M. Peter, Aarthi Sekar, Mauro D'Amato, Megan Y. Dennis, Joshua M. Schmidt, Aida M. Andrés. Human local adaptation of the TRPM8 cold receptor along a latitudinal cline. *PLOS Genetics*, 2018; 14 (5): e1007298

Hennersdorf, P., Kleinertz, S., Theisen, S., **Abdul-Aziz, M**., Mrotzek, G., Palm, H., & Saluz, H. (2016). Microbial diversity and parasitic load in tropical fish of different environmental conditions. PLoS ONE, 11(3), e0151594-1-e0151594-18.

Oetama, V., Hennersdorf, P., **Abdul-Aziz**, M., Haryanti, H., Mrotzek, G., & Saluz, H. (2016). Microbiome analysis and detection of pathogenic bacteria of Penaeus monodon from Jakarta Bay and Bali. Marine Pollution Bulletin, 110(2), 718-725.

Philipp Hennersdorf., Grit Mrotzek., **Abdul-Aziz, M**., & Hanspeter Saluz. (2016). Metagenomic analysis between free-living and cultured Epinephelus fuscoguttatus under different environmental conditions in Indonesian waters. Marine Pollution Bulletin, 110(2), 726-734.

Abdul-Aziz, M., Cooper, A., & Weyrich, L. (2016). Exploring relationships between host genome and microbiome: new insights from genome-wide association studies. Frontiers in Microbiology, 7 (OCT), 1611-1-1611-9.

Abdul-Aziz, M. A., Schoefl, G., Mrotzek, G., Haryanti, H., Sugama, K., & Saluz, H. P. (2015). Population structure of the Indonesian giant tiger shrimp Penaeus monodon: a window into evolutionary similarities between paralogous mitochondrial DNA sequences and their genomes. Ecology and Evolution, 5(17), 3570-3584.

AWARDS

Small Research Grants Scheme ($1,500/- for lab work)
 May. 2017 – May. 2018
The Royal Society of South Australia,
Adelaide, Australia

International Post-Graduate Research Scholarship
 Oct. 2015 – Nov. 2018
($ 27,082/- p.a)
The University of Adelaide
Adelaide, Australia

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End of Studies University Subsidy
June. 2014 – Dec. 2014
(Eur. 3,000/-)
Freidrich Schiller University of Jena
Jena, Germany

CONFERENCE POSTERS AND PRESENTATIONS

Sanger Institute Human Evolution conference
Sept. 2017
Hixton, United Kingdom
Local adaptation in humans of the TRPM8 cold receptor follows
 a latitudinal cline
Aida Andres, Felix M. Key, **Muslihudeen A. Abdul-Aziz,**
Roger Mundry, Benjamin M Peter, Aarthi Sekar, Mauro D'Amato,
Megan Y. Dennis, Joshua M. Schmidt

Society for Molecular Biology and Evolution
July 2017
Austin, Texas
Felix M. Key, **Muslihudeen A. Abdul-Aziz**, Roger Mundry, Benjamin Peter,
Mauro D'Amato, Megan Y. Dennis, Joshua M. Schmidt Aida M. Andrés

Spanish society for Population Genetics and Evolution
Oct. 2016
Sitges, Barcelona, Spain
Local adaptation in humans: insights from modern and ancient genomes
Felix M Key, Joshua Schmidt, **Muslih. Abdul-Aziz**, Qiaomei Fu, Frédéric
Romagné, Benjamin Peter, Mauro d'Amato, Megan Dennis, Michael Lachmann,
Aida M Andrés

LEADERSHIP AND VOLUNTEER POSITIONS

Postgraduate Representative – SRC, University of Adelaide
Jan. 2018 – Dec. 2018
Adelaide, Australia

Postgraduate Representative – Academic Board, University of Adelaide
Jan. 2017 – Dec. 2017
Adelaide, Australia

President – University of Adelaide African Student Association
Jan. 2017 – Dec. 2017
Adelaide, Australia

Team member, Organising committee - TEDx Adelaide
Sept. 2016 – Oct. 2016

Adelaide, Australia

Team leader – Promotion team, Organising committee - TEDx FSUJena
Oct. 2014 – Feb. 2015
Jena, Germany

Team leader, International Relations Team - AIESEC in Germany
Oct. 2013 – Aug. 2014
Bonn, Germany

Vice President of Outgoing Exchange - AIESEC USM
Jan. 2009 – **June 2010** Penang, Malaysia

Director - Marketing, Organizing Committee, AIESEC USM
Mar. 2008 – July 2008
Penang, Malaysia

KEY COMPETENCIES

- **Languages**
  Native: Yoruba, Pidgin English
  Advanced Speaker: English, Arabic
  Intermediate: German, French and Malay

- **I.T Skills**
  R, Unix Command line (Bash), Python, Microsoft C++, Visual Basic,
  MySQL, Microsoft Office Suite, Linux (Ubuntu) O.S and Apple Mac O.S.

REFERENCES

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*Others available upon request.