Ancient metaproteomics: a novel approach for understanding disease and diet in the archaeological record

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Abstract

Proteomics is increasingly being applied to archaeological samples following technological developments in mass spectrometry. This thesis explores how these developments may contribute to the characterisation of disease and diet in the archaeological record. This thesis has a three-fold aim; a) to evaluate the potential of shotgun proteomics as a method for characterising ancient disease, b) to develop the metaproteomic analysis of dental calculus as a tool for understanding both ancient oral health and patterns of individual food consumption and c) to apply these methodological developments to understanding individual lifeways of people enslaved during the 19th century transatlantic slave trade. This thesis demonstrates that ancient metaproteomics can be a powerful tool for identifying microorganisms in the archaeological record, characterising the functional profile of ancient proteomes and accessing individual patterns of food consumption with high taxonomic specificity. In particular, analysis of dental calculus may be an extremely valuable tool for understanding the aetiology of past oral diseases. Results of this study highlight the value of revisiting previous studies with more recent methodological approaches and demonstrate that biomolecular preservation can have a significant impact on the effectiveness of ancient proteins as an archaeological tool for this characterisation. Using the approaches developed in this study we have the opportunity to increase the visibility of past diseases and their aetiology, as well as develop a richer understanding of individual lifeways through the production of molecular life histories.

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Author's Declaration

This thesis is the original work of the author. All contributions to this work are acknowledged below. This work has not been submitted for any other degree or award at any other institution. Aspects of this thesis have been published or are under review:

Sections of Chapter 2, Introduction to Methods, have been included in a book chapter, *Proteomics*, in the text-book *Archaeological Science*, scheduled for publication with Cambridge University Press at the end of 2015.

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Chapter Contributions

All chapters which are not explicitly mentioned below were the sole work of JH.

Chapter 5: Tandem mass spectrometer performance and archaeological proteomes:

- Proteomic extractions, data processing, analysis, interpretation, and manuscript preparation were completed by JH
- Christina Warinner, Camilla Speller, Sarah Fiddyment, David Ashford and Sophy Charlton contributed data
- Christina Warinner provided SEED profiles generated from previously published proteomic and genomic data (Warinner, Rodrigues, et al. 2014)
- Roman Fischer, Christian Trachsel and Matthew Collins provided insight into mass spectrometric results and interpretation

Chapter 6: The challenge of identifying tuberculosis proteins in archaeological tissues:

- Data processing, analysis, interpretation, and manuscript preparation were completed by JH
- David Ashford and Kai Yik Teoh performed proteomic extractions of mummified remains in 2011
- Helen Donoghue, Ildikó Pap, David Minnikin, and Mark Spigelman provided contextual information about the samples and comments
- Mike Buckley and Matthew Collins assisted with interpreting the results of Boros-Major et al. (2011)

Chapter 7: Poor preservation of antibodies in archaeological human bone and dentine:

- Proteomic extraction of St Helena dentine samples, data analysis, interpretation was completed by JH

- Ross Kendall extracted and analysed proteins from British samples, observed the absence of immunoglobulin G and initiated the published manuscript, with resources and input from Andrew Millard and Rebecca Gowland

Chapter 8: Direct evidence for milk consumption in ancient human dental calculus:

- Data processing and analysis of all samples, proteomic extractions for St Helena samples, data interpretation, stable isotope analysis, BLG modelling and substantial manuscript preparation was completed by JH
- Christina Warinner and Matthew Collins also prepared the manuscript
- Christina Warinner, Christian Trachsel, Camilla Speller, Roman Fischer, Sarah Fiddyment, Enrico Cappellini, Anna Fotakis, Rosa Jersie-Christensen, Jesper Olsen, Sophy Charlton, Jette Arneborg, and Meaghan Mackie contributed data
- Matthew Collins, Camilla Speller, Thomas Gilbert, Enrico Cappellini, Abigail Bouwman, Frank Ruhli, Dallas Swallow, Alessandro Canci, Oliver Craig and Niels Lynnerup provided samples and resources
- Lactase persistence map was generated by Dallas Swallow, Nicolas Montalva and Anke Liebert generated the lactase persistence data and map

Chapter 9: Metaproteomic analysis of an ancient oral microbiome:

- All data generation, analysis, interpretation and manuscript preparation was performed by JH
- Dana Fialová, Eva Drozdová, Radim Skoupý, Petr Mikulík performed Scanning Electron Microscope analysis and provided comments on observations
- Kirsten Ziesemer provided information on samples from the Netherlands for comparison of thermal age

Chapter 10: Osteological and biomolecular analysis of oral pathologies of 19th century re-captive Africans from St Helena:

- Collation of osteological data, proteomic extractions, data analysis, interpretation and manuscript preparation was completed by JH
- Ann-Sofie Witkin completed initial osteological investigations, published in Witkin (2011)
- Erna Jóhannesdottir supplemented this osteological investigation by providing insight into dental modification practices and contributed ideas for the manuscript

Appendix 3, Intrinsic Challenges in Ancient Microbiome Reconstruction Using 16S rRNA Gene Amplification

- JH performed St Helena DNA extractions and contributed to the manuscript

Chapter 1: Research Framework and Aims

1.1 Research Context and Significance

Studying the health of past populations has the power to reveal how social inequalities, population demographics, patterns of subsistence and cultural practices influence human health. Osteological assessments of pathological indicators have revealed much of what we understand about past disease. Using molecular approaches we have the opportunity to increase the visibility of past diseases and develop a richer understanding of past patterns of individual health in the production of "molecular life histories".

The transatlantic slave trade saw a period of unprecedented forced migration, resulted in the subjugation of millions of people and created a far-reaching legacy of social inequality. As part of this system, diseases spread, proliferated and infected new populations. Many diseases, carried via slave ships or spread through migrations along Atlantic coastlines, continue to affect communities in the Atlantic world. Historical accounts and archaeological research are revealing aspects of the lifeways of enslaved people. However, what is largely absent from this history, is an understanding of the lives of people prior to their forced transportation. Here we have a two-fold gap in knowledge; a) the nature and spread of diseases during this era, and b) the identities and biographies of enslaved people.

1.2 Aims

This PhD explores a novel approach for investigating ancient disease and diet in the archaeological record and uses this approach to explore disease and diet in the era of transatlantic slavery. This research has three primary aims;

1. To apply, explore and develop **shotgun proteomics as a method for characterising disease** in the archaeological record (Chapters 5, 6 and 7)

2. To develop the biomolecular analysis of **ancient dental calculus as a tool for understanding disease and diet in the past** (Chapters 8, 9 and 10). This will focus primarily on the analysis of ancient proteins, with a secondary focus on ancient DNA.

3. The application of these methods to understanding disease and diet of individuals during the 19th century **transatlantic slave trade**, specifically focussing on a unique skeletal population from the South Atlantic island of St Helena (Chapters 9 and 10)

The primary case study for this PhD is the population from St Helena, but additional samples have also been included in this work, including;

- Dental calculus from the United Kingdom (Chelsea, Fewston, Tickhill, Norton, Driffield Terrace, Melton, Dixon Lane), Germany (Dalheim), Woolly Rhinoceros *(Coelodonta antiquitatis)* cementum from Trou al'Wess, Belgium, and modern dental calculus from Zurich, Switzerland (Chapter 5)
- Mummified remains from the Dominican Church of Vác, Hungary (Chapter 6)
- Dental calculus from the United Kingdom (Melton, Driffield Terrace, Leicester, Norton-on-Tees, Wighill, Fewston), Denmark (Gjerrild, Öster Harup), Norway (Trondheim), Germany (Nersingen, Regensburg-Dechbetten, Dalheim), Hungary (Szöreg-C, Sziv Utca), Italy (Olmo di Nogara, Isola Sacra), Armenia (Hatsarat, Nerkin Getashen, Noraduz), Russia (West Caucasus, Marchenkova Gora, Bulanovo) and Greenland (Brattahlið, Sandnes) (Chapter 8)

1.3 Thesis Structure and Outline

This PhD is structured as a series of discrete articles, bracketed by introductory and discussion sections. This approach was chosen because of the rapid pace of research in biomolecular archaeology. This enables aspects of this research to be published or prepared for publication as journal articles at the point of formation during the PhD, rather than delaying until the entire thesis is completed. For chapters which have already been published or accepted for publication (Chapter 7, Chapter 8 and Appendix 3) the formatting, structure and bibliographic style have been altered from the published or accepted version to give consistency to the thesis as a whole. Readers of this thesis are asked to forgive any repetition of introductory details, sampling, or methodologies necessary to make each article a stand-alone work.

1.3.1 Chapter Outline

Chapter 2 introduces the methodological approaches adopted for this PhD; ancient genomics, ancient proteomics and the analysis of ancient dental calculus. **Chapter 3** provides the historical context for the main case study of the PhD, the analysis of individuals buried on the South Atlantic island of St Helena.

Chapter 4: Disease and dental calculus: a novel biomolecular perspective on the transatlantic slave trade outlines how the biomolecular analysis of dental calculus may be used to understand aspects of the transatlantic slave trade and offers some potential future directions and considerations for dental calculus research.

Abstract: The transatlantic slave trade has a profound legacy in shaping patterns of human health through the Atlantic world. In a fatal mix, the transatlantic slave trade resulted in the spread of disease, the exposure of vulnerable populations to new disease environments and the movement of disease carrying vectors. Contemporary populations are still exposed to many of the same diseases, spread as a result of the transatlantic slavery (Lammie et al. 2007). Given the significance of the transatlantic slave trade in the history of human health, it is important that emerging new methods are adopted for the study of disease in this period. Through the extraction of human and bacterial proteins and DNA from dental calculus (mineralised plaque) biomolecules from pathogens and their associated diseases from this era could be studied in detail for the first time. This gives us the potential to identify diseases hidden from both the historical and osteological record, to mine the historical oral microbiome, to identify remnants of diet and explore the interplay between host immunity and microbial virulence. The removal of mineralised microbial biofilms adhering to the surface of teeth offers a minimally invasive way to explore health from skeletal remains. As interest in the analyses of ancient dental calculus grows it is necessary to consider the ethical boundaries of studying novel deposits such as these to maintain best practice in the handling and analysis of human remains from this period.

Chapter 5: Tandem mass spectrometer performance and archaeological proteomes explores the effect that the performance of different tandem mass spectrometer platforms can have on the archaeological applications of ancient proteomics.

Abstract: Shotgun proteomics is increasingly being applied to archaeological samples following technological developments in tandem mass spectrometry. As interest grows it is necessary to assess the performance of available instruments with complex mixtures of ancient and degraded proteins. Proteins were extracted and analysed from 59 samples of

archaeological dental tissues (cementum, dentine, and dental calculus) and analysed across multiple tandem mass spectrometry instruments (maXis 3G UHR Q-TOF, LTQ Orbitrap Velos, Orbitrap Elite and Q Exactive). This study observed that a) the hybrid Quadrupole-Orbitrap (Q Exactive) outperformed all instruments tested in terms of the number of protein group identifications, b) the Orbitrap Velos was able to identify a greater number of protein families than the maXis 3G and c) CID fragmentation produced a greater number of protein identifications than HCD. The results demonstrate the value of using the most recent, high-accuracy, high-speed mass spectrometers, which can provide increased sequence coverage and higher rates of protein identification. While this study may not be a strict comparison of instruments in the truest sense, given the use of different sample loads, different high-performance liquid chromatography (HPLC) setups and experiments at different locations, it nonetheless highlights the significant difference in results obtained. This has downstream consequences for archaeological data interpretation and applicability. As instrumentation continues to advance in resolution it is inevitable it will broaden the scope and reach of ancient proteomics.

Chapter 6: The challenge of identifying tuberculosis proteins in archaeological tissues explores some of the methodological considerations in identifying proteins derived from *Mycobacterium tuberculosis* in archaeological samples.

Abstract: Following the report of *Mycobacterium tuberculosis* proteins found in archaeological bone by Boros-Major et al. (2011), we attempted to identify *M. tuberculosis* proteins in mummified lung tissues from which ancient DNA success had already been reported. Using a filter-aided sample preparation protocol modified for ancient samples we applied shotgun proteomics to seven samples of mummified lung, chest and pleura tissues. However, we only identified four peptides with unique matches to the *Mycobacterium*

tuberculosis complex, none of which were unique to *M. tuberculosis*, although we did identify a range of human proteins and non-mycobacterial bacterial proteins. In light of these results we question the validity of the peptide mass fingerprint (PMF) approach presented by Boros-Major et al. (2011), especially because the PMF spectra presented in this study has similarities to that of human collagen, the dominant protein in the tissue under investigation. We explore the challenges of using proteomic approaches to detect *M. tuberculosis* and propose that given the contentious outcomes that have plagued ancient protein research in the past, the susceptibility of ancient material to modern contamination, and the degradation inherent in archaeological materials, caution is needed in the acquisition, analysis and reporting of proteomic data from such material.

Chapter 7: Poor preservation of antibodies in archaeological human bone and dentine follows work by Ross Kendall, exploring how the degradation of antibodies in archaeological material may prevent their use as a tool for disease identification.

Abstract: The growth of proteomics-based methods in archaeology prompted an investigation of the survival of non-collagenous proteins, specifically immunoglobulin G (IgG), in archaeological human bone and dentine. Over a decade ago reports were published on extracted, immunoreactive archaeological IgG and the variable yields of IgG molecules detected by Western blots of 1D and 2D SDS-PAGE gels (Schmidt-Schulz and Schultz, 2004). If IgG can indeed be recovered from archaeological skeletal material it offers remarkable opportunities for exploring the history of disease, for example in applying functional anti-malarial IgGs to study past patterns of malaria. More recently, the field has seen a move away from immunological approaches and towards the use of shotgun proteomics via mass spectrometry. Using previously published techniques, this study attempted to extract and characterise archaeological IgG proteins. In only one extraction method were immunoglobulin

derived peptides identified and these displayed extensive evidence of degradation. The failure to extract immunoglobulins by all but one method, along with observed patterns of protein degradation, suggests that IgG may be an unsuitable target for detecting disease-associated antigens. This research highlights the importance of revisiting previously 'successful' biomolecular methodologies using emerging technologies.

Chapter 8: Direct evidence of milk consumption from ancient human dental calculus explores the proteomic analysis of ancient dental calculus as a method to uncover direct evidence of food consumption in the archaeological record.

Abstract: Milk is a major food of global economic importance and its consumption is regarded as a classic example of gene-culture evolution. Humans have exploited animal milk as a food resource for at least 8,500 years, but the origins, spread and scale of dairying remain poorly understood. Indirect lines of evidence, such as lipid isotopic ratios of pottery residues, faunal mortality profiles and lactase persistence allele frequencies, provide a partial picture of this process; however, in order to understand how, where and when humans consumed milk products, it is necessary to link evidence of consumption directly to individuals and their dairy livestock. Here we report the first direct evidence of milk consumption, the whey protein β-lactoglobulin (BLG), preserved in human dental calculus from the Bronze Age (ca. 3,000 BCE) to the present day. Using protein tandem mass spectrometry, we demonstrate that BLG is a species-specific biomarker of dairy consumption and we identify individuals consuming cattle, sheep and goat milk products in the archaeological record. We then apply this method to human dental calculus from Greenland's medieval Norse colonies, and report a decline of this biomarker leading up to the abandonment of the Norse Greenland colonies in the 15th century CE.

Chapter 9: Metaproteomic analysis of an ancient oral microbiome explores the application of metaproteomics to dental tissues from formerly enslaved Africans buried on St Helena as a tool by which to identify disease and diet in the archaeological record.

Abstract: Analysis of ancient proteins is proving to be an effective tool by which to characterise ancient disease and diets, and may be particularly useful in regions where DNA analysis may be hampered by poor preservation. Proteomic analysis was applied to samples of dental calculus and dentine from formerly enslaved individuals from the South Atlantic island of St Helena with the aim of investigating disease and food consumption during the 19th century transatlantic slave trade. This analysis reveals that the human proteome of these dental tissues is preserved in archaeological samples. In addition, metaproteomic analysis of dental calculus indicates the preservation of proteins derived from a suite of oral bacteria, both commensal and pathogenic. However, although this information may provide valuable insight into periodontal diseases, this analysis does not reveal evidence of diseases that directly relate to transatlantic slavery. Despite previous reported successes of microbial DNA derived from the cardiovascular and respiratory systems (Warinner, Rodrigues, et al. 2014), this study demonstrates that the proteomic analysis of dental calculus may have the most use for specifically understanding oral diseases. The detection of these microbial proteins also facilitated an exploration into bacterial survival, where it was found proteins from Gram-negative bacteria were much more abundant than Gram-positive in archaeological samples, in contrast to previous reports for ancient DNA. Results of this study also demonstrate that protein preservation varies substantially between individuals and proposes that understanding the mechanism of this preservation is vital in order to comprehend the degree to which ancient samples are a reflection of the living tissue.

Chapter 10: Osteological and biomolecular analysis of oral pathologies of 19th century re-captive Africans from St Helena, explores the oral health of individuals from St Helena in more detail and compares osteological and biomolecular indicators of disease.

Abstract: In the mid-19th century over 26,000 "liberated Africans" were received onto the small, South Atlantic island of St Helena, "liberated" by the British Royal Navy as part of their campaign to abolish the transatlantic slave trade. Recent excavations have revealed the skeletal remains of 325 victims of this trade, representing individuals who died as a result of their forced transportation across the Atlantic. In this study we have analysed the relationship between the prevalence of cultural dental modifications and oral pathologies observed in this population. In addition, to further understand the aetiology of these pathologies, we have applied shotgun protein analysis to deposits of dental calculus, revealing some of the specific oral bacteria responsible for these infections. This study demonstrates that when pulp cavities are exposed through dental modification localised oral pathologies may become prevalent. In addition, we demonstrate that using biomolecular approaches in conjunction with palaeopathological observation it is possible to gain a deeper insight into disease identification and aetiology, although the relationship between osteological and biomolecular disease identification is complex and requires further investigation.

Chapter 11 summarises the work, discusses the major findings of the overall research, comments upon the research design, outlines the implications and limitations of the research, and poses some future direction.

1.3.2 Appendices

Appendix 1 presents the protein identifications made for Chapter 9 and provides more detailed St Helena sample information. DNA work completed as part of this PhD has been included as appendices in order to not detract from the focus on proteomic analysis. **Appendix 2** summarises ancient DNA work completed as part of this PhD. **Appendix 3** follows on from these DNA methodologies by exploring how a 16S-targeted approach results in taxonomic bias in ancient microbiome analysis. Appendix 3 is in the form of a paper currently under review in Scientific Reports. Here, I have performed DNA experiments and contributed data, but this input does not warrant inclusion as a thesis chapter.

Appendix 3: Intrinsic challenges in ancient microbiome reconstruction using 16S rRNA gene amplification

Abstract: To date, characterisation of ancient oral (dental calculus) and gut (coprolite) microbiota has been primarily accomplished through a phylotyping approach involving targeted amplification of one or more variable regions in the 16S rRNA gene. Specifically, the V3 region (*E. coli* 341-534) of this gene has been suggested as an excellent candidate for ancient DNA amplification and microbial community reconstruction. However, in practice this phylotyping approach often produces highly skewed taxonomic frequency data. In this study, we use non-targeted (shotgun metagenomics) sequencing methods to better understand skewed microbial profiles observed in four ancient dental calculus specimens previously analysed by amplicon sequencing. Through comparisons of microbial taxonomic counts from paired amplicon (V3 U341F/534R) and shotgun sequencing datasets, we demonstrate that extensive length polymorphisms in the V3 region are a consistent and major cause of differential amplification leading to taxonomic bias in ancient microbiome reconstructions based on amplicon sequencing. We conclude that systematic amplification bias confounds attempts to

accurately reconstruct microbiome taxonomic profiles from 16S rRNA V3 amplicon data generated using universal primers. Because *in silico* analysis indicates that alternative 16S rRNA hypervariable regions will present similar challenges, we advocate for the use of a shotgun metagenomics approach in ancient microbiome reconstructions.

1.4 Research Network: EUROTAST

This research stands within EUROTAST, an EC Marie-Curie Initial Training Network. Across 13 doctoral research projects EUROTAST seeks to use novel and scientific approaches to understanding the history and legacy of the transatlantic slave trade (Marchant 2011). In particular, this PhD research has close involvement with the work of three doctoral students also researching the individuals buried at St Helena. These doctoral researchers are exploring the geographical and population origins of these individuals using strontium isotope analysis and ancient DNA analysis, as well as researching the cultural dental modifications found in this population and what this might mean in terms of cultural practices and identities.

1.5 Statement on Language

Many words and terms connected with slavery and people of African descent have been and continue to be considered offensive or categorising, including terms used in academic contexts. Amongst all people with a history of marginalisation, the terms we use have the power to give and deny dignity and have strong meaning. Thus, the choice of language is both sensitive and consequential, and the decisions I have made in choosing specific terms are addressed here. The issues and problems surrounding the use of this language have been addressed and explored by EUROTAST researchers and the terms I have chosen have, in part,

originated from those discussions. I have also followed some of the recommendations outlined by the Understanding Slavery Initiative (http://www.understandingslavery.com/), which aims to support the teaching of the transatlantic slave trade and its presence in museums and collections.

Terms referring to people:

- When referring to people of African ancestry who have been enslaved I have chosen to use the term "enslaved African" or "enslaved woman/man/child" rather than "slave". The use of this term is part of a broader political and linguistic trend which seeks to emphasise that slavery was something that was done *to* someone, and seeks to bring some humanity to what is considered a categorising term. The use of the term "enslaved man/woman" seeks to separate the individual (i.e. "man/woman/African") from the label (i.e. "enslaved") more so than the term "slave" is able to do.
- Racially imbued terms, such as "Negro", are only used when they relate to a specific historical context or quote.
- Regarding the individuals buried on St Helena, we do not know what they called themselves during life. Most often I have chosen to simply use the term "individual", which has no label or origin attached and avoids assigning their identity as "enslaved" or even "liberated". However, to be consistent with previous publications on this population (Pearson et al. 2011), I have occasionally used the term "liberated Africans" or "re-captive Africans".
- When referring to a historic or archaeological population, I have chosen to use the term "people of African descent" rather than the term "black", which is a word that has become imbued with socio-political meaning, particularly in the post-slavery period, making it problematic to apply to a historical or archaeological context. In some cases

I have used the term “African diaspora” to mean people of African descent who live outside the African continent as a result of forced migration during the period of transatlantic slavery.

Other terms:

- The use of the word “Africa” in the context of the transatlantic slave trade has the danger of presenting the continent as a homogenous place, and does not do justice to the incredible cultural and linguistic traditions throughout the continent that existed prior to, during, and after the transatlantic slave trade. In this thesis I use the term “Africa” because we are largely unaware of where many individuals subject to transatlantic slavery originate, which is in turn a driving question in the study of the transatlantic slave trade. In many cases I have chosen to use the term “West Central Africa”, as this broadly encompasses the regions where individuals from St Helena are most likely to have originated. Where it is possible to be more specific about events and origins of people, I have deliberately avoided the use of “Africa” as a blanket term.
- Where appropriate I have used the term “transatlantic slave trade” rather than “slave trade” to emphasise that fact that there have been and continue to be many trades in enslaved people.
- I have chosen the term “Atlantic world” to mean the three continents of Africa, Europe and the Americas (both North and South) at any point in time.

1.6 Data Deposition

All proteomic data published at the time of thesis submission has been deposited in PRIDE (Proteomics IDEntifications, a data repository part of European Molecular Biology

Laboratory, the European Bioinformatics Institute – EMBL-EBI) via the ProteomeXchange (Vizcaíno et al. 2014). Dataset identifiers are listed below.

Chapter	Published title	PRIDE Identifier
7	Poor Preservation of Antibodies in Archaeological Human Bone and Dentine	PXD002295
8	Direct Evidence for Milk Consumption from Ancient Human Dental Calculus	*St Helena* PXD001357 *Northern Southwest Asia* PXD001359 *Central Europe* PXD001360 *Northern Europe* PXD001361 *UK* PXD001362

Chapter 2: Introduction to Methods

This chapter introduces the methodological approaches of this thesis, establishing the context for the methodological developments that constitute the basis of this PhD. The fields of palaeopathology and palaeomicrobiology will be briefly introduced (2.1) and how these fields are studied will be outlined (2.2). Ancient genomics (2.3) and proteomics (2.4) will then be introduced in more detail and their archaeological applications broadly reviewed. Subsequently, research concerning the archaeological applications of dental calculus analysis will be discussed (2.5).

2.1 Introduction to Palaeopathology

Palaeopathology, a subdiscipline of bioarchaeology, is broadly defined as the study of ancient disease, and is an enormous discipline with many approaches and applications. Most commonly, palaeopathology refers to the study of ancient disease by analysis of primary evidence from the skeletal remains of past individuals. Comprehensive outlines and reviews of the field can be found in Roberts and Manchester (2007), Waldron (2008) and Pinhasi and Mays (2008), and the development of the discipline has been reviewed in Buikstra and Roberts (2012).

Palaeopathology contributes to understanding the prevalence and nature of diseases in the past. At an individual level, it helps us to reconstruct the lifeways and biographies of individuals, but it can also be used to explore how patterns of dietary, social and cultural factors may have influenced the onset, severity or nature of particular diseases. Palaeopathology can also examine how diseases manifest within and between populations

(palaeodemography or palaeoepidemiology), which may illuminate the social, cultural or environmental factors which influence these manifestations (e.g. Barnes et al. 2011). By examining palaeopathology through space and time it is also possible to gain an understanding of how human beings can act as vectors for disease transmission and movement, which in the face of increasing global mobility is a major contemporary health challenge.

2.1.1 Palaeomicrobiology, Infectious Diseases and the Microbiome

A major component of palaeopathology is the study of infectious diseases, disease caused by pathogenic microorganisms. This constitutes the growing field of palaeomicrobiology, the study of microorganisms in the past. Infectious diseases comprise a major portion of the vast number of illnesses which impact human health, especially in the absence of modern antibiotics. However, understanding past infectious diseases has been largely confined to the study of infections which cause sufficiently diagnostic skeletal lesions, including tuberculosis (e.g. Roberts and Buikstra 2008), leprosy (e.g. Roberts, Lewis, and Manchester 2002) and treponemal disease (e.g. Baker and Armelagos 1988; Livingstone 1991).

Palaeomicrobiology has experienced rapid growth in the last two decades, in part due to a) the development and application of high-throughput next-generation sequencing (NGS) technologies which enable the characterisation and study of microorganisms, and b) a better understanding of how to control genomic microbial contamination, given the abundance of microorganisms in the archaeological and laboratory environment. Developments in these practices, workflows, sequencing capabilities and bioinformatic approaches have allowed the field to move beyond simple detecting pathogenic presence or absence, but to understand pathogenic evolution (e.g. Schuenemann et al. 2013).

By extension, there is a growing realisation that not only strictly pathogenic microbes are implicated in disease. The human "microbiome" is the suite of microorganisms which live on and in human beings, and the importance of symbiotic, mutualistic and antagonistic relationships between microbes and their human hosts is becoming increasingly recognised as an integral part of human health (Cho and Blaser 2012). For example, the microbiome of the gastrointestinal tract is involved in host immune response, development and nutritional processes (Eloe-Fadrosh and Rasko 2013; Round and Mazmanian 2009; Kau et al. 2011). The Human Microbiome Project in particular, which surveys the genetic information of these microorganisms, has highlighted the diversity and abundance of microbial organisms which are integral to human health (Turnbaugh et al. 2007). It is now thought that the composition of these microbe communities, as well as changes to this composition, may be indicative of disease states and may be used to diagnose diseases or susceptibility, such as obesity, cardiovascular disease, Irritable Bowel Syndrome and asthma (Cho and Blaser 2012). In a healthy microbiome these communities exist in a symbiotic relationship with the host, but communities in a disease-causing state are termed 'dysbiotic' (Lepage et al. 2013).

Given the technological improvements enabling the detection of microorganisms and the understanding that the microbiome is an integral part of human health, ancient microbiomes (microbiomes isolated from archaeological contexts) are beginning to be explored (Warinner et al. 2015). While so far this has focussed largely on methodological developments which facilitate this exploration, understanding the ancient microbiome may enable a greater understanding of how microorganisms and their human hosts have co-evolved and spread under the influence of changing cultural practices and environments. Since humans travel with their microbes, individual species within the microbiome have been studied in order to illuminate human origins and migrations. For example, modern strains of *Heliobacter pylori*, a

common gut bacteria, have been examined as a genetic tracer for human migrations (Dominguez-Bello and Blaser 2011; Moodley et al. 2009).

2.2 The Study of Past Diseases

Past diseases may be studied by three broad methods. Firstly, historical sources offer insight into societies which documented medical afflictions and practices in text or art. Secondly, osteological studies of skeletal remains offer a way to identify particular diseases directly from past individuals. Thirdly, biomolecular approaches, usually applied to human remains, involve the identification and study of pathogenic microorganisms and the human genome, offering an approach by which to identify some of the diseases which may be invisible to osteological approaches. The following section will examine in more detail how each of these approaches is used to understand ancient disease, as well as some of the limitations and challenges that each of these approaches face. How these approaches have been used to understand disease during the transatlantic slave trade, and the potential for future use, is specifically explored in Chapters 3 and 4.

It is worth noting that understanding *health*, defined by the World Health Organization as the "...state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" (WHO 1948), as opposed to *disease*, in past populations is much more complex, and to understand ancient health to the extent defined here is largely out of the range of palaeopathology.

2.2.1 Historical Records

Historical records, in the form of text or art, can provide detailed information into the nature and prevalence of ancient diseases. Such documents may contain clinical descriptions and ideas of disease aetiology and transmission as well as information on treatments, cures and preventatives (e.g. Winterbottom 1803). In addition to written and visual documents, there is also evidence from relevant objects and materials, such as medicine bottles, labels and medical implements, many of which can be found in the archaeological record (e.g. Otto and Burns 1983; Samford 1996). Historical records may also document rates of mortality or morbidity which can give evidence of palaeoepidemiology (e.g. Great Britain 1789).

However, analysing ancient disease through historical records can often be problematic (Mitchell 2011). Firstly, they are confined to populations who documented disease. Secondly, they can be limited in detail and may be limited to diseases which cause dramatic enough symptoms which warrant noting. Fundamentally, sources are limited by contemporary medical knowledge, may be biased and can be difficult to interpret into medically useful information (Dutour et al. 2003).

2.2.2 Osteological Observation

Analysis of human skeletal remains is the most common way that disease can be identified directly from individuals in the past. Through the examination of skeletal morphologies and lesions, osteological observations may reveal a range of pathologies, including metabolic diseases (such as rickets and scurvy), infectious diseases (such as tuberculosis), trauma and congenital diseases. Past medical treatments may also be revealed directly from skeletal remains, such as tooth fillings or trepanation. Such palaeopathological approaches have vastly

increased our understanding of ancient disease, especially in populations which cannot be studied using text-based sources (Roberts and Manchester 2007; Pinhasi and Mays 2008; Waldron 2008).

However, the study of diseases through osteological observation also has limitations. While there are many diseases which do affect bone tissue, there are many more that do not. Even if an individual is afflicted with such a disease, the individual may not have been affected for long enough for the disease to manifest skeletally and acute diseases may result in death before skeletal lesions have time to form. Since diseases may take a long time in an individual's life to manifest themselves on bone, a skeleton with extensive skeletal pathologies may actually represent an individual with greater life expectancy. Thus, using skeletal remains to understand disease prevalence in a population can be challenging because a skeletal population is unlikely to reflect disease prevalence in the living population. Collectively, these challenges and limitations are termed the "osteological paradox" (DeWitte and Stojanowski 2015; Wood et al. 1992; Wright and Yoder 2003). In addition, it can be challenging to consistently and acculately compare skeletal obvservations generated by different researchers, given the subjective nature of osteological observations (Roberts and Cox, 2003).

Well preserved, mummified remains may contain enough soft tissue to observe manifestations of disease (Lynnerup 2007). Of particular interest may be afflictions which cannot be observed osteologically, i.e. those which are acute or do not affect bone (Lowenstein 2004). In addition, they may also contain highly preserved biomolecules with which to study diseases (Anastasiou and Mitchell 2013). However these remains are rare, being limited in time and to environments which favour this preservation.

2.2.3 Biomolecular Palaeopathology

The biomolecular study of ancient disease facilitates the identification of pathogenic microorganisms, their implication in disease manifestation and, in the case of DNA, may be used to explore pathogenic evolution. Ancient biomolecules may be extracted from a number of sources, such as bone and teeth (e.g. Adler et al. 2011), soft tissues (e.g. Donoghue et al. 2011), as well as other sources such as coprolites (fossilised faeces) (Loreille et al. 2001) and museum specimens (Rohland, Siedel, and Hofreiter 2004). When applied demographically, biomolecular approaches may also be used to understand the speed and nature of pathogen evolution, as well as gain insight into the interaction between diseases and new environments and the coevolution of hosts and pathogens (Gagneux 2012).

It should be noted that the detection of a biomolecule does not necessarily indicate a cause of death, and individuals may carry pathogenic species without being infected or showing signs of being infected, the situation being partially analogous to the 'osteological paradox'. DNA based methods may be sufficiently sensitive to detect the presence of non-lethal concentrations of pathogens. Thus, the identification of disease is not confirmed by the presence of particular biomolecules, rather it provides information on the pathogen ecology of the population under investigation

However, just like the two approaches discussed above, the study of ancient biomolecules associated with disease is not without its challenges, limitations and controversies. Firstly, biomolecules must enter skeletal material during life in order to be detected archaeologically in this material. Even while the individual was alive, biomolecules from pathogens may be present in very small amounts, making their detection challenging (e.g. Barnes and Thomas 2006), although Schuenemann et al. (2013) found that without recourse to enrichment 40% of all DNA reads generated from one bone matched to *Mycobacterium leprae*. Secondly,

recovering pathogenic bacterial markers in the skeletal record may be challenging owing to degradation. Differences in preservation mean that the presence of biomolecules in ancient samples is often not consistent (Schuenemann et al. 2013; Der Sarkissian et al. 2015). Thirdly, identifying pathogenic species in the sea of microorganisms that inhabit the environment, the laboratory and the researcher mean that maintaining the rigorous standards necessary for biomolecular research is paramount (Cooper and Poinar 2000; Gilbert et al. 2005; Roberts and Ingham 2008). Thus, the application of biomolecular palaeopathology requires an understanding of sample preservation, biomolecular taphonomy (how biomolecules have degraded) and control of contamination.

The following sections will discuss in more detail two biomolecular methods and associated case studies which are used for biomolecular disease identification; genomics (2.3) and proteomics (2.4).

2.3 Ancient DNA and Ancient Genomics

Ancient DNA as a discipline began in earnest with reports of the identification of two mitochondrial DNA fragments from the skin of the extinct quagga (Higuchi et al. 1984). The advent of the polymerase chain reaction (PCR) (Mullis and Faloona 1987) enabled the detection of low levels of DNA from archaeological bone, the first being a common European mtDNA fragment from archaeological bone (Hagelberg, Sykes, and Hedges 1989; Pääbo 1989). After the development of NGS in 2005 it became possible to obtain very large amounts of DNA and reconstruct ancient genomes and, more recently, to characterise ancient metagenomes - the multi-species genomic information from complex samples. Now, ancient DNA is becoming a recognised sub-discipline of genetics which provides a temporal perspective to research in human, plant, animal and bacterial evolution, population

movements, kinship and patterns and timing of domestication, among others. Ancient DNA has been most recently reviewed in Orlando, Gilbert, and Willerslev (2015), Hagelberg, Hofreiter, and Keyser (2015), Der Sarkissian et al. (2015) and Brown and Barnes (2015).

2.3.1 Introduction to DNA

DNA (deoxyribonucleic acid) is a linear polymer biomolecule containing the code for the synthesis of protein sequences and control regions which regulate expression. DNA is made up of four monomer units, called nucleotides. Each nucleotide contains a phosphate component, a five-membered ring (deoxyribose) and one of four nitrogenous bases - guanine (G), adenine (A), thymine (T), cytosine (C). These monomer units are joined by phosphodiester bonds between the phosphate and the deoxyribose sugar. The polymer forms a double helix structure based on two polymer chains, comprised of a backbone of the sugar-phosphate with the bases directed inwards of the helix. Held by hydrogen bonds, bases from opposite chains pair up (A with T, and C with G) as 'base pairs' (Fig. 2.1).

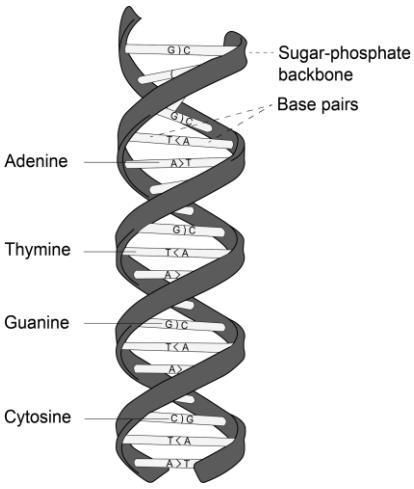

Figure 2.1. Basic structure of DNA

An organism's complete set of DNA is its genome. In prokaryotes (bacteria and archaea) the genome is found as a single, typically circular molecule within the cell, and the genome is organised by proteins, acting in a similar way to nucleosomes in the eukaryotic cell. Bacterial genomes vary in length from 0.5 to 10 million base pairs (in contrast, the human genome is three billion base pairs). In eukaryotes DNA is present in separate organelles, in the case of animals as both a nuclear and mitochondrial genome, whilst in plants it is also found in chloroplasts. The mitochondrial genome, although smaller in size (16,569 base pairs as opposed to three billion base pairs in the nuclear genome), is present in multiple copies (100-10,000 copies per cell).

2.3.2. Ancient DNA

DNA has been extracted from a range of different human tissues, including skeletal material (bones, and teeth) (Adler et al. 2011), as well as mummified or well preserved tissues such as hair (Carpenter et al. 2013; Gilbert et al. 2007; Rasmussen et al. 2010; Rasmussen et al. 2011). In addition, ancient DNA can be extracted from coprolites (Loreille et al. 2001; Speller et al. 2010; Tito et al. 2012), soil (Smith et al. 2015; Willerslev et al. 2003) and plant remains (Cappellini et al. 2010; Jaenicke-Després et al. 2003). Although a rapidly expanding field, accessing metagenomic DNA is currently achieved by multiple approaches; a targeted approach, such as the targeting of variable regions of the 16S rRNA gene (e.g. Adler et al. 2013) or by a shotgun approach, which sequences every extracted DNA fragment (e.g. Warinner, Rodrigues, et al. 2014). Recently, a capture based method for pathogenic DNA was also developed (Devault et al. 2014; Bos et al. 2015). All approaches involve four main steps; extraction, library preparation, amplification, and sequencing. A number of downstream

bioinformatic processes enable these data to be assessed and interrogated (Ginolhac et al. 2011).

DNA from archaeological samples is preserved in small quantities, is degraded and may contain inhibitors which hinder amplification. Successful analysis requires methods which remove inhibitors and protect DNA from further damage induced by reagents. A number of extraction methods have been developed in order to work around these challenges (Rohland and Hofreiter 2007; Rohland, Siedel, and Hofreiter 2010; Dabney et al. 2013; Yang et al. 1998). Following extraction, DNA libraries are prepared, which involves the attachment of primers (different primers may be used to target specific genomic regions or species), the attachment of indices, and the attachment of adapters, which facilitate the amplification via PCR, and sequencing (Meyer and Kircher 2010; Gansauge and Meyer 2013). Following library preparation, amplification via PCR is necessary given that the amount of endogenous DNA extraction from archaeological samples is typically small. Using a starting mix of polymerase, primers and nucleotides, PCR involves cycles of temperature changes which causes strands of DNA to be copied. Subsequently, the amplified library is sequenced, revealing the order of nucleotides. Sanger sequencing, based on the chain termination method, is the traditional method to identify the sequence of an extracted fragment. However, this is now being supplanted by faster and cheaper NGS technologies (Schuster 2008). This approach is particularly suited to ancient DNA given that ancient DNA is typically of a fragment length suited to this approach (Mardis 2008). As a result, the number of ancient genomes published in one study has rocketed, from the first human genome in 2010 (Rasmussen et al. 2010) to 101 genomes only five years later (Allentoft et al. 2015).

2.3.3 Applications of Ancient DNA

Ancient DNA is used to answer different questions about the past. Some of these will be briefly outlined, before a discussion on ancient DNA in the study of disease. Applications of ancient DNA are abundant, so where appropriate I have cited a recent example of such an application.

DNA extracted from extinct animals has been used to identify phylogenetic relationships and patterns of animal populations, including the moa (Cooper et al. 1992; A. J. Baker et al. 2005), the woolly mammoth (Miller et al. 2008; Poinar et al. 2006) and the cave bear (Hofreiter et al. 2002; Hänni et al. 1994). More recently, Orlando et al. (2013) analysed the genome of a Pleistocene horse in order to understand the evolution of this genus, currently the oldest sequenced complete genome (500-700 kya). Ancient DNA can also be used for species identification and for palaeoenvironmental reconstruction. For example, using both temperate and permafrost sediments Willerslev et al. (2003) were able to identify DNA derived from plants, megafauna and extinct animals.

Ancient DNA can also be used to explore the characteristics of past individuals, inclduing kinship and population identity (Haak et al. 2008). The sex of skeletons, which can sometimes be difficult to determine osteologically, is merely a by-product of NGS data (Skoglund et al. 2013). Ancient DNA can be used to demonstrate possible relationships between modern and ancient groups (Lazaridis et al. 2014) and the dispersal of populations (Haak et al. 2015; Allentoft et al. 2015) and individuals (Schroeder et al. 2015). In addition, DNA analysis extracted from early modern humans can be used to reconstruct early population movements, confirming the Out of Africa hypothesis and our genetic relationship to other hominids (Noonan et al. 2006; Prüfer et al. 2014; Stoneking and Krause 2011).

Ancient DNA analysis may also be used in the study of diet, agricultural developments and their relationship with the human genome. For example, the lactase persistence phenotype has been explored as both a method to understand the adoption of milk consumption into adulthood and as an example of how dietary practices can influence the human genome (Curry 2013). Ancient genetics can also give information about the origin and timing of domestication of plants and animals by comparing short tandem repeats, facilitating the identification of closely related species (Jones and Brown 2000). This method has been used to investigate a range of plant domesticates, such as maize (Pääbo et al. 2004; Jaenicke-Després et al. 2003) and animals, such as cattle (Scheu et al. 2015; Speller et al. 2013).

2.3.4 Ancient DNA and Disease

Ancient DNA is used to identify disease-causing organisms in archaeological samples, in order to identify population level exposures, make diagnoses in individuals or to understand the evolution, origin and phylogeny of particular pathogenic species. A selection of case studies are presented in Table 2.1, indicating the range of species identified and demonstrating the emphasis on *Mycobacterium tuberculosis* and *Mycobacterium leprae* as well as *Yersinia pestis*. In addition, Table 2.1 highlights the more recent movement from identifying single organisms, to more complex microbial systems. Reviews of the field can be found in Drancourt and Raoult (2005), Anastasiou and Mitchell (2013) and Roberts and Ingham (2008), who found that despite established protocols for ensuring authenticity many studies do not conform to these protocols.

Organism	Disease	Source	Reference
		Single organisms	
Ascaris	Roundworm	Parasite eggs in coprolites	Loreille et al. 2001; Leles et al. 2008
Bartonella quintana	Transmitted by ectoparasites, may cause a variety of diseases	Tooth	Drancourt et al. 2005
Clonorchis sinensis	Chinese river fluke	Parasite eggs in gall bladder	Liu et al. 2007
Enterobius vermicularis	Pinworm	Parasite eggs in Coprolite	Iñiguez et al. 2006
Escherichia coli	Infection	Mummified remains	Fricker, Spigelman, and Fricker 1997
Helicobacter pylori	Usually non pathogenic	Mummified remains	Swanston et al. 2011
Hepatitis B virus	Hepatitis B	Mummified remains	Kahila Bar-Gal et al. 2012
HTLV-1 virus	Viral infection	Mummified remains	Li et al. 1999
Influenza virus (viral RNA)	Spanish flu	Museum specimen - lung tissue	Taubenberger et al. 1997
Leishmania donovani	Leishmaniasis	Mummified remains	Zink et al. 2006
Mycobacteria tuberculosis	Tuberculosis	Mummified and skeletal remains	e.g. Salo et al. 1994; Jaeger et al. 2012; Bouwman et al. 2012; Bos et al. 2014; Müller, Roberts, and Brown 2014;
Mycobacterium leprae	Leprosy	Skeletal material	e.g. Rafi et al. 1994; Taylor et al. 2009; Schuenemann et al. 2013; Mendum et al. 2014
Plasmodium falciparum	Malaria	Mummified and skeletal remains	Sallares and Gomzi 2001; Khairat et al. 2013;
Salmonella enterica serovar Typhi	Typhoid fever	Dental pulp	Papagrigorakis et al. 2006; Shapiro, Rambaut, and Gilbert 2006
Treponema pallidum	Venereal syphilis, congenital sphillis, yaws, pinta	Skeletal material	Kolman et al. 1999; Bouwman and Brown 2005; von Hunnius et al. 2007
Trichuris trichiura	Whipworm	Parasite eggs in sediment	Oh et al. 2010
Trypanosoma cruzi	Chaga's disease	Mummified remains	Fernandes et al. 2008;

			Aufderheide et al. 2004
Yersinia pestis	Bubonic plague	Dental pulp	Drancourt et al. 1998; Gilbert et al. 2004; Bos et al. 2011
Streptcoccus mutans	Denta caries	Teeth	Simón et al. 2014
Human	Beta-thalassaemia mutation	Skeletal remains	Filon et al. 1995
		Metagenomics	
Plasmodium falciparum, Toxoplasma gondii	Malaria, Toxoplasmosis	Mummified remains	Khairat et al. 2013
Oral bacteria	Oral flora; Periodontal disease	Dental calculus	Adler et al. 2013; Warinner, Rodrigues, et al. 2014
Gastrointestinal bacteria	Gut bacteria	Mummified remains	Ubaldi et al. 1998; Cano et al. 2000; Cano et al. 2014; Tito et al. 2012

Table 2.1 Selected studies using ancient DNA for the study of past disease

Table 2.1 highlights that fact that the vast majority of studies concerning the identification of pathogenic species derive from mummified remains and parasitic eggs, both which are abundant in endogenous DNA. However, mummified remains are limited in time and space, and parasitic remains are rare and are unable to be linked directly to individuals.

2.3.5 Challenges of Ancient DNA Research

Issues of contamination and lack of authenticity are major concerns in ancient DNA research. Criteria of authenticity were established by Cooper and Poinar (2000), largely in response to the publication of unrepeatable results, such as the report of DNA derived from an 80 million year old dinosaur (Woodward, Weyand, and Bunnell 1994). Key criteria were established to ensure the quality publication of ancient DNA results, including measures to avoid

contamination from modern sources, reduce problems associated with the degraded nature of ancient DNA and minimise issues of working with small amounts of DNA (such as performing research in physically isolated laboratories, performing replicates from the same sample and undertaking independent replications). Ancient DNA research and its authentication has been reviewed since these initial criteria were established (Hofreiter et al. 2001; Willerslev and Cooper 2005; Gilbert et al. 2005; Pääbo et al. 2004), and specifically for palaeopathology in Roberts and Ingham (2008). Gilbert et.al (2005) noted that five years later few papers adopted all nine criteria and researchers should be critical when assessing their own or other's conclusions.

Using ancient DNA to answer questions about particular diseases is not without its difficulties. Some of the studies in Table 2.1 have been subject to scrutiny and very few of them have undertaken independent replication. Contaminating DNA from other sources can lead to false identification and interpretation. This can arise from DNA present in the burial environment, for example *Mycobacterium* spp. in soil (Wilbur et al. 2009; Pontiroli et al. 2013), by the handling of samples, from previous PCR reactions and from reagents and lab equipment (Yang and Watt 2005). In addition, as stated above, some diseases may not leave a biological signature in bones or teeth that survive on an archaeological time scale (the biomolecular paradox). For example, Wilbur et al. (2009) were unable to detect ancient DNA derived from venereal syphilis in a study of 46 bones from the 9th to the 19th century. This was mirror by Barnes and Thomas (2006) and von Hunnius et al. (2007). Gilbert et al. (2004) also reported that no DNA fragments of *Yersinia pestis* could be isolated in a study of 61 individuals which, the authors argue, makes it difficult to interpret previous studies of apparently ancient *Yersinia pestis* DNA (Drancourt et al. 1998; Drancourt and Raoult 2002).

2.4 Ancient Proteins and Ancient Proteomics

Whilst ancient DNA has become well established as a discipline research involving ancient proteins has lagged behind, despite having a much longer history as well as the widespread application of ancient proteins to radiocarbon dating and stable isotope analysis (Cappellini, Collins, and Gilbert 2014). Proteins are the product of the genetic code and play an integral role in cellular and biological functions, as well as comprising the major components of cellular and extracellular structures. They perform an enormous variety of functions, ranging from intracellular processes, transport, and metabolism, to structural support and immunity. In archaeology, studies of ancient proteins have been used to answer different questions about the past, and in particular, investigations of disease and diet. The following sections will introduce proteins (2.4.1) and will outline the development of ancient proteins as a discipline (2.4.2). Subsequently, the methods by which ancient proteins are studied will be outlined (2.4.3). Following this, archaeological applications of ancient proteins will be outlined (2.4.4), and their application to understanding ancient disease will be reviewed (2.4.5).

2.4.1 Introduction to Proteins and the Proteome

Proteins are long chain organic polymers made up of individual units of amino acids. Each amino acid has a characteristic structure, containing a central carbon atom (C), a hydrogen (H), a carboxyl group (COOH), an amino group (NH_2) and a variable side chain (R), where the variation in this side chain gives each amino acid characteristics (e.g. acidity, hydrophobicity) (Fig. 2.2). Amino acids are linked by peptide bonds, which form by the reaction of a carboxyl group and an amino group of a second molecule. By this reaction (condensation), long chains of amino acids are formed, with a carboxyl group (the C-terminus) and amino group (the N-terminus) at each end of the peptide.

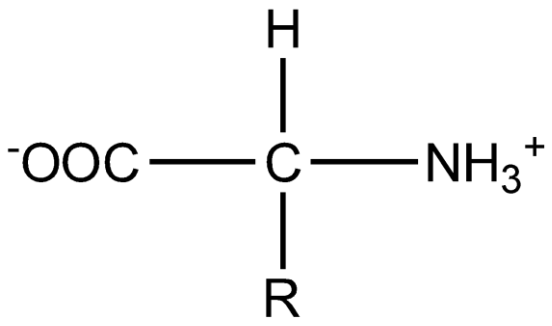

Figure 2.2. Essential structure of an amino acid.

Amino acid sequences are encoded by DNA. The genetic code consists of triplet nucleotide codes (codons), where each is an instruction for a specific amino acid or an instruction to stop. First, during *transcription*, the DNA double helix is unwound, and the sequence copied by RNA polymerase to produce an RNA sequence (messenger RNA), which is identical to DNA apart from the replacement of T (thymine) with U (uracil). Secondly, in a process called *translation*, the mRNA sequence is 'read' by the ribosome to produce the chain of amino acids. Adaptor molecules called transfer RNA (tRNA) are each linked to their own amino acid, and these form a bridge between the codon of the mRNA strand and the amino acid. This process builds up a chain of amino acids as defined by each codon.

After the initial synthesis, peptide chains may be chemically modified to give them their necessary biological functions, termed post-translational modifications (PTMs). However, post-translational modifications, such as oxidation, hydrolysis, deamidation and elimination, also result from external chemical, physical or biological processes acting on proteins in archaeological contexts. Such PTMs can be detected and used to indicate authentically old proteins. Deamidation, the removal of an amino group as ammonium, is one of the most common post-translational modifications in proteins and is one of the most rapid on the geological timescale (Bada and Miller 1968). Asparagine and glutamine are the amino acids most commonly affected, but the rate of deamidation of asparagine can be up to ten times

faster than for glutamine (Daniel, Dines, and Petach 1996; Terwilliger and Clarke 1981). The deamidation of glutamine can be used as an indicator of collagen degradation in bone (van Doorn et al. 2012; Wilson et al. 2012) and keratin degradation in textiles (Solazzo et al. 2014). More recently, hydroxylysine glucosylgalactosylation was detected in collagen extracted from 120,000 year old extinct Bison (Hill et al. 2015). It may be possible that other oxidative processes, such as the oxidation of cysteine, methionine and tryptophan, may also be indicative of age-related protein degradation.

Proteins are 3D molecules and exist in characteristic shapes which enable their function. Proteins can be thought of in four levels of structure. A chain of amino acids (the primary structure) folds into different conformations (the secondary structure) where the two main types are α-helices and β-sheet, which are stabilised by hydrogen bonds between chains. The tertiary structure is the folded protein in its 3D shape, where this folding is facilitated by interactions between the variable side chains (R - Fig. 2.2). Proteins may be composed of several polypeptide subunit structures (the quaternary structure). For example haemoglobin, the protein responsible for the movement of oxygen in blood, contains four globin proteins to make up its quaternary structure.

Just as the genome refers to a complete set of genes, the proteome, a term first coined by Wilkins et al. (1996), refers to the complete set of proteins expressed in a cell or biological system. Studying this proteome enables an understanding of cellular roles, functions or structures in a way that would not be possible using DNA alone. For example, proteins may vary in nature and abundance at different stages in disease progression. In archaeology this term has been largely adopted, perhaps misleadingly, to refer to methods of protein analysis by mass spectrometry. As a higher order to this, metaproteomics is a developing field exploring a community level proteome, i.e. proteins expressed from multiple organisms (e.g. from

microbial ecosystems). This field is burgeoning owing to the growing interest in the microbiome and the development of "omic" approaches (namely genomics, transcriptomics and proteomics) in characterising activities of microbial communities *in-situ* (Ram et al. 2005; Wilmes and Bond 2006; Verberkmoes et al. 2009; Xiong et al. 2015).

2.4.2 The Development of Ancient Proteins

Investigations of ancient proteins began with the identification of individual amino acids (Abelson 1954). Given that the composition of amino acids can be characteristic of particular proteins, this approach may still be used as a simple method for determining biological sources, for example, in detecting collagen (Palmqvist et al. 2003) and identifying the species of shell beads (Demarchi et al. 2014). The chirality of amino acids is exploited as a dating mechanism, amino acid racemization (Demarchi and Collins 2014).

Beyond identifying amino acid compositions, proteins can be detected using antibody based approaches. Antibodies (which are proteins in themselves) bind to a small, accessible region (an epitope) of a target protein (an antigen). Antibody-antigen binding is highly specific, so the positive binding of an antibody can be used to positively identify the presence of a particular protein (acting as the antigen).

Whilst antibodies are used widely across many fields, including medicine and the food industry, the approach is often limited when the protein has been denatured (the loss of higher order protein structures) and degraded. In archaeological contexts there are a myriad of physical, chemical and biological processes acting upon the protein, making the survival of epitopes highly variable. In addition, given the presence of other biomolecules present in archaeological contexts, cross-reactivity can generate false positive identifications. Thus,

although this approach has been used in a variety of archaeological applications (reviewed in Malainey 2011), it remains controversial (Cattaneo et al. 1992; Lowenstein 1981; Loy and Hardy 1992; Tuross, Barnes, and Potts 1996).

2.4.3 Protein Mass Spectrometry

First applied to ancient samples by Ostrom et al. (2000), mass spectrometry enables the identification of amino acid sequences, leading to a vast broadening of the scope and potential of ancient proteins (Cappellini, Collins, and Gilbert 2014). Mass spectrometry is based on the principle that molecules of different mass can be detected owing to differences in the behaviour of ions in electric and magnetic fields. Mass spectrometry based protein identification is an extremely diverse field, reviewed in Phizicky et al. (2003), Tyers and Mann (2003), Zhu, Bilgin and Snyder (2003), Bensimon, Heck and Aebersold (2012) and Bantscheff et al. (2012).

A typical shotgun proteomics workflow is outlined in Steen and Mann (2004, Figure 1). Broadly, this involves extraction of proteins, fragmentation into shorter peptide fragments, peptide separation, analysis and detection via mass spectrometry and subsequent downstream data analysis. Extraction of proteins from archaeological samples is largely dependent on the nature of the source. Typically, this extraction involves some kind of demineralisation, liberating the proteins from a mineral phase.

Prior to mass spectrometric analysis, protein or peptide separation reduces complexity prior to analysis via mass spectrometry. 1D and 2D electrophoretic gels have conventionally been used to separate protein mixtures, which separate whole proteins based on their electrophoretic mobility (predominantly dependent on protein size). Aside from the gels of Schmidt-Schultz

and Schultz (2004; 2015), separation of ancient protein mixtures have been challenging, owing to insufficient protein yield to enable precipitation and smearing due to protein fragmentation (Dobberstein et al. 2009). In more recent proteomics approaches extracted proteins are broken up with a protease (typically trypsin) into peptides, prior to the separation of these peptides using chromatography (Niessen 2006). Given the fast and high-resolution spectrometers now available, separation of peptides via liquid chromatography is often integrated with mass spectrometry instrument workflows (Gevaert and Vandekerckhove 2011).

Analysis of peptides involves three fundamental components; an ion source, a mass analyser and a detector. Firstly, separated peptides are ionised by a number of different methods, such as Matrix Assisted Laser Desorption Ionization (MALDI) or Electrospray Ionization (ESI), which enables the mobilisation of peptides into the mass analyser. The charged fragments can then be separated by size and charge (the mass to charge ratio, *m/z*). Different instruments use a variety of strategies to analyse these ions, which vary in their accuracy, speed, resolution and sensitivity (Forner, Foster, and Toppo 2007). These include measurements based on the behaviour of the ion after acceleration (Time-of-Flight, TOF), by separating ions as they flux in a field comprised of four electromagnetic rods (quadrupole), or separation by electrostatically trapping the ions in an orbit around a central, spindle shaped electrode (an Orbitrap). These masses are detected as spectra, with each peak corresponding to one *m/z*.

The resulting distribution of peptide masses (termed 'peptide mass fingerprint', PMF) is compared to a reference library of known spectra (Henzel, Watanabe, and Stults 2003). This distinctive distribution will be different between organisms because differences in the amino acid sequence of a peptide result in a change in mass. The method is of limited value in complex mixtures of proteins as numerous peptides may have similar masses. However, for single peptides this represents an inexpensive and rapid method for protein identification. For

example, this approach is utilised in ZooMS, Zooarchaeology by Mass Spectrometry (Buckley et al. 2009), where peptide mass fingerprints are used to distinguish the species origin of extracted collagen from archaeological bone.

In order to access peptide sequence information and for the analysis of complex mixtures of proteins, it is necessary to further fragment and analyse these ions in an additional mass analyser, an approach termed tandem mass spectrometry (MS/MS). Individual peptides identified by the first mass spectrometer (MS1) are further fragmented and analysed in a second mass spectrometer (MS2). This secondary fragmentation can be achieved by a number of approaches, for example by collision induced dissociation (CID) or electron-transfer dissociation (ETD). The second fragmentation generates a series of masses that reveal the sequence of the ionised peptide. Figure 2.3 demonstrates how the sequence of amino acids is derived from this approach, where the y-ion series is generated when the charge resides on the carboxyl-terminus, and the b-ion series is generated when the charge resides on the amino-terminus.

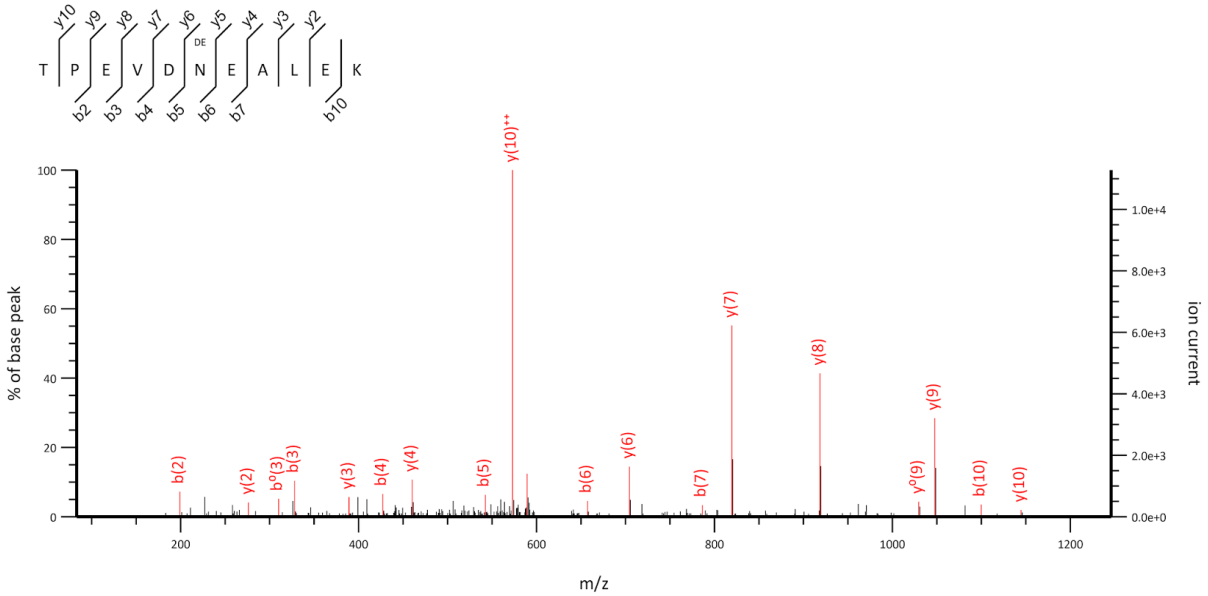

Figure 2.3. An example of how an MS/MS spectrum generates a peptide sequence. Dissociation leads to two fragments, one of which retains the charge. If this charge is retained

on the N-terminal fragment the ion is classified as the *a, b or c* ion-series. If the charge is retained on the C-terminus the ion is classified as the *x, y or z* ion series. A b-ion and y-ion series is shown here as an example.

This increased complexity clearly requires more advanced computational approaches, reviewed in Cottrell (2011). A database matching approach enables spectra generated via tandem mass spectrometry to be interpreted into peptide sequence information. This involves matching experimentally generated spectra against spectra which are theoretically derived *in silico* from protein sequences uploaded to sequence databases (Eng, McCormack, and Yates 1994). The basic procedure for this approach is as follows;

1. Protein sequences (typically translated from genomic data) are 'digested' *in silico* into peptides based on the known cleavage patterns of protease enzymes.
2. All *in silico* peptides with masses identical (within defined error) to the precursor ion are included in the approach. Additional peptides can be considered, for example those containing post-translational modifications, if the combined mass matches the mass of the precursor ion. However the larger the number of possible *in silico* peptides which may match the precursor ion, the slower the downstream bioinformatics search and the greater the chance of false-positive matches.
3. For every *in silico* peptide selected all possible dissociation ions are generated.
4. Masses generated via MS/MS are compared to masses generated from these digested *in silico* peptides and ranked according to the correspondence between observed and predicted masses.

Identifications are expressed as a probability of the match but this probability is strongly influenced by the size of the database and the quality of the spectra. Despite the large size of

databases, they only contain a very small fraction of proteins that exist from all forms of life. In addition, these databases may have a bias towards pathogenic or commercially relevant species. A number of computational programs exist to facilitate this database-matching approach, such as SEQUEST (Eng, McCormack, and Yates 1994), and Mascot (Perkins et al. 1999).

2.4.4 Applications of Ancient Proteins

Studies of archaeological proteins are beginning to be used for addressing different questions about the past, including investigations into ancient disease, diet and subsistence patterns, identification of species and the species phylogeny of extinct animals (recently review in Cappellini, Collins and Gilbert (2014) and Service (2015). Ancient proteins can be isolated from a range of archaeological samples, for example human and animal bones (Buckley et al. 2009; Richter et al. 2011; Buckley et al. 2010), mummified tissue (Corthals et al. 2012; Maixner et al. 2013), hair (Kempson et al. 2003) teeth (Buckley and Kansa 2011; Warinner, Rodrigues, et al. 2014; Tran et al. 2011), potsherds and ceramic vessels (Solazzo et al. 2008), wool (Solazzo et al. 2013) antler (von Holstein et al. 2014), eggshell (Stewart et al. 2014; Stewart et al. 2013) and soil derived archaeological house floors (Oonk, Cappellini, and Collins 2012).

The use of mass spectroscopy has opened up new avenues in archaeological research that were not possible using immunological methods. For studies of the distant past and the Quaternary period (Nielsen-Marsh et al. 2002; Welker, Soressi, et al. 2015; Cappellini et al. 2012; Orlando et al. 2013) collagen may provide a method of species identification and phylogenetic reconstruction, as it has a longer half-life than ancient DNA (Smith et al. 2003). For example,

collagen sequences were recently used to phylogenetically assign extinct South American megafauna (Welker, Collins, et al. 2015).

Proteomics has been used for identifying species from collagen extracted from bone fragments (Buckley et al. 2009; Buckley and Kansa 2011; Richter et al. 2011; Buckley et al. 2014). For example, the ability of a PMF approach to distinguish between sheep and goat bones (which are morphologically identical) (Buckley et al. 2010) enabled an assessment of husbandry practices in Neolithic Greece (Vaiglova et al. 2014). Similarly, proteomics has been used to identify the animal origin of textiles (Solazzo et al. 2011), Scandinavian antler combs (von Holstein et al. 2014) as well as clothing (Hollemeyer et al. 2008), skin (Brandt et al. 2014), and parchments (Kirby et al. 2013).

Proteins have also been isolated from ceramic vessels, which help to identify aspects of ancient diet and patterns of consumption. Immunological methods were used by Craig et al. (2000) to identify bovine α-caesin, a biomarker of milk, from vessels of the Iron Age. Using mass spectrometry, evidence of milk was isolated from Chinese vessels (Hong et al. 2012), from a food residue in a vessel dating to the Early Bronze Age (Buckley, Melton, and Montgomery 2013) and in food residues associated with mummified remains in China (Yang et al. 2014). Proteins were used to identify the consumption of marine mammals in Alaska from 1200-1400 AD (Solazzo et al. 2008) and have also been applied to identifying botanical remains (Cappellini et al. 2010).

Proteins undergo post-translational modifications both after synthesis in the cell in order to form particular biological functions and as a result of degradation by a number of mechanisms. These modifications appear as shifts in peptide masses and can be used to assess the level or nature of protein degradation, giving insight into the diagenesis of archaeological

proteins (Cleland, Schroeter, and Schweitzer 2015; Cappellini et al. 2012; van Doorn et al. 2012).

2.4.5 Ancient Proteins and Disease

Proteomics of anceint proteins has also been used in the study of ancient disease. However, given that the field is relatively recent, immunological methods are dominant with only a few more recent studies adopting proteomic methods. Immunological methods have been used to identify a range of different pathogens, including *Plasmodium falciparum*, the causative agent of malaria, in an Early Dynastic Period Egyptian mummy (Bianucci et al. 2008) and mummified remains from 16th century Italy (Fornaciari et al. 2010), *Yersinia pestis* (Bianucci et al. 2009), schistosoma infection (Miller et al. 1992), *Taenia solium* (pig tapeworm) in a Ptolemaic period mummy (Bruschi et al. 2006) and *Treponema pallidum* in skeletal remains from Easter Island (Kolman et al. 1999). Detection of the smallpox virus has also been reported (Fornaciari and Marchetti 1986).

More recently, methods employing mass spectrometry have been applied. Boros-Major et al. (2011) reported the identification of *Mycobacterium* spp. proteins in bone samples from skeletons 8th to the 16th century. Using gel separation and mass spectrometry by matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI TOF) peptide mass fingerprinting, and MALDI TOF/TOF tandem mass spectrometry proteins derived uniquely from *Mycobacterium tuberculosis* were identified. A similar approach was also taken by Hajdu et al. (2012). However, analysis of their results may indicate contamination from collagens and that the use of peptide mass fingerprinting may not be useful for the detection of specific pathogenic proteins (explored in Chapter 6). Schmidt-Schultz and Schultz (2004)

reported the detection of circulating antibodies (immunoglobulin G), extracted from human bone. Variation in the abundance of this protein led the authors to interpret this as a secondary immunodeficiency as result of scurvy in the individual with the lowest antibody count. However, caution should be noted with this approach and interpretation given a) the exceptionally clean SDS-PAGE gels presented in this work are highly unusual in ancient material, and b) the preservation of noncollagenous proteins in bone is not well understood.

More recently, proteomic analysis was applied to buccal swabs and cloth samples from two 500 year old Andean mummies (Corthals et al. 2012). This study detected proteins associated with the immune system as well the detection of *Mycobacterium* spp. using ancient DNA, which led to the conclusion that one of these individuals was fighting an active respiratory infection at the time of death. Proteomic analysis was also applied to the preserved brain of Ötzi, an ice-age mummy found in the Italian Alps, where 502 different proteins were identified, including those relating to wound healing (Maixner et al. 2013).

Although it is clearly in its infancy compared with ancient DNA, the application of ancient proteins to palaeopathology may offer different information to ancient DNA analysis and could hold some advantages. Whilst the detection of pathogenic microorganism indicates the presence of that species, the identification of proteins may facilitate that observation of the biological processes that are occurring in the tissue. In the examples above, Corthals et al. (2012) identified a suite of proteins involved in the immune response to lung infection, and Maixner et al. (2013) identified a proteome that may be reflective of wound healing. Another advantage is that proteomic analysis requires no amplification step so it may be less subject to contamination, although precautions to minimise contamination should clearly still be applied. Additionally, proteins may persist for a longer time period in the archaeological record (e.g.

Welker et al. 2015), allowing the application of biomolecular palaeopathology into deeper time periods than may be possible with ancient DNA.

However, despite these theoretical findings, the identification of ancient proteins associated with disease have only been applied to mummified remains as in the case of Corthals et al. (2012) and Maixner et al. (2013), or have adopted methodological approaches which may be problematic (Schmidt-Schultz and Schultz 2004; Boros-Major et al. 2011; Hajdu et al. 2012). In order to have greater applicability as a method for ancient disease characterisation, it is necessary to find a suitable archaeological substrate which is found across the archaeological record and advance methodological approaches.

2.5 Dental Calculus

Dental calculus (mineralised plaque) is an abundant, ubiquitous archaeological resource with high potential for studying ancient disease. Whilst dental calculus has been extensively studied in terms of indentifying microfossils derived from the diet and the environment, the biomolecular potential of this deposit is just beginning to be explored (Adler et al. 2013; Warinner, Rodrigues, et al. 2014; Warinner, Speller, and Collins 2015). The following section will introduce and describe dental calculus (2.5.1), before describing and reviewing its place in the archaeological record (2.5.2). Following this, recent approaches exploring the biomolecular potential of this resource will be discussed (2.5.3).

2.5.1 Introduction to Dental Calculus

Dental calculus, also known tartar, is plaque which has become mineralised on the tooth surface (Jin and Yip 2002). Incidentally, plaque has a significant place in the history of microbiology as the source of the first observed microorganisms, identified by Anton van Leeuwenhoek in 1676, and as the first surface bacterial biofilm to be studied. Plaque is formed by the initial deposition of pellicle, a layer of salivary and gingival proteins. A bacterial biofilm is quickly established by colonising bacteria, which aggregate and develop by binding to the pellicle. These bacteria establish in layers, between a dense, biomolecular extracellular matrix (Fig. 2.4). In terms of oral health, a variety of hypotheses have been proposed for the relationship between oral health and plaque (Loesche 1987; Marsh 2003).

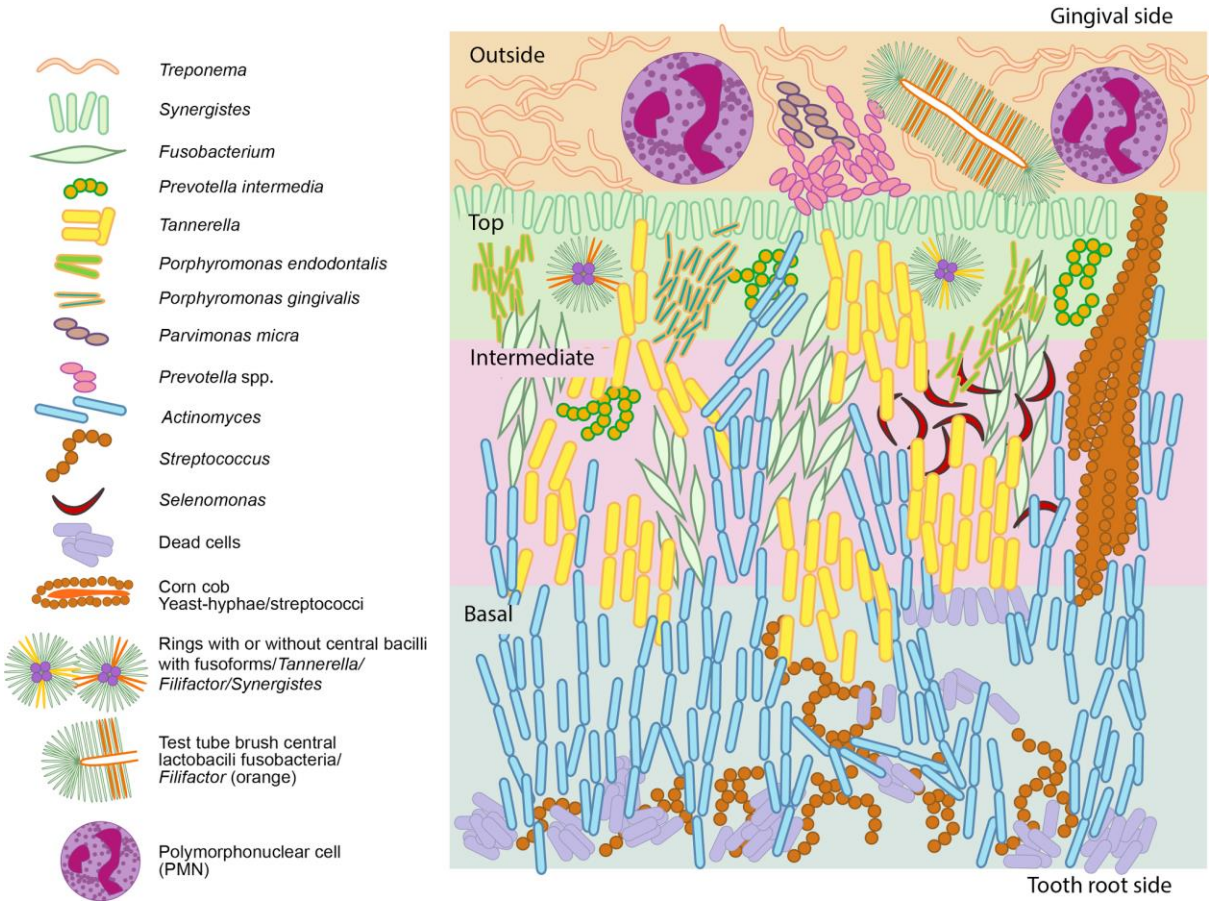

Figure 2.4. Schematic of mature biofilm architecture, indicating key species of the biofilm, from (Jakubovics and Palmer 2013, Figure 4.9)

Plaque becomes dental calculus as a result of mineralisation, as saliva and gingival fluids are supersaturated with calcium and phosphate. This process seems to occur both along bacterial surfaces and within microbial cells. In initial deposits brushite ($CaHPO_4.2H_2O$) is the dominant mineral then, as the calculus matures, hydroxylapatite ($Ca_5(PO_4)_3(OH)$) becomes dominant (Abraham et al. 2005), as well as whitlockite ($Ca_9Mg(PO_4)_6(HPO_4)$) and octacalcium phosphate ($Ca_8H_2(PO_4)_6{\cdot}5H_2O$).

Dental calculus can occur in two forms, subgingival and supragingival (Hillson 2005). Supragingival calculus, which forms above the gum line, is associated with both healthy and periodontitic teeth, whilst subgingival calculus, which forms on the surface of the tooth root as the gum line recedes, is associated with periodontitis. Both of these forms also differ in their crystalline phases (Roberts-Harry and Clerehugh 2000). The rate of formation of dental calculus is not yet well understood, but plaque can mineralise within a few days (Lieverse 1999). Several factors are implicated in this rate of growth, including the species composition of the biofilm, pH, oral fluids, the concentrations of phosphorus and calcium, as well as the presence of silicon (Jin and Yip 2002).

Owing to continued biofilm development, dental calculus grows as a deposit through a cycle of mineralisation and plaque deposition. During this mineralisation process, food debris and other particles which are inhaled or consumed also become entrapped. A deposit typically contains a 15-20% dry weight organic component, made up of amino acids, peptides, glycoproteins, carbohydrates and lipids (Jin and Yip 2002). Thus, dental calculus is an ectopic deposit made of a mix of organic food remains, oral bacteria and other biomolecules, trapped within a stable, densely mineralised matrix.

2.5.2 Dental Calculus as an Archaeological Resource

As it forms, dental calculus preserves microorganisms *in-situ* and traps debris which enters the mouth. Since dental calculus is a mineralised deposit this material survives well archaeologically, making it abundant across past populations and through time. Beginning with Armitage (1975), who observed phytoliths in ungulate dental calculus, dental calculus has been studied extensively microscopically, revealing a range of micro-debris. Most commonly, these include microfossils derived from plant remains, including phytoliths and starches (Henry and Piperno 2008; Hardy et al. 2009; Henry, Brooks, and Piperno 2011; Hardy et al. 2012; Dudgeon and Tromp 2012; Power et al. 2014; Tromp and Dudgeon 2015; Leonard et al. 2015) and other plant fibres (Blatt et al. 2011). Fungi have also been detected (Power et al. 2015). These remains of plants can be diagnostic of consumed foods, and in the case of starch granules, may reveal food preparation processes in the taphonomic alteration to starch granules (Henry, Hudson, and Piperno (2009), but see Collins and Copeland (2011)).

Using scanning electron microscopy (SEM), bacterial morphologies were observed in ancient dental calculus, leading to the idea that in archaeological deposits this substance could represent a novel source of information on ancient oral ecology (Friskopp and Hammarström 1980; Dobney and Ervynck 1986; Dobney and Brothwell 1988; Pap et al. 1995; Arensburg 1996; Meller et al. 2009). Linossier, Gajardo and Olavarria (1996) developed this SEM-based approach by analysing bacterial forms in calculus from different population groups, observing that a greater diversity in bacterial forms were recovered in individuals from an agricultural population than from hunter-gatherers. Bacteria were also observed in dental calculus using Gram-staining, indicating the presence of preserved bacterial cell walls (Charlier et al. 2010; Warinner, Rodrigues, et al. 2014). Dental calculus has also been used as a substrate for carbon and nitrogen stable isotope analysis of dietary composition (shuler and Poulson 2012; Poulson

et al. 2013), but this approach is dubious as bulk isotopic values will most likely derive from a microbial biofilm and not be reflective of the diet of the host (Salazar-García et al. 2014).

2.5.3 Biomolecular Applications of Dental Calculus

Given the already established value of dental calculus as an archaeological resource and the clear potential to study ancient oral microbiology, biomolecules trapped within dental calculus were subsequently targeted as techniques became available. These developments have recently been reviewed in Warinner, Speller and Collins (2015), Warinner et al. (2015), Weyrich, Dobney and Cooper (2015) and Huynh et al. (2015).

Preus et al. (2011) used gold-labelling to observe that DNA was preserved within calcified bacterial cell walls and noted the potential of dental calculus as a reservoir for oral and transient pathogens entering the body through the oral cavity. Adler et al. (2013) used a targeted 16S rRNA approach to identify bacteria in the dental calculus of 34 individuals from the Mesolithic through to the present day. They reported a decrease in oral microbiome diversity over time and the creation of what they term a more "disease-associated configuration" (2013, pg. 450). They also found an increase in *Porphyromonas gingivalis* from the Mesolithic to the Neolithic and an increase in *Streptococcus mutans* through time, which they attribute to the rise in carbohydrate consumption. However, it should be noted that in assessing bacterial diversity through time Adler et al. (2013) chose to exclude Gram-negative bacteria which may be biasing any observations of changing microbial diversity. In addition, as a result of ongoing methodological developments significant biases in this 16S targeted approach have been observed (Appendix 3). De La Fuente, Flores, and Moraga

(2013) developed and applied PCR primers to isolate five species of oral bacteria *(Actinomyces naeslundii, Fusobacterium nucleatum, Porphyromonas gingivalis, Streptococcus gordonii* and *Streptococcus mutans)* in samples of ancient dental calculus from 4520 - 500 BP from South America. They observed amplicons of at least one bacterium in 16 of 38 samples and note the higher abundance of *F. nucleatum* than other bacterial species.

Following these developments, Warinner, Rodrigues, et al. (2014) applied a suite of methods for exploring the biomolecular potential of dental calculus as a reservoir for disease and dietary information. This study applied DNA analysis via a targeted 16S approach as well as shotgun sequencing approach, proteomic analysis, microfossil analysis, and Raman spectroscopy to four samples of dental calculus from individuals from Medieval Germany. This study found that dental calculus yielded proteins and DNA from a suite of commensal and pathogenic bacteria from the oral and respiratory environment and detected DNA derived from dietary sources. This study also found that biomolecules were exceptionally well preserved and confirmed that dental calculus appears to be a relatively diagenetically stable reservoir of ancient biomolecules.

2.5.4 Potential of Dental Calculus

Dental calculus has potential for identifying aspects of ancient disease and diet, through accessing pathogenic bacteria, and ancient oral microbial communities, as well as species-specific DNA derived from consumed foodstuffs. Until these studies, exploring the interactions of microbes and their hosts has typically required exceptionally well preserved samples such as mummified remains. In contrast, dental calculus survives well on skeletal

material owing to its mineralisation, and is found on archaeological teeth worldwide throughout time.

Dental calculus has the potential to act as a reservoir for ancient pathogens, given both the observations in the studies highlighted above and the documentation of numerous transient pathogenic bacteria in the oral cavity. Diseases caused by such pathogens include tuberculosis (Eguchi et al. 2003; Wood et al. 2015), respiratory pathogens (Russell et al. 1999), leprosy (Martinez et al. 2011), as well as a number of viral pathogens (Slots and Slots 2011). In addition, dental calculus has the potential to act as a reservoir for understanding the ancient oral microbiome. Given that the oral microbiome is increasingly recognised as integral to human health (Wade 2013a), this represents an accessible resource to study the composition and change in these microbial communities through time.

In the United Kingdom, plaque and dental calculus are not included in the Human Tissues Act (2004) because they are ectopic deposits, which may have implications in terms of handling this material, in contrast to other human remains. In addition, it offers a method for studying diseases without needing access to the entire skeleton, allowing at least some information to be gained from isolated crania.

With the exception of Boros-Major et al. (2011) and Hajdu et al. (2012), to my knowledge, proteomics has not been applied to skeletal material in order to investigate ancient pathogens and disease. Given the potential of ancient proteomics as a method for uncovering palaeopathological data, the technological developments in tandem mass spectrometry, as well as the clear demonstration that dental calculus contains well preserved ancient biomolecules, there is clearly scope for a marriage of these approaches.

Chapter 3: Historical Background and Introduction to Case Study

This chapter will introduce the historical context for the main case study of this thesis, the biomolecular analysis of dental calculus from formerly enslaved individuals buried on the South Atlantic island of St Helena. This chapter will begin by briefly outlining the period of transatlantic slavery, with a focus on the history of slavery in the 19th century and its abolition (3.1). More specifically, it will describe the history of abolition in terms of its European history to comprehensively show the role of St Helena within this context. Following this, the history of St Helena and the archaeological excavations of the "Liberated African Establishment" will be introduced (3.2). Subsequently, how disease during the era of transatlantic slavery has been studied will be outlined through a review of historical sources as well as osteological approaches (3.3). Based on these approaches, rates of mortality (3.4) and the diseases present during the era of the transatlantic slave trade will be discussed in detail (3.5). The chapter will conclude with a discussion of the diseases afflicting "liberated Africans" at St Helena as documented by the analysis of both osteological indicators and historical records (3.6).

3.1 Introduction to the Transatlantic Slave Trade

In contrast to any system of slavery before it, the transatlantic slave trade transformed the social, political and economic character of whole continents and continues to have an enormous legacy across the Atlantic world. The transatlantic slave trade has its origins in the European explorations of the African continent, in particular, with Portuguese explorations in

West Africa in the 15th century. The exploitation of African people as enslaved workers began with the raiding and capture of African people to bring back to Portugal, and gradually the process of enslaving African people became integrated with Africa's existing systems of slavery. Although slavery was present before European contact, the presence of European traders dramatically changed the nature of the establishment, creating unprecedented demand for labour, a complex web of trade and contributing to the growth and flux of large, centralised African states (Thomas 1997; Walvin 2001; Rawley and Behrendt 2005; Walvin 2007).

In general, the transatlantic slave trade operated as a three-sided trading system. African people were captured by European-owned slave ships and transported across the Atlantic to the Americas, most often to work as plantation labourers (predominantly in sugar production), from which goods were transported back to the European continent (Thomas 1997). However, the web of this trade stretched beyond the Atlantic, with goods produced by enslaved people having a global presence, especially throughout the expanding British Empire (Walvin 2001). By the British abolition of the transatlantic slave trade in 1807 Britain alone had captured and transported 3.25 million African people to the Americas. In total, it is estimated that 12 million people were loaded onto slaving ships intended for the Americas, with approximately 10 million people surviving this journey (Walvin 2014). For Britain, an industry based on the exploitation of African people for labour became a cornerstone of the 17th and 18th century economy (Eltis 1987).

3.1.1 The Transatlantic Slave Trade in the 19th Century and its Abolition

At the end of the 18th century, the drive for the abolition of the transatlantic slave trade was gaining pace in Europe and the Americas. In the last three decades of the 18th century, in the

wake of the American War of Independence, increasing attention was paid to the morality of slavery. For example, the Somerset case of 1771-2, which determined that a British enslaved person could not be forcibly removed from the country against their will, spearheaded the abolitionist cause in the law courts (Hinks, McKivigan, and Williams 2007). In addition, the case of the *Zong*, where 133 enslaved African people were thrown overboard, resulting in the owner claiming insurance on his loss of "property", was one example of where gruesome particulars of the slave trade brought the abolitionist cause into public notice (Walvin 2011). In addition, the growing number of rebellions of enslaved Africans, which were often brutally suppressed, had a profound impact on abolition (Matthews 2006).

The strength of the parliamentary and public campaign in Britain, although it did not result in abolition in the 18th century, did result in legislative changes involving the regulation of the trade. For example, the Dolben's Act (1788) limited the number of people which could be boarded onto slave ships, required the presence of a ship doctor and required all causes of death to be recorded. Throughout the end of the 18th century and into the 19th century, public anti-slavery sentiment grew. For example, 519 petitions to parliament were submitted in 1792 alone (Turley 2004). In 1807, with a new parliament, the trade of enslaved African people became illegal in Britain with the passing of '*An Act for the Abolition of the Slave Trade'.*

Although the trading in enslaved people was abolished by this act, the institution of slavery in British colonies continued. On August 1st 1834 all children under six years old were freed and individuals older than six years old became apprentices, working up to six years with no pay. Enslaved people received no compensation, although slave-owners were granted over £20 million (£1.6 billion in today's money), representing approximately 40% of government expenditure that year (Draper 2009; Hall et al. 2014).

In 1838 slavery was abolished in British colonies and approximately 750,000 people were emancipated (Walvin 2002). However, there were enormous political, economic and social consequences of enslavement and "liberation", which contributed to the downward economic trajectory in these former colonies. Work was often irregular, with very low wages. With formerly enslaved people choosing different forms of employment, plantation owners no longer gained the labour they needed to be profitable (Thomas 1997). An indentured Indian labour scheme, officially endorsed until the First World War, saw half a million Indian people come to work in the British Caribbean as contract labourers (Northrup 1995). In addition, racist ideas of white superiority, used to justify the slave trade when it was active, were extremely prevalent and resulted in, for example, the exclusion of the black majority in the political landscape (McGlynn and Drescher 1992).

Despite the widespread abolition of the transatlantic slave during the 19th century, millions of people were still captured and enslaved during this century (Emory University 2008). In particular, the Brazilian and Cuban trades flourished during the early to mid-19th century. In the Portuguese governed areas in West Central Africa the transatlantic slave trade may have actually increased in response to the fear that the Royal Navy was going to end Portuguese trading. During this period, increasing proportions of captured enslaved people were children, possibly because this enabled as many people as possible to be placed on slave ships (Lovejoy 2006). Between 1811 and 1867, more than 41% of enslaved people boarded onto ships were children and in the Angolan trade this number was as high as 59% (Miller 1997).

3.1.2 The West-African Squadron

Following 1807, Britain established the anti-slavery West African Squadron as part of the Royal Navy, initially to intercept illegal British traders and later to intercept foreign vessels (Lloyd 2012). A number of bilateral treaties were established in order to facilitate this campaign. For example, in 1815 Portugal agreed to cease trading in enslaved people above the equator, an agreement patrolled by the West African Squadron, resulting in the southward movement of major ports to Angola and Congo. In 1839 the *'Slave Trade (Portugal) Act'* enabled British vessels to detain all Portuguese ships, and later all unmarked ships, intended for the trade in enslaved people (Lloyd 2012).

These treaties were enforced through the trial of vessels at Vice-Admiralty Courts (which dealt with enforcement of British law) or at Mixed Commission Courts (which involved both British and non-British judges) stationed around the Atlantic. If a vessel was found guilty of illegal trading, the ship was sold and the people on board "liberated". Between 1820 and 1870 £40 million pounds (between 0.3 - 1% of government expenditure) was spent on suppressing the trade through the West African Squadron and over 1600 ships were captured (Huzzey 2012). It is estimated that this resulted in the "liberation" of approximately 90,000 enslaved Africans between 1836 and 1866 (Huzzey 2012).

3.2 Introduction to St Helena

The history of St Helena, a remote island in the South Atlantic Ocean, is entwined with the history of the last decades of the transatlantic slave trade. Originally occupied by the Portuguese as a station for provisions and recuperation, St Helena came under British control in the 18th and 19th centuries (Pearson et al. 2011). St Helena occupied a strategic maritime

position, lying on ocean currents between the Cape of Good Hope and Cape Verde Islands and provided a useful stopping point for ships sailing along this route (Fig. 3.1).

Figure 3.1. Location of St Helena, showing movements of enslaved Africans (blue arrows) and ocean currents (white arrows), based on Eltis and Richardson (2010).

St Helena became the location of a Vice-Admiralty Court in 1840, following the enactment of the 1839 '*Act for the Suppression of the Slave Trade'*. Ships captured in the vicinity of St Helena were brought to the island for trial and the people on board "liberated" (Pearson et al. 2011). Given that the Portuguese/Brazilian trade had largely moved south of the equator after 1815, St Helena was the location of many adjudications during this time. Between 1845 and

1851 a large number of captured ships arrived at St Helena as a result of the increased pressure of the Royal Navy against the Brazilian slave trade. From 1845 the Aberdeen Act *('Act to carry into execution a Convention between His Majesty and the Emperor of Brazil, for the Regulation and final Abolition of the African Slave Trade')* enabled British Naval ships to capture all Brazilian vessels deemed to be participating in the trade in enslaved Africans (Lloyd 2012).

The number of enslaved Africans coming to the island fluctuated wildly. In a three month period in 1845 – 1846 2110 African people landed in St Helena, but in April and May 1846 the influx was so low that the governor thought to discharge half the establishment's staff (Pearson et al. 2011). After the transatlantic slave trade was abolished in Brazil in 1850, the St Helena establishment was almost disbanded. However, a renewal of the Cuban slave trade owing to the high demand for labour in Cuba's sugar plantations in the late 1850s saw St Helena again play a role in the "liberation" of people captured for this trade. Historical documents indicate that St Helena was usually understaffed and undersupplied (Pearson et al. 2011) and the arrival of slaving vessel, some of which contained 400 – 500 people, put a large strain on the island's resources.

3.2.1 The "Liberated African Establishment"

Throughout out the period 1840 - 1872 a 'Liberated African Establishment' was present on the island, initially at Lemon Valley until 1843 then at Rupert's Valley until the arrival of the last ship in 1865, to treat and house Africans "liberated" from slave ships (Pearson et al. 2011). A similar establishment also operated at the Mixed Commission Court at Sierra Leone (Huzzey 2012). As a result of the horrifying conditions of the transatlantic slave trade and the Middle

Passage (the voyage of enslaved Africans across the Atlantic Ocean), many people did not make it to St Helena alive or died shortly after arrival. Thus, St Helena also contained a large cemetery in Rupert's Valley, in conjunction with the settlement (Pearson et al. 2011).

The vast majority of these individuals did in fact continue to the Americas as indentured labourers in British colonies (Pearson et al. 2011). This second transportation was funded by either private individuals or the "importing" colony. Most African people who passed through St Helena were emigrated to Demerara, Jamaica, Trinidad and British Guiana. Some were also relocated to South Africa and Sierra Leone, and a small number remained on St Helena. In one case the recaptives were placed directly on a ship bound for British colonies in the West Indies without even landing on St Helena. No descendants of "liberated Africans" are present on St Helena today (Pearson et al. 2011).

3.2.2 Archaeological Excavations

In 2007 and 2008, as part of the construction of an airport for the island, excavations conducted by Andrew Pearson and the University of Bristol uncovered part of the cemetery associated with this settlement. A large collection of human remains (325 discrete individuals) were uncovered and have been documented in Witkin (2011). Demographically the population is very young with just under 50% over 18 years old and a large proportion (about one third) under 12 years old. 82% of individuals who had their sex estimated (n=160) were male. Preservation of these remains was variable due to shallow burials and subsequent exposure. Excavated remains were analysed on site and subsequently curated on the island. The human remains were examined again in June/July 2012, where samples of dental calculus were

obtained by Judy Watson (University of Bristol). Dental calculus from 117 individuals was removed from teeth using a dental pick and stored in 2.0mL Eppendorf tubes until analysis.

The skeletal assemblage is unique for several reasons; a) it is one of only a few excavations containing the remains of first generation enslaved Africans (Schroeder et al. 2009; Schroeder, Haviser, and Price 2014; Cook et al. 2015), and b) it is the only excavation, to my knowledge, of individuals who may have died as a result of the Middle Passage. This unique excavation offers insight into the lives of African people who endured the Middle Passage, and presents a rare opportunity to uncover individual life stories on the origins, health and lifeways of enslaved people.

3.3 The Study of Disease in the Transatlantic Slave Trade

This section will discuss how both the analyses of historical records and osteological research have been used to understand the health of enslaved Africans during the era of transatlantic slavery. In this section I have primarily focussed on diseases present during the Middle Passage because individuals buried on St Helena are people recently disembarked from slave ships. Disease and medical care of enslaved Africans in the Americas have been discussed in a number of sources, including Kiple (1984), Bankole (1998), Savitt (2002), McCandless (2011), and Rathbun and Steckel (2002).

In some ways the history of disease during the era of slavery is part and parcel of the history of transatlantic slavery as a whole. Europeans establishing economic enterprises in the Americas believed that European labourers were not suited to the tropical climate (Walvin 2002). Thus, it was the perceived tolerance that African adults showed to tropical diseases that led to the initial demand in African labour. The short life span of enslaved individuals and the

fact that populations of enslaved Africans were, for the most part, not self-sustaining meant that the enslaved population had to be continually replenished. For example, towards the end of the transatlantic slave trade the average life expectancy for an enslaved man in Brazil was 23 years, a figured influenced by high infant mortality (Klein and Vinson 2007). Thus, as the transatlantic slave trade escalated it was this extremely poor health that meant the transatlantic slave trade was maintained for so long.

Studying disease in the era of slavery largely began with efforts to estimate the number of people crossing the Atlantic and to estimate the number of people who died during the Middle Passage. Rates of mortality in the Middle Passage became accessible due to the work of slave trade databasing. Philip Curtin's pioneering work on epidemiology (1968; 1969) was the first of its kind to assess mortality rates during the transatlantic slave trade, where ship's records and financial transactions were used to estimate the number of people crossing the Atlantic, as well as the ports of origin, countries involved and mortality rates in the specifically in the Middle Passage. Since this time historians have sought to find and clarify particular periods of varying mortality and assess the cause of this variation.

According to Sheridan (1985), approaches to understanding disease in the transatlantic slave trade became influenced by ecology movements, which attempted to explore the relationship between different populations, diseases and the environment, as well as understanding the differences in health of European and African descendant populations in the Americas. In-depth research into particular disease afflictions and into the health and nutrition of enslaved Africans was carried out in the 1980s (Kiple 1984; Sheridan 1981; 1985). This research focussed on the spread of diseases across the Atlantic, links to the decimation of Native American populations, and changing disease/pathogen environments (Crosby 1991; Curtin 1993a; Kiple and King 2003). In addition, genetic research into modern pathogens contributed

to understanding how the transatlantic slave trade resulted in the spread of microorganisms, and in understanding differential immune responses of different population groups (Schroeder, Munger, and Powars 1990; Zimmerman et al. 1994; Arauz-Ruiz et al. 1997; Cox 2002; Hume, Lyons, and Day 2003).

3.3.1 Historical Sources

Evidence from primary, historical sources form the bulk of current knowledge on disease during the period of slavery. These sources are numerous, but vary in both their political standpoint (i.e. pro or anti-slavery) as well as in their focus and detail.

Personal narratives from European and African voices are first-hand accounts of experiences in the transatlantic slavery. Many written in the late 18th century were used by the abolitionist movement as powerful evidence of the horrors of the transatlantic slave trade, like the account of John Newton (1788). Narratives of an African voice, for example of Olaudah Equiano (1789), are much rarer given that few enslaved people were literate and even less had the opportunity to write a narrative. These texts offer glimpses into different aspects of the trade: life in West or West Central Africa before capture, the trek to the coast, the Middle Passage and life in the New World. However, conditions of slavery in Africa are largely unknown from these sources, although narratives from West Africa have recently been compiled and translated by Greene (2011).

Ship's surgeon's records offer information on common ailments, causes of death, rates of mortality and treatments. After 1788 with the enactment of the Dolben's Act it was a requirement that all mortality rates and causes of death were recorded on every voyage (Steckel and Jensen 1986). The Act also provided an incentive for surgeons to keep mortality

rates low as they would receive a premium when mortality did not rise above 2 or 3% per voyage (Sheridan 1981). In addition, evidence collected prior to the enactment of the Dolben's Act facilitates the reconstruction of disease and health in the late 18th and 19th century British slave trade. Instruction manuals for surgeons also document diseases present, such as that of Dr T. Aubrey (1729). Other parliamentary enquiries, summarised in Hogg (2014), also reveal details on diseases present during the transatlantic slave trade, and parliamentary enquiries following the passing of the Dolben's Act also used ship's surgeon's records as evidence for entire abolition (e.g. Colonial Office, Great Britain 1789).

Abolitionist publications from the 18th and 19th century further reveal details about diseases afflicting enslaved people. For example, Dr Alexander Falconbridge, a British surgeon and abolitionist published his '*Account of the Slave Trade on the Coast of Africa*' in (1788). In this document he details the hold of the slave ship, and the diseases and suffering experienced by those on board. He also documents how the enslaved are treated and sold after docking in the Americas. Similarly, Dr George McHenry, who was stationed as the surgeon on St Helena between 1840 and 1843, also published a series of pamphlets for the British and Foreign Anti-Slavery Society in 1863. He describes his duties and experiences as a surgeon for Africans on board foreign slaving vessels caught by the Royal Navy (McHenry 1863). In the Americas, plantation owners further documented disease afflicting enslaved people. For example, Thomas Thistlewood, a plantation owner in Jamaica, records diseases he observes and documents efforts to cure these afflictions (Thornton 2011). It is clear that this motivation derives from the desire to create a healthy, economically profitable workforce.

There are biases to using historical evidence in understanding disease in the era of slavery. For example, in Thomas Thistlewood's documentation, a child was not recorded as having been born until they had lived 9 days, which skews estimates of infant mortality rates. In addition,

the cause of death was only typically recorded if that case was clear. Hence accidents, suicides and violence seem to compose a great number of deaths in the records, 24% in the case of Thomas Thistlewood's plantation (Thornton 2011). Although these texts are numerous and often detailed they are often more concerned with a) documenting the loss of enslaved people through death or b) providing evidence of the inhumane conditions of the transatlantic slave for abolitionism. They generally do not indicate specific conditions or aetiologies. Additionally they are limited by the contemporary medical knowledge of the 18th and 19th centuries. Most often written by Europeans, they also have a very strong colonial filter or were inherently racist. For example, the idea that there were humoral differences between people of African and European descent often supported the notion that African people were more suited to the conditions of enslavement (Smith, 2014). For example, Dr Collins, in '*Practical Rules for the Management and Medical Treatment of Negro Slaves in the Sugar Colonies*' believes that the need to sweat was an inherent part of the African body (Collins 1803).

Concerning the establishment at St Helena, records from the Colonial Office document the correspondence between the governor and London, which include details of mortality and health as documented by the medical officers on the island (e.g. Colonial Office, Great Britain 1850). In addition, Vice-Admiralty records for St Helena and Sierra Leone can be found in the Foreign Office archive, details of financial transactions are found in Audit Office archives, and the House of Commons parliamentary papers also contain references to St Helena. In terms of understanding disease in this population, surgeon's reports, which form part of the Governor's report, include details of disease prevalence, rates of mortality and methods of treatment. Other personal accounts also provide some insight, such as that of Dr George McHenry, documented above.

3.3.2 Osteological Investigations

Primary evidence of disease during the era of slavery can also be found through the analysis of the human remains of enslaved populations. With the growth of African-American heritage sites in the 1960s, interest also grew in bioarchaeological analysis of the African diaspora. Blakely (2001) argues that by the 1990s there were two distinct approaches to this field, which came to a head during the excavation of the New York African Burial Ground; a 'biocultural approach', which incorporated cultural and historical data to create a greater understanding of the population in question, or a forensic approach, concerned with individual identifications and biological features of individuals. Blakely argues that the latter approach, which he describes as racialized and ahistorical, is a danger to understanding African diaspora bioarchaeology as the approach is "so reminiscent of the early years of physical anthropology to as to be at best puzzling, at worst repugnant to many African-Americans…" (2001, pg. 415). Shuler (2011) argues that bioarchaeological research in the Caribbean in particular is hampered by poor preservation and sampling bias, but has much to offer in understanding disease during this period. These approaches have the potential to reveal pathogenic exposure, stresses from physical activity and violence, as well as direct evidence of rates of mortality.

Osteological investigations which concern diseases afflicting enslaved individuals and populations are summarised in Table 3.1. It is clear that the majority of sites are located in North America, although the excavated remains from the Newton plantation, Barbados, have also been studied extensively. What is also clear is that evidence of harsh physical labour and poor quality of life is a major finding of almost all of these studies.

Study	Site, period	Major observations
	North America	
Martin, Magennis, and Rose 1987	Cedar Grove, Arkansas, mid-late 19th century	80 individuals: dietary deficiencies and infectious diseases observed
Rathbun 1987	Belleview Plantation, South Carolina, mid-late 19th century	36 individuals: females generally healthier, childhood nutritional deficiencies and anaemia prevalent, evidence of demanding physical labour
Owsley et al. 1987	New Orleans, early 18th-early 19th century	29 individuals: poor oral health, arthritic conditions commonly observed
Angel et al. 1987	First African Baptist Church, Philadelphia, 19th century	75 adults: nutritional deficiencies, and lower age at death of females observed
Kelley and Angel 1987	25 sites in Maryland, Virginia and Carolinas, 17-18th century	Nutritional stresses, occupational stress, lead content of bones
Rathbun and Steckel 2002	Philadelphia and South Carolina, 19th century	Health varies through time more so than between different descendent groups
Blakey and Rankin-Hill 2004	New York African Burial Ground, early 18th - late 18th century	408 individuals: high infant mortality rate, differential African-descent and European-descent mortality profiles
Lambert 2006	Eaton's Estate, north Carolina, mid-late 19th century	17 individuals:evidence of tuberculosis and syphilis
De la Cova 2011	Three anatomical collections, mid-late 19th century	651 skeletons from 3 anatomical collections, African American skeletons had significantly higher tuberculosis and treponematosis than other populations
	Caribbean	
Corruccini et al. 1982; Corruccini et al. 1987; Jacobi et al. 1992; Handler 2006a; Handler 2006b; Shuler 2011; Schroeder, Shuler, and Chenery 2013; Shuler and Schroeder 2013;	Newton plantation, Barbados, 17th-19th century	104 individuals: extensive palaeopathological analysis - congenital syphilis, lead poisoning, possible evidence of alcohol related birth defects
Watters 1994	Harney Site Slave Cemetery, Montserrat, late 18th century	17 individuals: traumas and severe malnutrition
Armstrong and Fleischman 2003	Seville Plantation, Jamaica, 18th century	4 individuals: evidence of labouring, general poor health
South America		

Khudabux 1999	Plantation Waterloo, Suriname, mid-late 19th century	57 individuals: physical strains, nutritional and infectious diseases observed
Okumura 2011	Skeletal material collected in 19th century Suriname, housed at University of Cambridge	8 individuals: poor oral health, pipe facets
Cook et al. 2015	Pretos Novos, Rio de Janeiro, Brazil, late 18th century - early 19th century	32 fragmentary teeth: little evidence of poor oral health, suggestion of the use of chewing sticks
	Africa	
Sealy et al. 1993	Vergelegen, South Africa, 18th century	1 individual: general little evidence of poor health, excluding extensive osteoarthritis
Ledger et al. 2000	Corben St, Capetown, South Africa, late 18th century	29 individuals: osteoarthritis and trauma: evidence of physical labour
Appleby et al. 2012	Le Morne cemetery, Mauritius, 1830s	11 individuals: little evidence of dietary deficiencies or poor health, although health likely to be very poor

Table 3.1. Selected summary of osteological investigations of enslaved Africans.

Two investigations in particular have substantially increased the understanding of the palaeopathology of transatlantic slavery; the excavation of individuals buried in the New York African Burial Ground, and from the Newton plantation cemetery, Barbados.

The New York Burial Ground, excavated between 1991 and 1992, is the largest skeletal assemblage of enslaved individuals to be excavated, containing 408 individuals dating between 1600 and 1794 (Blakey and Rankin-Hill 2004). One of the most striking results of the New York Burial Ground investigation was the discovery that, when combined with colonial census data, English-descendant men and women lived five-times longer than African men and women. Another striking feature of the cemetery was the high infant mortality rate, with 21% of the burials comprising of infants. Like other sites, the palaeopathological study of these remains, as well as the early age at death (22.5 years old) and observed disrupted growth, indicates that infectious diseases, combined with nutritional deficiencies, may have resulted in the poor health of this population (Mack et al. 2004; Null et al. 2004).

In the late 17th century Barbados was the richest of Britain's colonies with an economy based on sugar produced by enslaved people. The Newton plantation was one such sugar plantation. The cemetery associated with this plantation was excavated first in the early 1970s (Handler, Lange, and Riordan 1978) and represents one of the earliest bioarchaeological approaches to understanding the health, nutrition and demography of enslaved people. In this early study only craniodental remains were examined, until subsequent excavations in the late 1990s (Shuler 2011). Jointly, these osteological investigations offer some of the most detailed insight into the osteology and palaeopathology of an enslaved population.

Although the assemblage was co-mingled and poorly preserved, Corruccini et al. (1982) noted widespread periodontal disease, extensive enamel hypoplasia (which is linked to malnourishment or disease during tooth development (Hillson, 2005)), high infant mortality and a life expectancy of 29 years. In further analysis, Corruccini et al. (1987) proposed that the high prevalence of tooth root hypercementosis (89.4%) found at Newton may be linked to trauma, geophagy, periodontal disease or nutritional deficiencies. Handler (1986) used atomic absorption spectroscopy to reveal a high amount of lead in bone which correlated with the age of the individual. This was linked to historically reported symptoms of lead poisoning and is likely to be linked to the use of lead in rum distillation, although it should also be noted that lead can be post-depositionally absorbed from soils into skeletal tissues (Waldron 1983).

From excavations in the late 1990s, analysis of post-cranial remains derived from 46 individuals added further insight to the population (Shuler 2011). Age at death at the Newton plantation was re-evaluated to be just below 20 years old. Like early investigations this analysis revealed a very high infant mortality rate. Females appeared to have a younger age at death, probably due to death during childbirth or additional strains during pregnancy. Skeletal remains revealed that 41% had lesions possibly derived from infectious disease, with adult

males having the highest frequency of lesions. Of individuals with infectious lesions 37% were active at the time of death.

3.4 Rates of Mortality in the Transatlantic Slave Trade

Based on research generated using the approaches discussed in section 3.3, rates of mortality during the era of the transatlantic slave trade will be discussed. Rates of mortality varied heavily throughout the history of transatlantic slavery. In general, over the course of the transatlantic slave trade rates declined due to improvements in ships, which increased the speed of the Atlantic crossing (Cohn 1985; Haines and Shlomowitz 2000). For example, Miller (1997) estimates that mortality on ships dropped from 25-30% in the 18th century to 5 to 10% in the 1820s in the Portuguese trade from Angola.

Rates of mortality also varied at different stages of the trade. What is still largely unknown is the rate of mortality at the time that people were seized within the African continent and during the march to the coast. This lack of understanding is a reflection of the fact that internal routes and systems of slavery within the African continent are not well-understood. Miller (1997) estimates that death rates during the internal trade to the coast may have been between 400-600 per 1,000 people per annum, the result of malnutrition, starvation, disease and psychological suffering. Eltis (1989), echoing 19th century medical officials from St Helena (Colonial Office, Great Britain 1850), noted that keeping enslaved people in extremely unsanitary confinement prior to loading onto the ship was as deadly as being on the ship itself. In addition, the longer the ships were stationed off the West African Coast, the greater were the levels of mortality (Rawley and Behrendt 2005).

During the Middle Passage it appears that the most likely factors influencing the number of deaths of enslaved Africans were a) the African point of origin, b) the season of embarkation and c) the length of the voyage (Steckel and Jensen 1986). In particular, the Bight of Biafra is

one region where rates of mortality are higher than other embarkation points in West Africa (Northrup 1978). Klein et al. (2001) note the higher mortality rates for enslaved people on board ships crossing the Atlantic in comparison to other contemporary transoceanic voyages, and like Northrup (1978) note the variability in mortality rates between different ports of departure.

Conditions of the Middle Passage were highly conducive to the spread of pathogenic microorganisms (Cowley and Mannix 1994; Mustakeem 2008). The enslaved were kept in hot, crowded conditions where infections would have been rife owing to contact with faecal material and a weakened immunity. In particular, this would have been exacerbated if landings or crossings were delayed and when supplies of water and food became critically short. The crew were often, but not always, also affected by the same diseases afflicting the enslaved (Steckel and Jensen 1986; K. Brown 2011). Medical reports in documentation relating to St Helena illustrate these conditions;

> *"four or five hundred negroes enclosed in the limited space of slaver's 'between-decks' are exposed first to the depressing moral influences of fear and anxiety, and secondly to this of an intensely hot and vitiated atmosphere. To every variety of physical inconvenience arising from the pressure, constraint, want of room, is to be added the atmosphere, polluted with animal effluvia and loaded with carbonic acid" (Colonial Office, Great Britain 1850, pg.74).*

During the suppression of the transatlantic slave trade by the Royal Navy the length of time at sea was often extended during pursuits, also resulting in an increase in the number of deaths of people on board (Northrup 1978). One major problem facing the colonial establishment at Sierra Leone was that sailing time from West Central Africa to Sierra Leone was just as long as the sailing time across the Atlantic, owing to sailing against the prevailing current and wind

(Eltis 1989). Mortality of individuals coming to St Helena was also extremely high and is a chief concern of officials on St Helena. In a governor's report from 1850, the governor discusses the worrying high mortality rate (around 32%), but stresses that this is not from negligence or neglect (Colonial Office, Great Britain, 1850).

3.5 Diseases Associated with the Transatlantic Slave Trade

Throughout the transatlantic slave trade enslaved Africans suffered from a range of different diseases. These afflictions include infectious diseases, such as dysentery, smallpox and malaria, and those associated with nutrition, such as scurvy and anaemia. Parasitic diseases, such as hookworm, guinea worm and trypanosomiasis were also prevalent. In addition, enslaved people also died as a result of a system of labour which was inherently violent. In general, these diseases relate to conditions of enslavement, including malnourishment, crowded conditions, changing disease environments and the psychological effects of enslavement.

3.5.1 Infectious Diseases

Dysentery

According to the historical record, dysentery was the biggest killer of enslaved Africans throughout the period of transatlantic slavery (Klein 2010). Dysentery, also termed contemporaneously as the "bloody flux", is the inflammation of the membranes and glands of the large intestines, resulting in cramping, diarrhoea accompanied with blood and mucous discharges and vomiting, leading to death by dehydration. It exists in two forms; a bacillary dysentery caused by infection of *Shigella* bacteria (shigellosis), and amoebic dysentery,

caused by infection by *Entamoeba histolytica* (Brent, Davidson, and Seale 2014). Given the confined nature of a slave ship, surgeons would have struggled to control outbreaks of dysentery, spreading easily from person to person on board.

A slave ship infected with dysentery would have been extremely unpleasant for those on board. Several narratives, including that of Dr Alexander Falconbridge (1788), provide graphic descriptions of the experience.

> *"The deck, that is, the floor of their rooms, was so covered with the blood and mucus which had proceeded from them in consequence of the flux, that it resembled a slaughter-house"* (Falconbridge 1788, quoted in Dow 1927, pg. 146)

As well afflicting individuals on St Helena and those trapped on slave ships, dysentery was also prevalent among New World populations, particularly when enslaved Africans first arrived in on the continent (Campbell 2006). Together with a weakened immune system from malnourishment and overwork, dysentery caused high mortality among the enslaved Africans in the Americas.

Dr Isaac Wilson, a prominent naval surgeon with an anti-slavery position, thought that melancholia was the primary cause of death among the slaves (Sheridan 1981; Thomas 1997). He writes;

> *"The symptoms of melancholy are lowness of spirits and despondency; refusing their proper nourishment still increases these symptoms; at length the stomach gets weak, and incapable of digesting their food: Fluxes and dysenteries ensue; and, from the weak and debilitated state of the patient, it soon carries him off."* (Dr Issac Wilson, quoted in Sheridan 1981, pg. 605)

Yellow and Dengue Fever

Yellow fever is caused by infection of an RNA virus of the *Flavivirus* genus and is spread by a mosquito vector, *Aedes aegypti* (Gill and Beeching 2011). The disease in its initial stages results in a high fever, muscle pains and headaches. After a few days of no symptoms the infection is characterised by internal haemorrhage and black vomit, giving the disease its alternative name, 'vomito negro' (Kiple and Kiple 1977a).

Yellow fever occurred as recurring epidemics when there was a large enough population of non-immune individuals and a great enough number of mosquito vectors to spread infection (McNeill 2010). Like other diseases which originated on the African continent, yellow fever spread to the Americas via the transatlantic slave trade. Bryant et al. (2007) argue the strains currently present in Central and South America emerged once the virus was present in the Americas. Because some of the symptoms are similar to other African fevers the exact date of this introduction is uncertain, although the first confirmed report is in the 1640s (Curtin 1993b). Epidemics in port cities were common and the timing of epidemics may be linked to sugar production and shipments, as the mosquito can live on sucrose (Goodyear 1978). Curtin (1989) argues that the prevalence of yellow fever among European populations in the tropical Atlantic affected British political strategies in the Caribbean and Africa.

The nature of immunity of people of African descendent to the yellow fever virus is matter of contention. Kiple and Kiple (1977a) have argued that the African inhabitants of West Africa possessed an innate genetic immunity to this disease. This approach has been criticised as racially deterministic, for example, by Watts (2001), although see response by Kiple (2001). Watts (2001) also argues that such a view of genetically inherited immunity may hamper efforts for this disease's eradication in Africa, as yellow fever epidemics still occur in

contemporary tropical Africa. It may be the case that children acquire immunity when exposed to the virus at a young age (Taylor 2014).

Dengue fever, infection by the Dengue fever virus, is another RNA virus of the *Flavivirus* genus also transmitted by the *Aedes* mosquito. Epidemics of dengue were reported in port cities in the 18th to 20th centuries (Gubler 2006), and despite an *A. aegypti* eradication scheme in the 1950s and 1960s, dengue fever continues to be a global pandemic (Lounibos 2002).

Smallpox

Smallpox, an infection by the *Variola* virus, devastated Old and New World populations throughout the post-medieval period (Glynn and Glynn 2005; Hopkins 2002; Robertson 2001). The disease is caused by one of two viral species, *Variola major* and *Variola minor*, with the former being the more aggressive. The disease is highly virulent, is spread by person-to-person contact with no intermediate vector, and results in vomiting, fever and characteristic eruption of pustules over the body. It is uncertain where smallpox originated, although it was thought to be endemic in West Africa before European contact, possibly spread by Arab traders (Glynn and Glynn 2005). One infection by either species of *Variola* causes either death or life-long immunity.

The outbreak of a smallpox epidemic on board a slave ship could decimate both the enslaved and the crew. Smallpox epidemics in Brazil have been noted to correspond to peaks in imports of enslaved people, which in turn have been related to periods of drought in Africa, which increased the ferocity of the disease (Alden and Miller 1987). Inoculation against smallpox was practised by West African groups by deliberate infection via skin contact. Europeans routinely inoculated enslaved people from the late 18th century, carried out on slave ships from the 1720s by the British, and by French ships and plantations by the 1770s. A devastatingly

more brutal method for stopping the outbreak was to throw sick individuals overboard, as occurred in the case of the *Zong* in 1781. The quarantine of ships before enslaved people disembarked was also practiced. Although inoculation in the 18th and 19th century was a growing practice, which went some way to preventing the spread, there were still outbreaks occurring, particularly in ports, on ships, and among slave caravans (Glynn and Glynn 2005). That said, Steckel and Jensen (1986) observe that smallpox is almost absent as a listed cause of death in ship surgeon's documents compiled after 1788.

Smallpox was absent in the Americas until colonisation, but it is thought that the virus was brought to Central and South America by introductions from Europeans and enslaved Africans (Alden and Miller 1987). The first smallpox epidemics broke out in Haiti (then Hispaniola) in 1507 and subsequently in 1518 among enslaved African mineworkers, where the infection spread to the nearby Native American population (Hopkins 2002). Together with yellow fever and falciparum malaria, smallpox decimated the native populations of the Americas. For example, in the period following Spanish contact with Mexico it has been reported that the native population was reduced from 30 million to less than 2 million people in only 50 years (Geddes 2006), although see Brooks (1993).

Cholera

Epidemics of cholera, an infection in the small intestine from *Vibrio cholerae*, have also been documented during the era of the transatlantic slave trade. In the Caribbean, Curtin (1969) argues that during the 1850s the total population of the Caribbean declined owing to cholera epidemics. Further south, in the 19th century, a cholera epidemic occurred in Rio de Janeiro with the enslaved and free poor having the highest mortality in the population (Kodama et al. 2012).

Treponemal Diseases

Treponemal diseases, particularly yaws (caused by infection of *Treponema pallidum* subspecies *pertenue*) and venereal syphilis (caused by infection of *Treponema pallidum* subspecies *pallidum*), are also documented to have afflicted enslaved Africans. In a skeletal assemblage from the Waterloo Plantation in Suriname 27% have lesions consistent with treponemal infections (Khudabux 1989). Syphilis was also documented in the skeletal assemblage from the Newton plantation (Jacobi et al. 1992). Curtin (1993b) argues that yaws probably moved from Africa into the Americas via the transatlantic slave trade, but that venereal syphilis did not.

Respiratory Infections

Ship journals compiled after the passing of the Dolben's Act reveal that respiratory diseases were also present during the Middle Passage, affecting both the crew and enslaved (Steckel and Jensen 1986). However, from these records it is difficult to identify the aetiology of these respiratory infections. Once enslaved people were living in the Americas, respiratory diseases were common, especially in damp, draft and unsanitary accommodation (Savitt 2002). In the osteological analysis of enslaved Africans from the Newton plantation, Shuler (2011) noted that while there was an absence of specific diagnostic lesions, the historical documentation notes a high prevalence of infections of tuberculosis. The absence of specific, diagnostic markers of infectious disease, evidence from historical records, as well as the high mortality of young adults suggests that infectious diseases were likely to be the major cause of death in this population. This resulted in death before the infection could leave skeletal evidence, and indicates a very low quality of life for the enslaved on Barbados.

Mycobacterium tuberculosis was detected using ancient DNA in the remains of enslaved individuals from Pretos Novos (Jaeger et al. 2013) in Rio de Janeiro, Brazil and Anse Sainte-Marguerite on the island of Grande-Terre, Guadeloupe (Lösch et al. 2015). At Nossa Senhora do Carmo Church, also in Rio de Janeiro, no individuals with haplogroups of African ancestry tested positive for tuberculosis, although over 50% of individuals with European ancestry did test positive for the IS6110 insertion sequence (Jaeger et al. 2012). However, the positive detection of *Mycobacterium tuberculosis* complex (MTBC) in sediment samples should be cautiously interpreted, given the abundance of non-tuberculosis mycobacteria in soils (Pontiroli et al. 2013). At Pretos Novos, a site documented to be comprised of predominantly first-generation enslaved Africans (Cook et al. 2015), 25% of individuals (4/16) tested positive for ancient DNA derived from MTBC (Jaeger et al. 2013). As a result, the authors suggest that MTBC in these individuals was derived from the African continent. Lösch et al. (2015) used the same targeted approach and found that one individual of eleven tested positive for MTBC in a cemetery of enslaved Africans from Guadeloupe, although the authors note that DNA degradation may have generated false-negative results. All three studies target the insertion sequence IS6110, although this insertion sequence may in fact also be present in other, environmentally derived mycobacteria (Müller, Roberts, and Brown 2015).

Although *Mycobacterium africanum* has been documented to be an infective agent in populations in West Africa, there has been no evidence for this particular *Mycobacterium* species in modern populations in the Americas (Gagneux 2012). De Jong et al. (2010) suggest that a bottleneck effect occurred during the Middle Passage, where people infected with *M. africanum* did not make it to the Americas, or that *M. tuberculosis* may have outcompeted this species.

Oral Diseases

Poor oral health also afflicted enslaved people. Alexander Falconbridge (1788) documents the practice of inspecting teeth prior to people being shown to European purchasers, and Dr Rawlins on St Helena observed what he simply termed "bad mouths" (Colonial Office, Great Britain 1850, pg. 92). Osteological analysis of the remains of enslaved people has also documented poor oral health. For example, at the Newton plantation cemetery tooth root hypercementosis had a prevalence of 89% (Corruccini et al. 1987) and periodontal disease was observed to be widespread (Corruccini et al. 1982).

However, analysis of fragmentary dental remains from Pretos Novos in Rio de Janeiro revealed relatively good oral health (Cook et al. 2015). Pretos Novos was a cemetery founded in 1769, and it remained in use until 1830 as the primary burial ground of enslaved people who died before being sold. Of the 6119 people recorded as being buried during this time, 95.5% were enslaved Africans. The cemetery was rediscovered in 1996, where the remains of enslaved people were found buried in mass graves. Dental pathologies are relatively rare in this population, including a lack of dental calculus deposits compared to other remains of enslaved Africans. This, combined with high levels of polishing, may be the result of the use of chewing sticks, a common West African custom to the present day (Cook et al. 2015).

Ophthalmia

Contagious eye inflammations, which in contemporary sources are termed "ophthalmia", are documented for the period of the transatlantic slave trade. For example, in 1819 of an outbreak of ophthalmia resulted in the blinding of enslaved people and the entire crew of a French brig (Philadelphia Yearly Meeting of The Religious Society of Friends, 1824). Dr Rawlins

comments that ophthalmia is common among the African people in St Helena (Colonial Office, Great Britain 1850).

Other Infectious Diseases

Leprosy was also recorded, although has not been explored in detail. For example, leprosy, accompanied by smallpox infections, was documented in Sao Paulo in 1768 (Alden and Miller 1987). Phylogenetic studies of *Mycobacerium leprae* in the Atlantic suggest that leprosy was introduced to the Caribbean and South America from West Africa via the transatlantic slave trade (Monot et al. 2005).

3.5.2 Parasitic Diseases

A number of parasitic diseases were documented to have been afflicting enslaved Africans, including hookworm, Guinea worm, tapeworm, lymphatic filariasis, onchoecerciasis and African trypanosomiasis (Kiple 1984; Coelho and McGuire 1999;. Savitt 2002; Lammie et al. 200; Drisdelle 2010) Not all of these parasites appear to have survived the journey to the Americas, although some (such as malaria) today represent significant global health concerns as a result of these historical transmissions.

Malaria

Malaria is caused by infection of *Plasmodium protozoa*, spread via *Anopheles* mosquitoes. Given the density of populations in the tropical African coast, as well as the favourable environment for *Anopheles* mosquitoes in these regions, malaria was extremely prevalent in the tropical Atlantic, where it remains prevalent today (Gill and Beeching 2011). Although

there are several types of plasmodium protozoa (*Plasmodium vivax, P. malariae and P. ovale and P. falciparum*) it is *P. falciparum* which is the most deadly and the most prevalent throughout the African continent. It is widely believed, through multiple lines of evidence, that malaria was transmitted to the Americas from tropical Africa as a result of the transatlantic slave trade (de Castro and Singer 2005; Webb 2009; Yalcindag et al. 2012). Once present in the Americas, ecological changes associated with the development of plantations also increased the abundance of mosquitoes, for example, in the creation of irrigation systems (McNeill 2010). In the historical record, it can be challenging to identify malarial infections from other infections which result in similar symptoms (i.e. fevers, vomiting and diarrhoea).

The prevalence of malaria, along with other infectious diseases such as yellow fever and smallpox, on the West coast of Africa led to this area being termed "the White Man's Grave", owing to the very high mortality rate of Europeans entering the area (Curtin 1961). Mortality for arriving Europeans in West Africa was about estimated at approximately 50% in the 18th century (Kiple 1984). In the Americas, Europeans noticed that African populations experienced a degree of immunity. Together with a possible degree of immunity from yellow fever it was this apparent strength, in comparison to European susceptibility, which perpetuated the racist notion that African people were more suited to labour in the tropical Americas. Today, some populations, especially in sub-Saharan Africa have some immunity to *P. vivax* due to the absence of an antigen in red blood cells. Sickle cell anaemia, common to people of West and Central African descent, also provides some immunity to malarial infection (McNeill 2010).

African Trypanosomiasis

African trypanosomiasis, also termed "African sleeping sickness", is caused by infection of *Trypanosoma brucei gambiense* (which is concentrated in West and Central Africa) or

Trypanosoma brucei rhodesiense (which is concentrated in East and Southern Africa), and is spread via the tsetse fly. African trypanosomiasis was observed by doctors and medical officers during the era of transatlantic slavery (Steverding 2008) and epidemics continued in West Central Africa in the 20th century. In the Congo these epidemics were exacerbated by the effects of Belgian colonisation, which saw the displacement and malnourishment of populations (Lyons 2002). The parasite does not appear to have survived the Middle Passage and there is no evidence of its introduction to the Americas.

Schistosomiasis

Schistosomiasis is caused by an infection with *Schistosoma* trematodes, where freshwater snails act as the vector (Gill and Beeching 2004). Whilst historical documents do not seem to have recorded this parasite, evidence from modern strains suggest a spread of this parasite during the transatlantic slave trade. For example, Desprès et al. (1992) and Desprès, Imbert-Establet, and Monnerot (1993) found that South American strains of *Schistosoma mansoni* were phylogenetically similar to African strains. Morgan et al. (2005) also found that strains isolated from the Americas were closely related to parasites isolated from West Africa. In the archaeological record, *S. mansoni* eggs have been reported from a European latrine, possibly occurring from an infected human host with possible connections with the transatlantic slave trade (Bouchet et al. 2002).

3.5.3 Nutritional Diseases

Understanding the nutritional status of enslaved people has been a controversial study and has often been explored in the context of understanding the profitability of the institution. Fogel and Engerman (1974) in the controversial study '*Time on the Cross'*, argued that the diet of enslaved people in the United States was calorically substantial. However, Kiple and Kiple (1977b) argue that nutritional deficiencies were present in enslaved children owing to an

adaptation to an "African" diet, and Sutch (1975) argues that estimates by Fogel and Engerman (1974) have been over calculated. The prevalence of enamel hypoplasia in many osteological analyses of enslaved African human remains (Table 3.1) suggests that nutritional deficiencies were prevalent (e.g. Blakey, Leslie, and Reidy 1994; Wood 1996). Estimates of the heights of enslaved people, from both historical records (e.g. Margo and Steckel 1982; Steckel 1986) and osteological analyses (e.g. Kelley and Angel 1987), have also been used as evidence of nutritional deficiencies during childhood development.

A range of health problems were caused by dietary deficiencies (Kiple 1984; Handler 2006b). Scurvy, defective collagen formation as a result vitamin C deficiency, in particular was recorded during this time. For example, in 1731 753 enslaved people on board a slave ship died of scurvy (Rawley and Behrendt 2005). It became so common among enslaved people from Angola that it was known as the *mal de Loanda*, the Luanda sickness (Miller 1997).

Anaemia resulting from iron deficiency has been recorded osteologically (e.g. Kelley and Angel 1987). In general, nutritional diseases are less well documented in the historical record, possibly overshadowed by the presence of diseases that have more characteristic symptoms. Together with poor water supplies, poor diet would have exacerbated gastro-intestinal diseases like dysentery.

3.6 Diseases at St Helena

3.6.1 Diseases Documented in Historical Sources

Historical records (reviewed in section 3.3.1) give valuable insight into the conditions and medical treatment of "liberated Africans" arriving at the establishment on St Helena.

According to Dr Rawlins in 1849, prevalent conditions include dysentery, opthalmia, poor oral health, chest affections, fevers, ulcers, anasarea (dropsy), diseases of the heart, genito-urinary diseases, variolous diseases (smallpox), craw-craw (onchocerciasis), acute and chronic rheumatism, epilepsy, apoplexy and accidents (Colonial Office, Great Britain 1850, pg. 92). Dr Vowell notes similar affections but in addition he notes "dropsy of the brain, heart, lungs and abdomen" and 'caheetic' diseases (wasting/malnourishment), as well as an epilepsy-like conditions with an inflammatory attack. These are in line with other sources on the diseases of the enslaved from this and other periods, reviewed above. Dr Vowells notes that men, more so than women, suffer from dysentery, also commenting that dysentery is also prevalent among those working on St Helena and among officers. He laments the number of rats on St Helena and recommends that liberated Africans should eat greater quantities of better cooked food. He also notes that the clothing, made of coarse flannel or serge, irritates the skin.

Dr George McHenry provides possibly the most graphic description of the conditions experienced by African people landing at St Helena. He expresses horror and terror at the thought of having to enter the hold of slave ship. He describes that the air, filled with the smell of the dying, diseased, urine and faeces was "…the most destructive weapon in the armoury of death" (quoted in Pearson et al. 2011, pg. 166). His account, graphic and raw in its description, documents the arrival of a captured slave ship to St Helena;

> *Some exhibit huge scabs, the result of blows, over different parts of the body; or thick crusts, formed from the drying of the humours of the craw-craw, a loathsome cutaneous eruption. A few, still able to crawl, may be marked with the incipient pustules of the smallpox; while others are conspicuous from the size of the scorbutic tumours with which they are affected, or hideous and repulsive from the extent of the gangrenous sores eating into their flesh. Among the throng are to be found a few*

> *unable to move, from the rack of rheumatism, the stab of pleurisy, or the tortures of a broken bone; or in the last stage of emancipation, oozing out their lives with the constant flux of dysentery; or perhaps just dead."* (McHenry, 1863, quoted in Pearson et al. 2011, pg. 165)

Medical Treatment on St Helena

A number of medical officers were stationed on St Helena throughout the period of the establishment. In 1840 when the Vice-Admiralty court was established, a Colonial surgeon, a surgeon for the 91st regiment (who arrived with the first governor, although was later dismissed) and a health officer were provided for the island's population of 4000 (Pearson et al 2011). Throughout the period of the establishment the number of medical staff on St Helena seems to have varied, although a surgeon was a permanent position on the island.

It is clear from historical records that relationships between the medical officials and the governor were strained and that medical supplies and facilities were often pushed to the limit. For example, in the early period of the establishment two hulks were used for smallpox quarantine. Dr Rawlin highlights other difficulties in treatment, as a result of the language barrier between doctor and patient, the wariness among African people to subscribe to his treatments, as well as an overarching desire not to appear sick through concealing disease. Overall, Dr Rawlin finds recommended treatments ineffective, and the mortality rate on St Helena remained very high, despite these efforts.

3.6.2 Osteologically Identified Diseases

Osteological analysis also reveals information on the diseases suffered by these individuals. Results of the osteological analysis of 325 articulated indiviauls are summarised in Witkin (2011), but will be briefly repeated here.

Many of the observed osteological pathologies are associated with nutrient deficiencies. Evidence of general nutritional deficiencies was inferred from the high prevalence of cribra orbitalia (41.38% of total individuals) and porotic hyperostosis (10.59% of the total individuals). Scurvy was identified in 20.87% of individuals. Rickets was also identified in 11.53% of the population and may be the result of maternal malnourishment. Enamel hypoplasia was also observed in 22.11% of total individuals, although it is likely that deposits of dental calculus may have obscured this recording. In comparison, Rathbun (1987) observed a frequency of 92% (in males) of enamel hypoplasia in a skeletal assemblage of 19th century African Americas, and Blakey et al. (1994) observed that all individuals displayed enamel hypoplasia in a group of 27 enslaved individuals from the southern United States.

Infections were also observed osteologically in this population. Treponemal infections were identified in nine individuals, which Witkin (2011) suggests may be yaws or bejel (endemic syphilis). Non-specific infections were inferred from the presence of periostitis, identified in 24.28% of individuals over 1 year old, being most prevalent in the lower limbs. Similarly, Shuler (2011) observed a high prevalence of non-specific infections in the lower limbs at the Newton plantation, although at the Newton plantation overall rates of non-specific infections were higher (41%). Rib lesions, which may be indicative of pulmonary infections were identified in 3.74% (12/321) of individuals. Maxillary sinusitis, which could also be the result of respiratory disease, was documented in 21.91% (55/251) of individuals who had at least one sinus cavity preserved.

Osteoarthritis occurred in 6.8% (10/147) of the skeletal population, whilst amongst mature adults the rate was 17.65% (3/17). Spinal joint disease was observed in 29.41% of mature adults, and in 18% of the total population. Trauma was also observed, including a potential ankle injury, possibly caused by a shackle, and a possible sharp force trauma on the scapula of an adult male. Four individuals also had evidence of healing fractures.

This population also experienced a range of dental and oral diseases, including caries (19.5% (59/303) of individuals), antemortem tooth loss (12.5% (36/287) of individuals) periodontal disease (13.6% (39/287) of individuals) and abscesses (24.2% (45/186) of individuals). Deposits of dental calculus were extremely abundant, with 96.69% of individuals over 1 year old with dentition (293/303) displaying dental calculus. Although not strictly pathological, of particular note is the presence of cultural dental modifications, which is the largest assemblage of excavated remains which display these modifications. Although this skeletal assemblage is unique, these osteological observations are generally comparable to other osteological investigations of enslaved Africans.

3.6.3 Ethical Considerations

Accessing and studying human remains always requires ethical considerations, especially in understanding how studying remains may impact descendent groups. In the context of the transatlantic slave trade and slavery, this study has sometimes been fraught. For example, there was considerable controversy during the initial excavations at the New York African Burial Ground, where local descendent groups disagreed with the forensic-based approach taken in the study of these remains. Research was only able to move forward after the community gained control of the analysis, along with congressional involvement to generate

an appropriate research design (Blakey 2001). It is interesting to note that, like the New York African Burial Ground, there was also an initial denial of the presence of burials on St Helena (Andrew Pearson, pers. comm., 2015)

In St Helena there is no clear descendant community due to the transient nature of people entering and leaving the island. No descendants of "liberated Africans" remain on St Helena. Nevertheless, permission for the analysis of human remains from this site was granted by the island's governor before EUROTAST research commenced. Only samples of dental calculus, and teeth for ancient DNA and stable isotope analysis were removed from the island. The legacy of the excavated human remains on St Helena is uncertain.

3.7 Legacies of Diseases of the Transatlantic Slave Trade

The transatlantic slave trade, as an intercontinental "institution", caused a change in world demographics and had profound legacies for individuals and populations throughout the Atlantic world. One aspect of this legacy is that the transatlantic slave trade brought together different disease environments. Slave ships, in addition to being vessels of human suffering, were a vector for the transmission of pathogens to new environments. Across the Atlantic diseases believed to be derived from Europe, such as smallpox, measles, gonorrhoea and syphilis, become prevalent. In addition, diseases derived from Africa, such as yellow and dengue fever, malaria, and tropical parasitic infections were also spread. As well as clearly afflicting enslaved Africans, diseases carried on slave ships decimated populations of native Americans, who had no acquired immunity to such diseases. European traders in West Africa also had an extremely high mortality rate (Curtin 1961). The movement of people within Africa, where captured individuals from the interior were moved towards the coast, also exposed people to new infections. In all, the transatlantic slave trade saw the combination of

"congestion, dietary imbalance and multiple disease environments in a potentially lethal mixture" (Sheridan 1981, pg. 602).

In addition, the spread of mosquitoes also occurred during this time, being carried on board slave ships. Brown et al. (2014) argues that human movements have shaped recent evolution in *Aedes aegypti*, and that genetic data from this species indicates its spread through trade routes. As well as the movement of the vectors themselves, it may also be the case that dengue fever carried by enslaved people from West Africa was seeded in American mosquitoes (Morens and Fauci 2013).

Thus, this period saw the spread of infectious disease, the mixing of disease environments, and the interplay between the environment, culture and biology (Prothero 1977; McMichael 2002). In the light of talk of reparations (e.g. Clegg 2014), Lammie et al. (2007) argues that "the elimination of diseases that are a consequence of this trade would represent a tangible contribution to the health and wellbeing of people and communities who, arguably, still suffer from the residual effects of slavery" (2007, pg. 71).

Although beyond the scope of this PhD, it is worth noting the controversies surrounding the idea that the transatlantic slave trade has affected non-communicable diseases in contemporary African-American populations. In 1991, noting the higher incidences of hypertension among African American than European populations in North America, Wilson and Grim (1991) proposed that the transatlantic slave trade was responsible for a "natural selection" process. They argued that that individuals with a genetic ability to conserve salt had an advantage in surviving the dehydration brought on by the Middle Passage, and therefore dietary problems in African-American communities today (such as higher rates of hypertension) may be related to this selection. This argument was also taken up by Jared Diamond (1991). This "salt-retention" hypothesis was criticised by both historians (Curtin 1992), and geneticists (Jackson

1991). Jackson (2006) argues that while it is worthwhile studying the links between the Middle Passage and contemporary health of African Americans, it is also necessary to include cultural, history and environmental factors into the genetic study of population groups. This example is part of a broader debate on the use and validity of racial categories in medicine, and the genetic basis for disease disparities in different populations (Braun et al. 2007).

Chapter 4: Disease and Dental calculus: A Novel Biomolecular Perspective on the Transatlantic Slave Trade

After outlining methodological approaches to biomolecular palaeopathology (Chapter 2) and summarising disease during the period of transatlantic slavery (Chapter 3), this chapter brings these two aspects together to explore how the biomolecular analysis of dental calculus may increase our understanding of diseases during the transatlantic slave trade.

Abstract

The transatlantic slave trade has a profound legacy in shaping patterns of human health through the Atlantic world. In a fatal mix, the transatlantic slave trade resulted in the spread of disease, the exposure of vulnerable populations to new disease environments and the movement of disease carrying vectors. Contemporary populations are still exposed to many of the same diseases, spread as a result of the transatlantic slavery (Lammie et al. 2007). Given the significance of the transatlantic slave trade in the history of human health, it is important that emerging new methods are adopted for the study of disease in this period. Through the extraction of human and bacterial proteins and DNA from dental calculus (mineralised plaque) biomolecules from pathogens and their associated diseases from this era could be studied in detail for the first time. This gives us the potential to identify diseases hidden from both the historical and osteological record, to mine the historical oral microbiome, to identify remnants of diet and explore the interplay between host immunity and microbial virulence. The removal of mineralised microbial biofilms adhering to the surface of teeth offers a minimally invasive way to explore health from skeletal remains. As interest in the analyses of ancient dental

calculus grows it is necessary to maintain best practice in the handling and analysis of human remains from this period.

4.1 Health and Disease in the Transatlantic Slave Trade

The era of transatlantic slavery saw the reshaping of environments, the movement of people on an intercontinental scale and the proliferation of infectious diseases. The transatlantic slave trade saw the transport of people, pathogens and non-human vectors like mosquitoes into new tropical environments and vulnerable populations (Curtin 1993b). The conditions of enslavement, including overcrowding and inadequate hygiene and medical treatment, resulted in the proliferation of infectious diseases like dysentery, smallpox and yellow fever (Thomas 1997). Parasitic diseases such as hookworm and guinea worm were also prevalent (Sheridan 1981). Together with overwork, dietary deprivation resulted in a range of nutritional deficiencies.

Analyses of historical texts provide a powerful way to explore rates of mortality and prevalence disease. However, these can often be limited by contemporary medical knowledge, ambiguous medical details and often have a strong colonial bias. In addition, historical sources related to disease in Africa, prior to enslavement in the Americas, are lacking and thus there is a limited understanding of the nature and prevalence of diseases on this side of the Atlantic.

Analysis of human remains is a direct way of understanding individual patterns of disease and life conditions during transatlantic slavery (e.g. Handler and Corruccini 1983; Rathbun 1987; Shuler 2011; Okumura 2011; Schroeder, Shuler, and Chenery 2013). Skeletal analyses have revealed a pattern of chronic malnutrition and stress, documenting the harsh and violent system of exploitation (Handler 2009). Although osteological analysis is a powerful tool,

many diseases are not skeletally manifested and, while some diseases do affect bone tissue, few produce diagnostic lesions. Even if a disease is documented to cause specific diagnostic lesions the individual may not have been affected for long enough for the disease to manifest on the skeleton (Wood et al. 1992).

4.1.1 Biomolecular Approaches

Analysis of ancient DNA and proteins may be an alternative approach for understanding disease during the period of transatlantic slavery. Ancient DNA analysis from skeletal remains can reveal molecular evidence of disease, enabling the identification of particular strains and a method by which to explore pathogenic evolution (e.g. Bos et al. 2011; Schuenemann et al. 2013). In terms of transatlantic slavery, ancient DNA analysis has been applied to identifying tuberculosis in the remains of enslaved individuals in the Americas. At Nossa Senhora do Carmo Church in Rio de Janeiro, Brazil no individuals with haplogroups of African ancestry tested positive for tuberculosis, although over 50% of individuals with European ancestry did test positive for the IS6110 insertion sequence (Jaeger et al. 2012). At Pretos Novos, also in Rio de Janeiro, 25% of individuals (4/16) thought to be first-generation enslaved Africans tested positive for ancient DNA derived from the *Mycobacterium tuberculosis* complex (MTBC) (Jaeger et al. 2013). As a result, the authors suggest that MTBC in these individuals is derived from the African continent. Lösch et al. (2015) used the same targeted approach and found that one individual of eleven tested positive for MTBC in a cemetery of enslaved Africans from Anse Sainte-Marguerite on the island of Grande-Terre, Guadeloupe, although the authors note that DNA degradation may have generated false-negative results.

Ancient DNA can be subject to debilitating issues of preservation and contamination. Especially in warm climates, poor DNA preservation can prohibit this analysis (Smith et al. 2003), although recent capture-based methods (Carpenter et al. 2013) have proven successful for analysis of human DNA from the Caribbean (Schroeder et al. 2015). Ancient proteins, which can be more robust than DNA in the archaeological record (e.g. Welker et al. 2015) may also provide an alternative approach for identifying disease.

Genetically based investigations examining modern disease epidemiology have often highlighted the transatlantic slave trade as a mechanism for disease movement. For example, the yellow fever virus (Bryant, Holmes, and Barrett 2007), hepatitis (Arauz-Ruiz et al. 1997; Tanaka 2000), human papillomavirus (Chan et al. 1992), sickle cell anaemia (Gonçalves, Nechtman, and Figueiredo 1994), onchocerciasis (Zimmerman et al. 1994) and malaria (Hume, Lyons, and Day 2003) are among those studied. Analysis of ancient biomolecules, isolated from the specific time and place, may contribute to this understanding of the nature of current disease epidemiology.

A greater understanding of the role played by the human microbiome, i.e. microbial communities associated with the human body, in human health is emerging by the application of the same sequencing technologies (Cho and Blaser 2012). DNA from ancient microbiomes is turning from a trickle to a flood, and is being recovered from mummified tissues (Ubaldi et al. 1998; Khairat et al. 2013), palaeo-faeces (Cano et al. 2000; Tito et al. 2008; Tito et al. 2012; Santiago-Rodriguez et al. 2013) and dental calculus (Adler et al. 2013; Warinner, Rodrigues, et al. 2014). As we begin to explore ancient microbiomes these might shed light upon changes in our physiology and pathophysiology.

4.2 Dental Calculus: A Fossilised Mouth Swab?

Calculus, also known as tartar, is a microbial biofilm which has mineralised on the only stable surface in the oral cavity, the tooth (Jin and Yip 2002). The deposit is formed by the development of plaque on the tooth surface, where a layer of oral proteins on the surface quickly becomes colonised by microbes, which aggregate to form a biofilm. This biofilm grows in Gram-positive and Gram-negative dominated layers, self-organised by the metabolic demands of different microorganisms (Marsh 2005). This self-organisation is perturbed if conditions permit the formation of stable mineral phases, composed principally of calcium, phosphate and carbonate ions, associated with bacterial membranes of the biofilm. On this stable mineral layer the cycle of plaque formation and mineralisation is repeated. Mineral formation can occur within a few days of plaque formation, but then will undergo further diagenetic transformation to more stable phases (Lieverse 1999). During mineralisation, food remains, human and dietary biomolecules (such as proteins, peptides, nucleic acids, glycoproteins, carbohydrates and lipids), and other inhaled debris, can become entrapped.

Owing to this mineralisation, archaeological dental calculus is a reservoir of preserved dietary and environmental debris, human and microbial biomolecules, incorporated into this matrix at the time of formation. As a consequence of the diagenesis of the mineral phase over the lifetime of the biofilm, it will consist of larger crystals of stable hydroxyapatite than are present in bone and dentine, and may undergo burial diagenesis more slowly than bioapatite. It is also one of the few archaeological materials containing preserved bacterial and human biomolecules. Given that calculus is present in both living and ancient populations, particularly in individuals suffering from poor oral health and after the advent of grain agriculture, this deposit represents a medium through which ancient biomolecules can be

accessed across space and time, offering the potential to provide a detailed picture of disease and diet in individuals, as well as across and between populations.

Dental calculus is abundant in microscopic debris, and the microscopic analysis of this material is well established. Microscopic, diagnostic preserved plant remains such as starches, phytoliths and tissues have been widely explored for understanding food consumption (Reinhard et al. 2001; Piperno and Dillehay 2008; Henry and Piperno 2008; Hardy et al. 2009; Wesolowski et al. 2010; Blatt et al. 2011; Hardy et al. 2012; Dudgeon and Tromp 2012). In the case of starch granules it is possible that processing, such as boiling and parching, may be identified as taphonomic alteration to these granules (Henry, Hudson, and Piperno 2009), although these may also be modified by diagenesis (Collins and Copeland 2011). In addition to diet, this debris may hold information for answering a range of different questions from environmental change (Dudgeon and Tromp 2012) to trade (Blatt et al. 2011). For example, microscopic analysis of dental calculus is being used to look for phytoliths of chewing sticks from a skeletal assemblage of first generation enslaved Africans from Pretos Novos (Cook et al. 2015). Dental calculus also preserves bacterial morphology (Friskopp and Hammarström 1980), ancient DNA (Preus et al. 2011; Adler et al. 2013), and ancient proteins (Warinner, Rodrigues, et al. 2014).

4.2.1 Ancient Dental Calculus and the Transatlantic Slave Trade

For the study of disease in the transatlantic slave trade, dental calculus research may add to knowledge acquired from bioarchaeological and historical approaches. Through the analysis of biomolecules this approach may identify pathogens and their associated diseases hidden from these two sources. The extraction of biomolecular information may also reveal the active

processes occurring in the mouth at the time of calculus formation, giving the opportunity to view microbial virulence and the host response to that attack. This has the potential to identify the state of the immune system, giving a more definitive idea of health than is possible using osteological methods.

A biomolecular analysis of dental calculus also facilitates the identification of the commensal oral microbes understood to play significant roles in human health (Dewhirst et al. 2010). Differences in the gut microbiomes of remote and developed societies (Yatsunenko et al. 2012; Obregon-Tito et al. 2015) support the idea that microbial ecosystems adapt to differences in diet, medication and hygiene. As the field develops we have the opportunity to document the influence of population movement, diet and lifestyle on the microbiome in the past, contributing to the growing body of knowledge about the interactions of commensal and pathogenic microbes and human hosts.

Microbiomes differ in their temporal variability, diversity (the plaque microbiome is second only to the gut) and heritability (Lozupone et al. 2012). Modern oral health interventions, such as widespread fluoridation, have presumably lead to significant recent changes in the plaque microbiome and may make it more difficult to compare ancient and modern microbiomes. If it is possible to sequence (or concatenate) sufficiently long gene fragments it will be possible to track populations of the most abundant members of the fossilised plaque microbiome and establish if these are vertically or horizontally transmitted. Both types of organisms are useful, the former could track maternal genealogy and the latter could be used to establish the extent of isolation of populations. The oral microbiome as a whole also shows evidence of some vertical transmission (Li et al. 2007; Corby et al. 2007; Nasidze et al. 2009). By tracking these vertically transmitting microbes, it may be possible to document population movement in the past (Dominguez-Bello and Blaser 2011; Moodley et al. 2009). This could offer one

alternative to human genomic research for studying movements of people in the transatlantic slave trade.

Taking the recent excavations on the South Atlantic island of St Helena as an example, what are the more specific areas where the analysis of dental calculus could inform our understanding of the transatlantic slave trade? In 2007 and 2008 excavations on St Helena, a small island in the South Atlantic Ocean, uncovered part of the "Liberated African Graveyard" associated with the mid-19th century transatlantic slave trade (Pearson et al. 2011). The skeletal population excavated here is unique, representing individuals who experienced the Middle Passage but did not make their intended destinations in the Americas. In this population biomolecular analysis of dental calculus could supplement historical documentation and osteological analysis. Given that knowledge of diseases prevalent on the African side of the Atlantic during the period of transatlantic slavery is particularly limited, analysis of dental calculus in this population may be one way to examine disease exposure and dietary patterns which derive specifically from Africa. Similarly, there may be differences in disease and food consumption between individuals buried on St Helena and enslaved individuals in the Americas, which may assist in understanding the dietary and pathological changes occurring as individuals are moved from one continent to another. Additionally, analysis of dental calculus could also be used to corroborate or expand upon information derived from historical texts concerning St Helena. For example, are there detectable molecular traces of diseases which were not documented by historical texts? Similarly, is there any biomolecular evidence of medical treatment?

4.2.2 Future Directions

As the field grows and the interest in dental calculus research develops (Warinner, Speller, and Collins 2015; Weyrich, Dobney, and Cooper 2015), there are new directions to be tested and explored. For dietary analysis, it has been claimed that calculus may be a potential material for carbon and nitrogen isotope analysis (Scott and Poulson 2012; Poulson et al. 2013). This seems a dubious claim, as bulk isotopic values derived from a complex mixture of biomolecules which have been microbially generated and processed within biofilms will be extremely difficult to interpret (Salazar-García et al. 2014). In addition, the amount of material required for analysis compared to the total amount of calculus per individual, as well as the rich source of other information available in this material, means that this practice should probably be avoided in the future. We would not conduct (a more robust) collagen isotope analysis if we had to destroy 20% of the whole skeleton to obtain it. A more promising avenue may be as a reservoir for inorganic isotopes and trace elements. There are two particular advantages and one disadvantage to this approach. We assume (although this has yet to be proven) that calculus is more diagenetically stable that bone or dentine. The mineral has not been biopurified by the individual, as is the case in mineralised collagen, and should not be depleted in trace elements relative to the soil. Both factors suggest it may be possible to derive a more accurate environmental signature of diet from calculus. The one disadvantage is that the mineral is controlled by an active microbial biofilm, and whilst this is unlikely to discriminate between heavy isotopes (Ca, Sr), it will almost certainly influence light isotope distribution (C,H,N,O) and trace element concentrations, relative to the oral cavity. This approach is yet to be established, and requires a) an understanding of the nature and speed of calculus formation in mouths that lack basic oral hygiene and widespread fluoridation, b) patterns of calculus diagenesis, and c) extraction methods that can both recover organic, inorganic and microfossil data from the same sample.

Although the potential for calculus DNA (Adler et al. 2013; Warinner, Rodrigues, et al. 2014), protein (Warinner, Rodrigues, et al. 2014) and lipid analysis (Hardy et al. 2012) has been established, further methodological aspects need to be investigated. What is the best practice for maximising data extraction? At present each of these methods is conducted in isolation of all but the target compound, but, given the range of analyses, multiple data should be collected from the same sample. Additionally, how important is sample size in calculus research? Will it be possible to interpret the layers within calculus as a longitudinal record?

There are differences in the ways that populations treat materials which are associated with the human body but do not constitute human tissues. In the UK, plaque and dental calculus are not covered in the Human Tissues Act (2004). Given that the material is composed of mostly microbial remains, is this material defined as a human tissue? If so what are the implications of how we handle this material from archaeological populations? We need to understand if or how descendent populations value ectopic deposits from ancestral remains so that we can ensure engagement and agency with descendant populations in archaeological practices.

In the study of human remains of the African Diaspora it is paramount that descendant groups have agency over research, that authority over remains are maintained, and that care and consideration is taken with handling and analysis. As these novel approaches for understanding past health are developed, we propose questions for consideration to develop best practice as this field develops. Firstly, does this approach offer a less obtrusive way to study health of individuals than a skeletal assessment? A calculus deposit is relatively easy and quick to remove for analysis, requiring only access to the teeth. This allows at least some information to be gained from isolated crania and teeth, found in skeletal collections or in archaeological deposits. However, in the absence of osteological evidence (age, sex, pathologies) how useful is this approach for representing the biological profile of the

population? Secondly, what are the ethical boundaries in studying these kinds of ectopic deposits, given that the material is largely microbial, grows on an external dental surface and is removed voluntarily in modern populations? Should dental calculus deposits be subject to the same procedures for repatriation as bone samples?

4.3 Conclusion

The analysis of biomolecular information on health and disease may facilitate new insights into transatlantic slavery, facilitating the identification of diseases invisible in the historical and osteological record, uncovering the interplay between the immune system and microbial virulence, and exploring the historical oral microbiome. Given the significance and legacy of the transatlantic slave trade in global health histories, it is vital that new methods are adopted to understand the health of enslaved people, as well as the pathogens afflicting them. In addition, analysis of dental calculus may enable an understanding of individual patterns of health, disease and food consumption, where the experiences of these individuals may help us to understand the period of transatlantic slavery as a whole. However, as the field advances and the ability to extract and examine these aspects of disease and health improves, the questions proposed here should be considered for best practice.

Chapter 5: Tandem Mass Spectrometer Performance and Archaeological Proteomes

The previously chapters outlined the development and potential of ancient proteins in archaeological applications and how this progress is linked to technological developments in tandem mass spectrometry. In the wake of this growing interest, this chapter explores the performance of available tandem mass spectrometers with complex mixtures of ancient proteins and explores how differences in spectrometer performance have implications for the use of ancient proteins as a tool for understanding the past.

Abstract

Shotgun proteomics is increasingly being applied to archaeological samples following technological developments in tandem mass spectrometry. As interest grows it is necessary to assess the performance of available instruments with complex mixtures of ancient and degraded proteins. Proteins were extracted and analysed from 59 samples of archaeological dental tissues (cementum, dentine, and dental calculus) and analysed across multiple tandem mass spectrometry instruments (maXis 3G UHR Q-TOF, LTQ Orbitrap Velos, Orbitrap Elite and Q Exactive). This study observed that a) the hybrid Quadrupole-Orbitrap (Q Exactive) outperformed all instruments tested in terms of the number of protein group identifications, b) the Orbitrap Velos was able to identify a greater number of protein families than the maXis 3G and c) CID fragmentation produced a greater number of protein identifications than HCD. The results demonstrate the value of using the most recent, high-accuracy, high-speed mass spectrometers, which can provide increased sequence coverage and higher rates of protein

identification. While this study may not be a strict comparison of instruments in the truest sense (given the use of different sample loads, different high-performance liquid chromatography (HPLC) setups and experiments at different locations), it nonetheless highlights the significant difference in results obtained, and the impact of technical advances on the analysis of complex mixtures of ancient proteins. This has downstream consequences for archaeological data interpretation and applicability. As instrumentation continues to advance in resolution it is inevitable it will broaden the scope and reach of ancient proteomics.

5.1 Introduction

In comparison to the accelerating impact of ancient DNA in archaeology, ancient protein research has lagged behind (Cappellini, Collins, and Gilbert 2014), despite the fact that the bone protein collagen is the dominant material used for stable isotope analyses and radiocarbon dating of human and animal remains (Fig. 5.1). Proteins from archaeological samples were first detected through the bulk analysis of amino acids (Abelson 1955) and subsequently via immunoassays (Westbroek et al. 1979; Lowenstein 1981), although methodological challenges with immunological approaches limited the scope of ancient protein research and in some cases led to contentious outcomes (Downs 1995; Fiedel 1996; Newman et al. 1997; Custer, Ilgenfritz, and Doms 1988). However, first by Edman degradation (Huq, Tseng, and Chapman 1990) and then by mass spectrometry (Ostrom et al. 2000), the ability to generate peptide sequences has dramatically increased the scope of ancient protein research. In addition, protein mass spectrometry, with its ability to detect post-translational modifications (PTMs), offers further insight into the degradation of proteins in archaeological contexts (Hill et al. 2015; Cleland, Schroeter, and Schweitzer 2015). Due to the development of high throughput, high sensitivity tandem mass spectrometry it has recently

become possible to analyse complex mixtures (proteomes and metaproteomes), as opposed to single, dominant proteins in archaeological material (Cappellini et al. 2012; Cappellini et al. 2014; Warinner, Rodrigues, et al. 2014; Warinner, Hendy, et al. 2014).

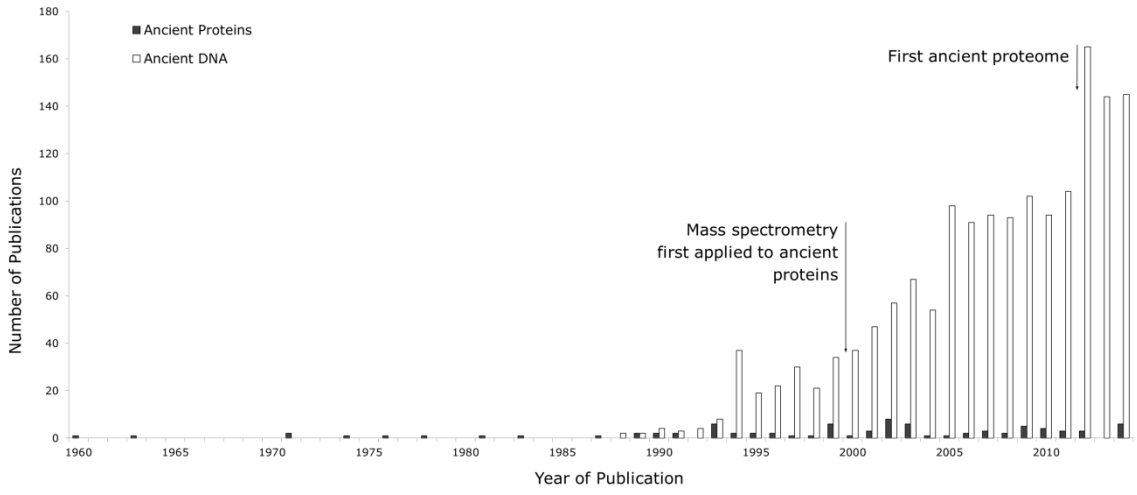

Figure 5.1. Number of articles published per year on ancient DNA and ancient protein research, indicating the first using of MALDI (Ostrom et al. 2000) and MS/MS (Cappellini et al. 2012) for archaeological samples. Publications were compiled using a Web of Science search using key terms 'Ancient DNA', and 'Ancient Proteins' found in the title, abstract, or keywords in the fields 'Palaeontology', 'Archaeology', 'Anthropology', 'Evolutionary Biology', and 'History'.

Increasingly, ancient proteins are finding a number of niches in archaeological and paleontological research, addressing problems of phylogenetic and nomenclature assignments (Cappellini et al. 2014; Welker et al. 2015), and the identification of non-diagnostic bone fragments (Buckley et al. 2009; Buckley et al. 2010; Buckley and Kansa 2011; Richter et al. 2011) and other proteinaceous materials from archaeological contexts (Hollemeyer et al. 2008; Solazzo et al. 2011; von Holstein et al. 2014; Brandt et al. 2014). Ancient proteins also act as biomarkers of foodways (Craig et al. 2000; Solazzo et al. 2008; Hong et al. 2012; Buckley, Melton, and Montgomery 2013; Warinner, Hendy, et al. 2014) and have been used

to identify patterns of health and disease in past populations (Corthals et al. 2012; Maixner et al. 2013; Warinner, Rodrigues, et al. 2014). Ancient proteins have been isolated from a diverse range of archaeological sources, such as animal and human bone and dentine (Buckley et al. 2009; Buckley et al. 2010; Buckley and Kansa 2011; Richter et al. 2011; Cappellini et al. 2012; Warinner, Rodrigues, et al. 2014), potsherds and ceramic vessels (Craig et al. 2000; Solazzo et al. 2008; Buckley, Melton, and Montgomery 2013), eggshell (Stewart et al. 2013; Stewart et al. 2014), soil (Oonk, Cappellini, and Collins 2012), antler (von Holstein et al. 2014), wool (Solazzo et al. 2013), mummified tissue (Corthals et al. 2012; Maixner et al. 2013), and preserved food remains (Cappellini et al. 2010; Yang et al. 2014; Shevchenko et al. 2014). Most recently, archaeological dental calculus (mineralised dental plaque, tartar) is emerging as one of the most promising reservoirs of ancient proteins, giving insight into the oral microbiome, host immunity and ancient diets (Warinner, Rodrigues, et al. 2014; Warinner, Hendy, et al. 2014).

Archaeological samples range in their proteomic complexity. Both radiocarbon dating and stable isotope analyses primarily focussed on one protein, type I collagen, the most abundant and well-preserved bone protein. More recently however, the field has begun to move away from the analysis of single target proteins, such as collagens, to the analysis of simple and complex proteomes using "bottom-up" approaches of shotgun proteomics. Such studies have included the analysis of bone and dentine proteomes of humans and animals (Cappellini et al. 2012; Wadsworth and Buckley 2014; Warinner, Rodrigues, et al. 2014), preserved foodstuffs and residues (Cappellini et al. 2010; Hong et al. 2012; Yang et al. 2014; Shevchenko et al. 2014; Warinner, Hendy, et al. 2014), mummified human remains (Maixner et al. 2013; Corthals et al. 2012) and microbial communities (Warinner, Rodrigues, et al. 2014). Among these studies, several have focused on relatively simple proteomes, such as the bone proteome of animals with well-characterised genomes (e.g. bovine species and proboscideans)

(Cappellini et al. 2012; Wadsworth and Buckley 2014), while others have targeted highly complex metaproteomes, such as from dental calculus, an ecosystem with immense microbial diversity (Warinner, Rodrigues, et al. 2014). Given the complexity already posed by this mineralised oral biofilm *in vivo*, the complexity posed by an ancient sample is manifold. In all ancient samples there is the additional challenge of degradation, with multiple degradation pathways acting upon the sample through time and resulting in protein denaturation, PTMs and fragmentation. Thus, ancient dental calculus arguably represents one of the most challenging samples in proteomics. However, if we can access these ancient proteins, we have the ability to explore a vast array of questions surrounding the nature of the ancient oral microbiome, food consumption and health.

Proteomic analysis is key to understanding biological processes in both modern and ancient biological systems because it reveals important functional aspects of the tissues or substrates that cannot be determined from genomic data alone. For example, a key finding of the Human Microbiome Project was that although the taxonomic makeup of the body site microbiomes varied between individuals, gene functional profiles were largely invariant, suggesting a high degree of functional redundancy (Cho and Blaser 2012; Wade 2013b). However, genomic functional potential does not directly correlate with gene expression levels, and thus genomes do not equate to transcriptomes or proteomes (Maier, Güell, and Serrano 2009; de Sousa Abreu et al. 2009; Schwanhäusser et al. 2011). Although some success with ancient RNA has been demonstrated (Fordyce et al. 2013; Smith et al. 2014), reconstructing gene expression in antiquity through transcriptomics is beyond the reach of current ancient biomolecule technology. In contrast, tandem mass spectrometry makes ancient proteomics feasible. The complex relationship between genomes and proteomes is illustrated by comparing SEED (http://pubseed.theseed.org/) annotation profiles of metagenomic and metaproteomic data

generated from the same sample (Fig. 5.2), in this case two previously published, well-preserved medieval dental calculus samples from individuals with periodontal disease (G12, B61) (Warinner, Rodrigues, et al. 2014). SEED profiles generated from metagenomics data are highly consistent between the two samples, with the largest number of genes assigned to the functions ‘clustering based subsystems’ and ‘protein metabolism’. In contrast, SEED profiles generated from metaproteomic data are more variable and are dominated by processes related to ‘carbohydrate metabolism’, ‘protein metabolism’ and ‘microbial virulence’. The differences between the two data sets reveal differences in gene expression levels that are informative about biofilm behaviour, pathogenicity and disease progression.

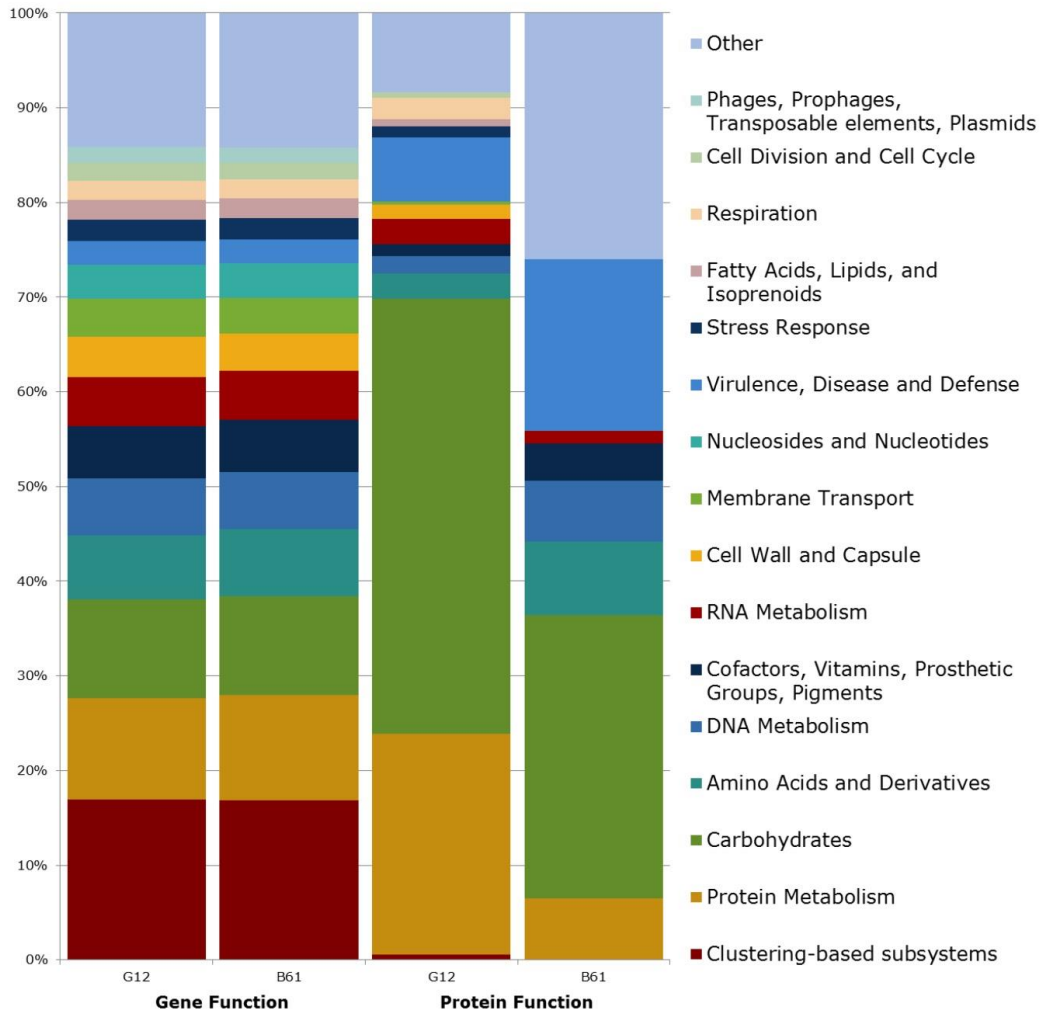

Figure 5.2. Comparison of gene and protein function as revealed by metagenomic analysis and protein mass spectrometry, respectively, of two samples of dental calculus (G12 and B61). Data from Warinner, Rodrigues, et al. (2014) was entered into MEGAN (Mitra et al. 2011) and function was compiled using SEED.

Ancient protein research has advanced in step with improvements in the resolution and sensitivity of mass spectrometry instruments. Consequently, it becomes necessary to understand the relationship between instrumental strategies and the complex mixtures of

degraded proteins that comprise many archaeological materials. This study reviews the performance of four instruments, the maXis 3G (Bruker), LTQ Velos, LTQ Elite and Q Exactive (Thermo-Scientific) with 59 samples of ancient dental tissues. Additionally, in the case of the Orbitrap Velos we compare two fragmentation techniques, collision induced dissociation (CID) and higher energy C-trap dissociation (HCD). This study also compares the performance of a simpler proteome with a well-characterised host genome (in samples of dentine and cementum) and a more complex proteome (dental calculus). We find that instrument selection has a major impact on the number of downstream protein identifications.

5.1.1 Tandem Mass Spectrometry

Enzymatically digested peptides are typically separated by hydrophobicity via nano-liquid chromatography (LC) which delivers them to the ion source, where the peptides are ionised. Subsequently, each peptide is analysed twice: first as a precursor ion in MS1 and then following collision into fragment ions in MS2 (hence *tandem* mass spectrometry, MS/MS). The mass (m) and charge (z) data from MS1 and MS2 are then compared to a database to infer the original peptide sequence and ultimately the identity of the original protein. Although the principle of MS/MS is consistent across instruments, the specific approaches used to isolate and measure the precursor and fragment ion *m/z* differ (Table 5.1). Differences in the efficiency, speed and resolution of these approaches lead to downstream differences in the protein identification rates between instruments.

LC system	Instrument	Ion Selection (MS1)	Analyser (MS2)	Collision Type	Release year
nano-Acquity UPLC (Waters)	*maXis 3G*	Quadrupole	Time of Flight (TOF)	CID	Ketterlinus and Sanders 2008
nano-Acquity UPLC (Waters)	*LTQ Orbitrap Velos*	Linear Ion Trap	Orbitrap	HCD, ETD, CID	Olsen et al. 2009
EASY-Spray UPLC + Dionex Ultimate 3000 nUPLC	*Q Exactive*	Quadrupole	Orbitrap	HCD or CID	Michalski et al. 2011
nano-Acquity UPLC (Waters)	*LTQ Orbitrap Elite*	Linear Ion Trap	Orbitrap	HCD, ETD, CID	Michalski et al. 2012

Table 5.1. Main features and release year of hybrid tandem mass spectrometers explored in this study.

5.1.2 MS/MS Instruments

We compared four alternative hybrid MS/MS instruments (Table 5.1). The maXis 3G combines a quadrupole mass filter and a TOF mass analyser, which has a very high repetition rate and therefore can analyse many ions "on the fly". In MS1, precursor ions are separated nearly instantaneously in real time, first by passage through a quadrupole (which selects a narrow mass range) and then in MS2 by the flight time of the fragment ions (following collision) in the TOF. The Q Exactive is a quadrupole-Orbitrap hybrid, which, like the maXis, uses a quadrupole mass filter in MS1 to select and transmit precursor ions to the collision cell. However, in place of a TOF, the Q Exactive uses an Orbitrap mass analyser for MS2 (Michalski et al. 2011) which separates ions based on electrostatic trapping in an orbit around a central, spindle shaped electrode. In contrast, the LTQ Orbitrap Velos (Second et al. 2009) and LTQ Orbitrap Elite (Michalski et al. 2012) use linear ion traps in MS1 to trap the precursor ions "in time". After collision, MS2 takes place in an Orbitrap. The Orbitrap Elite is

an improvement on the LTQ Orbitrap Velos, which results in double the resolution (resolution improves the ability to discriminate between two peaks of similar *m/z* ratio) and increased speed (Michalski et al. 2012). For the Orbitrap Velos, we also compared two alternative collision methods CID and HCD.

5.2 Methods

5.2.1 Materials

Three dental tissue types were analysed: dentine, cementum, and dental calculus. Dentine is a living dental tissue comprised of a mineral and organic composite and forms the majority of the tooth root structure which surrounds the soft tissues (pulp) (Hillson 2005). Cementum is a less mineralised tissue of similar composition that forms in incremental layers and facilitates tooth attachment to the periodontal ligament. In humans, cementum formation is limited to the tooth root, while in many species of animals, such as the horse, *Ovis* species and Pleistocene woolly rhinoceros analysed in this study, cementum encases the entire exposed tooth surface, including both the root and crown (Hillson 2005). Dental calculus is calcified dental plaque, a complex microbial biofilm formed through the colonisation of the tooth surface by members of the oral microbiome. Saliva is supersaturated with respect to calcium and phosphate, and precipitates both around and within bacterial cell membranes, forming a crystalline structure that preserves morphology and biomolecules, and simultaneously traps other oral debris and host molecules (Jin and Yip 2002).

Samples of ancient animal cementum were obtained from Germany - horse and *Ovid/Caprid*, n = 2 (Warinner, Rodrigues, et al. 2014) and Belgium (Pleistocene woolly rhinoceros, *Coelodonta antiquitatis*, from Trou al Wess, Belgium, n = 1 (Miller et al. 2005). Samples of

archaeological human dentine (n = 4) were obtained from Germany (Warinner, Rodrigues, et al. 2014). Cementum and dentine samples were selected because proteomes of these tissues are expected to be relatively simple, being primarily composed of mineralised collagen, and because the host genomes are well characterised (or in the case of *Coelodonta antiquitatis* the genome of the related Southern White Rhinoceros, *Ceratotherium simum simum,* can be substituted). Samples of archaeological dental calculus (n = 50) were obtained from a range of archaeological sites in the UK, Germany, and from the South Atlantic island of St Helena (Table 5.2). Samples of modern dental calculus (n = 4, two extracts each from two samples) were obtained from dental patients in Zurich, Switzerland. These samples were selected because the proteome of dental calculus is highly complex, being composed of microbial, host, and dietary proteins, and because genomic information for the oral microbiome is incomplete. Both simple and complex proteomes were selected to determine if certain instruments perform better when the target proteome is less or more complex.

Region	Date	Sample type	Species	No. of samples	Sample ID (Extraction ID)	Comparison
Britain						
Chelsea	1829 – 1853CE	Dental calculus	Human	2	SK43 (Z42), SK54 (Z43)	1
Fewston	1835-1896 CE	Dental calculus	Human	2	SLF09:SK342 (Z44); SLF09:SK351 (Z45);	1
				2	SLF09:SK053 (FW053), SLF09:SK435 (FW435)	3
Tickhill	ca. 1400-1550CE	Dental calculus	Human	4	Tickhill SKA(TKAC), Tickhill SKC (TKDC), Tickhill SKE (TKEC), Tickhill SKF (TKFC)	3
Norton	ca. 500-910 CE	Dental calculus	Human	6	2006:1500:18 (NEM18); 2006:1500:99 (NEM099); 2006:1500:93 (NEM093);	3

					NBS:03:SK262 (NBS262); NBS:03:SK325 (NBS325); NBS:03:SK410 (NBS410)	
Driffield Terrace	ca. 44-410 CE	Dental calculus	Human	1	YORYM:2004.354.SK54 (3DT54)	1, 2
				5	YORYM:2004.354.SK21 (3DT21); YORYM:2004.354.SK26 (3DT26); YORYM:2005.513.SK21 (6DT21); YORYM:2005.513.SK03 (6DT3); YORYM:2005.513.SK07 (6DT7)	3
Melton	ca. 800 BCE to 80 CE	Dental calculus	Human	4	OSA04EX03 SK1032 (ML1032); OSA04EX03 SK1489 (ML1489); OSA04EX03 SK1823 (ML1823); OSA04EX03 SK3890 (ML3890)	3
Dixon Lane	1100-1400CE	Dental calculus	Human	1	SK116	1,2
Germany						
Dalheim	ca. 950-1200 CE	Dental calculus	Human	2	G12(Z1), G12(Z2)	4
				1	B61 (Z46)	1
		Dentine	Human	4	G12 (Z23), B17 (Z24), B61 (Z25), B78 (Z26)	2
		Cementum	Cow	1	F5 (Z33)	2
			Sheep/goat	1	F1 (Z31)	2
Switzerland						
Zurich	2011	(Modern) dental calculus	Human	2*	CPMAIR (Z5, Z6); CPNATR (Z7, Z8)	2
West Central Africa						
St Helena	1840-1872	Dental calculus	Human	18	STH212 (JH1), STH218 (JH2), STH237 (JH3), STH343 (JH4), STH359 (JH5), STH421 (JH6), STH430 (JH7), STH474 (JH8), STH219 (JH10),STH268 (JH11),	3

					STH276 (JH12), STH306 (JH13), STH319 (JH14),STH389 (JH15), STH419 (JH16), STH449 (JH17), STH414 (JH19), STH423 (JH20)	
				2	STH8804 (B621-3, OTV3348), STH8726 (OTV3350)	1, 2
Belgium						
Trou al Wess	Pleistocene	Cementum	Woolly rhinoceros	1	R9721(B621-1, OTV3347)	1

Table 5.2. Samples of dental calculus and cementum analysed in this study. * Two extraction fractions were analysed separately for each sample.

5.2.2 Sample Preparation

Tryptic peptides were extracted using a modified filter-aided sample preparation (Wiśniewski et al. 2009) previously used for mineralised archaeological samples (Warinner, Rodrigues, et al. 2014). Samples were ground, weighed to between 20 and 50 mg, and decalcified in 1 mL of EDTA (0.5 M) overnight. To the supernatant, 9 mL of UA solution (8 M urea in 0.1 M Tris/HCl.) was added and the solution filtered in 10 K Amicon filters. To the pellet, 200 µL of lysis solution (4% SDS, 0.1 M DTT, 0.1 M Tris/HCl) was added, vortexed and heated at 95 ^{o}C. This second supernatant fraction was added to the Amicon filters and washed with 2 mL of UA solution. 500 µL of 2-chloroacetamide (CAA) (0.05 M) was added to alkylate cysteine residues. Filters were subsequently washed with 1 mL of UA and 1 mL of ammonium bicarbonate solution (ABC) (0.05 M), followed by a further addition of 100 µL of ABC. Retentate was suspended with 300 µL of ABC and digested using 4 µL of trypsin (0.5 µg/µL) at 37 ^{o}C overnight, with an additional trypsin digest (1 µL, 0.5 µg/µL) for four hours. Samples were then centrifuged to remove the peptides from the filter and 30-50 µL trifluoroacetic acid (TFA) (10% TFA, 90% ddH_2O) was added to stop the trypsin digest. Peptides were

immobilised on C_{18} stage tips, and washed with 150 µL methanol, 150 µL of EB80 solution (80% acetonitrile, 0.5% acetic acid, 19.5% ddH_2O) and 150 µL acetic acid (0.5% acetic acid, 99.5% ddH_2O) solution. Immediately prior to MS/MS analyses the peptides were eluted from the tips using 40 µL EB40 solution (40% acetonitrile, 0.5% acetic acid, 59.5% ddH_2O), 40 µL EB60 solution (60% acetonitrile, 0.5% acetic acid, 39.5% ddH_2O) and 40 µL EB80 solution. The extracts were evaporated to 2-4 µL, and resuspended in TFA before MS/MS analysis.

5.2.3 MS/MS Analysis

We performed three sets of comparisons. For all comparisons tryptic peptides were extracted from samples and extracts were split between the instruments.

Comparison 1. For seven samples of human dental calculus and one sample of ancient woolly rhinoceros cementum (Table 5.2) we compared the **maXis 3G**, with the **LTQ Orbitrap Velos**. The cementum was selected in order to compare the sequence coverage of collagen extracted from this source.

LC-MS/MS analysis on the maXis 3G (Bruker) used a nanoAcquity UPLC system equipped with a Symmetry C_{18}, 180 µm x 20 mm, 5 µm trap and a BEH130 C_{18}, 75 mm x 250 mm, 1.7 µm column (all Waters). The trap was washed for 5 minutes with 0.1% formic acid at 10 µL/min before switching. The gradient was 5% to 30% acetonitrile in 0.1% formic acid over 125 min at 300 nL/min and 60 °C. Positive ESI- MS and MS/MS spectra were acquired using AutoMSMS mode. Instrument settings were; ion spray voltage: 1,400 V, dry gas: 4 L/min, dry gas temperature: 160 °C, MS: acquisition range: 50-2200 *m/z*, 0.5s, MS/MS: (CID with N_2) precursor range: *m/z* 300-1500, 8 precursor ions, absolute threshold 1000 counts, 0.1seconds acquisition for precursor intensities above 100,000 counts, increasing linearly to 1 second for

precursors of 1000 counts. Collision energy and isolation width were set automatically via AutoMSMS. Preferred charge states: 2 – 4, singly charged ions excluded. A single MS/MS spectrum was acquired for each precursor and former target ions were excluded for 30 seconds.

Analyses using the LTQ Orbitrap Velos instruments were performed at two locations. Samples from Germany, Switzerland and Britain (Fewston and Chelsea) were analysed at the Functional Genomics Centre Zürich (FGCZ), while samples from Britain, Belgium and West Central Africa were analysed at the Mass Spectrometry laboratory of the Target Discovery Institute (TDI), Oxford. At the FGCZ, full-scan MS spectra (300+1700 *m/z*) were acquired in the Orbitrap with a resolution of 30,000 at 400 *m/z* after accumulation to a target value of 1,000,000. Higher energy collision induced dissociation (HCD) MS/MS spectra were recorded in data dependent manner in the Orbitrap with a resolution of 7500 at 400 *m/z* after accumulation to a target value of 100,000. Precursors were isolated from the ten most intense signals above a threshold of 500 arbitrary units with an isolation window of 2 Da. Three collision energy steps were applied with a step width of 15.0% around a normalised collision energy of 40% and an activation time of 0.1 millisecond. Charge state screening was enabled, excluding non-charge state assigned and singly charged ions from MS/MS experiments. Precursor masses already selected for MS/MS were excluded for further selection for 45 seconds with an exclusion window of 20 ppm. The size of the exclusion list was set to a maximum of 500 entries.

Samples from Britain, Belgium and West Central Africa were analysed at the Mass Spectrometry laboratory of the Target Discovery Institute. Tryptic peptides were separated on a nanoAcquity UPLC (Waters) using a 75 µm x 250 mm, 1.7 µm particle size BEH C18 column with a gradient of 1-40% Acetonitrile over 60 minutes and a flow rate of 250 nL/min.

MS1 spectra were acquired between 400 and 1800 *m/z* with a resolution of 60000 at 400 *m/z* in MS1 and 7500 in MS2 scan mode; ion targets were set to 1E6 in MS1 and 50000 in MS2 respectively. The most abundant 20 precursor ions were selected for MS2 using a normalised collision energy of 28% and dynamic auto exclusion for 27 seconds.

Comparison 2. Using both LTQ Orbitrap Velos instruments detailed above, we compared the performance of different fragmentation methods using five samples of ancient dental calculus, two samples of modern calculus, four samples of ancient human dentine and three samples of ancient animal cementum (Table 5.2). We compared the conventional collision-induced dissociation (**CID**), with higher energy collision dissociation (**HCD**), a stronger fragmentation which takes place in a separate collision cell, before returning to the C-trap before analysis in the Orbitrap.

The HCD analyses were performed as described above, while the CID analyses were performed with the following modifications. Full-scan MS spectra (300+1700 *m/z*) were acquired in the Orbitrap with a resolution of 60,000 at 400 *m/z* after accumulation to a target value of 1,000,000. CID MS/MS spectra were recorded in data dependent manner in the linear trap after accumulation to a target value of 100,000. Precursors were isolated from the 20 most intense signals above a threshold of 1000 arbitrary units with an isolation window of 1.2 Da. Precursors were activated with 35% normalised collision energy for 10 milliseconds with an activation Q of 0.25. Charge state screening was enabled excluding non-charge state assigned and singly charged ions from MS/MS experiments. Precursor masses already selected for MS/MS were excluded for further selection for 90 seconds with an exclusion window of 20 ppm. The size of the exclusion list was set to a maximum of 500 entries.

Comparison 3. Following the superior results of the LTQ Orbitrap Velos from comparison 1, we explored the advantage of a linear-ion trap to enhance the recovery of low abundance

peptides in a complex mixture by comparing the performance of two Orbitrap instruments; an **Orbitrap Elite** (performed in CID fragmentation mode) and a **Q Exactive** with 39 samples of dental calculus (Table 5.2).

Q Exactive analysis was performed after UPLC separation on an EASY-Spray column (50 cm x 75 µm ID, PepMap RSLC C_{18}, 2 µm) connected to a Dionex Ultimate 3000 nUPLC (all Thermo Scientific) using a gradient of 2-40% acetonitrile in 0.1% formic acid and a flow rate of 250 nL/min at 40°C. MS spectra were acquired at a resolution of 70,000 at 200 *m/z* using an ion target of 3E6 between 380 and 1800 *m/z*. MS/MS spectra of up to 15 precursor masses at a signal threshold of 1E5 counts and a dynamic exclusion for 7 seconds were acquired at a resolution of 17,500 using an ion target of 1E5 and a maximal injection time of 50 milliseconds. Precursor masses were isolated with an isolation window of 1.6 Da and fragmented with 28% normalised collision energy. Orbitrap Elite analysis was performed under similar LC conditions using a nanoAcquity UPLC (1.7 µm BEH130 C_{18}, 75 µm x 250 mm). MS spectra were acquired at a resolution of 120000 at 400 *m/z* using an ion target of 5E5 between 300 and 1800 *m/z*. MS/MS spectra of up to 200 precursor masses at a signal threshold of 1000 counts and a dynamic exclusion for 15 seconds were acquired in the linear ion trap using rapid scan and an ion target of 5E4. Precursor masses were isolated with an isolation window of 1.5 Da and fragmented with 35% normalised collision energy.

Comparison 4. Following the superior results of the Q Exactive, we compared the performance of the Q Exactive with the Orbitrap Velos (CID) at the FGCZ from comparison 2 using two extracts of one sample of ancient dental calculus (Table 5.2). The Q Exactive results from this comparison have been previously published (Warinner, Rodrigues, et al. 2014). The Oribtrap Velos (CID) analysis was performed using the same instrument parameters as described above in Comparison 2.

5.2.4 Peptide Identification and Evaluation

Raw MS/MS spectra were converted to searchable Mascot generic format using Proteowizard version 3.0.4743 using the 200 most intense peaks in each MS/MS spectrum. MS/MS ion database searching was performed on Mascot (Matrix Science^{TM}, version 2.4.01) against the UniProt (2014) database and the Human Oral Microbiome Database (Chen et al. 2010). Searches were performed against a decoy database to generate false discovery rates. In every platform the search parameters included carbamidomethylation as a fixed modification (owing to the addition of 2-iodoacetmide during protein extraction), and acetylation (protein N-terminus), deamidation of glutamine and asparagine, glutamine to pyroglutamate, methionine oxidation and hydroxylation of proline as variable modifications (Cappellini et al. 2012). Peptide tolerance was 10 ppm, and with a semi-tryptic search with up to two missed cleavages. MS/MS ion tolerance was 0.5 Da (Velos and Elite CID), 0.07 Da (Q Exactive and Velos HCD) and 0.1 Da (maXis 3G).

Scaffold software (version 4.2.1, Proteome Software Inc., Portland, OR) was used to elucidate the numbers of peptides shared between samples and instruments. Mascot results for each compared group were pooled using the MuDPIT option. Simple and complex proteomes were pooled in separate categories. Results were searched against a concatenated database of UniProtKB/Swiss-Prot (v.20121031; total 1076779 sequences), all proteins available in the Human Oral Microbiome Database as of 2012/10/11 (total 4476028 sequences; http://www.homd.org/), and all proteins available for Genbank genome accessions of bacteria and archaea in which the word “soil” appears in the metadata (total 2843972 sequences) as of 2012/02/22. Peptide matches were accepted if the match was greater than 95.0% probability as specified by the PeptideProphet algorithm, and protein identifications were accepted at greater than 99.0% ProteinProphet probability and contained at least two identified peptides.

5.2.5 Contamination Control

Archaeological material can be susceptible to contamination from modern proteins so it is important to take precautions and monitor for contamination and minimise false positive identifications. All protein extractions were performed in a laboratory dedicated to ancient biomolecules. Blank controls were included with every batch of protein extractions. Nitrile gloves were worn to avoid contamination with latex (a natural rubber which contains plant proteins).

5.3 Results and Discussion

In samples of ancient dental calculus, proteins related to the oral environment were identified, including host proteins (dominated by immune system proteins) and oral bacterial proteins of commensals and pathogens. In modern samples, a similar taxonomic and functional profile was observed (see Warinner, Rodrigues, et al. 2014). In samples of cementum, collagen was dominant, and in samples of human dentine identified proteins are consistent with the proteome observed in modern samples of dentine (Salmon et al. 2013).

5.3.1 Comparison 1: MaXis 3G and Orbitrap Velos

The performance of the Bruker maXis 3G and Thermo Fisher Scientific Orbitrap Velos was compared by analysing seven samples of ancient dental calculus and one sample of ancient cementum. We observe that the maXis 3G produced a broader range in the number of spectra assigned to peptides by Mascot compared to the Velos, although the mean number of assigned spectra appears to be similar (Fig. 3a). However, when comparing the *proportion* of spectra

that are assigned (i.e. the number of assigned spectra over the total number of spectra produced), Mascot is able to assign a greater proportion of spectra generated from the Velos than the maXis 3G (Fig. 3b). Analysis of data generated by the Velos also results in a greater number of protein family identifications (Fig. 3c).

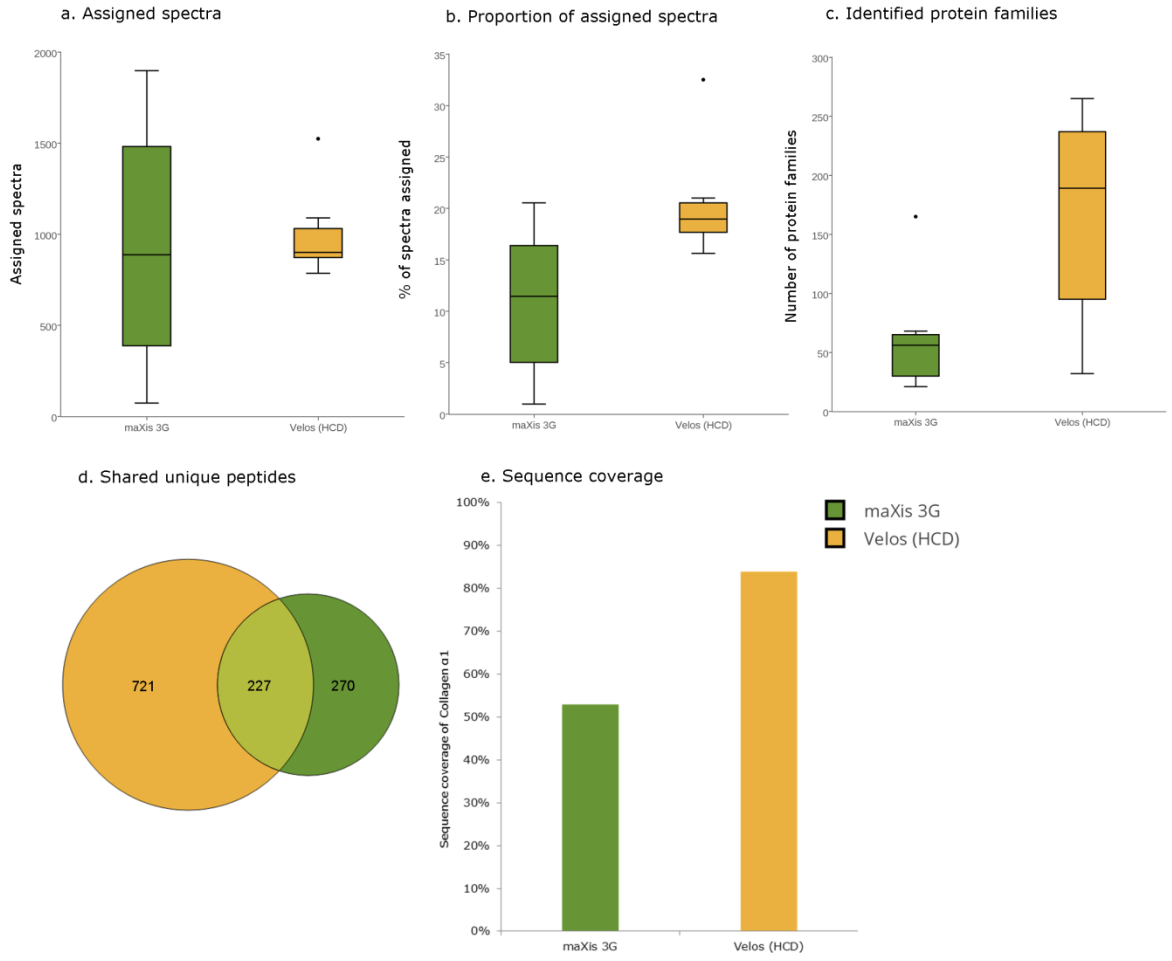

Figure 5.3. Comparison of seven samples of ancient dental tissues in the maXis 3G and Orbitrap Velos (HCD), showing a) the range of the number assigned spectra, b) the proportion of total spectra that were assigned c) the range of the number of Mascot identified protein families, d) the number of shared unique peptides and e) the sequence coverage of collagen sequence coverage of Pleistocene woolly mammoth collagen extracted from ancient cementum, matched to modern *Ceratotherium simum simum* alpha-1 collagen.

We analysed one sample of ancient cementum, a sample predicted to have a simpler proteome and found that the maXis 3G was able to identify 36 protein families, while 32 proteins were identified using the Orbitrap Velos. To further explore this we performed a search against our own database consisting only of collagen sequences, and compared sequence coverage of peptide matches to collagen alpha 1 of White Rhinoceros (*Ceratotherium simum simum*). Here, we found that the maXis 3G was able to identify 56% sequence coverage to collagen alpha 1, while the Velos (HCD) was able to produce sequence coverage of 84% (Fig. 5.3e). We assume that this greater sequence coverage of the Velos is due to the ability of the ion trap to concentrate and therefore query lower abundance ions more effectively than the maXis which lacks an ion trap. Maximising sequence coverage could be vital, for example, in the high-resolution taxonomic placement of extinct species such as these (e.g. Welker et al. 2015). Similarly, for samples with greater complexity (samples of ancient dental calculus) the Orbitrap may be able to identify a greater number of peptides because it is able to pick up ions which are much lower in abundance.

5.3.2 Comparison 2: Orbitrap Velos - HCD and CID

The method by which precursor ions are fragmented after MS1 has important consequences for the mass of the resulting fragment ions that will be analysed in MS2. For the Orbitrap family of mass spectrometers three fragmentation methods are available: collision induced dissociation (CID), higher energy collisional dissociation (HCD), and electron transfer dissociation (ETD). CID involves the isolation of precursor ions followed by bombardment with neutral gas atoms. Vibrational energy as a result of these collisions causes the weakest covalent peptide bonds to break (Frese et al. 2011). HCD involves fragmentation in the ion trap collision cell (Olsen et al. 2007), and the ions are then passed back into the C-trap for

analysis in the Orbitrap. To determine the effect of fragmentation method on downstream protein identification rates we compared the conventional CID fragmentation method with HCD. ETD, which is used primarily for post-translational modification characterisation, was not tested.

Across all samples of dental tissue (modern dental calculus, n = 4; ancient dental calculus, n = 6; ancient cementum, n = 3; ancient dentine, n = 4) CID fragmentation resulted in a greater number of identifications than HCD (Fig. 5.4), a statistically significant observation (paired t-test, $p < 0.05$). We grouped samples into two categories; simple proteomes (consisting of dentine and cementum) and complex proteomes (ancient and modern dental calculus) to test whether one fragmentation is superior for complex or simple proteomes. We found that analysis of samples produced by CID produce more assigned spectra and a greater number of identified protein families HCD for both groups

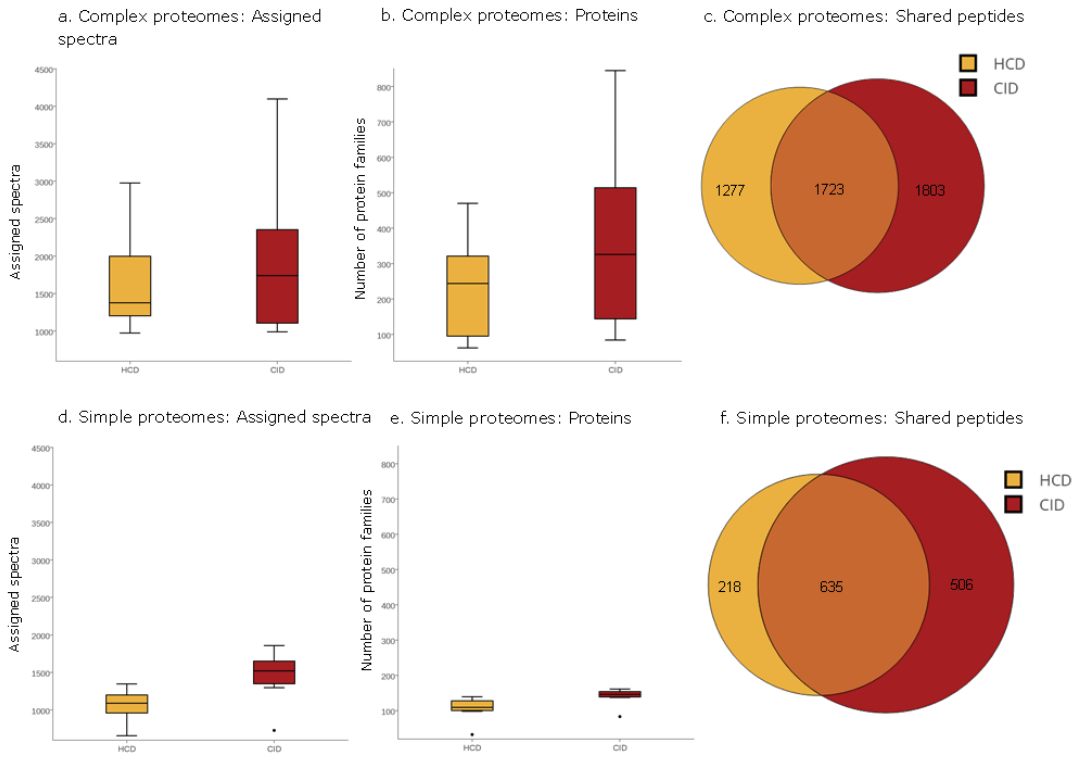

Figure 5.4. Comparison of ancient simple (n=7) and complex proteomes (n=10) analysed by higher energy collision dissociation (HCD) and collision induced dissociation (CID) in an Orbitrap Velos, showing a) the number of assigned spectra, b) the number of identified protein families and c) the number of shared unique peptides for complex proteins, and d) the number of assigned spectra, e) the number of identified protein families and f) the number of shared unique peptides for simple proteins.

Our results appear contrary to some previous studies, where HCD was found to generate more peptide identifications than CID (Sun, Zhu, and Dovichi 2013; Frese et al. 2011; Nagaraj et al. 2010). However, in the case of a whole mouse brain phosphoproteome (Jedrychowski et al. 2011), CID produced a larger data set. Although resolution is higher for HCD, acquisition speed is about half that of CID. As was noted by Jedrychowski et al. (2011) it may be the case that speed is more important than resolution when dealing with complex proteomes.

5.3.3 Comparisons 3 & 4: Q Exactive, Orbitrap Elite and Orbitrap Velos

We compared the performance of a linear ion trap instrument (Orbitrap Elite) with one that uses a quadrupole for precursor ion selection (Q Exactive) using 39 extracts of ancient dental calculus. We found that the use of the quadrupole for ion selection outperformed the linear ion trap instrument, where the mean number of protein families identified in the Elite was 64, and in the Q Exactive was 118 (Fig. 5.5). A paired t-test indicates that this is a statistically significant difference ($p < 0.05$).

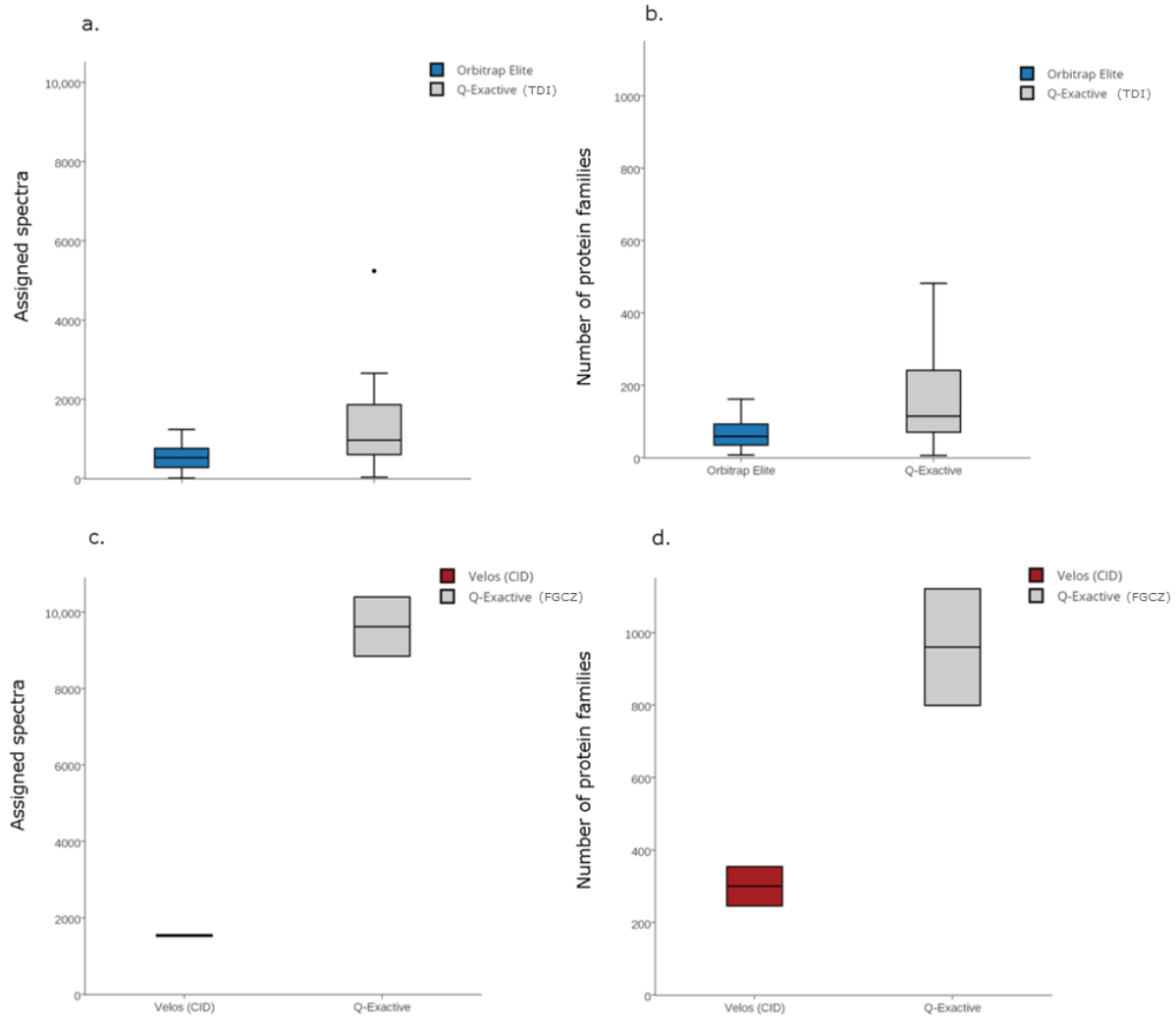

Figure 5.5. Comparison of a) the number of assigned spectra and b) the number of protein families on the Q Exactive and Orbitrap Elite with 39 samples of ancient dental calculus, and comparison of c) the number of assigned spectra and d) the number of protein families on the Q Exactive and Orbitrap Velos using two extracts of one sample of ancient dental calculus.

We also compared the performance of the Q Exactive with the Orbitrap Velos using two extracts of one exceptionally well preserved sample (G12) of ancient dental calculus. Like the comparison with the Elite, the Q Exactive outperforms the Orbitrap Velos (Fig. 5.5). In the richer extract of the two extracts the Orbitrap Velos identified 354 protein families, while the Q Exactive identified 1121 proteins families, an increase of over 200%.

The Q Exactive is an instrument where the coupling of a quadrupole mass filter to an Orbitrap mass analyser results in high mass accuracy and increased resolution (Michalski et al. 2011). Comparisons using modern material have also demonstrated the improved performance of the Q Exactive compared with older Orbitrap instruments. For example, in a study comparing varying concentrations of cell lysates the Q Exactive outperformed the Velos in terms of identifications across all sample sizes because of its increased speed and higher sensitivity (Sun, Zhu, and Dovichi 2013). This was also observed in plasma proteomic experiments (Jones et al. 2013). Our results corroborate these studies.

For complex mixtures the hybrid Quadrupole-Orbitrap (Q Exactive) outperformed other platforms tested in terms of number of protein identifications. When comparing maXis 3G with an Orbitrap instrument (Velos), we observed that data generated from the maXis 3G produced a lower proportion of assigned spectra as well as a lower number of identified protein families, even though the total number of assigned spectra was sometimes higher. Differences were also observed with fragmentation mode, with CID fragmentation producing more peptide matches than HCD.

5.3.4 Challenges of Ancient Samples

Compared to modern samples, archaeological proteins present distinct challenges. Samples are typically rare (especially those of very ancient material) and only very small sample sizes are typically available. They may be sourced from museums and may have gone through curation and treatment to aid their preservation (such as being coated in glues and resins). Analytically, they may be complex, containing a mixture of biomolecules of which the composition may be poorly understood. Archaeological samples also face the additional complexity of degradation.

Thus, the rarity, scarcity and degraded nature of ancient proteins means that optimal processing and analysis is very important if ancient proteins are to be applied in answering questions about the past.

5.3.5 Archaeological Significance

It may be obvious from a methodological standpoint that the most recent (and most technologically advanced) instrument would produce the highest rate of protein identifications. However, demonstrating the scale of this difference is important as it can have downstream consequences on the value of ancient proteins as a tool for addressing archaeological questions. Two examples of such consequences will be highlighted here.

The oral microbiome is one of the most diverse microbiomes in the body (Wade 2013b) and through the analysis of ancient dental calculus it has become possible to analyse the oral microbiome through time (Adler et al. 2013; Warinner, Rodrigues, et al. 2014; Warinner, Speller, and Collins 2015; Warinner et al. 2015). One of the key aspects of understanding both ancient and modern microbiomes is characterising both taxonomic and functional diversity. For example, Adler et al. (2013) argue that modern oral microbiomes are less diverse than historic microbiomes, possibly due to increased sugar consumption at the time of the Industrial Revolution. However, if instrumentation prohibits an understanding of this diversity (whether taxonomic or functional) this represents a major confounding factor for understanding the diversity of ancient oral microbiomes. Taking one sample of dental calculus from this study as an example (8804, St Helena), analysis of data generated from the maXis 3G identified 12 members of the human oral microbiome, whilst on the Orbitrap Velos (HCD) the same sample identified 64 species (identifications based on Mascot search against the Human Oral

Microbiome Database (Chen et al. 2010), with a significance threshold of less than 0.05 and a peptide ion score of greater than 25). Clearly, these differences could have implications for how we might interpret past microbiome diversity.

As well as understanding overall diversity, the isolation of single proteins within this complex reservoir may reveal archaeologically valuable data. This was recently highlighted by Warinner, Hendy, et al. (2014) who identified a species-specific biomarker for dairy consumption in the form of the milk protein beta-lactoglobulin (BLG). Dietary-derived components constitute a very small proportion of biomolecules present in calculus (for example, dietary proteins from St Helena dental calculus represent only 1% of all proteins identified (Chapter 9)). Thus, isolating these sequences from a substance highly dominant in bacterial or host biomolecules represents a technical challenge requiring high sensitivity. Although the sample size is limited here, we observe that of the 21 UK samples from comparison 3, the Q Exactive yielded BLG in four samples of dental calculus, whilst the Orbitrap Elite identified two. Clearly, there will be an instrumental limit to detection. Exploring this limit is essential in order minimise false-negative data, enabling the interpretation of past dairy consumption practices as accurately as possible.

5.4 Conclusion

This study quantitatively demonstrates that archaeological samples benefit the most recent technological advances in mass spectrometry, finding that the Q Exactive produced the highest number of protein identifications of all instruments tested. While this study is not a strict instrumental comparison in its truest sense given the use of different sample loads, different high-performance liquid chromatography (HPLC) setups and experiments at different

locations, this study nonetheless highlights that instrumental strategies have downstream consequences on the archaeological applicability of ancient proteins. Given the limited availability of archaeological specimens, it is important to choose the best techniques for the sample to maximise such results. In the case of archaeological proteomics there are bound to be many factors influencing protein identifications, such as the method of extraction, the presence of other biomolecules and taphonomic processes acting in the post-depositional environment, among others. A full understanding of these issues is necessarily as ancient proteomics moves forward to increasingly complex questions and materials. As instrumentation continues to advance (e.g. Beck et al. 2015) this technology will no doubt continue to broaden the scope of ancient proteomics.

Chapter 6: The Challenge of Identifying Tuberculosis Proteins in Archaeological Tissues

This chapter continues exploring methodological developments for the application of shotgun proteomics to archaeological materials. This chapter presents a proteomic analysis of mummified remains with the aim of identifying the presence of *Mycobacterium tuberculosis*. Proteomic extraction of mummified remains was performed by David Ashford and Kai Yik Teoh in 2011 and the data analysis and interpretation of these extractions were performed as part of this PhD. These results prompted a discussion into the best practice for identifying *M. tuberculosis* through the analysis of ancient proteins. In particular, this discussion occurs in the light of the approaches taken by 'Boros-Major et al. (2011). New perspectives in biomolecular palaeopathology of ancient tuberculosis: a proteomic approach. *Journal of Archaeological Science*, 38(1), pp.197–201.' A form of this paper is currently under reivew in Journal of Archaeological Science.

Abstract

Following the report of *Mycobacterium tuberculosis* proteins found in archaeological bone by Boros-Major et al. (2011), we attempted to identify *M. tuberculosis* proteins in mummified lung tissues from which ancient DNA success had already been reported. Using a filter-aided sample preparation protocol modified for ancient samples we applied shotgun proteomics to seven samples of mummified lung, chest and pleura tissues. However, we only identified four peptides with unique matches to the *Mycobacterium tuberculosis* complex, none of which

were unique to *M. tuberculosis*, although we did identify a range of human proteins and non-mycobacterial bacterial proteins. In light of these results we question the validity of the peptide mass fingerprint (PMF) approach presented by Boros-Major et al. (2011), especially because the PMF spectra presented in this study has similarities to that of human collagen, the dominant protein in the tissue under investigation. We explore the challenges of using proteomic approaches to detect *M. tuberculosis* and propose that given the contentious outcomes that have plagued ancient protein research in the past, the susceptibility of ancient material to modern contamination, and the degradation inherent in archaeological materials, caution is needed in the acquisition, analysis and reporting of proteomic data from such material.

6.1 Introduction

The detection of tuberculosis in the archaeological record reveals past patterns of this infection (Roberts and Buikstra, 2008; Roberts, 2012; Pálfi et al. 2015). Osteologically, tuberculosis can be identified typically by changes to the spine (Pott's disease). A number of other non-specific infectious lesions can be suggestive of tuberculosis, such as pitting and new bone growth on visceral rib surfaces and osteomyelitis at the end of the long bones and associated joints (Roberts and Buikstra, 2008). Skeletal lesions occur in approximately 3-5% of untreated tuberculosis cases, appearing in secondary infections when the bacteria is reactivated after being present in the body but not actively causing an infection (latent). As a result of the low frequency of skeletal invovlement during infection alternative methods to detect tuberculosis have been sought.

One approach to the study of tuberculosis in the archaeological record has been the use of amplified ancient DNA fragments (Fletcher, Donoghue, Holton, et al. 2003; Hershkovitz et al.

2008; Donoghue et al. 2011; Minnikin et al. 2012). Extended information from genomic data can reveal the phylogeny of this genus (Bouwman et al. 2012; Chan et al. 2013) and its evolution across time and space (Spigelman et al. 2015). DNA based methods have not been without controversy. Caution has been recommended in the use of the insertion sequence IS6110 as a biomarker specific to *M. tuberculosis* complex (MTBC) bacteria (Müller et al. 2015), and in the use of spoligotyping (Warren et al. 2002). Additionally, the importance of adherence to ancient DNA authentication protocols and concerns over destructive analysis have been expressed, especially in the light of adequate palaeopathological assessment (Roberts and Ingham, 2008; Donoghue et al. 2009; Wilbur et al. 2009). Consequently alternative biomarkers have been sought.

The detection of cell wall lipids, such as mycolic, mycocerosic and mycolipenic acids, is one approach (Gernaey et al. 2001; Minnikin et al. 2015, 2012), where the concentration of these lipids may be a function of the biomass of the pathogen. However this approach too has been controversial, for example, in the use of matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI TOF) mass spectrometry to detect mycolic acids (Márk et al. 2010; Minnikin et al. 2010; Márk et al. 2011). Corthals et al. (2012) adopted both ancient DNA and proteomic analyses from mouth swabs of two 500 year old Inca mummies. Targeting the *hsp65* gene using *Mycobacterium* specific primers, they identified *Mycobacterium* sp. in one of the mummies and used shotgun proteomics to argue that the same individual demonstrated an active anti-bacterial immune response to a pulmonary infection.

Boros-Major et al. (2011) reported the detection of proteins from *M. tuberculosis* in bone samples from three skeletons from Hungary, dating from 700-1600 AD. In this study the authors extracted proteins from bones of three individuals where positive *M. tuberculosis* identifications had been previously reported using ancient DNA and mycolic acids, as well as

from a number of "healthy" control samples. Using SDS-PAGE separation followed by MALDI TOF peptide mass fingerprinting and tandem mass spectrometry using MALDI TOF/TOF, proteins derived uniquely from *M. tuberculosis* were identified. A similar approach was subsequently adopted by Hajdu et al. (2012). More recently, Schmidt-Schultz and Schultz (2015) applied Western blot testing in order to identify antigen-85 (Ag85) proteins from *M. tuberculosis*, proposing that this method may be an alternative approach to identifying active tuberculosis infections in bone, although the mycobacterial specificity remains to be determined.

In light of these findings, we applied a proteomic approach to identify *M. tuberculosis* proteins from naturally mummified remains from sealed crypts at the Dominican Church of Vác, Hungary (Pap et al. 1999). We selected well-preserved, reasonably modern (18^{th}-19^{th} century) samples that had previously tested positive and negative for *M. tuberculosis* by ancient DNA amplification (Donoghue, unpublished data).

6.2 Materials and Methods

6.2.1 Materials

Mummified tissues were obtained from remains discovered in sealed crypts in the Dominican Church, Vác, Hungary (Pap et al. 1999), where around 60% of the 265 individuals uncovered were totally or partially naturally mummified (Fletcher, Donoghue, Holton, et al. 2003). These remains are those of local families and clerics interred between 1731 and 1838, and for many individuals historical records of their identities and medical histories are available; thus, this assemblage represents a rich source of information on disease in this post-Medieval population.

Ancient DNA derived from *M. tuberculosis* has been extensively studied in this population. Pap et al. (1999) initially suggested that DNA derived from *M. tuberculosis* might be well-preserved in this assemblage, and in a subsequent study 51% of 168 individuals tested positive for the IS6110 insertion sequence, suggesting the widespread presence of MTBC (Fletcher, Donoghue, Holton, et al. 2003). Further work using spoligotyping suggested variation in strains infecting a mother and her two daughters (Fletcher, Donoghue, Taylor, et al. 2003). By examining multiple sites from the body, Donoghue et al. (2011) was able to suggest that 26 of 93 (35.6%) had an infection that had disseminated to sites outside of the lungs (extra-pulmonary tuberculosis). Chan et al. (2013) used metagenomics to analyse reads of *M. tuberculosis*, revealing a mixed strain infection in a young adult female in the family group mentioned above, and Szikossy et al. (2015) used targeted PCR to identify *M. tuberculosis* in two mummies with no physiological indications of tuberculosis. More recently, Kay et al. (2015) identified 12 distinct *M. tuberculosis* genotypes in 26 individuals from 18th century Vác, and most bodies yielded more than one genotype.

For proteomic analysis, seven samples of 18^{th}-19^{th} century mummified lung tissue were selected (Table 6.1). Five samples had positive results for the presence of *M. tuberculosis* DNA and two samples did not (Donoghue, unpublished data).

Samples	Sex	Age-at-death	Tissue Type	DNA evidence of MTB infection
L6	M	41	Lung tissues	Y
L23	M	20-49	Lung tissues	Y
L36	F	60	Lung tissues	N
L64	M	30	Lung tissues	Y
C25	M	58	Chest tissues	Y
C30	F	40	Chest tissues	N
P17	M	46	Pleura tissues	Y

Table 6.1. Mummified lung tissue samples (Donoghue, unpublished data). Results of L23 have been previously published in Fletcher, Donoghue, Holton, et al. (2003).

6.2.2 Methods

Approximately 20 mg of mummified tissue were pulverised using a sterile mortar and pestle. 100 µl of SDS lysis buffer (comprised of 6 M Tris-HCl, pH 8.0, 0.1 M dithiothreitol, 4% SDS) was added to all samples, which were then heated in boiling water for 5 minutes. We then applied a filter-aided sample preparation (FASP) digestion approach (Wiśniewski et al. 2009) which has been previously been applied to ancient samples (Cappellini et al. 2014). The resulting tryptic peptides were separated with a nano-Acquity UPLC system, followed by analysis with a maXis 3G (UHR-Q-TOF) mass spectrometer (Bruker Daltonics) as described previously (Warinner, Hendy, et al. 2014).

MS/MS spectra were converted to searchable Mascot generic format using Proteowizard version 3.0.6839 using the 200 most intense peaks in each MS/MS spectrum. MS/MS ions were searched using Mascot version 2.5.1 against both UniProt (2014) and a database comprised of human sequences and *M. tuberculosis*. Peptide mass tolerance was set at 10 ppm, and fragment mass tolerance at 0.1 Da. Based on previous observations for ancient proteins (Cappellini et al. 2012), modifications included carbamidomethylation as a fixed

modification (due to treatment with iodoacetamide), and acetylation (protein N-terminus), deamidation of asparagine and glutamine, glutamine to pyroglutamate, methionine oxidation and hydroxylation of proline as variable modifications. Mascot results were filtered using a significance threshold of $p < 0.05$ and an ion score cut off of 25. Given the high number of false positive matches observed using the database comprised of *M. tuberculosis* and human sequences, we only considered identifications from UniProt (2014). For peptide matches to be considered positive at least two spectral matches to each peptide were required. BLASTp was used to verify single peptides derived from multiple spectra. These were only retained when they were uniquely matched from all non-redundant protein sequences. All peptides derived from mycobacteria were verified using BLASTp against all non-redundant protein sequences. Functional networks of identified human proteins were analysed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database version 9.0 (Jensen et al. 2009) with all default clustering and confidence settings.

6.3 Results

6.3.1 Detection of Mycobacterial Proteins

Shotgun proteomics of mummified lung tissues revealed a suite of proteins predominantly derived from the human host. In terms of mycobacterial protein detection only one sample, L64, yielded weak evidence of organisms from MTBC (Table 6.2). Within the complex *M. tuberculosis* is the most common causative agent of tuberculosis in humans, although *Mycobacterium bovis*, *Mycobacterium canettii*, *Mycobacterium orygis* and *Mycobacterium africanum* can also cause infections (Galagan, 2014). Three mycobacterial proteins were

identified, but only one was specific to MTBC bacteria and this was not unique to *M. tuberculosis.*

Samples P17 and L23 also showed evidence of peptides derived from mycobacteria, although these were either not unique beyond the genus *Mycobacterium* or were of insufficient quality. No mycobacterial proteins were detected in samples L36 and C30, which had also tested negative for MTB DNA, nor in C25. We have displayed all identified peptides, even those with poor scores, derived from mycobacterial proteins in Table 6.2.

Number of Proteins	MTBC Proteins	Gene	Protein Score	Peptide	Number of Spectra	Peptide score
P17						
69	Putative uncharacterized protein	Rv2991	45	**R.SWDHRKLGLPHM.S + Oxidation (M)***	**58**	**50**
	NAD kinase	nadK	38	**T.LDVVVR.Q**	**3**	**41**
				R.VLSAEAVDRG.S*	1	12
L6						
52	No MTBC proteins					
L64						
106	ESX-3 secretion system protein eccA4	eccA3	52	R.SGFAALTR.V	1	52
				Q.HIGETEAK.T	1	15
				K.ITLGTALAR.L	2	18
	ESX-3 secretion system protein eccC3	eccC3	47	**R.VAFTVLDR.R***	**2**	**43**
				R.SIGDVPTGIDLTK.V**	1	22
				R.TFGELEQLLLSR.Q*	1	43
				R.AQAAEQRASALWSHPD.P + 2 Deamidated (NQ)**	1	1
	Catalase-peroxidase	katG	31	**R.SPTMLATDLSLR.V + Oxidation (M)****	**2**	**40**
				R.VDLVFGSNSELR.A	1	22
				R.VDLVFGSNSELR.A + Deamidated	1	31

				(NQ)		
	DNA-binding protein HU homolog	hup	25	**R.QATAAVENVVDTIVR.A + Gln->pyro-Glu (N-term Q)***	**3**	**27**
				R.QATAAVENVVDTIVR.A + Deamidated (NQ)*	1	26
L36						
84	No MTBC proteins					
C30						
55	No MTBC proteins					
L23						
43	Peptidoglycan endopeptidase	ripB	41	T.AQATQTTLDLGR.Q + Deamidated (NQ)*	1	41
	Uncharacterized protein	MT2954	16	R.LSGNHSHG.R**	1	16
C25						
101	No MTBC proteins					

Table 6.2. Protein matches using Mascot Mowse score derived from samples of mummified tissue, where * indicates a match to level of the *Mycobacterium* genus and ** indicates a match to *M. tuberculosis* complex bacteria. Low scoring and single spectra peptide matches are only included for completeness and are unbolded.

6.3.2 Detection of the Lung Proteome

Across all samples of mummified lung tissue 66 distinct human proteins were identified. A number of proteins derived from the immune system were detected, including; eosinophil peroxidase, eosinophil cationic protein, azurocidin, neutrophil elastase, alpha-1-antitrypsin, myeloperoxidase, myeloblastin, alpha-1-antichymotrypsin, superoxide dismutase [Mn], Ig kappa chain C region, Ig kappa constant, S100-A8, and S100-A9. One particular immune protein of note is eosinophil peroxidase, an enzyme formed in eosinophil granules which demonstrates significant inhibitory activity towards *M. tuberculosis* (Borelli et al. 2003). All identified human proteins are displayed in Supplementary Table S6.1.

In the individual that yielded tentative evidence of infection with MTBC bacteria (L64), we used STRING to assess whether any functional networks between human proteins are present in these tissues. From 25 human proteins detected in this sample, leukocyte migration, defence response to fungus, collagen catabolic processes (defined as the breakdown of collagen in the extracellular matrix, usually the result of nearby proteases) and wound healing were the equally most dominant Gene Ontology (GO) Biological Processes (Ashburner et al. 2000). In comparison, C25, which did not yield any evidence of respiratory bacteria but yielded an abundance of human proteins (n=38) was also analysed using STRING, indicating extracellular matrix disassembly as the dominant GO Biological Process. In the former individual, it appears the proteomic profile is reflective of a host response to infection, a profile not observed in individual C25.

However, although this difference could indeed be the result of differences in respiratory health, variation could also be observed owing to the different tissue types these samples represent. Without more samples it is difficult to make any assessments on the effect that tissue type has on a) the preservation of proteins or b) the preservation of *M. tuberculosis*. Corthals et al. (2012) used a similar approach to suggest that the variation in proteomic profile observed in two Inca mummies indicates that one was actively responding to an infection at the time of death. While this approach could be extremely useful for identifying active infections at the time of death, protein preservation is likely to play a major role in the identification of this proteome. For example, a large difference in spectral counts was observed in the two swab samples (288 spectra from the 'Boy', and 5499 spectra from the 'Maiden'). Further work is needed to explore the effect of biomolecular preservation on proteomic profiles.

6.3.3. Other Bacterial Proteins

Other bacteria were also detected, although these appear to be derived from environmental sources, (such as *Nanoarchaeum equitans*, Cyclobacteriaceae, *Geobacillus*, *Lachnoclostridium phytofermentans*, *Acidiphilium cryptum*, *Salmonella enterica* and Pasteurellaceae). Peptide sequences of bacteria which were not environmentally derived were searched using BLASTp against a database of all non-redundant protein sequences. In L64, *Mycoplasma pneumoniae* and *Streptococcus pyogenes* were detected, both of which can be pathogenic. *M. pneumoniae* is a major cause of primary atypical pneumonia and may cause severe lower respiratory illness, especially in children, adults under 45 years old and the elderly (Atkinson et al. 2008). *S. pyogenes* is usually a commensal on the skin and mucous membranes, only causing pathogenesis in the respiratory system after the bacteria adheres to respiratory epithelial cells (Pechère and Kaplan, 2004). It is possible that this individual could have experienced a co-infection with multiple pathogenic bacteria, with MTBC being the principle pathogen (Wu and Rong, 2012). The age of this individual (30) also correlates with the most likely adult age group to be infected by *M. pneumoniae* (Foy, 2013). No bacterial species known to infect humans was confidently identified in any other sample.

6.3.4. Environmental Proteins

Following the observation of environmentally derived bacterial proteins, we additionally analysed all confidently identified peptide sequences in UniPept (Mesuere et al. 2015) in order

to assess the taxonomic origin of all proteins identified and the degree of environmental contamination in these samples. Following Mascot searches, all the confidently identified peptide sequences (those with an ion score greater than 25 and derived from more than one spectra) from all samples were combined into a single FASTA file and duplicates removed to speed up analyses, before being searched using UniPept. UniPept identifies peptides to the lowest common ancestor (LCA) by searching all peptides against a database of UniProtKB (2014) and the NCBI Taxonomy Database (Federhen, 2012).

A number of probable environmental contaminants were identified, although the number of peptides identified in each of these phyla is small. In addition to a number of environmentally derived bacteria (listed above), other potential contaminants included plants (three peptides identified to the level of Viriplantae), fungi (two peptides identified to the level of Fungi), and insects (three peptides identified to the level of Arthropoda) (Fig. 6.1).

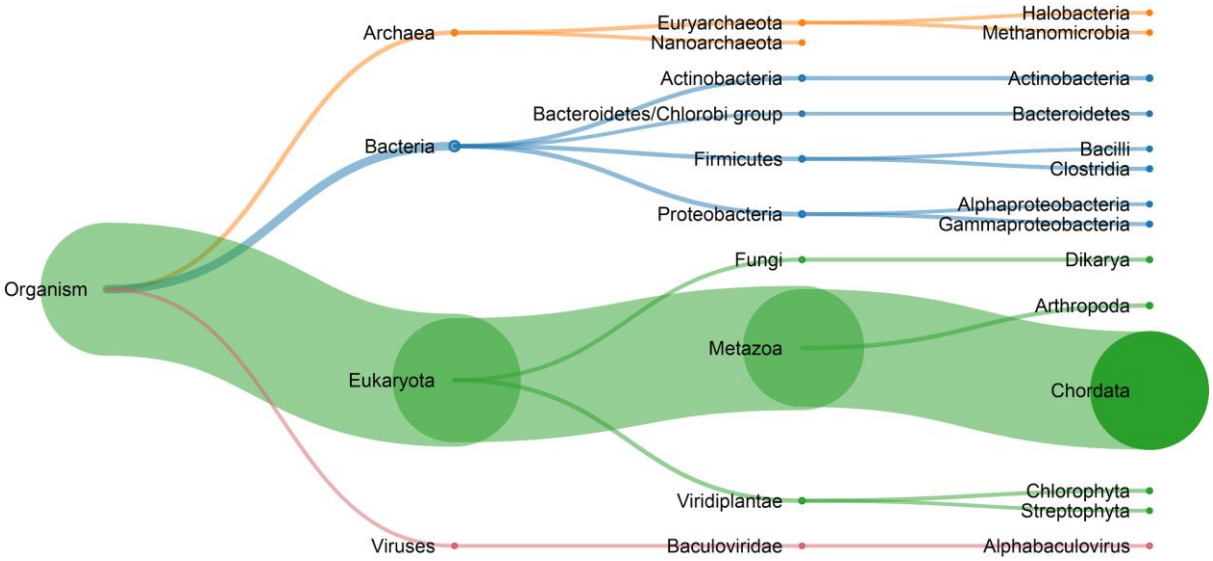

Figure 6.1. Taxonomic distribution of peptides identified across seven samples of mummified lung tissue. For ease of display the tree is restricted to the phylum level. The chordata phylum is dominant in endogenous human proteins.

6.4 Discussion

LC-MS/MS analysis of seven samples of mummified lung tissue revealed a suite of human, bacterial and environmental proteins. However, we failed to confidently detect proteins uniquely derived from *M. tuberculosis*. Only one of seven samples yielded results which could be suggestive of MTBC. Although two other individuals also yielded mycobacterial proteins (P17and L23), these must be discounted based on the lack of specificity beyond the *Mycobacterium* genus (for P17) and peptide matches of insufficient quality (for L23).

We were able to identify human proteins derived from the lung proteome, and differences in this proteome between two individuals could potentially indicate variation in respiratory health. However, we also identified a range of proteins likely to be derived from contamination from the immediate environment; from environmental bacteria, plants, insects and fungi. This suggests that, although biomolecular preservation of these remains has yielded

successful results (Donoghue et al. 2011; Fletcher, Donoghue, Taylor, et al. 2003; Fletcher, Donoghue, Holton, et al. 2003), they are by no means free from the presence of other microorganisms.

In this study we analyse well preserved mummified tissue, utilising an extraction method which has previously been successful (e.g. Cappellini et al. 2014; Warinner, Rodrigues, et al. 2014). We identify a number of non-*M. tuberculosis* specific mycobacterial peptides, as well as an abundance of proteins derived from the environment. However, in contrast to Boros-Major et al. (2011) we were unable to confidently detect proteins from *M. tuberculosis*. In light of these findings, we explore a number of methodological considerations in using ancient proteomics for the detection of *M. tuberculosis.*

6.4.1 Protein Separation

Protein or peptide separation is vital to reduce complexity prior to analysis via mass spectrometry. This can be achieved through separation of the whole protein based on its physico-chemical properties via gel based separations such as SDS-PAGE. This involves protein denaturing by sodium dodecyl sulfate (SDS), followed by separation using polyacrylamide gel electrophoresis (PAGE), which separates proteins based on their electrophoretic mobility (predominately dependent on protein size). In more recent proteomic approaches proteins are typically enzymatically digested into peptides prior to the separation of these peptides using chromatography. Given fast and high-resolution spectrometers now available, separation of peptides via liquid chromatography integrated with mass spectrometry instrument workflows may remove the need to separate proteins on gels entirely (Gevaert and Vandekerckhove, 2011).

In the analyses described by Boros-Major et al. (2011) SDS-PAGE gels are crucial for the isolation of the protein. However, for archaeological proteins, the use of PAGE to separate whole proteins can be problematic. Archaeological proteins are typically degraded. Given the resulting abundance of protein/peptide fragments, these result in smears across a gel when these fragments are separated. Smearing has been noted in extracted collagen found in archaeological bone (Dobberstein et al. 2009; Tuross et al. 1980), although is absent in gels reported by Schmidt-Schultz and Schultz (2004, 2015). As Boros-Major et al. (2011) do not display their gels the degree of protein fragmentation and how this manifests in terms of protein separation cannot be assessed. In addition, it is unclear which bands were "of interest" and how this interest was determined.

6.4.2 Peptide Mass Fingerprinting

Whilst peptide mass fingerprinting (PMF) is used in a number of approaches for ancient protein analysis (e.g. Buckley and Kansa, 2011; Richter et al. 2011; Stewart et al. 2013; Welker et al. 2015), it is less applicable for the detection of single proteins within a complex mixture. A PMF approach is based on the comparison of the distribution pattern of peptide masses between an unknown sample and a reference library of known spectra. Whilst this is particularly useful when samples are dominant in a single protein, it is less applicable for complex mixtures of proteins as numerous peptides may have similar masses which cannot be distinguished from one another.

Given the abundance of collagen in bone and the likelihood that proteins from *M. tuberculosis* would only be present in low abundance, we hypothesise that the PMF results presented in Boros-Major et al. (2011) may contain peaks that can be attributed to collagen peptides. To

test this, we aligned the MALDI TOF spectrum presented in Figure 1 of Boros-Major et al. (2011) with a typical MALDI TOF spectrum of human bone collagen (Fig. 6.2).

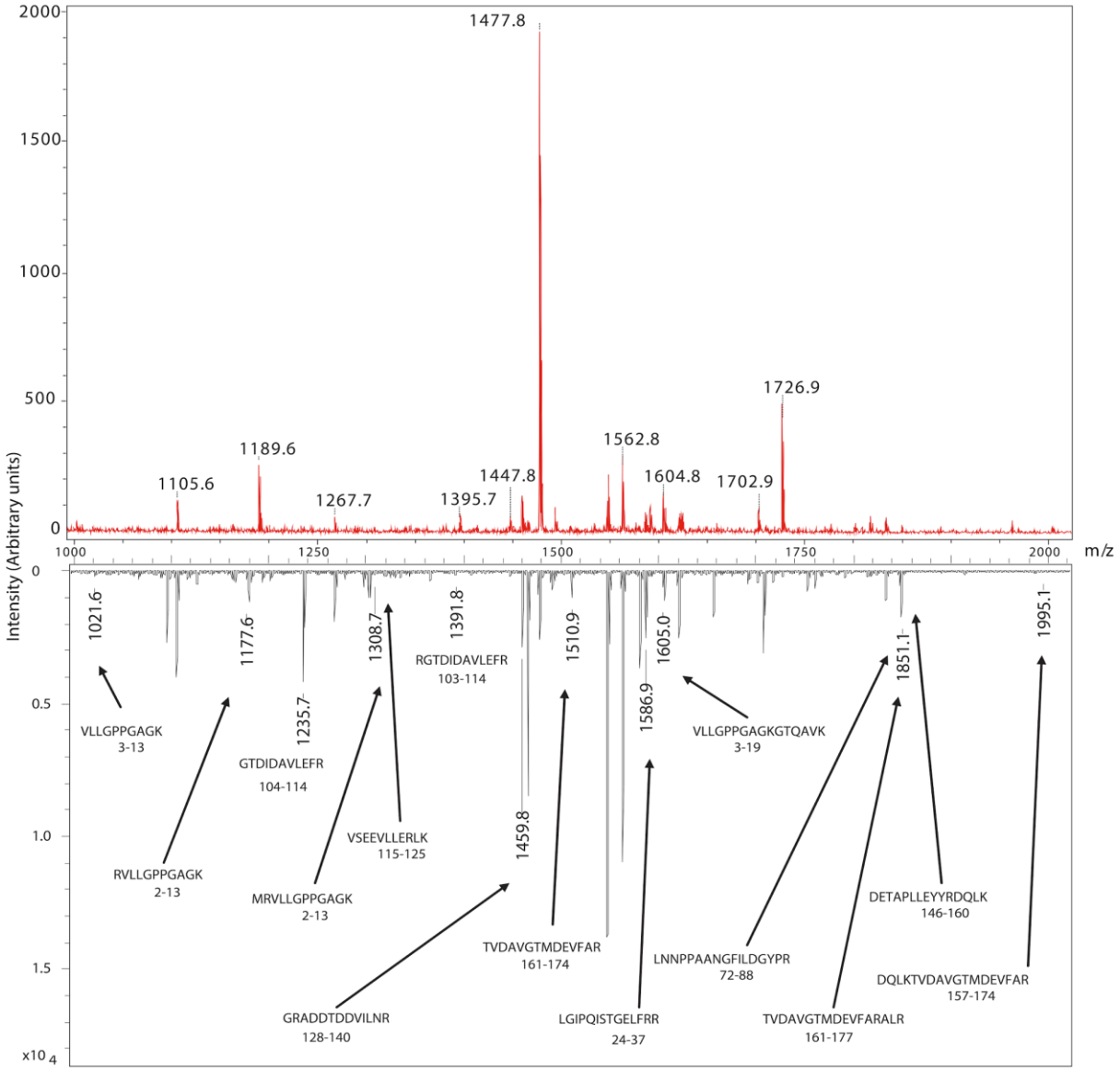

Figure 6.2. Alignment of a PMF typical for human collagen (top) with the PMF presented in Figure 1 of Boros-Major et al. (2011).

Figure 6.2 demonstrates that many *m/z* ratios are shared between both spectra. For example, the peaks observed at 1105.6, 1267.7, 1562.8, 1604.8, and 1702.9 *m/z* in human collagen (red) are all observed in the Boros-Major et al. (2011) spectrum, although they are not labelled. In

addition, a number of points are unclear; a) although the mass range acquired was 800-5000 *m/z*, only a range between 1000 and 2000 *m/z* is considered here, b) many of the most intense peaks in the spectrum are unassigned and c) some peaks that have been assigned are very small peaks or almost non-existent. In addition, three peaks appear to be incorrectly assigned or labelled. The peptide labelled at 1021 *m/z* would give an ion at 908 *m/z*; 1177 *m/z* would give an ion at 1064 *m/z*, and that at 1605 *m/z* would give an ion instead at 1492 *m/z*. All these values are incorrect by an increment of 113 *m/z*, the molecular weight of leucine or isoleucine. It is unfortunate that the authors do not provide any MS/MS spectra in support of any of these peptide identifications, especially given that this low abundance protein is so crucial to the message of the study.

6.4.3 Challenges of the Proteomic Detection of M. tuberculosis

We find a range of other bacteria in the lung samples we have analysed and it is anticipated that this would be even more problematic for bones lying in soil. Given that most human remains are buried in soil, distinguishing between pathogenic and environmental mycobacteria (Pontiroli et al. 2013) is crucial. Sequence analysis, either by DNA or proteins, is one way that this can be achieved, where variation in sequences of *Mycobacterium* species enables these two classes to be distinguished. Given the abundance of non-mycobacterial proteins found in our samples and the range of microorganisms that inhabit biological tissues and the environment, it is curious that *M. tuberculosis* was the only bacterium identified in their study. Some sequences reported by Boros-Major et al. (2011) are not specific to *M. tuberculosis*, although two sequences are conserved between *M. tuberculosis* and *M. bovis* (Table 6.3). We also note that three reported peptides fail to give a 100% match to any sequenced MTBC and perhaps represent sequences from novel environmental mycobacteria (Pontiroli et al. 2013).

Peptide	Specificity	E-value
VLLGPPGAGK	Not unique to mycobacteria	2.8
RVLLGPPGAGK*	Not unique to mycobacteria	3.2
GTDIDAVLEFR	Unique to *Mycobacterium* spp.	0.048
MRLVLLGPPGAGK*	Not unique to mycobacteria	0.16
VSEEVLLERLK	Unique to *M. tuberculosis, M. kansaii, M. bovis*	0.093
GRADDTDDVILNR	Unique to *Mycobacterium* spp.	4.00E-04
RGTDIDAVLEFR	Unique to *Mycobacterium* spp.	0.004
TVDAVGTMDEVFAR	Unique to *Mycobacterium* spp.	9.00E-06
LGIPQISTGELFRR	Unique to *Mycobacterium* spp.	6.00E-05
VLLGPPGAGKGTQAVK	Unique to *Mycobacterium* spp.	1.00E-05
LNNPPAANGFILDGYPR*	Unique to *Mycobacterium* spp.	4.00E-06
TVDAVGTMDEVFARALR	Unique to *M. tuberculosis, M. bovis*	3.00E-08
DETAPLLEYYRDQLK	Unique to *M. tuberculosis, M. simiae, M. bovis*	6.00E-07
DQLKTVDAVGTMDEVFAR	Unique to *M. tuberculosis, bovis*	2.00E-09

Table 6.3. Taxonomic specificity of peptide sequences reported in Boros-Major et al. (2011), where * denotes peptides which failed to give a 100% match against all non-redundant protein sequences using BLASTp (amino acid change is underlined). The E-value is defined as the number of hits one could expect to see by chance. Generally, the lower the E-value the more significant is the match.

In our study we also find an abundance of human proteins, most of which are structural, (collagens and keratins), some of which are tissue specific (e.g. pulmonary surfactant-associated protein B) whilst others are linked to cellular activity (e.g. prosaposin) and still others to disease (e.g. anti-trypsin). In most studies of bone proteins collagen is dominant (e.g. Wadsworth and Buckley, 2014). It is curious that this protein is only reported in "healthy" samples especially when analysis of their PMF spectrum appears to reveal peptides at the appropriate mass for human collagen. In the light of the above comments it would have been helpful if the mass spectrometry data had been made available to enable further analysis, not

least because more peptides may be discovered if the data are reanalysed in future years when more microbial genomes are available.

6.5 Conclusion

Using shotgun proteomics we analysed proteins extracted from the soft tissue of naturally mummified, post-medieval remains, targeting chest cavity tissues, lung tissues and calcified pleura, some of which were positive and others negative for *M. tuberculosis* DNA. Despite this promising material we did not identify peptides unique to *M. tuberculosis* and only tentative evidence of MTBC. Most identified proteins were derived from high abundance human extra-cellular matrix proteins, although some immune system and catabolic proteins were identified.

In our study we were unable to confidently detect proteins from *Mycobacterium tuberculosis*, in contrast to Boros-Major et al. (2011) and Hajdu et al. (2012). We are confident based upon our more recent work (Warinner, Rodrigues, et al. 2014; Warinner, Hendy, et al. 2014), that the use of higher resolution, higher sensitivity mass-spectrometric platforms will yield even more information. However, we believe it would be helpful if all archaeological journals, including the *Journal of Archaeological Science*, adhered to minimum standards for reporting protein mass-spectrometric data, including a requirement to deposit MS/MS spectra. This is not merely so that other groups are able to re-analyse the data, but recognises the fact that only a small percentage of microbial genomes have yet been sequenced and later re-analysis of the same data set may reveal additional peptides which may aid in identification. Whilst shotgun proteomics represents a potential new avenue for exploring ancient tuberculosis, the field is

very much in its infancy and considerable challenges need to be surmounted as the field moves forward.

Supplementary Table S6.1

Protein	Gene name	Score	Spectra	Sequences
P17				
Collagen alpha-2(I) chain	COL1A2	921	68	13
Collagen alpha-1(I) chain	COL1A1	630	61	17
Neutrophil defensin 1	DEFA1	127	17	2
Collagen alpha-1(III) chain	COL3A1	120	12	3
Apolipoprotein D	APOD	67	3	2
Keratin-associated protein 3-3	KRTAP3-3	70	2	1
Protein S100-A9	S100A9	66	2	1
Keratin, type I cytoskeletal 9	KRT9	58	6	3
Alpha-1-antichymotrypsin	SERPINA3	57	4	3
Dermatopontin	DPT	52	5	3
Pantothenate kinase 4	PANK4	42	4	1
CUB and sushi domain-containing protein 1	CSMD1	40	8	1
L6				
Collagen alpha-2(I) chain	COL1A2	708	70	12
Collagen alpha-1(I) chain	COL1A1	499	51	11
Ferritin light chain	FTL	187	9	2
Neutrophil defensin 1	DEFA1	89	14	3
ADP/ATP translocase 1	SLC25A	70	4	3
Protein S100-A9	S100A9	62	2	1
Serum albumin	ALB	58	2	1
Alpha-1-antichymotrypsin	SERPINA3	55	7	3
Keratin, type II cytoskeletal 1	KRT1	49	3	3
Dermatopontin	DPT	45	2	1

Mitochondrial 2-oxoglutarate/malate carrier protein	SLC25A11	42	2	1
C-type lectin domain family 17, member A	CLEC17A	34	2	1
L64				
Protein S100-A8	S100A8	1120	82	6
Protein S100-A9	S100A9	966	108	11
Collagen alpha-2(I) chain	COL1A2	764	70	13
Azurocidin	AZU1	713	69	12
Collagen alpha-1(I) chain	COL1A1	486	44	13
Neutrophil defensin 1	DEFA1	455	140	7
Myeloperoxidase	MPO	284	34	13
Eosinophil cationic protein	RNASE3	143	17	3
Keratin, type II cytoskeletal 1	KRT1	113	17	8
Myeloblastin	PRTN3	98	15	3
Complement component C9	C9	95	4	2
Keratin, type I cytoskeletal 10	KRT10	94	9	6
Collagen alpha-1(III) chain	COL3A1	88	11	3
Erythrocyte band 7 integral membrane protein	STOM	87	14	6
Resistin	RETN	84	6	2
Alpha-1-antitrypsin	SERPINA1	79	3	1
Caveolin-1	CAV1	79	7	2
Histone H4	HIST1H4A	76	4	2
Keratin, type I cytoskeletal 9	KRT9	62	3	2
Apolipoprotein D	APOD	59	5	3
Neutrophil elastase	ELANE	51	2	2
Pulmonary surfactant-associated protein B	SFTPB	50	3	1
Kv channel-interacting protein 2	KCNIP2	39	2	1
Voltage-dependent anion-selective channel protein 2	VDAC2	39	2	2
Ferritin light chain	FTL	38	2	1

Guanine nucleotide-binding protein G(t) subunit alpha-3	GNAT3	36	2	1
L36				
Collagen alpha-2(I) chain	COL1A2	633	47	11
Collagen alpha-1(I) chain	COL1A1	426	42	11
Collagen alpha-1(X) chain	COL10A1	202	13	2
Natriuretic peptides A	NPPA	197	7	2
Neutrophil defensin 1 or 3	DEFA1 or DEFA3	105	11	2
Ferritin light chain	FTL	102	8	1
Keratin, type I cytoskeletal 9	KRT9	92	5	5
Protein S100-A9	S100A9	74	4	3
Apolipoprotein D	APOD	69	6	2
Keratin, type II cytoskeletal 1	KRT1	57	10	5
Alpha-1-antitrypsin	SERPINA1	55	3	1
Alpha-1-antichymotrypsin	SERPINA3	54	7	4
Keratin, type I cytoskeletal 16	KRT16	49	6	4
Collagen alpha-1(II) chain	COL2A1	49	4	3
Dermatopontin	DPT	49	3	1
Protein S100-A8	S100A8	42	6	2
Collagen alpha-1(III) chain	COL3A1	35	8	4
C50				
Collagen alpha-2(I) chain	COL1A2	913	75	17
Collagen alpha-1(I) chain	COL1A1	782	76	22
Alpha-1-antichymotrypsin	SERPINA3	122	17	6
Alpha-1-antitrypsin	SERPINA1	111	5	3
Neutrophil defensin 1 or 3	DEFA1 or DEFA3	106	12	2
Collagen alpha-1(III) chain	COL3A1	104	14	3
Protein S100-A8	S100A8	62	3	2
Tumor necrosis factor ligand superfamily member 11	TNFSF11	60	3	1

Protein S100-A9	S100A9	40	3	1
Fibrillin-1	FBN1	30	2	2
L23				
Collagen alpha-2(I) chain	COL1A2	455	35	10
Protein S100-A8	S100A8	247	16	3
Protein S100-A9	S100A9	229	24	6
Collagen alpha-1(I) chain	COL1A1	225	24	7
Keratin, type I cytoskeletal 9	KRT9	75	2	1
Collagen alpha-1(III) chain	COL3A1	53	4	3
Neutrophil defensin 1 or 3	DEFA1 or DEFA3	46	9	2
Alpha-1-antichymotrypsin	SERPINA3	45	4	4
C25				
Zymogen granule membrane protein 16	ZG16	1125	119	16
Keratin, type II cytoskeletal 1	KRT1	666	77	20
Collagen alpha-2(I) chain	COL1A2	620	59	12
Eosinophil peroxidase	EPX	490	54	18
Collagen alpha-1(I) chain	COL1A1	481	46	11
Alpha-1-antitrypsin	SERPINA1	385	36	9
Keratin, type I cytoskeletal 10	KRT10	319	40	17
Ferritin light chain	FTL	251	18	3
Keratin, type I cytoskeletal 9	KRT9	189	22	8
Protein S100-A9	S100A9	178	14	4
Prosaposin	PSAP	147	9	3
Eosinophil cationic protein	RNASE3	135	13	2
Neutrophil defensin 1 or 3	DEFA1	130	25	3
Erythrocyte band 7 integral membrane protein	STOM	110	8	3
Collagen alpha-1(III) chain	COL3A1	110	11	5
Anti-RhD monoclonal T125 gamma1 heavy chain	IGHG1	102	8	3

Serum amyloid P-component	APCS	99	7	3
Collagen alpha-2(IV) chain	COL4A2	89	4	2
Proteoglycan 3	PRG3	88	2	1
Ig kappa chain C region	IGKC	87	8	1
Dermatopontin	DPT	67	8	4
Fibrillin-1	FBN1	66	4	3
Biglycan	BGN	60	3	2
Apolipoprotein D	APOD	59	4	2
Protein S100-A8	S100A8	59	6	2
Superoxide dismutase [Mn], mitochondrial	SOD2	59	3	1
Alpha-1-antichymotrypsin	SERPINA3	58	14	7
Vitronectin	VTN	54	3	1
Serum albumin	ALB	53	2	2
Collagen alpha-3(VI) chain	COL6A3	50	3	3
Asporin	ASPN	50	2	1
60S ribosomal protein L13a	RPL13A	49	2	1
Prenylcysteine oxidase 1	PCYOX1	47	4	3
Chymase	CMA1	44	5	3
Chymotrypsin-like elastase family member 3A	CELA3A	44	5	4
Collagen alpha-1(IV) chain	COL4A1	39	4	2
Collagen alpha-2(VI) chain	COL6A2	35	5	3
Bone marrow proteoglycan	PRG2	33	7	3
Mimecan	OGN	32	3	2
Collagen alpha-1(V) chain	COL5A1	28	2	1

Supplementary Table S6.1. Full list of human proteins identified from all samples of mummified lung tissue

Chapter 7: Poor Preservation of Antibodies in Archaeological Human Bone and Dentine

Like the preceding chapter, this chapter explores the application of proteomics to understanding ancient disease. This chapter builds on the work of Ross Kendall, who explored the potential of using immunoreactive immunoglobulin G (IgG) antibodies to detect malarial infections in archaeological skeletal material (Kendall, 2014). As a part of this study he attempted to extract and characterise archaeological IgG proteins using two previously published extraction techniques, followed by 1D gel separation, gel excision, digestion and mass spectrometry. In this PhD immunoglobulin proteins were identified in the proteomic analysis of ancient dentine from St Helena. The failure to identify IgG in the former study and the data generated from this PhD prompted an exploration of the survival of this protein. A form of this chapter is accepted (awaiting publication) in *Science and Technology in Archaeological Research*.

Abstract

The growth of proteomics-based methods in archaeology prompted an investigation of the survival of non-collagenous proteins, specifically immunoglobulin G (IgG), in archaeological human bone and dentine. Over a decade ago reports were published on extracted, immunoreactive archaeological IgG and the variable yields of IgG molecules detected by Western blots of 1D and 2D SDS-PAGE gels (Schmidt-Schulz and Schultz, 2004). If IgG can indeed be recovered from archaeological skeletal material it offers remarkable opportunities for exploring the history of disease, for example in applying functional anti-malarial IgGs to

study past patterns of malaria. More recently, the field has seen a move away from immunological approaches and towards the use of shotgun proteomics via mass spectrometry. Using previously published techniques, this study attempted to extract and characterise archaeological IgG proteins. In only one extraction method were immunoglobulin derived peptides identified and these displayed extensive evidence of degradation. The failure to extract immunoglobulins by all but one method, along with observed patterns of protein degradation, suggests that IgG may be an unsuitable target for detecting disease-associated antigens. This research highlights the importance of revisiting previously 'successful' biomolecular methodologies using emerging technologies.

7.1 Introduction

Proteins are increasingly used as a powerful research tool for understanding ancient diseases, diets, and phylogenies (Cappellini, Collins, et al. 2014; Warinner, Hendy, et al. 2014; Welker et al. 2015). New high-resolution mass spectrometric technology is now providing access to archaeological proteins of low abundance preserved in a range of materials (e.g. Cappellini et al. 2012; Wadsworth and Buckley 2014; Warinner, Hendy, et al. 2014; Warinner, Rodrigues, et al. 2014). Early archaeological bone protein research (Gürtler et al. 1981; Hedges and Wallace 1978) isolated collagen, confirming it as the dominant protein in archaeological samples. More recent research has confirmed the longevity and stability of collagen in bone approximately 1.5 million years old (Buckley and Collins 2011). Bone and dentine also contain a number of non-collagenous proteins (NCPs), and it has been argued that the high affinity of some of these (e.g. osteocalcin, matrix gla protein) for bone mineral (bioapatite) may offer a degree of protection from diagenesis following death and inhumation (Collins et al. 2000; Freundorfer et al. 1995; Masters 1987; Nielsen-Marsh and Hedges 2000; Grupe and

Turban-Just 1996; Smith et al. 2005; Wiechmann et al. 1999) but see Buckley et al. (2008). Other studies have reported apparent success in detecting NCPs in archaeological bone (e.g. Brandt et al. 2000; Cattaneo et al. 1992; Schmidt-Schultz and Schultz 2004; Wadsworth and Buckley 2014; Wiechmann et al. 1999), including bone that has undergone post excavation treatment and museum curation (Tuross 1991), and human dentine and dental calculus (Warinner, Rodrigues, et al. 2014).

Immunoglobulin antibodies represent an important target NCP group for biomolecular palaeopathological investigation, offering enormous potential for the detection and characterisation of past disease, either through the confirmation of a suspected diagnosis, or the identification of latent conditions. Immunoglobulin G (IgG) antibodies are the most abundant antibody class, accounting for approximately 75% of serum immunoglobulins (Nezlin 1998). They are large molecules (approximately 150 kDa) consisting of a classic Y-shaped paired heavy and light chain structure connected by disulfide bonds, and are mostly active in the adaptive immune response (Janeway et al. 2001). IgG titers become elevated during periods of infection and disease-specific IgGs have been clinically shown to circulate long after infection and associated pathogenic molecules have been cleared from the body. Wipasa et al. (2010), for example, demonstrated long-term stable anti-malarial IgG titers in patients in the absence of reinfection. Thus, the presence of circulating disease-specific IgGs may be used to infer past infections long after the individual has recovered. The high affinity of IgG for bioapatite and its tendency to concentrate within bone mineral (Nakagawa et al. 2010; Omelyaneko et al. 2013) should, theoretically, provide increased protection against diagenetic factors, thus enhancing the possibility of IgG surviving in a functional state. Consequently, assuming that antibodies are indeed retained in the bone, they represent a more stable target for immunological analysis than pathogenic molecules, which are transitory.

Targeting ancient antibodies as markers of disease, however, makes a number of assumptions; a) that IgG is concentrated in sufficient quantity in skeletal material, b) that they can then be successfully extracted from this reservoir, and c) that they are sufficiently well preserved to remain immunoreactive. Considering their great potential for enhancing palaeopathology, surprisingly little research has concentrated on archaeological IgG. Cattaneo et al. (1992) concluded that the protein represents a poor choice for biomolecular analysis following attempts to detect IgG using ELISA. Since then there have been at least two independent reports of successful extraction of immunoreactive and well-preserved IgGs from archaeological human bone, assessed by ELISA (Kolman et al. 1999) and SDS-PAGE (Schmidt-Schultz and Schultz 2004). However, in two recent shotgun proteomic analyses of prehistoric bone (Wadsworth and Buckley 2014; Cappellini et al. 2012) only one found highly degraded IgG (in a 43,000 year old Mammoth sample from the Siberian permafrost, Cappellini et al. 2012). This dissonance of evidence prompted an investigation specifically targeting archaeological IgG. The primary aim of this study is to assess the survival of IgG in archaeological human bone and dentine using shotgun proteomic analyses based on published extraction and characterisation techniques (Schmidt-Schultz and Schultz 2004; Jiang et al. 2007; Cappellini, Gentry, et al. 2014; Warinner, Rodrigues, et al. 2014).

7.2 Methods

7.2.1 Site Selection

To provide a focus for our study we targeted archaeological individuals from two different environments where they are likely to have been exposed to malaria. Malaria is a debilitating disease which may lead to mortality, particularly if comorbid with other conditions (Dobson

and Smith 1997). Infected individuals have been shown to exhibit long-term elevated levels of circulating IgG (Wipasa et al. 2010).

UK sites were selected by Kendall based on an association with historically recorded presence of the malaria vector *Anopheline* species (Nuttall et al. 1901), proximity to likely *Anopheline* breeding grounds (Kaufmann and Briegel 2004), topography, and *cribra orbitalia* prevalence (Gowland and Western 2012) (Table 7.1). Preservation of each sample was characterised histologically by thick section, using the 0-5 categories of the Oxford Histological Index, following Hedges et al. (1995) and Millard (2001). Ribs, phalanges and cranial fragments were selected since the removal of these elements does not cause excessive destruction to the individual skeleton. Ribs were also selected since they retain a haematopoietic function throughout life and are, therefore, always rich in blood supply (Rodak et al. 2013) and extracellular blood serum proteins (including IgG).

Two samples of historic dentine were also selected (Table 7.1). These samples derive from individuals buried on the South Atlantic island of St Helena from 1840-1872 (Pearson et al. 2011). Dentine was selected rather than bone because of the permissions in place to remove skeletal material from the island. This skeletal material represents individuals likely to be of 19th century West Central African origin. Given the historically record presence of malaria in tropical West Africa and its documented presence during the transatlantic slave trade (Chapter 3) these individuals may have been exposed to *Plasmodium falciparum* infection prior to relocation.

Site	Period	Number of samples
Hoplands, Sleaford, Lincs.	Roman	33
Watersmeet, Huntingdon, Cambs.	Roman	20
Castledyke South, Lincs.	Anglo-Saxon	51
Edix Hill, Cambs.	Anglo-Saxon	25
Highfield Farm, Littleport, Cambs.	Anglo-Saxon	66
Orchard Lane, Huntingdon, Cambs.	Medieval	18
Hanging Ditch, Manchester	Post-medieval	26
Rupert's Valley, St Helena	19th-century	2

Table 7.1. Cemetery sites selected for investigation. Lincs: Lincolnshire; Cambs: Cambridgeshire.

7.2.2 IgG Extraction and Purification

Three published protein extraction methodologies were attempted. The first ('P1') followed a protocol developed by Schmidt-Schultz and Schultz (2004). Despite the warning in the published protocol that protein purification results in NCP loss, an antibody purification technique was introduced to eliminate collagen and concentrate target IgGs. Seven human bone samples (Table 7.2) were subjected to this extraction protocol (based on their histological preservation) and subsequent IgG purification.

The second extraction protocol ('P2') was based on a multi-stage protein extraction from fresh bone in preparation for proteomic analysis (Jiang et al. 2007). The P2 extraction followed the published protocol exactly, other than a change from 1.2 M to 0.6 M HCl, after Buckley et al. (2009), and Cleland, Voegele, and Schweitzer (2012) and addition of the IgG purification stage. Eleven human bone samples were subjected to the P2 extraction and subsequent IgG purification (Table 7.2).

Due to the generally low abundance of the target protein even in modern serum samples, antibody purification requires techniques that result in the highest yields. Kolman et al. (1999), for example, extracted IgGs from relatively recent archaeological bone and purified them using HPLC over protein A affinity columns, prior to ELISA against syphilis antigens. For each P1 and P2 extraction stage, sample supernatants were subjected to thiophilic purification using a Pierce Thiophilic Adsorption Kit (Thermo Scientific), following the manufacturer-supplied methodology.

A third extraction ('P3') was performed on the two St Helena dentine samples (Table 7.2) using a modified filter-aided sample preparation (FASP) protocol described by Cappellini et al. (2014) and Warinner, Rodrigues, et al. (2014) designed for samples of archaeological bone, dentine and dental calculus.

Sample	Site	Type	Extraction Protocol
CD120.2	Castledyke	Adult phalanx	P1
CD165.1	Castledyke	Sub-adult rib	P1
HDAP2	Hanging Ditch	Adult phalanx	P1
HDAP3	Hanging Ditch	Adult phalanx	P1
HDAP5	Hanging Ditch	Adult phalanx	P1
HDAP6	Hanging Ditch	Adult phalanx	P1
HDAR4	Hanging Ditch	Adult rib	P1
CD127.2	Castledyke	Adult cranium	P2
EH156.3	Edix Hill	Adult cranium	P2
HDAN5	Hanging Ditch	Adult rib	P2
HDAP5	Hanging Ditch	Adult phalanx	P2
HDAP5	Hanging Ditch	Adult phalanx	P2
HDAP5	Hanging Ditch	Adult phalanx	P2
HDAR2	Hanging Ditch	Adult rib	P2
HDAR9	Hanging Ditch	Adult rib	P2
HP154.1	Hoplands	Adult rib	P2

OL1104.3	Orchard Lane	Adult phalanx	P2
WM2316.1	Watersmeet	Sub-adult rib	P2
SH10806	St Helena	Adult dentine	P3
SH10809	St Helena	Sub-adult dentine	P3

Table 7.2. Samples selected for IgG extraction. All bone samples scored 5 on the Oxford Histological Index.

7.2.4 IgG Detection and Characterisation

Selected samples from the P1 and P2 extractions displaying the highest post-TAC protein concentration (determined by spectrophotometry) were subjected to 1D SDS-PAGE and subsequent gel band proteomic analysis in order to identify and characterise any surviving IgGs. After multiple trials utilising slightly different precipitation techniques and buffer/gel recipes, it was found that 8% TCA precipitation (after Schmidt-Schultz and Schultz 2004) followed by introduction into a 5% stacking/15% resolving gel resulted in the clearest bands for IgG (positive control) heavy and light chains. This configuration resulted in generally clear bands for both the positive control and resolved ancient proteins. The addition of the IgG positive control proved useful in determining the ideal polyacrylamide gel concentrations, at least in terms of modern IgGs. Reserved bands were chosen and excised based on molecular weights closely corresponding to IgG heavy chains and light chains (approximately 50 kDa and 25 kDa, respectively) and were analysed by nanospray liquid chromatography-mass spectrometry (nLC-MS/MS) in the Department of Biological and Biomedical Sciences, Durham University.

Proteomic analysis of P1 and P2 samples

Excised gel bands (Fig. 7.1) and two in-solution post-TAC eluted samples, CD120.2 and EH156.3 (two samples displaying excellent histological preservation and high post-TAC protein concentration), were chosen for proteomic analysis. Tryptic digestion of samples was performed using a ProGest robot (Genomic Solutions). 15 µl of each sample fraction of tryptic peptide digest was analysed using a Dionex Ultimate 3000 nano-flow HPLC coupled to a hybrid quadrupole-TOF mass spectrometer (QStar Pulsar *i*, Applied Biosystems) fitted with a nanospray source (Protana) and a PicoTip silica emitter (New Objective). Each sample was loaded and washed on a Zorbax 300SB-C_{18}, 5 mm, 5 x 0.3 mm trap column (Agilent) and online chromatographic separation was achieved over 2 hours on a Zorbax 300SB-C_{18} capillary column (15 cm x 3.5 x 75 µm) with a linear gradient of 0-40% acetonitrile, 0.1% formic acid at a flow rate of 200 nL/minute.

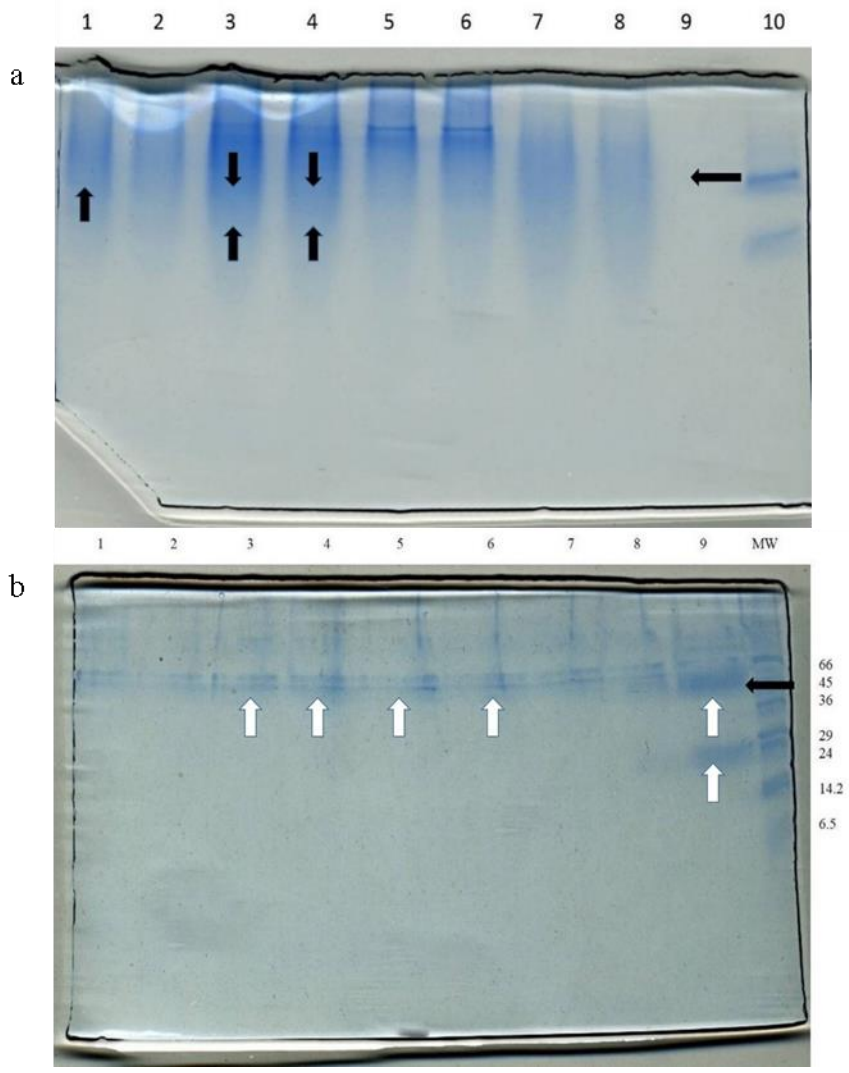

Figure 7.1. a) Example of P1 post-TAC Colloidal Coomassie stained gel. Lanes 1&2 – HDAP2; 3&4 – CD120.2; 5&6 – HDAP5; 7&8 – Castledyke 165.1; 9 - blank; 10 - IgG positive control, b) Example of P2 post-TAC Colloidal Coomassie stained gel. Lane 1 – WM2316.1; 2 – CD127.2; 3&4 – OL1104.2; 5&6 – HDAR9; 7 – HDAP5; 8&9 - IgG positive control. MW = molecular weight marker (kDa). Horizontal arrow indicates expected bands for IgG heavy chains. Vertical arrows indicate bands excised for proteomic analysis.

MS-MS data were acquired using 1 second survey scan and 3 x 3 second product ion scans throughout the peptide elution. Only ions with 2+ to 4+ charge state and with TIC > 10 counts were selected for fragmentation. Throughout the chromatographic run the mass spectrometer

cycled every 10 seconds between a 1.0 second survey scan (MS peptide parent ion mass) and 3 x 3.0 second MS-MS scans (3 peptides fragmented). ProteinPilot 2.0.1 (AB Sciex) was used to interrogate the SwissProt or human Trembl sequence databases (both accessed in September 2013) with the acquired mass data. ProteinPilot uses a probability-based (Paragon) algorithm to rank peptide-spectrum and provide protein scores, thus giving an indication of protein match confidence. The threshold for a protein identification is usually either 1.3 (one peptide at 95% confidence) or 2.0 (one peptide at 99% confidence). Additional database searching was performed using Mascot (Matrix Science), version 2.4.01, against all available sequences in SwissProt (accessed March 2015). These searches were performed against a decoy database to generate false discovery rates, with a peptide tolerance was 10 ppm and a semi-tryptic search with up to two missed cleavages. MS/MS ion tolerance was set to 0.1 Da. Based on previous observations of ancient proteome modification (Cappellini et al. 2012), carbamidomethylation was set as a fixed modification, and acetylation (protein N-term), deamidation of glutamine and asparagine, glutamine to pyroglutamate, methionine oxidation and hydroxylation of proline as variable modifications.

Proteomic Analysis of P3 (St Helena) Samples

LC-MS/MS analysis of tryptic peptides extracted from the St Helena samples was performed using a Q Exactive at the Mass Spectrometry Laboratory of the Target Discovery Institute, Oxford. Q Exactive analysis was performed after UPLC separation on an EASY-Spray column (50 cm x 75 μm ID, PepMap RSLC C_{18}, 2μm) connected to a Dionex Ultimate 3000 nUPLC (all Thermo Scientific) using a gradient of 2-40% acetonitrile in 0.1% formic acid and a flow rate of 250 nL/min at 40 °C. MS spectra were acquired at a resolution of 70,000 at 200 *m/z* using an ion target of 3E6 between 380 and 1800 *m/z*. MS/MS spectra of up to 15

precursor masses at a signal threshold of 1E5 counts and a dynamic exclusion for 7 seconds were acquired at a resolution of 17500 using an ion target of 1E5 and a maximal injection time of 50 ms. Precursor masses were isolated with an isolation window of 1.6 Da and fragmented with 28% normalised collision energy. Raw MS/MS spectra were converted to searchable Mascot generic format using Proteowizard version 3.0.6839 using the 200 most intense peaks in each MS/MS spectrum. MS/MS ion database searching was performed using Mascot against all available sequences in UniProt (2014) (accessed November 2014). Searches were performed against a decoy database to generate false discovery rates. Peptide tolerance was 10 ppm with a semi-tryptic search with up to two missed cleavages. MS/MS ion tolerance was set to 0.07 Da, with the same fixed and variable modifications as above. Mascot searches were filtered using an FDR of 2%, and an ion score cut-off of 25. BLASTp was used to verify matches to immunoglobulins.

7.3 Results and Discussion

IgG was not positively identified in any analysed samples from extractions based on Schmidt-Schultz and Schultz (2004) or Jiang et al. (2007) in searches using Protein Pilot or Mascot. Using a FASP-based approach on samples of ancient dentine peptides derived from immunoglobulins were identified, although these peptide fragments indicate post-depositional degradation. Despite the attempts to extract, purify, detect and characterise IgG, the detection of only highly degraded immunoglobulin using a single extraction methodology suggests that this protein would be unlikely to survive over archaeological time scales, let alone retain immunoreactive functionality and thus is an unsuitable candidate for detecting antigens. Some of the factors that may have influenced this detection, as well as some suggested future directions are discussed below.

Past studies attempting to extract archaeological IgGs have tested a variety of skeletal elements. Some favoured long bones, such as the femur, due to their inherently thicker cortices and inferred resistance to diagenesis. Others, such as Cattaneo et al. (1992), tested (hematopoietic) vertebral bodies in the anticipation that these would contain higher concentrations of IgG. Interestingly, none have tested dentine. Upon consideration of the results of this study, it would seem prudent that future IgG extractions should perhaps concentrate on dentine with precursory histological analysis to characterise preservation.

One of the most significant factors to overcome in the identification of NCPs is the masking of many of these proteins by collagen, invariably released during extraction. Insufficient detail in the published P1 protocol (Schmidt-Schultz and Schultz 2004) meant that it is unclear how this was overcome. Degraded collagen recovered from all extractions in our study served to mask proteins of lower concentration. Thus, it was impossible to evaluate the efficacy of the Schmidt-Schultz and Schultz (2004) protocol. Their exceptionally clean SDS-PAGE gels could not be replicated. It is possible that alternative methods of bone demineralisation and solubilisation may influence the extraction of NCPs over collagens (Cleland et al. 2012). However, despite the approaches attempted soluble collagen was present in all of the extractions, and revealed itself as a smear in all of the gels (Fig. 7.1).

7.3.1 IgG Purification and Detection

Despite the use of TAC purification designed to both remove contaminants and high abundance proteins from samples, and its potential for isolating fragmented antibodies, IgG-related peptides were not identified in any P1 or P2 sample. As suggested by Schmidt-Schultz and Schultz (2004), the introduction of a purification step may increase the opportunity for

loss of target proteins, particularly if they have a tendency to adsorb to any of the equipment or filters. However, they fail to provide a strategy for reducing the masking effect of high abundance proteins.

Proteomic analysis of SDS-PAGE gel bands failed to detect any NCPs, revealing only collagen and exogenous keratin. It is unlikely that SDS-PAGE gel bands contained sufficient concentrations for protein precipitation. Following Schmidt-Schultz and Schultz's (2004) reported detection of ancient IgGs (heavy chains) at a molecular weight approximating 55-60 kDa, it was assumed that analysis of bands around this weight range would produce positive results for IgG. However, considering the degraded state of IgGs detected in the St Helena samples, it is unlikely that any extracted IgGs would survive at the discrete molecular weights reported by Schmidt-Schultz and Schultz (2004) in their SDS-PAGE gels.

7.3.2 Proteomics (nLC-MS/MS)

Five NCPs (identified based on more than one peptide) were detected using MS/MS in the P2 (EH156.3) post-TAC in-solution eluted sample (Table 7.3). The range of post-TAC NCPs extracted and characterised in this study are a very small fraction of the whole extracted proteomes. It is likely that some of these NCPs were retained due to their close association with collagen. Biglycan, vitronectin, chondroadherin and lumican, for instance, all bind to, interact with, or form complexes with collagen (Schvartz et al. 1999; Månsson et al. 2001; Wiberg et al. 2002; Nikitovic et al. 2008).

Protein	Sequence Coverage (%)	Paragon Score	Sequence (number of spectra)	Modifications
Extraction: P1 (Sample: Castledyke (CD)120.2)				
Terminal uridylyltransferase 4	6.5	15	MDDFQLKGIVEEKFVK(1)	Oxidation(M); Lys->Allysine
Ankyrin repeat and SOCS box protein 18	9.2	13	GAHVDARNGRGETALSAACGAAR(1)	
Extraction: P2 (Sample: Edix Hill (EH)156.3)				
Vitronectin	12.1	16	DVWGIEGPIDAAFTR(1)	
		16	RVDTVDPPYPR(1)	
		16	FEDGVLDPDYPR(1)	
Chondroadherin	19.8	17	FSDGAFLGVTTLK(1)	
		14	SIPDNAFQSFGR(1)	Deamidated(N); Deamidated(Q)
Pigment epithelium-derived factor	24.6	18	DTDTGALLFIGK(3)	
		16	LAAAVSNFGYDLYR(2)	Deamidated(N)
		16	TSLEDFYLDEER(1)	
Biglycan	20.1	13	LGLGHNQIR(1)	Deamidated(N)
		16	PVPYWEVQPATFR(1)	
		14	VPSGLPDLK(1)	
Prothrombin	17.9	15	ELLESYIDGR(1)	
Protein argonaute-4	7.9	15	RPGLGTVGKPIR(1)	Deamidated(R); Oxidation(P)
Protein AHNAK2	11.1	13	GLQEDAPGRQGSAGR(1)	Deamidated(Q)
Alpha-2-HS-glycoprotein	14.4	17	HTLNQIDEVK(1)	Deamidated(N)
Osteomodulin	4.3	14	LLLGYNEISK(1)	Deamidated(N)
Lumican	10.9	13	FNALQYLR(1)	Deamidated(N)
Matrix Gla protein	36.9	18	RNANTFISPQQR(1)	Deamidated(N); Deamidated(N)
		14	YAMVYGYNAAYNR(1)	Deamidated(N); Deamidated(N)
		17	YAMVYGYNAAYNR(1)	Oxidation(M); Deamidated(N); Deamidated(N)

Table 7.3. Extracted endogenous human NCPs for P1 (CD120.2) and P2 (EH156.3) post-TAC samples. Protein scores determined by the Paragon Algorithm.

Of the sequences obtained from IgG (e.g. Fig. 7.2) in the St Helena sample, many displayed post-translational modifications (Table 7.4). These peptides display evidence of degradation, in the form of both non-tryptic cleavage and deamidation. Deamidation of glutamine and asparagine, post-translational modifications indicative of protein degradation (van Doorn et al. 2012), was observed in six of nine unique peptides. The two St Helena samples yielded 87 proteins of human origin, of which 74 were NCPs. The extraction of this relatively rich proteome suggests that these samples are reasonably well preserved, and that the degradation of IgG is not due solely to poor overall sample preservation. The detection of highly degraded IgG in these relatively recent (19th century) samples may be indicative of the poor longevity of the protein in archaeological remains. Amino acid modification of the variable light chain region (the antigen-antibody reaction site of the IgG molecule) may also limit the immunoreactivity of recovered immunoglobulins.

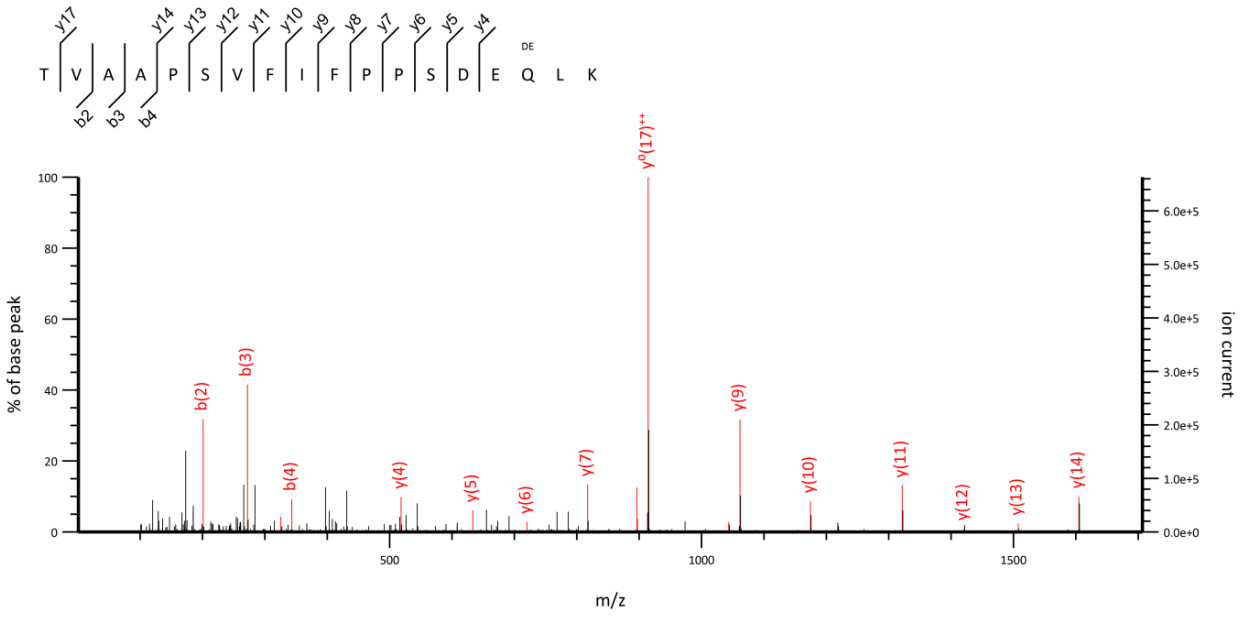

Figure 7.2. Mass spectrum from St Helena sample 10809, Ig kappa chain C region (Table 7.4).

Sample	Protein	Sequence (number of spectra)	Mascot score	Modifications
10806	Ig gamma-1 chain C region	K.FNWYVDGVEVH.N* (1)	45	
		K.FNWYVDGVEVHNAK.T* (1)	28	Deamidated (NQ)
		K.FNWYVDGVEVHNAK.T* (3)	54	Deamidated (NQ)
		K.FNWYVDGVEVHNAK.T* (2)	34	2 Deamidated (NQ)
		K.GFYPSDIAVEWESNGQPENNYK.T* (2)	52	3 Deamidated (NQ)
		K.GFYPSDIAVEWESNGQPENNYK.T* (1)	51	3 Deamidated (NQ)
	Ig lambda-1 chain C regions	Y.LSLTPEQWK.S (1)	36	
		Y.LSLTPEQWK.S (1)	52	Deamidated (NQ)
		S.SYLSLTPEQWK.S (1)	41	
10809	Ig kappa chain C region	-.TVAAPSVFIFPPSDEQLK.S* (1)	55	Deamidated (NQ)
	Ig gamma-1 chain C region	K.ALPAPIEK.T* (1)	30	
		R.VVSVLTVLHQD.W* (1)	44	Deamidated (NQ)
		R.EPQVYTLPPSR.D* (1)	29	Deamidated (NQ)

Table 7.4. Summary of Ig peptides identified in two St Helena samples of ancient dentine. *indicates a unique match to IgG.

We agree with other studies (e.g. Cappellini et al. 2012; Wadsworth and Buckley 2014) in suggesting that sample age and burial location are likely to be important factors in the survival of NCPs, including IgG. However, given the novelty of using shotgun proteomics for detecting NCPs, we do not yet fully understand the nature and extent of this preservation, especially across multiple extraction methods and instrumentation. For this study, it should be noted that making an accurate comparison of proteome preservation between St Helena and other sites is difficult given that a) a different mineralised tissue (i.e. dentine) was explored, and b) a different extraction procedure and mass spectrometer was utilised. Anglo-Saxon

proteomes were similar to those reported by Buckley and Wadsworth (2014), supporting the suggestion that certain proteins (e.g. biglycan and pigment epithelium derived factor) may preferentially survive. However, further research is necessary. Perhaps in the case of IgG it would be useful to perform sequential, high-resolution monitoring of IgG degradation in modern bone and dentine samples exposed to different burial conditions.

7.4 Conclusion

Evaluation of published protein extraction protocols (Schmidt-Schultz and Schultz 2004; Jiang at al. 2007) bone failed to yield antibodies from archaeological bone displaying excellent histological preservation. LC-MS/MS analysis of TAC elutions demonstrated non-specific binding of collagen and a small quantity of endogenous NCPs. The more sensitive modified FASP-based approach detected IgG in historic dentine, but revealed extensive degradation, despite the relatively rich proteome of these samples. This, combined with the failure to extract IgG from relatively well preserved bone samples, suggests that the poor preservation of immunoglobulins is not necessarily related to the overall structural and biomolecular preservation of these particular samples. It also calls into question the hypothesis that IgG may be preferentially protected from diagenetic factors due to its affinity for bioapatite.

It is evident that survival of IgG is not universal. Indeed, given the low levels of IgG detected, and the evidence from this and other studies of hydrolysis and deamidation, we would caution future researchers that approaches intending to use well-preserved IgG to confirm the presence of disease may not meet with the success reported over a decade ago (e.g. Kolman et al. 1999; Schmidt-Schultz and Schultz 2004). Given the evidence of non-tryptic peptide cleavage and deamidation, even in samples with rich proteomes, the challenge remains the extraction,

detection and the ultimate utilisation of an incredibly elusive biomolecule that offers high potential for palaeopathology. This research also highlights the importance of revisiting previously ‘successful’ biomolecular methodologies using the latest technologies in order to further assess their efficacy.

Chapter 8: Direct Evidence of Milk Consumption from Ancient Human Dental Calculus

This chapter explores dental calculus as resource of dietary information, demonstrating that dental calculus can act as a reservoir of dietary information, as well as the value of ancient proteomics for identifying food consumption in the archaeological record. Through the proteomic analysis of 98 samples of dental calculus, this research identifies the first direct, species-specific biomarker of milk consumption, the whey protein β-lactoglobulin (BLG), preserved in human dental calculus from the Bronze Age (ca. 3,000 BCE) to the present day. A form of this chapter is published with the reference: Warinner, Christina*, Jessica Hendy*, Camilla Speller, Enrico Cappellini, Roman Fischer, Christian Trachsel, Jette Arneborg, et al. 2014. "Direct Evidence of Milk Consumption from Ancient Human Dental Calculus." *Scientific Reports* 4 (November): 7104.*Equal author contribution.

Abstract

Milk is a major food of global economic importance and its consumption is regarded as a classic example of gene-culture evolution. Humans have exploited animal milk as a food resource for at least 8,500 years, but the origins, spread and scale of dairying remain poorly understood. Indirect lines of evidence, such as lipid isotopic ratios of pottery residues, faunal mortality profiles and lactase persistence allele frequencies, provide a partial picture of this process; however, in order to understand how, where and when humans consumed milk products, it is necessary to link evidence of consumption directly to individuals and their dairy livestock. Here we report the first direct evidence of milk consumption, the whey protein β-

lactoglobulin (BLG), preserved in human dental calculus from the Bronze Age (ca. 3,000 BCE) to the present day. Using protein tandem mass spectrometry, we demonstrate that BLG is a species-specific biomarker of dairy consumption, and we identify individuals consuming cattle, sheep and goat milk products in the archaeological record. We then apply this method to human dental calculus from Greenland's medieval Norse colonies, and report a decline of this biomarker leading up to the abandonment of the Norse Greenland colonies in the 15th century CE.

8.1 Introduction

Milk is a major nutritional resource. In addition to being a source of clean liquid (milk is 80-90% water), milk solids contain approximately 25-55% sugar (lactose), 25-45% fat, and 5-35% protein (caseins and whey proteins), as well as calcium, potassium and B-vitamins (U.S. Department of Agriculture Agricultural Research Service 2011). Adoption of animal milk consumption by humans typically requires behavioural adaptations, such as culturing and curdling techniques, to remove or reduce the lactose content of milk in order to make dairy products digestible after infancy. Additionally, populations with long pastoralist traditions in Europe and India, East Africa and the Arabian peninsula have also independently evolved lactase persistence (LP), a genetic adaptation in the regulation of the lactase gene (*LCT*) that allows continued adult digestion of milk (Ingram et al. 2009; Romero et al. 2011) (Fig. 8.1). LP is hailed as one of the clearest examples of gene-culture co-evolution in humans (Nielsen et al. 2007), yet many fundamental aspects of its evolution remain unknown (Burger et al. 2007; Gerbault et al. 2011; Lacan, Keyser, Ricaut, Brucato, Duranthon, et al. 2011; Lacan, Keyser, Ricaut, Brucato, Tarrús, et al. 2011; Sverrisdóttir et al. 2014) and the socioeconomic context and scale of prehistoric and historic dairying are only poorly understood.

The ability to directly identify milk consumption patterns in past populations would thus advance understanding of human dietary ecology, evolution and cultural agency. However, previous attempts to directly measure milk consumption using bone calcium isotopes have proven unsuccessful (Reynard, Henderson, and Hedges 2011). Other milk biomarkers offer only indirect lines of evidence. For example, isotopic inference of milk lipids from pottery residues (Evershed et al. 2008) is the most widely used approach to identify dairying, but this method cannot discriminate species of origin, and reuse of communal vessels and exploitation of cervid adipose tissue (Craig et al. 2012) both pose further challenges to interpretation. Milk proteins have also been recovered from food residues (Craig et al. 2000; Hong et al. 2012; Yang et al. 2014), but such finds are exceptional and rare, and animal bone evidence for the exploitation of secondary products is limited by the availability of large, well preserved assemblages to provide interpretable mortality profiles (McGrory et al. 2012; Vigne and Helmer 2007).

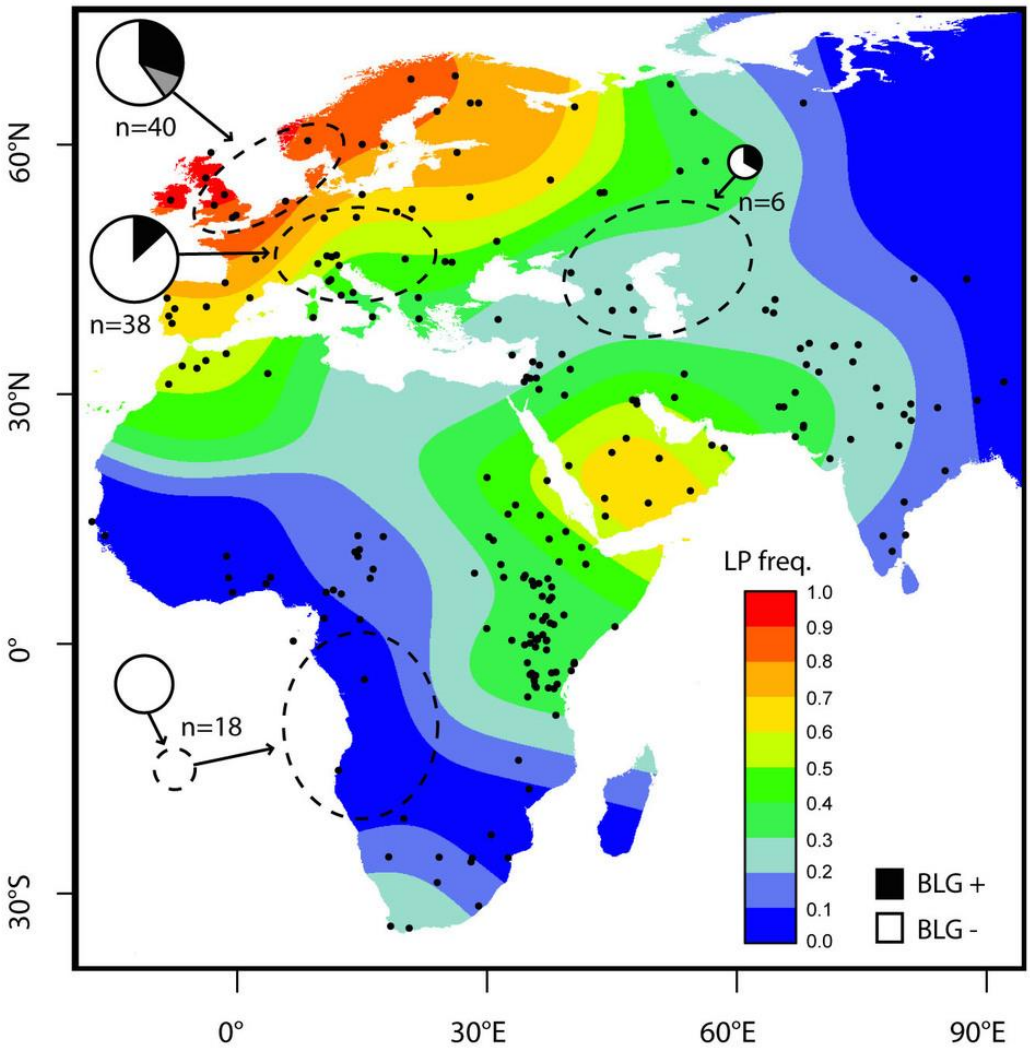

Figure 8.1. Locations of historic populations analysed in this study and contour map of present day lactase persistence frequency inferred from LP frequency data. Archaeological dental calculus samples analysed in this study were selected from regions (dashed ovals) where present day LP allele frequencies are high (Northern Europe: Britain, Norway, Denmark), moderate (Central Europe: Germany, Hungary, Italy), low (northern Southwest Asia: Armenia, Russia), and very low (Central West Africa, buried on the island of St Helena). Pie charts for each region are scaled by sample size and indicate the proportion of individuals from each region testing positive for milk BLG peptides (black) in dental calculus. A pooled sample of five individuals from Norway testing positive for BLG is shown in grey indicating the uncertainty of the number of BLG+ individuals. Interpolated contour map of lactase persistence frequencies were generated from allele frequencies of all five known LP causal alleles (-13907*G, -13910*T, -13915*G, -14009*G and -14010*C) in present day populations

in Europe, Africa, and northern Southwest Asia. Data points are shown as dots and interpolation may be inaccurate where there are few data points.

To address these problems this study turned to dental calculus, a mineralised form of dental plaque that serves as a long-term reservoir of dietary biomolecules and microfossils (Warinner, Rodrigues, et al. 2014). Nearly ubiquitous in archaeological populations and sourced directly from the oral cavity, dental calculus presents a unique opportunity to access primary evidence of ancient diets at an individual level. Warinner, Rodrigues, et al. (2014) identified the milk whey protein β-lactoglobulin (BLG) in the dental calculus of a modern Swiss dental patient using tandem mass spectrometry. This protein was also recently identified within preserved kefir cheese curds associated with the mummified remains of Bronze Age Xiaohe pastoralists (ca. 1980-1450 BC) in Xinjiang, China (Yang et al. 2014). BLG is a lipocalin within the calycin superfamily of proteins (Sawyer and Kontopidis 2000) and it is the dominant whey (milk serum) protein in ruminant milk, making up 11% of the total milk proteins and 50% of the whey proteins (O'Connor 1995). BLG offers many advantages as a milk biomarker (Sawyer and Kontopidis 2000; O'Connor 1995) including: a) humans do not produce BLG and therefore the presence of the protein in dental calculus excludes a host origin; b) BLG is present only in milk and thus it is a specific biomarker for this fluid; c) BLG is more resistant to enzymatic degradation and microbial proteolysis than other milk proteins (Bertrand-Harb et al. 2003); d) BLG lacks close bacterial orthologs, making it readily identifiable against a background of bacterial proteins; e) over half of the amino acid residues in BLG are variable among traditional dairy livestock, allowing genus and species discrimination between cattle, buffalo, sheep, goat, horse, donkey and reindeer, among others; f) BLG is the dominant protein in the whey fraction of milk and partitions with lactose during dairy processing, thereby making BLG a superior proxy for lactose compared to caseins or milk fats, which separate from the lactose-rich whey fraction during cheese and butter

production (O'Connor 1995); g) and finally, because BLG is identified directly from protein sequence data, uncertainties arising from indirect detection methods, such as isotopic analysis, are minimised.

This study employed shotgun protein analysis by liquid chromatography-tandem mass spectrometry to identify dairy consumption in the archaeological record. In this study we analysed 92 archaeological dental calculus samples selected from regions with (Europe and northern Southwest Asia) and without (Central West Africa) long-standing dairying traditions for the presence of BLG (Fig. 8.1; Table 8.1). After establishing that BLG can be detected in the dental calculus of archaeological populations from dairy-consuming regions, we then applied this approach to six additional medieval dental calculus specimens from the Norse Greenland sites of Brattahlið (Qassiarsuk) and Sandnes (Kilaarsarfik). These sites date to a period during which a major dietary shift from ruminant dairy to marine resources has been previously hypothesised on the basis of bone stable isotope and zooarchaeological evidence (Arneborg et al. 2006; Arneborg et al. 2012). We confirm that a decline in BLG observed at these sites is consistent with a dietary shift, and specifically diminished access to dairy products, leading up to the abandonment of the Norse Greenland colonies in the 15th century CE.

8.2 Methods

8.2.1 Interpolated Contour Map of Present Day Lactase Persistence

To assist in the selection of archaeological samples for analysis, we generated an updated interpolated contour map of present day LP frequency in Europe, northern Southwest Asia, and Africa (Fig. 8.1) using recently published LP genotype data (Itan et al. 2009; Ranciaro et

al. 2014). We then selected archaeological dental calculus samples from individuals from sites located within regions with high (Northern Europe), moderate (Central Europe), low (northern Southwest Asia) and very low (Central West Africa) present-day LP frequencies. Lactase persistence frequency data were estimated from allele frequency data assuming dominant inheritance and was taken, where possible, from full sequencing of the lactase enhancer to include all five published known functional LP variants (Jones et al. 2013).

Additional data taken from genotyping of the individual SNPs, where informative, as recorded in the GLAD database http://www.ucl.ac.uk/mace-lab/resources/glad (Itan et al. 2009), but revised and updated to include more recent publications through March 2014 (Ranciaro et al. 2014). Latitude and longitude of the data points were taken as near as possible to the collection sites where these were known. Where country alone was known these were estimated using major cities. The contour map was constructed in 'R' (v.3.1.0, 2014-04-10, "Spring Dance") using the spatstat package (Baddeley and Turner 2004) and included weighting for sample size. Interpolation smoothing was conducted at the lowest non-overflowing bandwidth (value of sigma) allowable from the heterogeneous data available. Interpolation may be inaccurate where there are few data points and it should be noted that neighbouring populations with different ancestry and life-style, in Africa particularly, sometimes have very different allele frequencies.

8.2.2 Samples and MS/MS analysis

Dental calculus samples (n=98) were obtained from diverse historic human populations in Eurasia, Africa, and Greenland dating from the Bronze Age to the present (Table 8.1). Dental calculus was removed using a dental scaler and stored in sterile 2.0 mL tubes until further analysis. Tryptic peptides were extracted from decalcified dental calculus using a filter-aided sample preparation (FASP) protocol modified for degraded samples (Cappellini et al. 2014)

according to previously published protocols (Warinner, Rodrigues, et al. 2014). The extracted peptides were then analysed using shotgun protein tandem mass spectrometry (LC-MS/MS) to detect the presence of β-lactoglobulin. MS/MS analyses of samples were performed at three independent laboratories in Switzerland, the UK, and Denmark.

Functional Genomics Center Zurich at the University of Zurich and Swiss Federal Institute of Technology

Samples from Greenland and Germany (Z1, Z2, Z27, Z46, and Z28) were analysed by tandem mass spectrometry at the Functional Genomics Centre Zürich (FGCZ) using an LTQ Orbitrap VELOS mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) coupled to an Eksigent-NanoLC-Ultra 1D plus HPLC system (Eksigent Technologies, Dublin (CA), USA). Solvent composition at the two channels was 0.2% formic acid, 1% acetonitrile for channel A and 0.2% formic acid, 100% acetonitrile for channel B. Peptides were loaded on a self-made tip column (75 µm × 80 mm) packed with reverse phase C_{18} material (AQ, 3 µm 200 Å, Bischoff GmbH, Leonberg, Germany) and eluted with a flow rate of 250 nL/min by a gradient from 0.8% to 4.8% of B in 2 min, 35% B at 57 min, 48% B at 60 min, 97% at 65 min. Full-scan MS spectra (300−1700 *m/z*) were acquired in the Orbitrap with a resolution of 30,000 at 400 *m/z* after accumulation to a target value of 1,000,000. Higher energy collision induced dissociation (HCD) MS/MS spectra were recorded in data dependent manner in the Orbitrap with a resolution of 7500 at 400 *m/z* after accumulation to a target value of $100,000$. Precursors were isolated from the ten most intense signals above a threshold of 500 arbitrary units with an isolation window of 2 Da. Three collision energy steps were applied with a step width of 15.0% around a normalised collision energy of 40% and an activation time of 0.1 milliseconds. Charge state screening was enabled excluding non-charge state assigned and singly charged ions from MS/MS experiments. Precursor masses already selected for MS/MS

were excluded for further selection for 45 seconds with an exclusion window of 20 ppm. The size of the exclusion list was set to a maximum of 500 entries.

Proteomics Discovery Institute at the University of Oxford

Samples from Britain, Germany (Y47, Y48, and Y49), St Helena and Italy (ODN19-1, ODN98-1, ODN207-1, ODN271-1, ODN361-1, ODN424-1, ODN458-1, SCR227-1, SCR250-1, SCR264-1, SCR323-1, SCR832-1, SCR5082-1, SCR5042-1, SCR5070-1) were analysed at the Target Discovery Institute, Oxford on Q Exactive and Orbitrap Elite tandem mass spectrometers.

Q Exactive analysis was performed after UPLC separation on an EASY-Spray column (50 cm x 75 µm ID, PepMap RSLC C_{18}, 2 µm) connected to a Dionex Ultimate 3000 nUPLC (all Thermo Scientific) using a gradient of 2-40% Acetonitrile in 0.1% Formic Acid and a flow rate of 250nL/min at 40°C. MS spectra were acquired at a resolution of 70,000 at 200 *m/z* using an ion target of 3E6 between 380 and 1800 m/z. MS/MS spectra of up to 15 precursor masses at a signal threshold of 1E5 counts and a dynamic exclusion for 7 seconds were acquired at a resolution of 17,500 using an ion target of 1E5 and a maximal injection time of 50 milliseconds. Precursor masses were isolated with an isolation window of 1.6 Da and fragmented with 28% normalised collision energy.

Orbitrap Elite analysis was performed under similar LC conditions as above using a nanoAcquity UPLC (1.7 um BEH130 C18, 75um x 250mm). MS spectra were acquired at a resolution of 120,000 at 400 *m/z* using an ion target of 5E5 between 300 and 1800 *m/z*. MS/MS spectra of up to 200 precursor masses at a signal threshold of 1,000 counts and a dynamic exclusion for 15 seconds were acquired in the linear ion trap using rapid scan and an

ion target of 5E4. Precursor masses were isolated with an isolation window of 1.5 Da and fragmented with 35% normalised collision energy.

Novo Nordisk Foundation Center for Protein Research at the University of Copenhagen

Samples from Norway, Denmark, Hungary, Germany (RISE 472 and RISE 473), Italy (RISE 466 and RISE 467), Armenia, and Russia were analysed by tandem mass spectrometry at the Novo Nordisk Foundation Center for Protein Research at the University of Copenhagen, Denmark using a Q Exactive mass spectrometer. The LC-MS system consisted of an EASY-nLC™system (Thermo Scientific, Odense, Denmark) connected to the Q Exactive (Thermo Scientific, Bremen, Germany) through a nano electrospray ion source. 5 µL of each peptide sample was auto-sampled onto and directly separated in a 15 cm analytical column (75 µm inner diameter) in-house packed with 3µm C_{18} beads (Reprosil-AQ Pur, Dr. Maisch) with a 130 minute linear gradient from 5% to 25% acetonitrile followed by a steeper linear 20 minute gradient from 25% to 40% acetonitrile. Throughout the gradients a fixed concentration of 0.5% acetic acid and a flow rate of 250 nL/min were set. A final washout and column re-equilibration added an additional 20 minutes to each acquisition. The effluent from the HPLC was directly electrosprayed into the mass spectrometer by applying 2.0 kV through a platinum-based liquid-junction. The Q Exactive was operated in data-dependent mode to automatically switch between full scan MS and MS/MS acquisition. Software control was Tune version 2.2-1646 and Excalibur version 2.2.42, and the settings were adjusted for 'sensitive' acquisition. Briefly, each full scan MS was followed by up to 10 MS/MS events. The isolation window was set at 2.5 Th and a dynamic exclusion of 90 seconds was used to avoid repeated sequencing. Only precursor charge states above 1 and below 6 were considered for fragmentation. A minimum intensity threshold for triggering fragment MS/MS was set at

1e5. Full scan MS were recorded at resolution of 70,000 at *m/z* 200 in a mass range of 300-1750 *m/z* with a target value of 1e6 and a maximum injection time of 30 milliseconds. Fragment MS/MS were recorded with a fixed ion injection time set to 108 millisecondss through a target value set to 2e5 and recorded at a resolution of 35,000 with a fixed first mass set to 100 *m/z*. Normalised collision energy was 25%.

8.2.3 Data Analysis

Raw MS/MS spectra were converted to searchable Mascot generic format using Proteowizard version 3.0.4743 using the 200 most intense peaks in each MS/MS spectrum. MS/MS ion database searching was performed on Mascot (Matrix Science, version 2.4.01) against all available sequences in UniProt and the Human Oral Microbiome Database (HOMD) (Chen et al. 2010). Searches were performed against a decoy database to generate false discovery rates. Peptide tolerance was 10 ppm, and with a semi-tryptic search with up to two missed cleavages. MS/MS ion tolerance was set to 0.07 Da. Based on previous observations of ancient proteome degradation (Cappellini et al. 2012), post-translational modifications were set as carbamidomethylation (fixed modification), and acetylation (protein N-term), deamidation of glutamine and asparagine, glutamine to pyroglutamate, methionine oxidation and hydroxylation of proline (variable modifications). Mascot search results were filtered using an ion score cut-off of 25 and significance threshold of $p<0.05$. BLASTp was used to verify matches to β-lactoglobulin, and taxonomic assignment is reported based on the consensus peptide assignments for each individual. The three-dimensional structure of bovine β-lactoglobulin protein, rendered from PDB 3NPO using Visual Molecular Dynamics software (Humphrey, Dalke, and Schulten 1996) VMDn v.1.9.1, http://www.ks.uiuc.edu/Research/vmd/current).

Contamination Exclusion

It is important to monitor and test for contamination because bovine proteins are used in some proteomics laboratories as instrument standards (e.g. bovine fetuin), among other purposes. In order to exclude such contaminants as the source of the BLG peptides in dental calculus, negative extraction controls, bovine fetuin protein standards and isopropanol wash steps were analysed with the experimental samples in parallel. No BLG peptides were observed in any non-template negative extraction controls (n=12), bovine fetuin standards (n=21), or isopropanol wash steps (n=9).

BLG Protein Modelling

The three-dimensional structure of bovine β-lactoglobulin protein was rendered from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) accession 3NPO (unliganded, DOI:10.2210/pdb3npo/pdb) using VMD v.1.9.1 (Humphrey, Dalke, and Schulten 1996). The mapped locations of all BLG peptide sequences identified by tandem mass spectrometry within archaeological dental calculus were then visualised in red, while unmapped regions were visualised in white.

Bone Collagen Stable Isotope Analysis

Bone collagen from Tjodhilde's Church individuals KAL1052 and KAL1064 was prepared for stable isotope $\delta^{13}C$ and $\delta^{15}N$ analysis as previously described (Craig et al. 2010). Duplicate collagen specimens (1 mg) were measured using a Sercon 20-22 Isotope Ratio Mass

Spectrometer coupled to a Sercon GSL Elemental Analyser in the Department of Archaeology at the University of York. The results for KAL1052 are as follows: $d^{13}C$, -18.7352, -18.5608; $d^{15}N$, 12.8049, 12.8799; C/N, 3.31. The results for KAL1064 are as follows: $d^{13}C$, -19.1342, -19.1654; $d^{15}N$, 13.1990, 13.3332; C/N, 3.51. Carbon isotopic values are reported relative to Pee Dee Belemnite (PDB); nitrogen isotopic values are reported relative to AIR. Mean bone collagen $d^{13}C$ and $d^{15}N$ for each sample are presented in Figure 8.3. Stable isotopic data for the Sandnes samples and the remaining Tjodhilde's Church samples were obtained from the literature (Arneborg et al. 2006; Arneborg et al. 2012).

8.3 Results

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8.3.2 BLG in Europe and Northern Southwest Asia

Seventy-four dental calculus samples were selected from 20 sites in Northern Europe (Britain, Denmark, and Norway; n=40), Central Europe (Germany, Hungary, and Italy; n=38) and northern Southwest Asia (Armenia and Russia, n=6) dating from the Bronze Age (ca. 3,000 BCE) through the 19th century CE (Table 8.1). Approximately one quarter (25.7%) of the Eurasian dental calculus samples tested positive for BLG peptides (Fig. 8.1). In total, 229 spectra (representing 37 unique peptide sequences) from the Eurasian dataset were assigned to BLG (Table 8.2), resulting in a reconstruction of 72% of the protein (Fig. 8.2). For each of the 19 Eurasian dental calculus samples that tested positive for BLG, the consensus BLG sequence could be assigned to ruminants of the Pecora infraorder of Artiodactyla and 18 samples contained bovid-specific (Bovidae) peptides. Among these samples, 4 samples

contained cattle-specific (*Bos* sp.) peptides, 3 samples contained sheep-specific (*Ovis* sp.) peptides, 2 samples contained goat-specific (*Capra* sp.) peptides, and 3 samples contained BLG peptides from multiple ruminant species (Table 8.2).

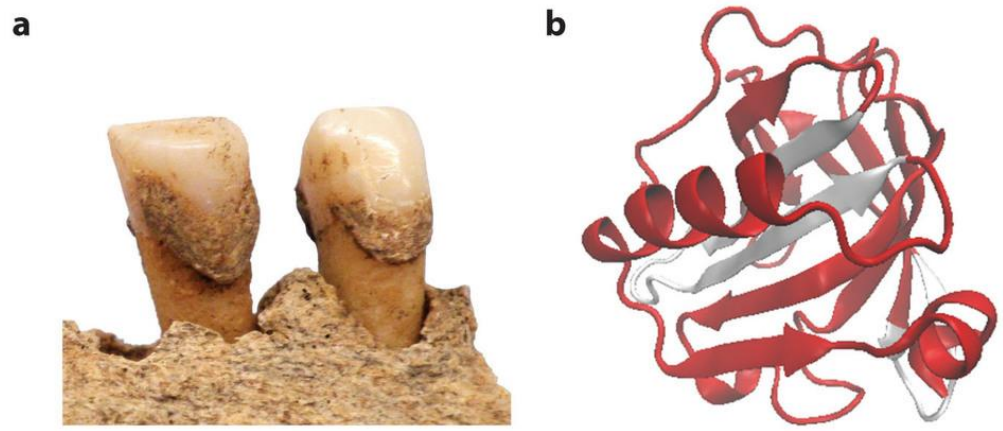

Figure 8.2. Protein coverage of β-lactoglobulin identified within Eurasian archaeological dental calculus, a) human dental calculus from the British Anglo-Saxon site of Norton-on-Tees (sample NEM18, ca. 6th century CE) found to contain seven β-lactoglobulin peptides, b) Three-dimensional structure of bovine β-lactoglobulin protein, rendered from PDB 3NPO using VMD v.1.9.1. The mapped locations of all BLG peptide sequences identified by tandem mass spectrometry within archaeological dental calculus are shown in red, resulting in coverage of 72% of the reconstructed consensus BLG protein.

8.3.3 Absence of BLG in Central West Africa

Eighteen dental calculus samples were selected from a 19th century cemetery on the island of St Helena, located approximately 2,000 km west of Angola in the southern Atlantic Ocean. The cemetery contains the remains of Central West Africans (Pearson et al. 2011) originating from a region with traditionally very low or no milk consumption (Fig. 8.1). As expected, BLG peptides were not identified in any of the West African samples (Fig. 8.1; Table 8.1).

8.3.4 BLG in Norse Greenland

Recent isotopic and faunal evidence suggests that Greenland Norse settlements shifted from an economy initially based on dairy to one increasingly reliant on marine mammals after the onset of the Little Ice Age ca. 1250 CE (Arneborg et al. 2012). To test this hypothesis, we analysed dental calculus from individuals buried at Tjodhildes Church, an early cemetery (ca. 985-1250 CE) at the Eastern settlement landnám site of Brattahlið (Qassiarsuk) established by Erik the Red, the founder of the Norse Greenland colonies (Arneborg et al. 2012), and at Sandnes (Kilaarsarfik), a high-status farm and church in the Western Settlement that continued to be in use until the abandonment of the settlement in the 15th century CE. The individuals analysed from Tjodhildes Church (n=2) exhibited strong evidence of dairy consumption (Fig. 8.3), with a total of 38 spectra matching BLG peptides (12 unique peptides including one *Bos*-specific sequence). Because these two individuals had not been previously analysed isotopically, we then performed carbon and nitrogen stable isotope analysis on bone collagen extracted from these individuals and confirmed that their isotopic values are consistent with a terrestrial diet (Fig. 8.3). Analysis of the Sandnes individuals (n=4) revealed that only one individual showed evidence of milk consumption (weak), as evidenced by a single spectrum (Fig. 8.3). This individual was previously determined to have consumed a primarily terrestrial diet on the basis of isotopic evidence (Arneborg et al. 2012). The remaining BLG-negative individuals exhibited bone stable isotope values consistent with increasing marine resource consumption (Fig. 8.3).

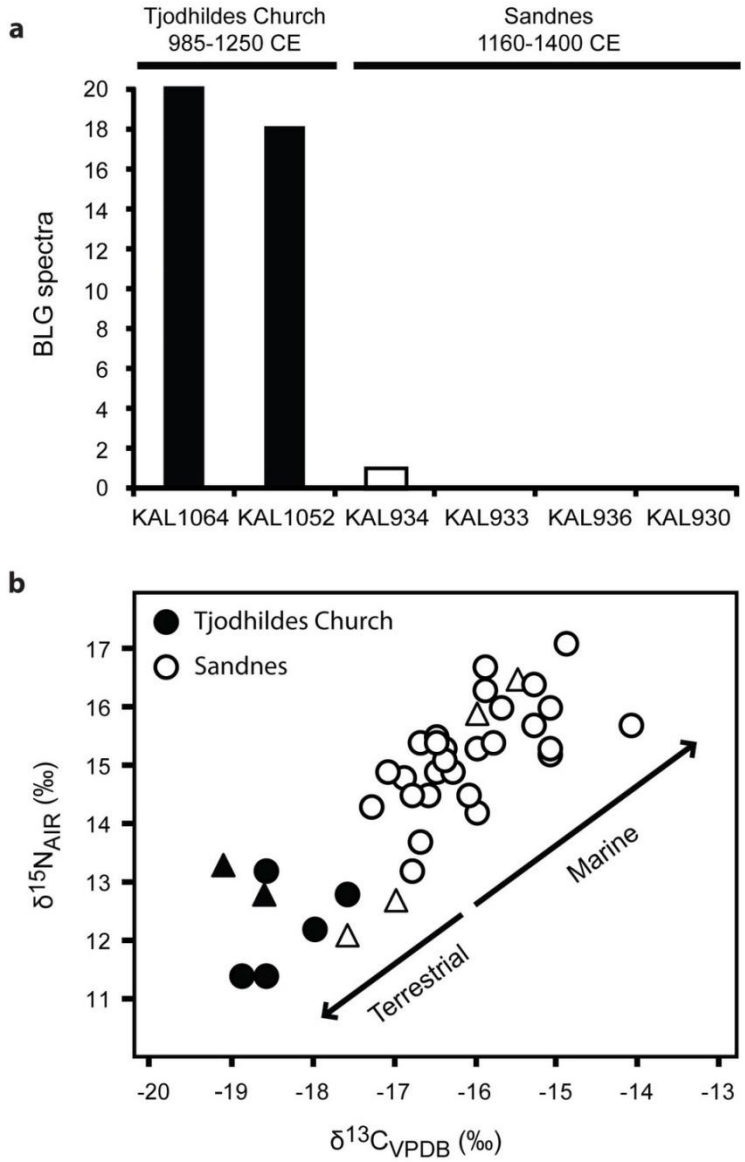

Figure 8.3. BLG pattern in dental calculus is consistent with bone collagen stable isotope evidence of a decline of the dairy economy in Norse Greenland with the onset of the Little Ice Age (ca.1250 CE), a) total spectra matching BLG peptides recovered from dental calculus samples from the earlier Tjodhildes Church at Ø29a Brattahlið in the Eastern Settlement (individuals KAL1064 and KAL1052) and from the later V51 Sandnes site in the Western Settlement (individuals KAL934, KAL933, KAL936, and KAL930), b) bone collagen carbon and nitrogen stable isotope values measured from burials at Tjodhildes Church (black) and Sandnes (white), showing a major dietary shift toward marine resources at the later Sandnes site. Isotopic values for individuals also analysed for dental calculus BLG peptides are represented by triangles, and from left to right on the x-axis are: KAL1064, KAL1052, KAL934, KAL933, KAL936, and KAL930. Isotopic data for KAL1064 and KAL1052 were measured in this study.

8.4 Discussion

Our results show that the milk protein BLG preserves in archaeological dental calculus and can be identified in specimens dating back to at least the Bronze Age (ca. 3,000 BCE) in Europe and northern Southwest Asia. Moreover, this study demonstrates that BLG is a species-specific milk biomarker that allows cattle, sheep and goat dairy product consumption to be distinguished. As expected, no BLG was detected in calculus samples from Central West Africa, where dairy consumption was historically very low or absent and current LP frequencies are very low.

In addition to establishing BLG as a biomarker of milk consumption in the archaeological record, the broad survey of dental calculus BLG conducted in this study reveals previously uncharacterised temporal and geographical complexities in dairy consumption. In Central Europe, for example, it is intriguing given the high prevalence of LP in both modern day (Fig. 8.1) and medieval (Krüttli et al. 2014) German populations that no BLG peptides were detected in Bronze Age or medieval German samples (0/9 individuals). This stands in contrast to the northern Italian and Hungarian samples where BLG peptides were detected at a relatively high frequency (5/11 individuals) (Table 8.2). These geographic patterns may reflect different dairy consumption levels or differential usage of high (e.g. soured milk, whey, ricotta, kefir) and low (e.g. cheese, butter) BLG dairy products. Using this approach it now becomes possible to explore specific cultural, social and environmental factors influencing past dairy economies at both a population and an individual level.

To explore a specific dairy economy in greater detail, we applied this approach to Norse Greenland to examine the hypothesis that this population underwent a dramatic dietary shift in

response to environmental change during the Little Ice Age (Arneborg et al. 2012). The medieval Greenland Norse economy was primarily based on animal husbandry and especially dairying. The short growing season and cold climate of Greenland precluded the successful establishment of agriculture but was sufficient for ruminant pastoralism. Like other Scandinavian populations, the medieval Norse utilised nearly all by-products of dairy processing, including BLG-rich whey (Gísladóttir 1992; Lanigan and Bartlett 2013). The use of sour whey (súrr) to pickle meats is described in the Norse Icelandic sagas (Byock 2001), and Scandinavian whey "cheese" (e.g. mysost, gjetost, and brunost), an evaporated whey concentrate with an exceptionally high lactose content (30-55%), was also likely consumed, but unfortunately the sagas lack detail on specific "cheese" (ost) types.

Our dental calculus BLG results confirm that dairy products were consumed by individuals buried in the early Norse cemetery at Tjodhildes Church (ca. 985-1250 CE), and bone collagen stable isotopic values from the same individuals are consistent with a terrestrial diet. By contrast, BLG was very low or absent in the dental calculus of individuals buried at the Sandnam site, which was occupied until ca. 1430 CE, and bone stable isotope values from these individuals indicate a dietary shift toward marine resources.

Diminished access to dairy products and a collapse of dairy herds would have had a strongly negative effect on the Norse economy, removing not only a major source of storable nutrition but also impeding the ability to preserve other perishable foods, such as meats, thereby exacerbating food instability especially during the winter months. Our findings support the hypothesis that climate change and the consequent decline of dairy herds contributed to the decline and ultimate abandonment of the Norse Greenland colonies in the 15th century CE.

In this study, we identify the protein β-lactoglobulin in archaeological dental calculus and demonstrate that it is a species-specific milk biomarker and an indicator of dairy consumption in the archaeological record. Nearly ubiquitous and obtained directly from the oral cavity of individuals, dental calculus provides a novel approach to detecting patterns of milk consumption and the dietary variables driving recent natural selection in humans.

Tables

Site	Period	Dates	Sample ID (Extraction ID)[a]	Site Reference
		Europe		
		Switzerland		
Zürich[b]	Present Day	2011 CE	CPMAIR (Z5, Z6); CPNATR (Z7, Z8)	Warinner, Rodrigues, et al. 2014
		Britain		
Melton	Iron Age	800 BCE to 80 CE	OSA04EX03 SK1032 (ML1032); OSA04EX03 SK1489 (ML1489); OSA04EX03 SK1823 (ML1823); OSA04EX03 SK3890 (ML3890)	Caffell and Holst 2011
Driffield Terrace	Roman	44-410 CE	YORYM:2004.354.SK21 (3DT21); YORYM:2004.354.SK26 (3DT26); YORYM:2005.513.SK21 (6DT21); YORYM:2005.513.SK03 (6DT3); YORYM:2005.513.SK07 (6DT7)	Muldner et al. 2011; Muldner 2013; Caffell and Holst 2012; Montgomery et al. 2011; Montgomery et al. 2010
Leicester	Roman	44-410 CE	A2-2013-SK1 (OX01); A2-2013-SK3 (OX03); A2-2013-SK4 (OX4); A2-2013-SK5 (OX05); A2-2013-SK6 (OX6); A2-2013-SK9 (OX09); A2-2013-SK10 (OX10); A2-2013-SK12 (OX12)	Keefe and Holst 2013; Morris 2010
Norton-on-Tees	Anglo-Saxon	ca. 500-910 CE	2006:1500:18 (NEM18); 2006:1500:99 (NEM099); 2006:1500:93 (NEM093); NBS:03:SK262 (NBS262); NBS:03:SK325 (NBS325); NBS:03:SK410 (NBS410)	Sherlock and Welch 1992; Johnson 2005

Wighill (Syningthwaite Priory Farm)	Medieval	ca. 1000-1550 CE	SYP08:SK1082 (WG1082); SYP08:SK1252 (WG1252); SYP08:SK1483 (WG1483); SYP08:SK1561 (WG1561); SYP08:SK1566 (WG1566); SYP08:SK1585 (WG1585)	Unpublished; site report in progress by Mike Griffiths & Associates, D9A The Raylor Centre, James Street, York YO10 3DW, UK
Fewston	Victorian	1835-1895 CE	SLF09:SK053 (FW053) SLF09:SK435 (FW435); SLF09:SK342 (Z44); SLF09:SK351 (Z45)	Henderson et al. 2013a; Henderson et al. 2013b
		Denmark		
Gjerrild	Neolithic	ca. 3000-1500 BCE	NMA44578 (RISE432)	Ebbesen 1985; Vandkilde 2003;
Öster Harup	Neolithic	ca. 2800-2400 BCE	Skive mus. journal no 74 (RISE460)	Unpublished; Report on file, Lab of Biological Anthropology, University of Copenhagen, AS 10/77
		Norway		
Trondheim	Medieval to 17th century	ca. 1100-1700 CE	SØVI SØXV, SØXXI, KA50, SK148 (CP247-CP249)	Turner-Walker et al. 2001
		Germany		
Nersingen	Bronze Age	ca. 3000-1500 BCE	Burial 1/6 (RISE 472)	Mackensen 1987
Regensburg-Dechbetten	Bronze Age	ca. 3000-1500 BCE	RISE 473	Unpublished
Dalheim	Medieval	ca. 950-1200 CE	G12 (Z1, Z2)[b]; B17 (Z27)[b]; B61 (Z46)[b]; B78 (Z28)[b]; B27a (Y47); B40 (Y48); B85 (Y49)	Warinner, Rodrigues, et al. 2014
		Hungary		
Szöreg-C (Sziv Utca)	Bronze Age	ca. 3000-1500 BCE	Grave 75 (RISE368); Grave 7 (RISE 363)	O'Shea 1996; Sprincz and Beck 1981
		Italy		
Olmo di Nogara	Bronze Age	ca. 2700-1350 BCE	Grave 89 (RISE466); Grave 41 (RISE467); ODN19 (ODN19-1); ODN98 (ODN98-1); ODN207 (ODN207-1);	Salzani 2005; Tafuri et al. 2009

			ODN271 (ODN271-1); ODN361 (ODN361-1); ODN424 (ODN424-1); ODN458 (ODN458-1)	
Isola Sacra (Portus Romae)	Imperial Roman	50-200 CE	SCR227 (SCR227-1); SCR250 (SCR250-1); SCR262 (SCR264-1); SCR323 (SCR323-1); SCR832 (SCR832-1); SCR5082 (SCR5082-1); SCR5042 (SCR5042-1); SCR5070 (SCR5070-1)	Prowse et al. 2004
Northern Southwest Asia[a]				
Armenia				
Hatsarat	Bronze Age	ca. 2000 BCE	15 (RISE417)	Marshall and Mkrtchyan 2011;
Nerkin Getashen	Bronze Age	ca. 1200-1300 BCE	13 (RISE415)	Khudaverdyan 2014; Marshall and Mkrtchyan 2011
Noraduz	Bronze Age	ca. 1200-1300 BCE	12 (RISE414)	Marshall and Mkrtchyan 2011
Noraduz	Iron Age	ca. 700 BCE	18 (RISE420)	Khudaverdyan 2014
Russia				
West Caucasus, Marchenkova Gora	Bronze Age	ca. 3000-2000 BCE	MG-03-D-13-EB-04 (RISE307)	Unpublished
Bulanovo	Bronze Age	ca. 3000-1500 BCE	Burial 8 (RISE387)	Morgunova and Khokhlova 2006
Central West Africa				
St. Helena				
Rupert's Valley	Victorian	1840-1872 CE	STH212 (JH1); STH218 (JH2); STH237 (JH3); STH343 (JH4); STH359 (JH5); STH421 (JH6); STH430 (JH7); STH474 (JH8); STH219 (JH10); STH268 (JH11); STH276	Pearson et al. 2011

			(JH12); STH306 (JH13); STH319 (JH14); STH389 (JH15); STH419 (JH16); STH449 (JH17); STH414 (JH19); STH423 (JH20);	
		Greenland		
Ø29a Brattahlið	Medieval	ca. 890-1230 CE	KAL1052 (Z39); KAL1064 (Z40);	Arneborg et al. 1999, 2012
W51 Sandnes	Medieval	ca. 1290-1430 CE	KAL930 (Z35); KAL933 (Z36); KAL934 (Z37); KAL936 (Z38)	Arneborg et al. 1999, 2012

Table 8.1. Samples of dental calculus analysed in this study; a) all extraction IDs preceded by "Z" were extracted at the Centre for Evolutionary Medicine at the University of Zürich, Switzerland. All extraction IDs preceded by "RISE" or "CP" were extracted at the Centre for GeoGenetics at the University of Copenhagen, Denmark. All remaining extractions were performed at the University of York, UK; b) sample data from Warinner, Rodrigues, et al. 2014.

ID	Total spectra assigned to BLG[a]	Identified peptide sequences[a]	PTMs	Ion Score	Protein taxonomic assignment
		Britain			
ML1032	8	A.LIVTQTMK.G*[c] (2)	Oxidation (M)	31, 34	Bovidae
		K.VLVLDTDYKK.Y* (3)		31, 38, 56	
		K.VLVLDTDYK.K*		28	
		K.TKIPAVFK.I		27	
		K.ALPMHIR.L		31	
ML1823	20	-.IIVTQTMK.G*[c] (3)	Oxidation (M)	27, 28, 30	*Ovis* sp.
		-.IIVTQTMK.G*[c]	Acetyl (Protein N-term); Oxidation (M)	30	
		A.SDISLLDAQSAPLR.V*		26	
		R.VYVEELKPTPEG.N* (2)		33, 46	
		R.VYVEELKPTPE.G* (3)		40, 50, 59	
		K.TKIPAVF.K		28	
		K.IDALNENK.V	Deamidated (NQ)	51	
		R.TPEVDNEALEK.F* (2)		29, 33	
		R.TPEVDNEALEK.F*[d] (4)	Deamidated (NQ)	38, 39, 49, 67	
		R.LAFNPTQLEG.Q*		44	
		R.LAFNPTQLEGQ.C*		29	
3DT21	23	-.IIVTQTMK.G*[c]	Oxidation (M)	29	Bovidae
		K.IDALNENK.V (5)	Deamidated (NQ)	28, 36, 38, 40, 50	
		K.IDALNENK.V	2 Deamidated (NQ)	29	
		R.TPEVDDEALEK.F*[d] (6)		30, 33, 34, 44, 54, 69	
		R.TPEVDNEALEK.F*[d] (10)	Deamidated (NQ)	29, 31, 32, 41, 42, 44, 44, 48, 51, 51	
OX4	12	A.LIVTQTMK.G*[c]	Oxidation (M)	27	*Bos* sp.
		A.LIVTQTMK.G*[c]	Deamidated (NQ); Oxidation (M)	39	
		A.SDISLLDAQSAPLR.V*		80	
		A.SDISLLDAQSAPLR.V* (2)	Deamidated (NQ)	33, 48	
		R.VYVEELKPTPEGDLEILLQK.W*[e]		35	

		K.IDALNENK.V		46	
		K.VLVLDTDYK.K* (2)		39, 70	
		K.VLVLDTDYKK.Y* (2)		34, 36	
		R.LSFNPTQLEEQCHI.-*		45	
OX06	2	R.VYVEELKPTPEGDLEILLQK.W*[e] (2)		43, 63	Bovidae
OX12	23	-.IIVTQTMK.G*[c]	Oxidation (M)	34	Caprinae
		-.IIVTQTMK.G*[c]	Deamidated (NQ); Oxidation (M)	46	
		Y.SLAMAASDISLLDAQSAPLR.V*		76	
		Y.SLAMAASDISLLDAQSAPLR.V* (2)	Oxidation (M)	59, 81	
		Y.SLAMAASDISLLDAQSAPLR.V* (2)	Deamidated (NQ); Oxidation (M)	37, 85	
		S.LAMAASDISLLDAQSAPLR.V* (2)	Oxidation (M)	77, 139	
		S.LAMAASDISLLDAQSAPLR.V* (2)	Deamidated (NQ); Oxidation (M)	62, 108	
		A.MAASDISLLDAQSAPLR.V*	Deamidated (NQ); Oxidation (M)	41	
		A.SDISLLDAQSAPLR.V* (2)		36, 66	
		A.SDISLLDAQSAPLR.V*	Deamidated (NQ)	67	
		R.VYVEELKPTPEGNLEILLQK.W*		25	
		R.VYVEELKPTPEGNLEILLQK.W*	2 Deamidated (NQ)	32	
		K.IPAVFK.I		25	
		K.IDALNENK.V		35	
		K.IDALNENK.V	Deamidated (NQ)	29	
		K.VLVLDTDYK.K*		52	
		K.VLVLDTDYKK.Y*		52	
		K.ALPMHIR.L	Oxidation (M)	33	
NEM18	22	A.LIVTQTMK.G*[c]	Oxidation (M)	36	Bovidae
		K.VLVLDTDYK.K* (3)		32, 53, 59	
		K.VLVLDTDYKK.Y* (2)		30, 31	
		K.TKIPAVFK.I (2)		30, 32	
		K.IDALNENK.V (3)	Deamidated (NQ)	28, 31, 44	
		K.ALPMHIR.L (6)		25, 29, 31, 31, 31, 33	
		K.ALPMHIR.L (5)	Oxidation (M)	25, 25, 26, 26, 31	
WG1082	7	A.SDISLLDAQSAPLR.V*		38	Bovidae

		F.KIDALNENK.V*	2 Deamidated (NQ)	26	
		R.TPEVDDEALEK.F*[d] (4)		47, 58, 63, 77	
		R.TPEVDDEALEKFDK.A*[d]		34	
WG1483	1	R.TPEVDDEALEKFDK.A*[d]		46	Bovidae
WG1561	6	A.SDISLLDAQSAPLR.V*		65	Bovidae
		K.TKIPAVFK.I		32	
		K.IDALNENK.V	Deamidated (NQ)	48	
		R.TPEVDDEALEK.F*[d] (3)		40, 47, 68	
FW435	4	K.VLVLDTDYKK.Y* (2)		33, 61	Pecora
		K.ALPMHIR.L		31	
		K.ALPMHIR.L	Oxidation (M)	37	
		Norway			
CP247-CP249	43	-.IIVTQTMK.G*[c]		53	*Bos* sp.
		-.IIVTQTMK.G*[c] (2)	Oxidation (M)	50, 52	
		-.IIVTQTMK.G*[c]	Acetyl (Protein N-term); Oxidation (M)	49	
		K.VAGTWYSLAMAASDISLLDAQSAPLR.V*		66	
		R.VYVEELKPTPEGDLEILLQK.W*[e] (2)		54, 56	
		R.VYVEELKPTPEGDLEILLQK.W*[e]	Deamidated (NQ)	81	
		K.KIIAEK.T (2)		52, 52	
		K.TKIPAVFK.I (3)		43, 43, 43	
		K.IDALNENK.V		55	
		K.VLVLDTDYK.K* (4)		51, 52, 70, 71	
		K.VLVLDTDYKK.Y* (7)		47, 49, 50, 52, 54, 64, 67	
		R.TPEVDDEALEK.F*[d] (5)		60, 68, 71, 72, 75	
		R.TPEVDDEALEKFDK.A*[d] (9)		56, 60, 63, 65, 67, 68, 76, 90, 94	
		R.LSFNPTQLEEQCHI.-* (3)		67, 69, 75,	
		R.LSFNPTQLEEQCHI.-*	Deamidated (NQ)	73	
		Hungary			
RISE368	38	-.IIVTQTMKGLDIQK.V*[c] (3)	Acetyl (Protein N-	38, 42,	*Bos* sp. and

			term); Oxidation (M)	48	*Ovis* sp.
		-.IIVTQTMKGLDIQK.V*[c] (2)	Acetyl (Protein N-term); Deamidated (NQ); Oxidation (M)	35, 60	
		A.LIVTQTMKGLDIQK.V*[c]	Oxidation (M)	28	
		K.IIAEKTKIPAVFK.I*		28	
		K.IDALNENKVLVLDTDYKK.Y*	Deamidated (NQ)	32	
		R.TPEVDNEALEKFDK.A* (3)		27, 34, 39	
		R.TPEVDNEALEKFDKALK.A*		55	
		K.ALKALPMHIR.L* (2)		31, 54	
		K.ALKALPMHIR.L* (4)	Oxidation (M)	47, 50, 53, 64	
		K.ALPMHIR.L	Oxidation (M)	45	
		I.RLSFNPTQLEEQCHI.-*	Deamidated (NQ)	31	
		R.LSFNPTQLEEQCHI.-* (4)		53, 54, 60, 81	
		R.LSFNPTQLEEQCHI.-* (4)	Deamidated (NQ)	37, 38, 54, 94	
		N.PTQLEEQCHI.-*		49	
		N.PTQLEEQCHI.-* (2)	Deamidated (NQ)	43, 61	
		N.PTQLEEQCHI.-*	2 Deamidated (NQ)	58	
		R.LAFNPTQLEGQCHV.-*		55	
		R.LAFNPTQLEGQCHV.-* (4)	Deamidated (NQ)	38, 38, 40, 49	
		R.LAFNPTQLEGQCHV.-*	2 Deamidated (NQ)	57	
			Italy		
ODN98	1	R.TPEVDDEALEK.F*[d]		53	Bovidae
ODN271	2	R.TPEVDDEALEK.F*[d] (2)		57, 73	Bovidae
ODN207	4	K.KIIAEK.T		34	*Capra* sp. and either Bovinae or *Ovis* sp.
		K.VLVLDTDYKK.Y*		29	
		R.TPEVDDEALEK.F*[d]		57	
		R.TPEVDKEALEK.F*		34	
ODN424	4	K.KIIAEK.T		28	*Capra* sp. and either Bovinae or *Ovis* sp.
		K.VLVLDTDYKK.Y*		27	
		R.TPEVDKEALEK.F*		51	
		R.TPEVDNEALEK.F*[d]	Deamidated (NQ)	36	
			Armenia		
RISE417	2	R.TPEVDNEALEKFDK.A* (2)		40, 43	*Ovis* sp.
			Russia		
RISE387	8	R.LSFNPTQLEEQCHI.-*		82	*Bos* sp.

		R.LSFNPTQLEEQCHI.-*	Deamidated (NQ)	31	
		R.LSFNPTQLEEQCHI.-* (2)	2 Deamidated (NQ)	39, 47	
		N.PTQLEEQCHI.-*		30	
		N.PTQLEEQCHI.-* (2)	Deamidated (NQ)	61, 62	
		N.PTQLEEQCHI.-* (1)	2 Deamidated (NQ)	40	
		Greenland			
Z39	18	K.KIIAEK.T		42	Bovidae
		K.IPAVFK.I (3)		31, 36, 36	
		K.IDALNENK.V		43	
		K.IDALNENK.V (4)	Deamidated (NQ)	28, 28, 31, 45	
		K.VLVLDTDYK.K* (2)		28, 48	
		R.TPEVDDEALEK.F*[d] (6)		48, 50, 54, 58, 66, 74	
		R.TPEVDDEALEKFDK.A*[d]		80	
Z40	20	-.IIVTQTMK.G*[c]	Acetyl (Protein N-term; Oxidation (M)	30	*Bos* sp.
		K.IPAVFK.I (2)		31, 41	
		K.IDALNENK.V (3)	Deamidated (NQ)	46, 46, 53	
		K.VLVLDTDYK.K* (2)		61, 71	
		K.VLVLDTDYKK.Y*		46	
		R.TPEVDDEALEK.F*[d] (2)		58, 76	
		R.TPEVDNEALEK.F*[d] (2)	Deamidated (NQ)	62, 80	
		R.TPEVDDEALEKFDK.A*[d] (2)		111, 117	
		R.TPEVDNEALEKFDK.A*[d] (4)	Deamidated (NQ)	36, 54, 69, 99	
		R.LSFNPTQLEEQCHI.-*	Deamidated (NQ)	31	
Z37	1	R.TPEVDDEALEK.F*[d]		59	Bovidae
		Switzerland			
Z5	4	K.VLVLDTDYKK.Y*		66	*Bos* sp.
		R.TPEVDDEALEKFDK.A*[d]		77	
		R.LSFNPTQLEEQCHI.-* (2)		82, 90	

Table 8.2. BLG peptides identified in samples of dental calculus; a) excludes spectra with a Mascot ion score <25. Peptides observed more than once are followed by parentheses indicating the total number of observations, b) based on an alignment of available BLG sequences in UniProt (http://www.uniprot.org) from the following taxa: *Bos taurus* (P02754),

Bos mutus (L8J1Z0), *Bubalus bubalis* (P02755), *Ovis aries* (P67976), *Ovis orientalis* (P67975), *Capra hircus* (P02756), *Rangifer tarandus* (Q00P86), *Sus scrofa* (P04119), *Turisops truncatus*, (Q7M2T1), *Equus caballus* (P02758, P07380), *Equus asinus* (P13613, P19647); as well as human glycodelin (P09466), the most closely related human protein to BLG. Note that equids produce two distinct BLG proteins: BLG I and BLG II. Camelids, like humans, do not produce BLG, c) current MS/MS data do not allow the peptides -.IIVTQTMK.G (Pecora, but not *Bos*) and A.LIVTQTMK.G (*Bos*) to be unambiguously distinguished, d) among Bovidae, Bovinae (cattle, yak, and buffalo) are distinguished from Caprinae (sheep and goats) by N→D or K→D at residue 149; because N deamidation to D is a common post-mortem modification, it is uncertain if the D at this residue is authentic (D) or a damage artefact (deamidated N), e) among Bovidae, Bovinae (cattle, yak, and buffalo) are distinguished from Caprinae (sheep and goats) by N→D at residue 71. Because N deamidation to D is a common post-mortem modification, it is uncertain if the D at this residue is authentic or a damage artefact, f) data from Warinner, Rodrigues, et al. 2014. All sequences have been verified for specificity by conducting a protein BLAST (blastp) search against the NCBI nr database and sequences that uniquely match BLG are marked with an asterisk (*). Only samples with at least one spectrum uniquely matching BLG are considered BLG+.

Chapter 9: Metaproteomic Analysis of an Ancient Oral Microbiome

The previous chapter highlighted the value of dental calculus as a tool for uncovering food consumption in the archaeological record. This chapter explores the value of dental calculus in terms of a resource for understanding past patterns of disease and health. This exploration takes place in the context of understanding disease during the 19th century transatlantic slave trade.

Abstract

Analysis of ancient proteins is proving to be an effective tool by which to characterise ancient disease and diets, and may be particularly useful in regions where DNA analysis may be hampered by poor preservation. Proteomic analysis was applied to samples of dental calculus and dentine from formerly enslaved individuals from the South Atlantic island of St Helena with the aim of investigating disease and food consumption during the 19th century transatlantic slave trade. This analysis reveals that the human proteome of these dental tissues is preserved in archaeological samples. In addition, metaproteomic analysis of dental calculus indicates the preservation of proteins derived from a suite of oral bacteria, both commensal and pathogenic. However, although this information may provide valuable insight into periodontal diseases, this analysis does not reveal evidence of diseases that directly relate to transatlantic slavery. Despite previous reported successes of microbial DNA derived from the cardiovascular and respiratory systems (Warinner, Rodrigues, et al. 2014), this study demonstrates that the proteomic analysis of dental calculus may have the most use for

specifically understanding oral diseases. The detection of these microbial proteins also facilitated an exploration into bacterial survival, where it was found proteins from Gram-negative bacteria were much more abundant than Gram-positive in archaeological samples, in contrast to previous reports for ancient DNA. Results of this study also demonstrate that protein preservation varies substantially between individuals and proposes that understanding the mechanism of this preservation is vital in order to understand the degree to which ancient samples are a reflection of the living tissue.

9.1 Introduction

Although in its infancy, ancient proteomics may prove to be an effective method for characterising ancient disease (Warinner, Rodrigues, et al. 2014; Corthals et al. 2012). Ancient proteomics has rapidly moved from identifying single proteins (Ostrom et al. 2000), to identifying ancient proteomes (Cappellini et al. 2012; Orlando et al. 2013; Cappellini et al. 2014; Bona et al. 2014) and even microbiomes (Warinner, Rodrigues, et al. 2014). In terms of disease, whilst the identification of single, specific organisms may be important for understanding infections where there is a clear causative agent, many diseases, such as periodontitis, are polymicrobial (Kinane and Mombelli 2012; Jakubovics and Palmer 2013). In addition, the role of the microbiome, the suite of commensal species which inhabit biological systems, is becoming increasingly recognised as an integral part of human health (Wade 2013a). Subsequently, understanding the evolution of our microbiomes is crucial for understanding what constitutes a healthy microbiome, and how microbiomes may be altered by both cultural practices and the environment (Warinner et al. 2015). However, accessing this information in archaeological material is challenging owing to the degradation, molecular complexity and rarity of archaeological materials. In the face of these challenges, how can we

access and understand this microbial complexity, especially in regions where biomolecular preservation is likely to be poor?

In this study ancient metaproteomics is used to characterise microbial communities and examine the health and diet of individuals enslaved in West Central Africa as part of the transatlantic slave trade in the 19th century. This study demonstrates that bacteria implicated in oral disease can be identified using a metaproteomic approach. This study also demonstrates that proteomic analysis may reveal host responses to infections. In addition, this study explores biomolecular survival in ancient dental calculus, assesses the factors that might be contributing to protein survival and considers the link between DNA and protein preservation in this novel resource.

9.1.1 Biomolecular Preservation

Biomolecules in archaeological material are typically degraded and thus require either sufficient molecular preservation (e.g. Schuenemann et al. 2013) or improved detection methods (e.g. Carpenter et al. 2013) in order to generate successful results. For example, almost all complete ancient nuclear genomes reported to date derive from regions where the cooler climate facilitates favourable DNA preservation (Fig. 9.1). Exceptions to this include the recent study by Schroeder et al. (2015) who used whole genome capture methods to reconstruct the genome of three enslaved individuals dating from 17th century Sint Maarten and the reconstruction of the genome of an Aboriginal Australian dating to the 19th century (Rasmussen et al. 2011). Although the technique is much more recent, ancient proteomes have only been extracted from samples originating from Europe (Fig. 9.1), even though we presume

that ancient proteins are more robust biomolecules than ancient DNA (Collins et al. 2000; Allentoft et al. 2012; Crisp et al. 2013; Welker et al. 2015).

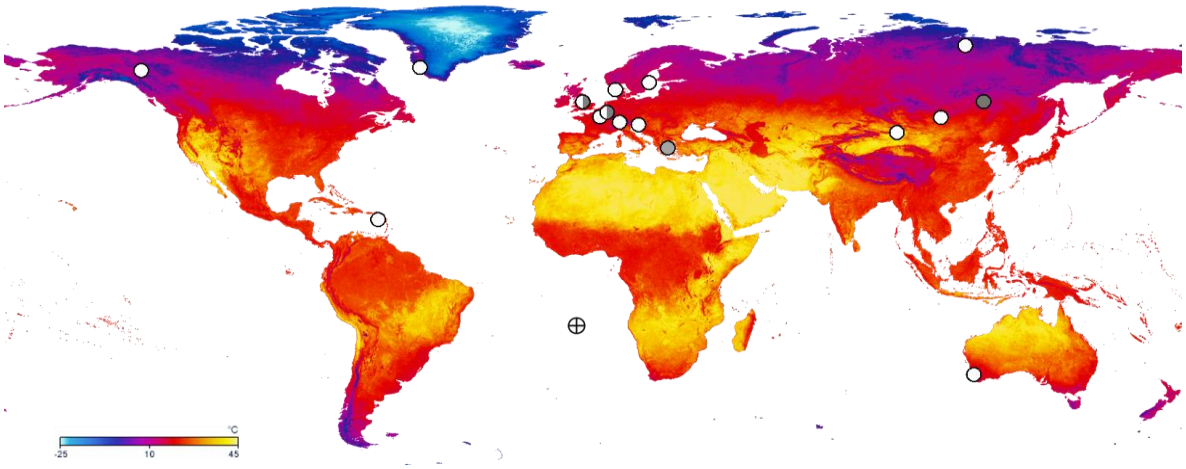

Figure 9.1. Global thermal map indicating the sample locations of complete ancient nuclear genomes (white circles), ancient proteomes (grey circles) and the location of the sample set used in this study (cross). The background thermal map of land surface was produced by NASA's Earth Observatory using data courtesy of the MODIS Land Group. This dataset depicts land surface temperature at the time of archaeological excavations on St Helena (December 2008).

Recent studies of dental calculus have demonstrated exceptional preservation of DNA (Adler et al. 2013; Warinner, Rodrigues, et al. 2014) and proteins (Warinner, Rodrigues, et al. 2014) from this material and that this resource can be used to access the ancient oral environment (Warinner, Speller, and Collins 2015; Weyrich, Dobney, and Cooper 2015). Given these successes, it may be possible that dental calculus will be a successful source of biomolecular

data from regions such as the tropics where biomolecular preservation can be a major debilitating issue.

9.1.2 Thermal Age Modelling

In 2007 and 2008 excavations on the South Atlantic island of St Helena revealed a skeletal assemblage representing individuals enslaved as part of the 19th century transatlantic slave trade (Pearson et al. 2011). In order to assess the potential for successful DNA analysis from this assemblage, sample information was run through a thermal age model (Smith et al. 2003) (Table 9.1). St Helena lies in the tropics (15°56′S 5°42′W) and its climate is classified as tropical monsoon (Am) by the Köppen classification system. Osteological preservation, graded using McKinley (2004), of human remains was highly variable, probably due to variations in the nature and depth of burials (Witkin 2011).

Thermal age was predicted to be up to 2.035 kyr@10 °C, meaning that DNA damage expected in these samples is equivalent to having been stored at a constant temperature of 10 °C for 2035 years; 10 times older than the real age of the sample (Table 9.1). For comparison, a site of similar age in the Netherlands (Middenbeemster) yielded a thermal age of up to 0.309 kyr@10 °C. This difference is reflected in the average DNA yield from each of these sites, where samples of Middenbeemster yielded over 30 times more DNA than samples from St Helena (Table 9.1).

	Rupert's Valley, St Helena	Middenbeemster, Netherlands
Conditions		
Date	1840 - 1872	1829-1866
Latitude	-15.9608	52.5497
Longitude	-5.651021	4.9131
Elevation (m)	60	-2
Excavation year	2008	2011
Burial environment	0.1m sandy soil, 3% water	2m fine sand 15% water
Storage temperature (°C)	21.5	8.3
DNA degradation		
Lambda	0.00527 - 0.00608	0.0003 - 0.0009
Thermal age (kyr@10°C)	1.764 - 2.035	0.106 - 0.309
Mean DNA yield (ng/mg)	1.617	53

Table 9.1. Thermal age (Smith et al. 2003) parameters and results for samples originating from St Helena and the Netherlands demonstrating the effects of environmental conditions on DNA preservation.

These predicted poor results for DNA analysis additionally suggest that ancient proteomics may be a valuable tool for exploring biomolecular evidence of disease in this population. Information of DNA extraction and analysis is outlined in Appendix 2.

9.2 Methods

9.2.1 Sampling

Twenty samples of dental calculus, two samples of dentine and two samples of burial soil were selected for protein extraction (Table 9.2). Samples of dental calculus were obtained from excavated skeletal material from St Helena in July 2012.

Samples of dental calculus were selected with the following criteria; a) sufficient dental calculus for multiple biomolecular approaches to be attempted, b) individuals with and

without osteologically identified oral and respiratory (maxillary sinusitis) pathologies, and c) a range of skeletal ages. Only one female was among the sample set because of the low numbers of females identified in this population (16% of individuals able to be sexed, Witkin 2011). Samples of dentine were obtained from two individuals, from which dental calculus was also obtained. Dental calculus was removed using a dental scaler and stored in sterile 2.0 mL tubes until analysis.

Individual	**Age**	**Sex**	**Oral and Respiratory Pathologies**
		Calculus	
430*	30 - 35	Possible Male	Caries, periodontal disease, antemortem tooth loss
218	25 - 28	Possible Male	None observed
474*	35 - 40	Possible Male	Periodontal disease, maxillary sinusitis
414	28 - 32	Male	Abscesses
421	8 - 12	Unknown	None observed
212*	10 - 12	Unknown	None observed
343*	35 - 40	Male	Periodontal disease
419*	22 - 25	Female	Lesion on palatine process
359	24 - 26	Male	Abscesses
237*	25 - 29	Male	Caries, Abscesses
319	12 - 14	Unknown	None observed
276	23 - 25	Male	Periodontal disease, abscesses, maxillary sinusitis
306	27 - 31	Male	Periodontal disease,
389*	22 - 24	Male	None observed
268	11 - 13	Unknown	None observed
219*	26 - 35	Male	Abscesses
423	35 - 39	Male	None observed
459	30 - 35	Male	Abscesses
449	25 - 32	Male	None observed
358	30 - 39	Male	None observed

			Dentine
430	30 - 35	Possible Male	Caries, periodontal disease, antemortem tooth loss
212	10 - 12	Unknown	None observed

Table 9.2. Individuals selected for this study, *indicates individuals where samples of dental calculus were analysed using scanning electron microscopy. More detailed osteological information is listed in Appendix 1, Table 2.

9.2.2 Analysis of Dental Calculus with Scanning Electron Microscopy

Eight samples of dental calculus were analysed using scanning electron microscopy (SEM) in order to visualise calculus mineralisation and assess the microscopic composition of dental calculus (Table 9.2). Only the side which adhered to the tooth was observed. Samples were attached to sample holders by conductive sticky tape and no plating was used. SEM examination of each fragment was performed by FEI Quanta 3D 200i in high vacuum 10^{-4} Pa using a secondary electron detector with tuneable field of view. The microscope was set on probe current 0.18 nA or 0.36 nA at accelerating voltage 20 kV. Images were collected by integrating 128 images in the dwell time of 100 ns or 300 ns.

9.2.3 Proteomic Analysis

Extraction of Dental Calculus

Tryptic peptides were extracted from dental calculus samples using a filter-aided sample preparation protocol modified for ancient samples (Cappellini et al. 2014; Warinner, Rodrigues, et al. 2014; Chapter 5).

Extraction of Dentine

Peptides were extracted from two samples of dentine. This extraction was identical to the procedure for dental calculus, although the lysis solution was replaced with a commercially available lysis buffer designed for mammalian cells (M-PER, Thermo Scientific). Analysis of dentine was performed in order to compare microbial and host proteome composition with dental calculus and to explore differences in the preservation of proteins in these two dental tissues. In addition, this is because dentine is typically sterile during life and any bacteria recovered could be proxies for post mortem contamination (Warinner, Speller, and Collins 2015).

Extraction of St Helena Soil

Two extractions were performed on soil samples taken from the burial context, using the same protocol as extraction for dental calculus. Soil was extracted in order to assess the level of potential burial matrix contamination within dental tissues. If bacteria derived from the soil were identified in archaeological tissues, then these bacteria could be excluded.

MS/MS Analysis

MS/MS analysis on all samples was performed on a Q Exactive spectrometer (Thermo Scientific) at the Mass Spectrometry Laboratory of the Target Discovery Institute, University of Oxford, previously described in Warinner, Hendy, et al. (2014) and Chapter 5.

Data Analysis

Spectra were converted to Mascot generic format using Proteowizard version 3.0.4743 using the 200 most intense peaks in each MS/MS spectrum. MS/MS ion database searching was performed using Mascot (Matrix Science), against the UniProt database (2014) (541762 sequences; searches) and Human Oral Microbiome Database (Chen et al. 2010) (3643231

sequences). Searches were performed against a decoy database to generate false discovery rates (FDR). Search parameters included carbamidomethylation as a fixed modification and acetylation (protein N-term), deamidation of glutamine and asparagine, glutamine to pyroglutamate, methionine oxidation and hydroxylation of proline as variable modifications, based on modification patterns observed in Cappellini et al. (2012) and Warinner, Rodrigues, et al. (2014). Peptide tolerance was 10 ppm with semi-trypsin as the enzyme with up to two missed cleavages. MS/MS ion tolerance was 0.07 Da.

Mascot generated results were filtered to 1% peptide FDR. When there were insufficient protein identifications to make a meaningful 1% FDR calculation, the lowest FDR possible was chosen (Appendix 1, Table 1). When the number of peptides matching to a protein was less than five each of these peptides was searched using BLASTp (Altschul et al. 1990) against all non-redundant protein sequences (consisting of all translated sequences from GenBank and all sequences from Refseq, PDB, SwissProt, PIR and PRF) to confirm a unique match. In some cases identification down to species level was not possible due to the identification of conserved domains. If the peptide could be identified to at least the level of genus the protein was retained in the analysis. Each protein was manually searched in UniProtKB or available literature in terms of its Gene Ontology (GO) Biological Process, and Cellular Location and expression as characterised by UniProtKB (Ashburner et al. 2000; The UniProt Consortium 2014).

GO terms were occasionally difficult to classify in a meaningful way, especially given that many proteins identified had multiple or ambiguous functions. Arbitrary categories based on GO Biological Functions were additionally created in order to make these data easier to present and examine. In cases where a particular protein had multiple or ambiguous functions, the protein was characterised by its most dominant known function according to available

literature. For proteins where this is not clear they have been classed as “Multiple functions” or “Unknown”.

STRING v.10 (von Mering et al. 2005) was also used to explore possible functional networks in human proteins in dental calculus and dentine. STRING uses proteomic data from multiple databases and repositories to map protein-protein interaction networks and allows enriched functional pathways and Gene Ontologies to be observed. STRING also provides a visual map of protein-protein interactions. Understanding these networks and pathways is a crucial aspect of understanding system-level biological processes. Analysis was performed using default settings of medium confidence (0.400). Enriched Gene Ontologies and pathways were sorted by their p-value (Rivals et al. 2007; Franceschini et al. 2013). In this study functional networks and Gene Ontologies were only accepted if the confidence was $p<0.01$.

Three extraction blanks were included in this analysis, two with dental calculus extractions and one with dentine extraction. Proteins identified in these blanks (listed in Appendix 1) were excluded from further analysis as were any proteins common in laboratory environments, dust and protein standards as listed in the Common Repository of Adventitious Proteins (http://www.thegpm.org/cRAP/).

9.3 Results and Discussion

SEM analysis of dental calculus from individuals from St Helena reveals the calcified remains of a biofilm (Fig. 9.2). Figure 9.2 demonstrates the range of bacterial morphologies and impressions found in dental calculus, showing a) impressions of cocci bacteria, b) a possible spirochete, c) the preservation of rod and filamentous bacterial morphologies and d) cocci bacteria embedded within the calculus mineral. The observed spirochete bacterial form is

similar in size and morphology to spirochete forms observed in plaque using SEM (Peters et al. 1999; Riviere et al. 1991). This is could be from a *Treponema* species, most likely *Treponema denticola*, a common oral pathogenic species which was detected using proteomics in this study (section 9.3.3).

Observations of these bacterial forms corroborate with previous modern and archaeological SEM analyses of dental calculus (e.g. Charlier et al. 2010; Hardy et al. 2012). No dietary or environmental debris was observed by SEM. The range of forms observed reflects the composition of a mature plaque biofilm, which is comprised of a conglomerate of different bacterial forms (Zijnge et al. 2013). Calcification appears to first occur on the membrane and growth epitaxially from the surface, often leaving an electron light hollow inside (e.g. Fig. 9.2a), although sometimes the mineral appears to have also grown into and filled the cellular void (e.g. Fig 9.2c).

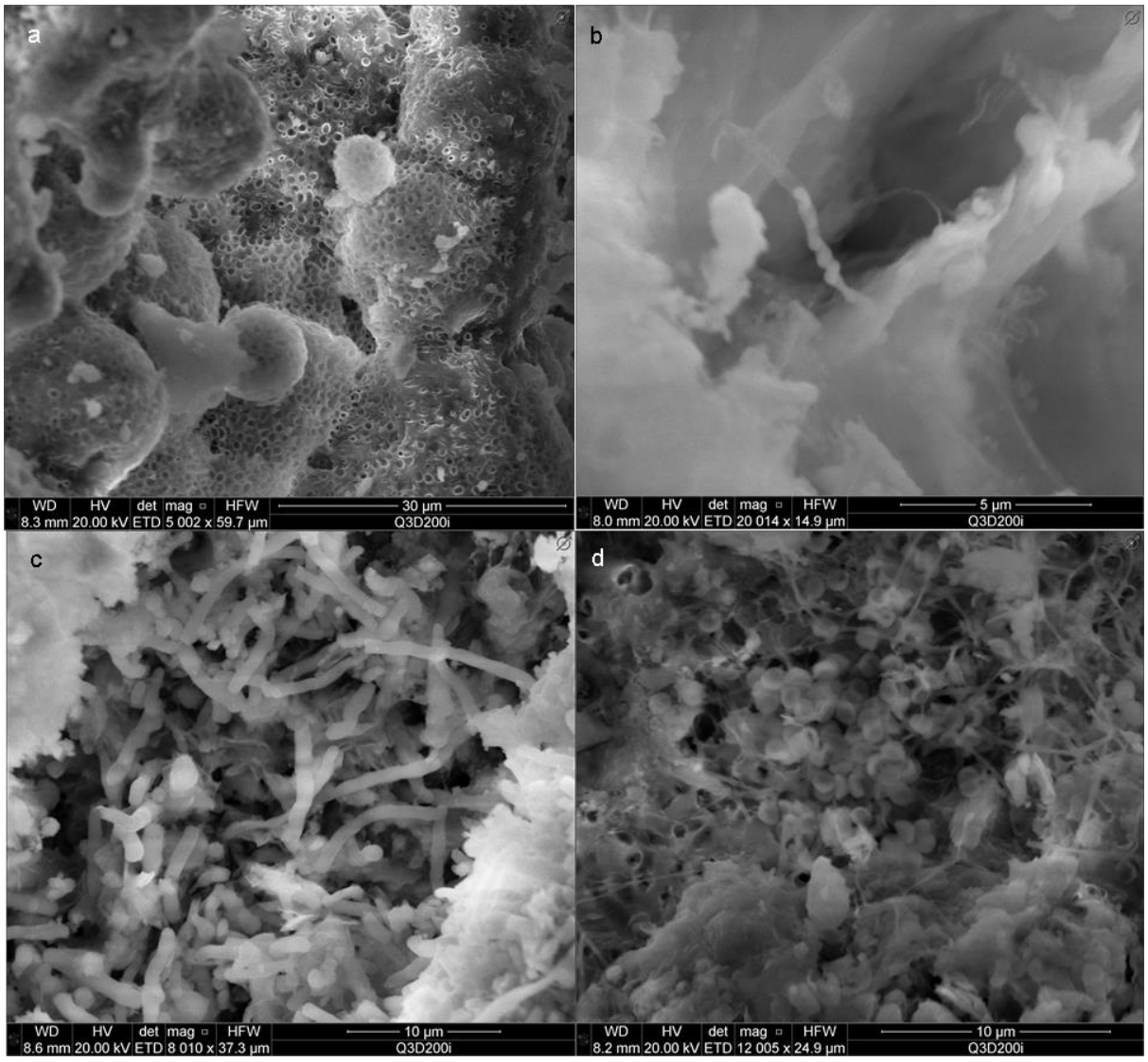

Figure 9.2. Calcified bacteria and bacterial impressions preserved in ancient dental calculus, showing a) impressions of cocci bacteria (individual 430), b) a possible spirochete (individual 212), c) the preservation of rod and filamentous bacterial morphologies (individual 430) and d) cocci bacteria embedded within the calculus mineral (individual 343).

Applying shotgun proteomics to this calcified biofilm this study has identified 160 different proteins (356 total proteins) from ancient human dental calculus (n=20), including proteins originating from the human host, commensal and pathogenic oral bacteria and proteins from potential dietary sources and the environment. The number of proteins identified for each

individual varies considerably (between 1 and 88). In samples of dentine (n=2) 64 proteins (98 total proteins) were identified, predominantly human derived. In soil samples (n=2) 7 proteins (11 total proteins) were identified. The origin of these proteins (bacterial, human, dietary, environmental or contamination) are presented for each material type in Figure 9.3.

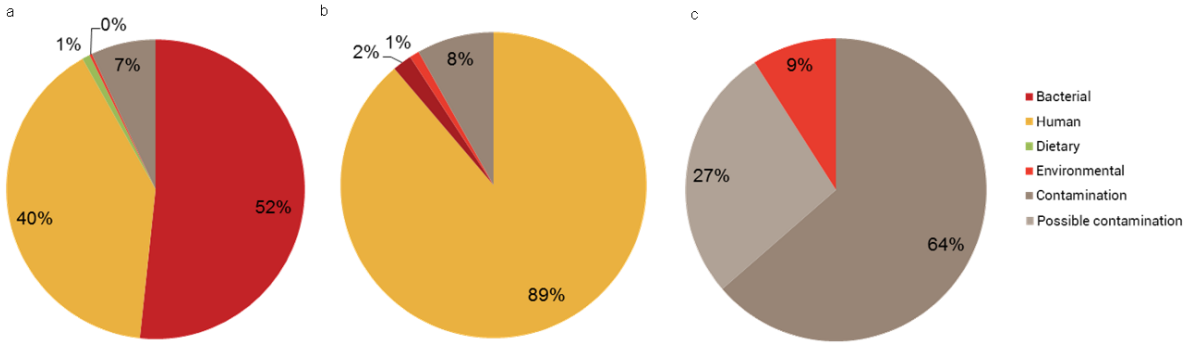

Figure 9.3. Sources of proteins identified in a) calculus, b) dentine and c) soil.

Comparing the origin of proteins extracted from these three sources, dental calculus is dominant in bacterial proteins, which suggests the preservation of a preserved biofilm. Unsurprisingly, dentine is comprised of 89% human proteins. In soil, of 7 total proteins only one bacterial protein was identified (*Pectobacterium carotovorum*, chaperone protein DnaK) and the remaining 6 are human. Of these, 5 (keratins and dermcidin) are likely to be contaminants derived from skin. Collagen (COL1A1) was also identified in soil as well as the negative control association with this extraction. The lack of proteins extracted and identified from soil is presumably because the extraction method performed on these samples is optimised for calcified biological tissues and the proteomic analysis of soils in archaeological contexts have had limited success (Oonk, Cappellini, and Collins 2012). In addition, our understanding of the soil microbiome lags behind that of the oral microbiome owing to the scale and diversity of environments occupied by soil bacteria. Thus, the lack of reference

genomes may also be hampering our ability to identify proteins from bacteria in this environment (Mendes et al. 2015).

The following section explores proteins identified in this study in terms of human proteins identified (7.3.1), bacterial proteins (7.3.2), proteins derived from food consumption (7.3.3) and proteins derived from the environment (7.3.4). All proteins identified in each individual are outlined in Appendix 1, Table 1.

9.3.1 Human Proteins

Dentine

This study identified 64 proteins (98 total proteins) from two samples of dentine. Of this total, 87 were human and 74 of these were non-collagenous proteins (NCPs), although the number of spectral matches to collagen is clearly high (e.g. 36% in 10806) given the dominance of this protein in dentine (Nanci 2014). Collagens were retained in this analysis, despite the presence of COL1A1 in one of the negative controls, because it is highly likely that the collagen observed in dentine is endogenous. Collectively, the proteins identified here constitute the dentine proteome, the suite of proteins that make up the bulk of the organic component of the tooth root. Each of these proteins has been studied in detail in terms of their function, location and interactions, and to explore each of these proteins individually would be exhaustive. The broad biological functions of the proteome will instead be explored and the proteome of these two individuals will be compared.

Dentine is one of the four major components of the tooth (the others being enamel, pulp and cementum) and is comprised of a (predominately) collagen matrix mineralised with

hydroxyapatite, formed by odontoblasts. Dentine is a porous structure, comprised of dentinal tubules which allow odontoblast processes and dentinal fluid to permeate the tissue.

Analysis of the biological functions of proteins extracted from dentine reveal that the dominant function present in this tissue is related to structural support (40%). Structural proteins, such as collagens, biglycan, fibronectin and lumican, provide cellular support or constitute the major extracellular components of tissues. Two such structural proteins identified (secreted phosphoprotein 24 and transforming growth factor beta-1) are specifically involved in bone remodelling. Several proteins also have active roles in biomineralisation (the process by which mineral structures are created from biological tissues), such as alpha-2-HS-glycoprotein, asporin, dentin matrix acidic phosphoprotein 1, mimecan, osteomodulin and tetranectin.

Some proteins detected are highly tissue specific. Matrix metalloproteinase-20 is secreted by odontoblasts and is localised in enamel, dentine and pulp tissue (Sulkala et al. 2002). Asporin is also specifically expressed in the periodontal ligament (Yamada et al. 2007). Dentin matrix acidic phosphoprotein 1 is also expressed specifically in the tooth, playing a role in dentine mineralisation (George et al. 1993). Proteins associated with the immune system were also detected (11%), including alpha-1-antichymotrypsin (associated with the acute phase response, the early phase of the immune system's response to inflammation or stress) S100A9, S100A7A, Ig gamma-1 chain C region, collectin-12, complement component C9, clusterin and fibroleukin. Proteins derived from the immune system may derive from proteins in the pulp or fluid in dentinal tubules (Knutsson, Jontell, and Bergenholtz 1994). Invasive bacterial products are able to permeate dentinal tubules, which can activate the immune system (Pashley 1996).

Additional data analysis using STRING reveals that the most common GO Biological Processes observed in ancient dentine are those related to blood coagulation, extracellular

matrix/structure support and platelet activities. Figure 9.4 highlights two functional networks from individual 212. Figure 9.4a depicts a cluster resulting from extracellular matrix/structure organisation and Figure 9.4b depicts a cluster originating from wound healing. These results indicate that the biological roles of proteins in dentine are still reflected in ancient samples.

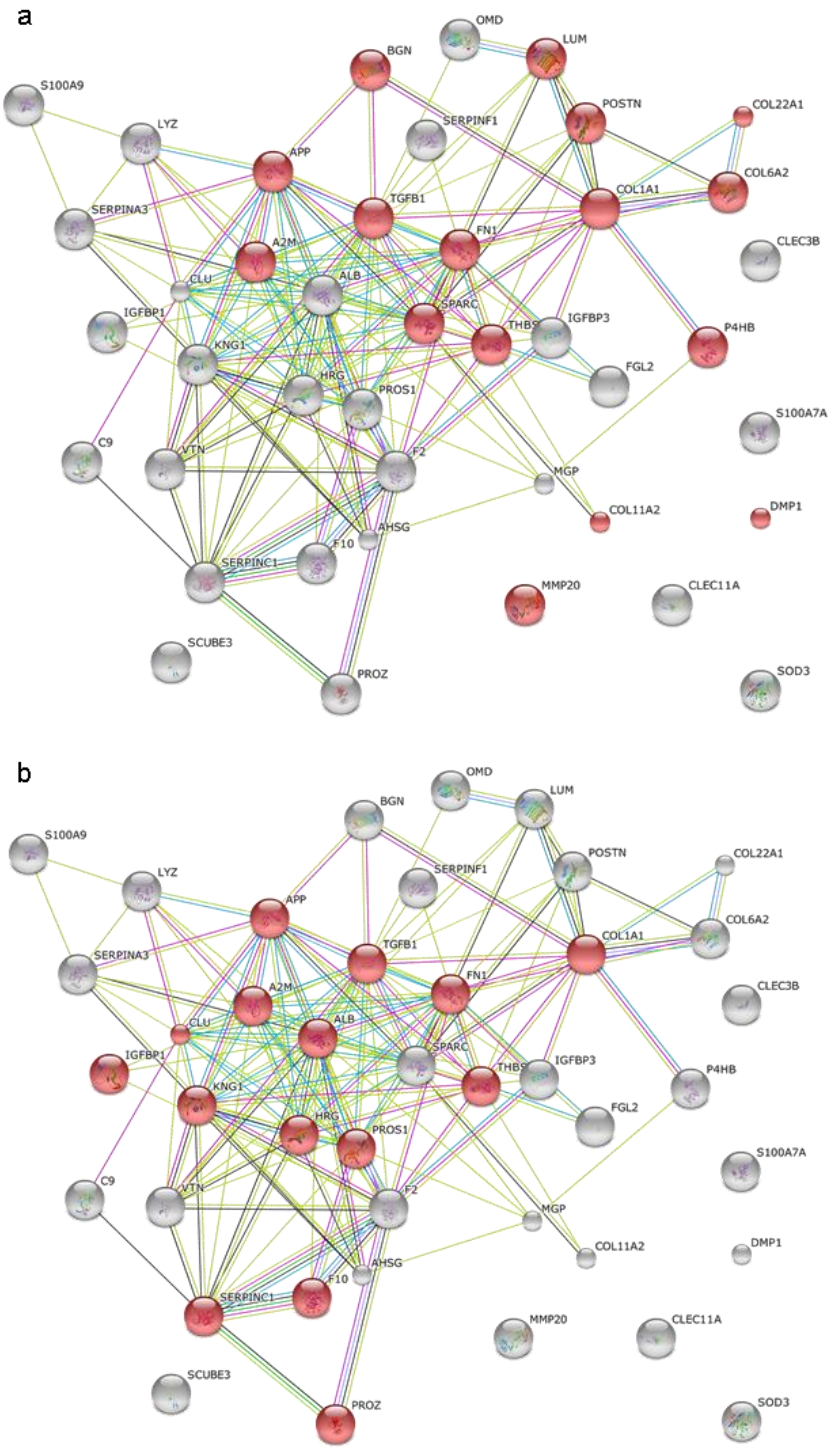

Figure 9.4. Functional networks identified by STRING for ancient dentine from individual 212, depicting a) the protein cluster involved in extracellular matrix support, and b) the protein cluster involved in wound healing. Each protein (represented by each circle) is annotated with its gene name. The lines between each protein represent a different aspect of biochemical

evidence which connects the two proteins, thus, the number of lines is a reflection of the strength of association between the two proteins.

The proteomic analysis of dental tissues is a large field of enquiry (e.g. Hu et al. 2005; Park et al. 2009; Chun et al. 2011; Jágr et al. 2012; Rezende et al. 2013). Proteomes derived from these tissues have been studied in order to understand the processes of tooth formation, as well as the response of the tooth structure to periodontal disease, and to identify markers for such disease progression (Silva, Rosa, and Lara 2004; Smith 2003). The proteome of ancient dentine has also been explored (Schmidt-Schultz and Schultz 2007; Warinner, Rodrigues, et al. 2014) by 2-D gels and immunological approaches in the former, and by shotgun proteomics in the latter. These studies have focussed on the most effective method for extracting NCPs from dentine and exploring the range and nature of NCPs surviving in this tissue. However, as yet there has been little interpretation of what the ancient dentine proteome might tell us about human health.

Now that an effective method for proteome characterisation from ancient dentine has been demonstrated in this and previous studies what are the potential applications for this approach? Schmidt-Schultz and Schultz (2004) proposed that identification of circulating antibodies may be one approach to identify specific past infections. However, as explored in Chapter 7, these proteins are unlikely to retain their immunological functionality. Shotgun proteomics was also used by Bona et al. (2014) to characterise biomarkers of tumour development in an osteogenic sarcoma. In the same study it was also observed that some proteins were over-expressed in the sarcoma compared to healthy bones. The authors propose that this approach could be used to detect early cancer progression where osteological analysis is not indicative of this disease. Thus, one major application could be in identifying diseases, infections or responses to

infections which are not osteological manifesting or the identification of early-stage disease onset.

Osteological analysis of individual 430 indicates that this individual suffered from periodontal disease, caries and antemortem tooth loss. Thus, does the proteomic profile differ between these two individuals to reflect this? STRING analysis of the dentine proteome from these two individuals revealed that there were no major differences in the ten most common GO Biological Processes observed between these two individuals (Fig. 9.5). To explore this further, the nature and number of proteins *uniquely* identified between these two individuals was examined, with the hypothesis that individual 430 may display more proteins related to an immune response or response to disease than individual 212. Similarly, there was no observable difference in the GO Biological Processes of these uniquely identified proteins when analysed using STRING. Thus, analysis of the whole proteome, as well as proteins that were uniquely found in each individual, did not demonstrate an enriched signal for an immune response or indicate proteomic evidence of periodontal disease in individual 430.

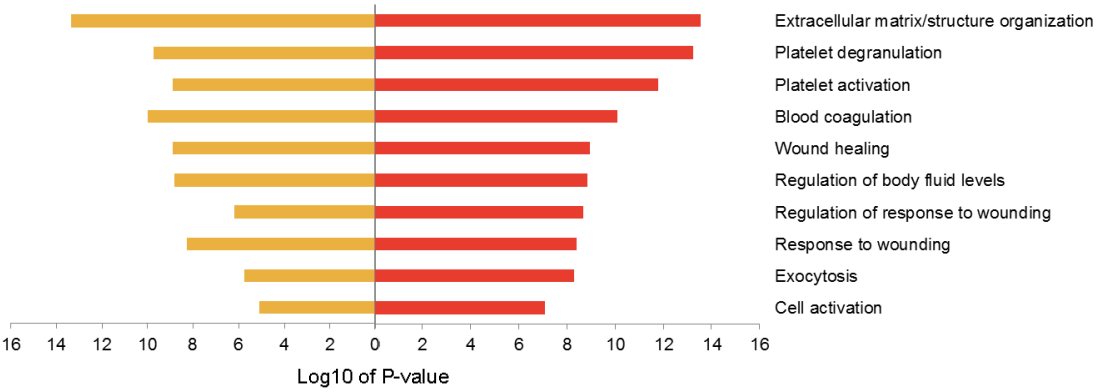

Figure 9.5. Dominant GO Biological Processes identified in STRING, individual 430 (left, yellow) and individual 212 (right, red).

Even if a difference was observed it should be noted that we do not yet understand the individual variability between proteins observed in ancient dentine, and to draw conclusions from only two samples would be problematic. It would also be worth considering that where in the tooth the dentine was sampled may also influence the proteomic profile, given that different proteins may have been entrapped at different stages in dentine development. If we wish to examine the differences in periodontal disease progression it is perhaps more informative to analyse alveolar bone or dental calculus which is adjacent to the gum-line.

Calculus

In samples of dental calculus from St Helena (n=20), 37 different proteins derived from the host were identified (140 total proteins). Collagen alpha-1(I) and anti-trypsin were the most commonly observed proteins, found in 15 of 20 individuals, followed by serum albumin in 11 individuals, and collagen alpha-2(I) in 9 individuals.

Protein functions were classified using the UniProtKB database and compared to those observed in dentine (Fig. 9.6). Proteins extracted from dentine reflect their role in maintaining structural support of the mineralised tissue (as discussed above). In contrast, proteins derived from dental calculus are more reflective of a dynamic process of host defence.

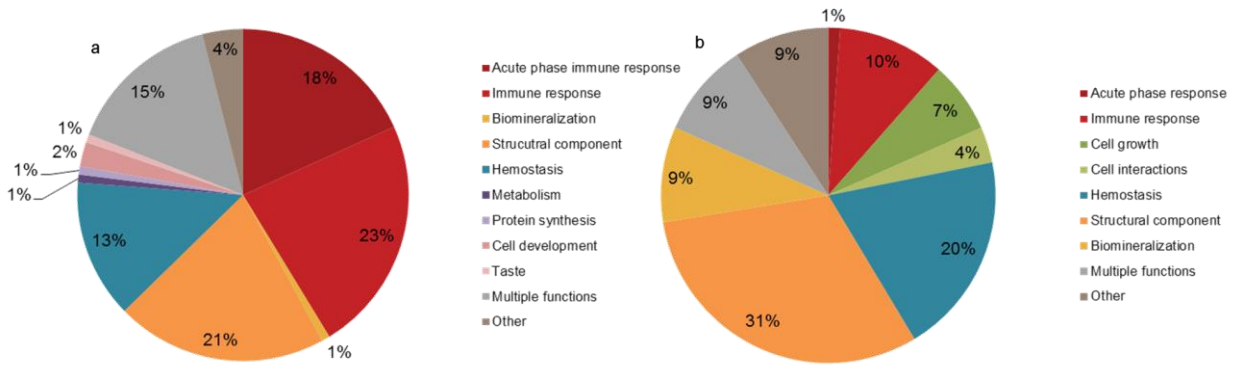

Figure 9.6. Biological functions of human-derived proteins detected in a) ancient dental calculus and b) ancient dentine.

To further investigate the biological function of human proteins observed in dental calculus all human proteins observed across all individuals were pooled and analysed using STRING. The ten most common GO Biological Processes observed are listed in Table 9.3. For an unknown reason, STRING was unable to identify immunoglobulins in its reference database so these proteins are excluded from this analysis.

Biological Process	**P-value**
Regulation of endopeptidase activity	7.980E-7
Defence response to bacterium	2.709E-6
Negative regulation of endopeptidase activity	2.709E-6
Retina homeostasis	2.709E-6
Negative regulation of peptidase activity	2.709E-6
Defence response to other organism	5.020E-6
Tissue homeostasis	1.349E-5
Negative regulation of proteolysis	2.209E-5
Response to transition metal nanoparticle	3.830E-5
Multicellular organismal homeostasis	4.289E-5

Table 9.3. The ten most enriched functional networks observed across all samples of dental calculus.

Human proteins found in dental calculus are predominantly associated with the immune system and responses to bacteria. These include serpin B10, resistin, S100-A9, S100-A8, peptidoglycan recognition protein 1, neutrophil defensin 1, neutrophil defensin 3, myeloperoxidase, lysozyme C, leukocyte elastase inhibitor, Ig lambda-2 chain C region, Ig lambda-3 chain C region, Ig kappa chain C region, Ig gamma-1 chain C region, Ig alpha-2 chain C region, eosinophil cationic protein, complement C3, bactericidal permeability-increasing protein, antileukoproteinase, alpha-1-antitrypsin and alpha-1-antichymotrypsin. Many of these (neutrophil defensin 1 and 3, serpin B10, resistin, S100-A9 and A8, myeloperoxidase, lysozyme C, bactericidal permeability-increasing protein and alpha-1-antitrypsin) constitute part of the proteome of neutrophils, cells which are among the first lines of defence for the host and normally circulate in the blood stream before responding to a site of stress or inflammation (Tomazella et al. 2009).

Proteins involved in structural support, either as a structural component or involved in biomineralisation, are also common (22%). This is largely owing to the abundance of collagens (COL1A1 and COL2A1) in most individuals, which could be derived from the gums or wounds in the mouth. Dr Vowells, a colonial surgeon on St Helena, observed that the tongue and cheeks of people on St Helena were covered in shed skin, which could explain this observation (Colonial Office, Great Britain 1850). Proteins extracted from dental calculus are reflective of a salivary environment, for example, containing proteins associated with taste (lipocalin-1, prolactin-inducible protein). However, it is noticeable that many salivary proteins were *not* detected. These include amylase (the enzyme responsible for starch digestion), statherin (which prevents calcium phosphate precipitation) or histatin (an antimicrobial protein) despite the fact that, in the case of the latter two, both have an affinity for binding to hydroxyapatite (Lee et al. 2013) and thus might be hypothesised to survival in calculus. The

proteome of ancient dental calculus is thus likely to constitute a palimpsest of proteins acquired from the oral environment, including proteins from acquired enamel pellicle (the first layer of proteins in the formation of the plaque biofilm), saliva, gingival fluid, as well as proteins derived from the gums, all of which become incorporated into the plaque biofilm.

Focussing on one individual where both the dentine and calculus proteome was analysed, the ten most commonly shared GO Biological Processes were compared (Fig. 9.7). In this one individual the different biological roles of these two tissues are clear and mirror the more general observations made above, where proteins derived from calculus are associated with the immune response and proteins derived from dentine are relate to structural support.

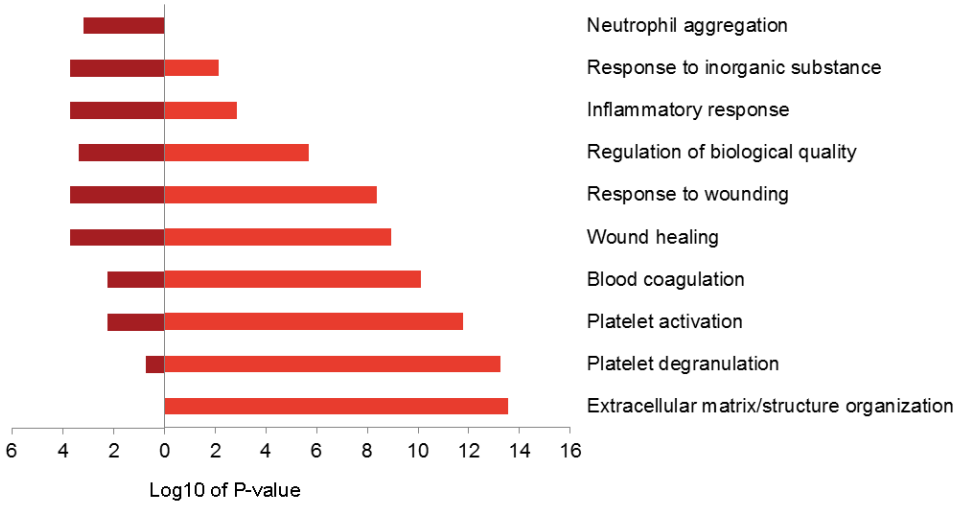

Figure 9.7. Dominant Biological Processes identified in STRING, calculus (left) and dentine (right) from individual 212.

STRING was also used to analyse whether proteins preserved in dental calculus display any differences in function between individuals. All 20 individuals were analysed, but only individuals where p-values for GO Biological Processes were less than 0.01 are included below. Only the GO Biological Process with the lowest p-value was recorded.

Individual	No. of human proteins used in STRING	GO Biological Process	P-value
212	12	inflammatory response; response to inorganic substance; wound healing and response to wounding	0.0001959
237	7	tissue homeostasis	0.00158
343	8	tissue homeostasis	0.00315
359	13	neutrophil aggregation; response to transition metal nanoparticle	0.00236
276	9	tissue homeostasis	0.00004259
358	25	defence response to bacterium; defence response to other organism	0.00001989

Table 9.4. The most enriched GO Biological Process from the proteome of ancient dental calculus from six individuals.

Table 9.4 demonstrates that there are differences in Biological Processes between individual samples of dental calculus. In individuals 237, 343 and 276 the dominant process reflected in the proteome is tissue homeostasis (proteins involved in maintaining the tissue structure). In contrast, individuals 358 and 359 have profiles related to the immune system, whilst individual 212 has a profile related to wound healing. These different profiles could be a reflection of differences in oral health. However, a major question here, which will only be answered as we develop our understanding of protein taphonomy and understand the method's sensitivity, is whether this a true reflection of processes occurring in the mouth or a reflection of which proteins are preferentially preserved. An additional aspect to explore will be the effect of time-averaging on the dental calculus signal. Dental calculus represents a palimpsest of protein signatures accumulated during the formation of this deposit. Thus, deciphering whether an infection represents a single event or an accumulation of events is challenging. These aspects are discussed in Chapter 11.

9.3.2 Bacterial Proteins

Two bacteria were identified in two samples of dentine *(Gluconacetobacter diazotrophicus* and *Anabaena variabilis).* Both these species have previously been isolated from environmental contexts (Saravan et al. 2008; Thiel, Lyons, and Erker 1997) and are likely to be derived from the burial environment (Hollund et al. 2013). Neither of these species are documented to have any associations with the human infections and no further interpretations about the presence of these bacteria have been made.

From 20 samples of dental calculus this study identified 110 different bacterial proteins (184 total proteins) from 46 bacterial species. Commensal species derived from the oral microbiome were the most common (42%) followed by proteins derived from pathogenic bacteria (31%) (Fig. 9.8). In some cases (17%), where a species was only able to be assigned to a genus level, it cannot be known whether the species derives from a pathogenic or commensal organism. The number of bacteria identified in each individual varied substantially, from no bacteria identified to 56.

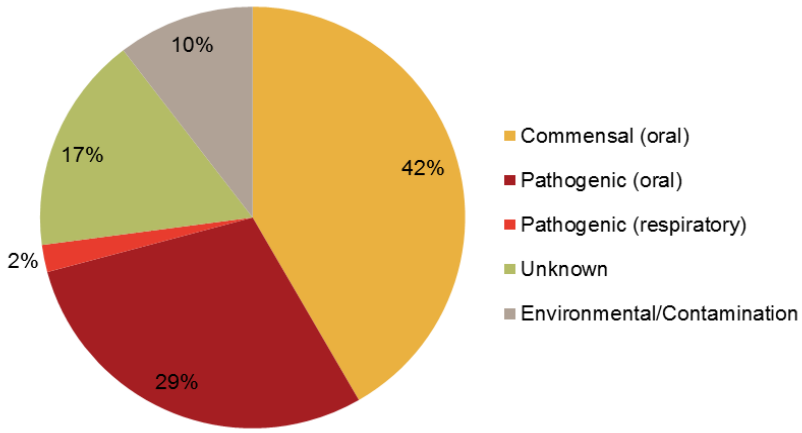

Figure 9.8. Composition of bacteria identified across 20 samples of ancient dental calculus

Commensal Species: The Oral Microbiome

42% of bacterial proteins identified in ancient dental calculus were derived from members of the oral microbiome. Genera include *Actinomyces, Aggregatibacter, Campylobacter, Fusobacterium, Neisseria, Prevotella, Selenomonas, Synergistetes, Bacteroidetes, Cardiobacterium, Eikenella, Kingella, Lachnospiraceae, Lactobacillus, Lautropia, Pyramidobacter, Shuttleworthia* and *Veillonella.* All identified species are listed in Appendix 1.

The oral microbiome is just one of the complex, microbial communities that inhabit the human body and is the second largest microbial community in the body, second to the gut (Paster et al. 2001; Chen et al. 2010). One millilitre of saliva contains over 100 million microorganisms and in dental plaque this number is even higher, with over 200 million bacterial cells per milligram (Socransky and Haffajee 2005). As of the year 2010, approximately 700 prokaryote species have been identified in the human oral environment, primarily existing as biofilms on oral surfaces (Chen et al. 2010). Microorganisms in this environment exist as part of biofilm communities, and the oral environment is host to a range of bacterial ecosystems each with their distinct community (Dewhirst et al. 2010). The dental plaque biofilm is one such community and is the most relevant to this study as dental calculus is the mineralised form of this biofilm. Dental plaque is one of the most complex biofilms found in nature and has many characteristic features of a bacterial biofilm, such as increased resistance to antibiotics, a role in host defences and increased resistance to localised shear forces (Marsh 2005).

The oral microbiome is integrally linked with the oral health of its host. The presence of commensal oral species limits colonisation from pathogens, a phenomenon termed ‘niche saturation’ (Sullivan, Edlund, and Nord 2001; Van Hoogmoed et al. 2008). Some commensal bacteria have also been identified as antagonists towards incoming pathogens (Wescombe et

al. 2009). Oral bacteria may also play a role in cardiovascular health by maintaining levels of nitric oxide, which has an anti-hypertensive effect on blood vessels (Govoni et al. 2008).

Characterisation of Biofilm Composition

Culture-independent methods, such as next-generation sequencing, have been instrumental in isolating and characterising the composition of the oral microbiome (Wade 2013b). However, as discussed above, the microbiome community exists and functions as a complete community in itself, so identifying individual species within the microbiome is only one part of exploring a much broader system. Hence, researchers have turned to metaproteomics and transcriptomics in order to understand community level behaviours, and the relationship between the microbiome and environmental, chemical, physical and biological processes (Siggins, Gunnigle, and Abram 2012; Kolmeder and de Vos 2014; Jorth et al. 2014). Proteomics is also used to explore bacterial composition of oral communities as a complement to genomic approaches (e.g. Rudney et al. 2010; Veloo et al. 2011). To my knowledge, apart from Warinner, Rodrigues, et al. (2014), the proteome of dental calculus has not been studied even in modern contexts, despite the fact that channels and lacunae created due to calcification could be reservoirs for pathogenic bacteria in the calculus of living people (Tan et al. 2004). However, the proteomic composition of saliva and plaque has been studied extensively (reviewed in Socransky and Haffajee 2005; Jenkinson 2011; Siggins, Gunnigle, and Abram 2012; Wade 2013a; Kolmeder and de Vos 2014)

Proteomic analysis of the oral microbiome has typically focussed on the analysis of particular oral bacteria within the microbiome, specifically addressing how their proteome may differ when the bacteria is isolated on its own and in a biofilm community (Macarthur and Jacques

2003). For example, Svensäter et al. (2001) found that *Streptococcus mutan*s expressed a different proteomic profile when it existed in a biofilm compared to its native state. Similarly, Kuboniwa et al. (2009) found that the virulent periodontitis-associated bacteria *Porphyromonas gingivalis* expressed proteins associated with physiological support when it inhabited a biofilm community. Proteomic analysis of *Tannerella forsythia*, another periodontitis-associated pathogen, displayed an up-regulated expression of proteins resistant to oxidative stress when the bacteria was growing in a biofilm, indicating that when functioning as a community this bacteria has enhanced resistance to host defences (Pham et al. 2010).

Function of the Oral Microbiome

In this study, in order to identify community-level functions of bacterial in ancient dental calculus functional profiles were determined by manually examining each detected protein in UniProtKB in terms of its Gene Ontology Biological Process. In the case of ambiguities, available literature was sourced or, if available, the function was obtained from the same protein in a species from the same genus. The use of STRING for this classification was not possible, as the tool requires the proteome to be derived from one organism. Bacterial proteins are less well characterised than human proteins, so the functions of 34% of bacterial proteins could not be identified (Fig. 9.9).

Functional profiles of bacteria from ancient dental calculus are dominant in glycolysis (19%) and metabolic processes (23%) (Fig. 9.9). Proteins involved in virulence are also present (8%). Proteins associated with transport, defined as the movement of substances across or within a cell or between organisms, are also observed (15%). Compared to the salivary metaproteome in modern studies, glycolysis was also identified as a dominant function of salivary bacteria

(Jagtap et al. 2012; Rudney et al. 2010). This is to be a reflection of bacteria metabolising human salivary glycoproteins.

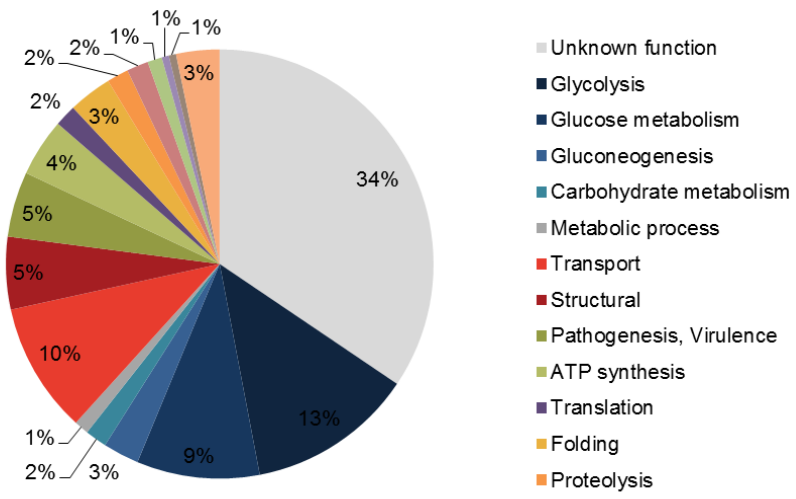

Figure 9.9. Bacterial protein functions observed in all samples of ancient dental calculus.

Bacteria proteins observed in ancient dental calculus also reflect their role in forming the extracellular biomolecular matrix of the biofilm. Bacteria inhabit biofilms within a complex biomolecular matrix comprised of extracellular polymeric substances which provide structural integrity to the biofilm (Flemming and Wingender 2010). For example, surface proteins act as adhesins, which provide adhesion either to oral surfaces, host proteins or to allow co-aggregation to other bacteria. In this study, surface proteins identified from *Campylobacter showae*, *Actinomyces* sp., *Neisseria* sp., *Porphyromonas gingivalis*, *Selenomonas* sp., *Synergistes* sp., *Tannerella forsythia* and *Veillonella* sp. were detected.

Pathogens and Opportunistic Pathogens

This study identified a number of proteins from documented pathogenic bacteria and opportunistic pathogens (Table 9.5).

Bacteria	Virulence factors	Disease association	Reference
Actinomyces graevenitzii	-	Isolated from pulmonary infections and has been reported to cause actinomycosis, may also be an early coloniser of the oral cavity	Könönen and Wade 2015; Hall 2008; Ramos et al. 1997; Sarkonen et al. 2000
Alloprevotella tannerae	-	Periodontitis associated	Lourenço et al. 2014; Serra e Silva Filho et al. 2014
Atopobium parvulum	GroEL (cross-reactivity with human heat shock proteins may be associated with disease)	May be implicated in oral disease, associated with halitosis	Kazor et al. 2003
Campylobacter showae	Major outer membrane protein	May be associated with periodontal disease	Macuch and Tanner 2000
Enterococcus spp.	-	Lactic acid producing bacteria implicated in caries, isolated from periodontitc dental plaque	Sedgley et al. 2005
Eubacterium yurii	Peptidase	May be involved in periodontal disease	Margaret, Heath, and Krywolap 1990
Filifactor alocis	-	Emerging as an important periodontal pathogen, associated with periodontal inflammation and a marker for periodontal disease, also associated with different forms of periradicular periodontitis	Gomes et al. 2006; Schlafer et al. 2010; Moffatt et al. 2011; Wang et al. 2013; Siqueira and Rôças 2003a
Leptotrichia buccalis	Flagellar motor protein	May be associated with caries and other oral infections	Eribe and Olsen 2008
Porphyromonas gingivalis	Pg-II fimbriae a, Zinc carboxypeptidase, Lys-gingipain, Hemagglutinin A, Major fimbrial subunit protein type-2, Major fimbrial subunit protein type-1.	Well characterised pathogen associated with periodontitis; part of the “red complex”	Reviews: Yilmaz 2008; Hajishengallis 2009; Holt and Ebersole 2005
Prevotella oris	-	Periodontitis associated, co-aggregates with *Porphyromonas gingivalis*	Dymock et al. 1996; Sato and Nakazawa 2014
Pseudoramibacter alactolyticus	-	Can be periodontitis associated, a possible endodontic pathogen and prevalent in infected apical root canal of periodontitis	Siqueira and Rôças 2003b; Chhour et al. 2005; Siqueira et al. 2009
Streptococcus mutans	-	Associated with dental caries	Loesche 1986
Tannerella forsythia	Surface layer protein B, Outer membrane	Periodontitis associated; part of the “red complex”	Holt and Ebersole 2005; Sharma 2010;

	protein 40		Tanner and Izard 2006; Shimotahira et al. 2013
Treponema denticola	-	Periodontitis associated; part of the "red complex".	Sela 2001
Selenomonas sp.	Flagellin, S-layer protein	Associated with gingivitis and periodontitis in some studies	Hespell, Paster, and Dewhirst 2006; Drescher et al. 2010; Gonçalves et al. 2012

Table 9.5. Pathogenic oral and respiratory species identified in ancient dental calculus, indicating virulence factors identified in this study.

In addition to the species listed in Table 9.5, a number of proteins were also identified which could only be assigned to the genus level. These include *Treponema*, *Actinomyces*, *Fusobacterium*, *Streptococcus*, *Enterococcus*, *Aggregatibacter*, and *Capnocytophaga.* Many of these genera contain pathogenic species but the identification of these cannot be confirmed.

The majority of identified pathogenic bacteria are those associated with periodontitis and gingivitis. *Streptococcus mutans,* the microorganism most implicated in caries development (Selwitz, Ismail, and Pitts 2007) was also observed in one individual. *Actinomyces graevenitzii* was the only potential respiratory pathogen identified although this may be an opportunistic pathogen (Sarkonen et al. 2000; Hall 2008; Fujita et al. 2012). Of particular note are *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia*, the so-called "red-complex" bacteria which are implicated in severe periodontal disease (Rôças et al. 2001).

This study identified a number of virulence factors (listed in Table 9.5), where a virulence factor is defined as "any moiety that is necessary for causing disease in the host" (Kumar, Mason, and Yu 2013, pg. 158). It is possible that the isolation of virulence factors is evidence that the host was under attack from the pathogen as some point during calculus formation. Fimbriae are important for pathogenic adhesion, adhering to proteins in the pellicle and

epithelial cells, stimulating a leukocytic response. For example, fimbriae from *Porphyromonas gingivalis* identified in this study have the ability to stimulate processes in the human host that lead to bone resorption (Hajishengallis 2009). Extracellular proteolytic enzymes (proteases) are also classed as virulence factors, causing hydrolysis of peptide bonds and resulting in the break-up of peptide chains. One of the most well-studied examples of such proteins are gingipains, the major virulence factors of *Porphyromonas gingivalis*, (Hajishengallis 2009). Two gingipains derived from *Porphyromonas gingivalis* were detected in this study (lys-gingipain and hemagglutinin A). Surface layer proteins are also implicated in virulence, as well as being integral parts of the biofilm matrix. For example, *Campylobacter* sp. surface layer proteins, one of which was observed in this study, avoid the host's immune system by preventing the binding of complement factor C3b to the bacteria (Thompson 2002). Two virulence factors were also identified from *Veillonella* sp. and *Aggregatibacter* sp., hemagglutinin and peptidoglycan-associated lipoprotein, respectively. These bacterial proteins could only be assigned to the genus level so it is uncertain whether these represent pathogenic species. However, the detection of these virulence factors could indicate pathogenicity.

In terms of individual patterns of oral disease, the composition of commensal and pathogenic bacteria was assessed for each individual by comparing the proportion of pathogenic to commensal bacteria (Table 9.6).

Individual	No. of pathogenic bacteria (No. of proteins)	No. of commensal bacteria (No. of proteins)	Proportion of pathogenic to commensal bacteria	Human proteins
212	3 (3)	5 (5)	0.60	inflammatory response; wound healing and response to wounding
218	0 (0)	1 (1)	0.00	
237	1 (1)	1 (1)	1.00	tissue homeostasis

343	2 (2)	5 (6)	0.40	tissue homeostasis
359	5 (5)	14 (29)	0.36	neutrophil aggregation;
421	0 (0)	1 (1)	0.00	-
430	1 (1)	4 (5)	0.25	-
474	3 (3)	7 (9)	0.43	-
219	1 (1)	3 (3)	0.33	-
268	0 (0)	0 (0)	-	-
276	0 (0)	3 (3)	0.00	tissue homeostasis
306	1 (1)	5 (8)	0.20	-
319	0 (0)	0 (0)	-	-
389	4 (6)	4 (9)	1.00	-
419	2 (2)	6 (8)	0.33	-
449	0 (0)	0 (0)	-	-
414	2 (2)	3 (3)	0.67	-
423	2 (2)	2 (2)	1.00	-
459	0 (0)	0 (0)	-	-
358	10 (15)	18 (41)	0.56	defence response to bacterium; defence response to other organism

Table 9.6. Number of pathogenic and commensal species observed in the dental calculus of 20 individuals, as well as the proportion of pathogenic to commensal species.

Individuals 414, 423, 389, 358 and 212 have the highest proportion of pathogenic to commensal species identified, with pathogenic bacteria comprising at least half of all identified bacteria in each individual. We could speculate from this that these individuals were experiencing periodontal infections at the time of calculus formation. Proteins derived from "red complex" bacteria, which are heavily implicated in periodontal disease and may serve as markers for this infection (Rôças et al. 2001; Holt and Ebersole 2005; Thurnheer, Belibasakis, and Bostanci 2014), were identified in several individuals (Table 9.6). The relationship

between pathogenic bacteria and osteological evidence for infection, and how this may be related to conditions of transatlantic slavery, is discussed in Chapter 10.

Whilst individual bacteria can be recognised as pathogenic, oral diseases are typically polymicrobial (Jenkinson and Lamont 2005; Marsh et al. 2009; Wade 2013a). Distinct groups of bacteria are associated with initiation and progression of different types of periodontal disease. However, untangling these networks constitutes ongoing research and it is challenging to even understand what constitutes a healthy consortium of oral bacteria (Aas et al. 2005). Whilst oral bacteria are clearly implicated in oral disease, the same microbiota have also been implicated in conditions outside the oral environment. These include pneumonia caused by oral periodontopathic bacteria (Scannapieco 1999), obesity (Goodson et al. 2009) and atherosclerotic plaque (Haraszthy et al. 2000).

9.3.3 Potential Dietary Proteins

One food source was identified in the analysis of dental calculus from St Helena. Proteins derived from peanut *(Arachis hypogaea)* were identified in two individuals. These proteins were galactose-binding lectin and conglutin, both of which are found in peanut seeds (Kottapalli et al. 2008). The peanut, although American in origin, was widespread in West Africa in the 19th century and became an important commercial crop during this time (Brooks 1975). Based on written documentation concerning food consumption on St Helena (Pearson et al. 2011; Great Britain 1789) there is no evidence of peanut consumption on St Helena. The identification of this protein is possible evidence of food consumption in Africa prior to the Middle Passage. It also further evidence that dental calculus can act as a reservoir for dietary

biomolecules (Warinner, Rodrigues, et al. 2014). This is the first plant-derived protein to be detected in ancient dental calculus.

Proteins derived from yeast (*Saccharomyces cerevisiae*, Brewer's yeast/Baker's yeast) were also identified in dental calculus. Three proteins were observed to the genus (*Saccharomyces* sp.) and family (Saccharomycetaceae) level. *Saccharomyces cerevisiae* and other *Saccharomyces* sp. have been identified in the healthy oral mycobiome (Ghannoum et al 2010) and in HIV patients (Aas et al 2007), although they may exist as part of the commensal oral microflora. Given that Saccharomycetaceae are ubiquitous in nature and may constitute commensal oral microflora, it may be difficult to interpret the presence of yeasts as dietary in origin. Yeasts were identified in dental calculus microscopically by Blondiaux and Charlier (2008), and similarly the authors conclude that yeast contamination from the environment cannot be ruled out. In addition, in the negative control associated with dentine protein extraction Saccharomycetaceae was identified.

9.3.4 Environmental Sources

Several proteins derived from environmental bacteria were identified in ancient dental calculus, and are mostly likely derived from the burial environment. These include *Corynebacterium efficiens*, *Desulfatibacillum sp*., *Methylovorus sp*. and *Stenotrophomonas maltophilia*. *S. maltophilia* has been identified in respiratory infections, but is present in the environment (including soil) and thus has been excluded from any further analysis. No further interpretation of these bacteria has been made. Bacteria identified from soil extractions and dentine extractions are mentioned in section 9.3 and 9.3.2.

9.4 Preservation

The most significant output of this study is the characterisation of an ancient oral microbiome composition and function. However, a key question in this characterisation is whether this composition and function is an accurate reflection of the living tissue, or whether biomolecular preservation has a major, confounding influence. In modern studies of the oral microbiome, proteomic approaches have focussed on understanding microbial composition (as a complement to DNA profiling) and for understanding the community-level function of the microbiome by exploring which proteins are expressed and up-regulated (e.g. Macarthur and Jacques 2003; Kuboniwa et al. 2009; Pham et al. 2010; Jagtap et al. 2012; Lee et al. 2013; Rezende et al. 2013). For the exploration of the microbiome in archaeological samples it may be challenging to adopt this metaproteomic approach outright because taphonomic processes will likely have a significant effect on the number and nature of observed proteins. In order to understand whether ancient samples are a reflection of protein expression in the living tissue it is vital to explore the nature of protein survival in dental calculus. The following section explores the preservation of bacteria (9.4.1), the nature of protein preservation (9.4.2) and the relationship between DNA and protein preservation in dental calculus (9.4.3).

9.4.1 The Preservation of Bacteria in Dental Calculus

A range of oral bacteria were identified from ancient dental calculus in this study. To explore whether particular bacteria appear to be preferentially surviving, the cellular structure and shape of bacteria identified in this study were explored.

Bacterial Cell Walls

Bacteria can be broadly classified based on the composition of their cellular membranes. Gram-negative bacteria have an external cell membrane comprised of lipopolysaccharides. This membrane is absent in Gram-positive bacteria, although Gram-positive bacteria have a peptidoglycan layer which is much thicker, with multiple layers (Silhavy, Kahne, and Walker 2010). In archaeological samples, it has been proposed that ancient DNA derived from mycobacteria is more resistant to degradation than other bacterial DNA (Donoghue and Spigelman 2006; Schuenemann et al. 2013). For example, Schuenemann et al. (2013) observed that *Mycobacterium leprae* displayed less DNA damage than *Yersinia pestis* and human mtDNA. They attribute this difference to the waxy cell wall present in *M. leprae*, which may provide an increased resistance to diagenesis, although, as Wilbur et al. (2009) argue, DNA degradation could in fact occur from nucleases and other biomolecules already present inside the bacterial cell walls. Along a similar argument to Schuenemann et al. (2013) and Donoghue and Spigelman (2006), one might hypothesise that Gram-positive bacteria might preferentially survive over Gram-negative species in archaeological contexts given the thicker layer of peptidoglycan in the cell envelope.

The dental plaque biofilm is a mixture of Gram-positive and negative species. In its early formation dental plaque is dominant in Gram-positive bacteria, particularly *Streptococcus* (Marsh and Bradshaw 1995). However, as the biofilm matures it becomes more dominant in Gram-negative bacteria (Kuramitsu et al. 2007). One might speculate by extension that dental calculus may be more dominant in Gram-negative bacteria since it forms when the plaque biofilm is mature, although intact Gram-positive membranes have been observed in ancient and modern dental calculus (Warinner, Rodrigues, et al. 2014).

Bacteria identified in this study were classified as Gram-positive or negative by searching available literature, and the number of proteins derived from both these classes identified. In addition, using previously published data from Warinner, Rodrigues, et al. (2014), Gram-positive and Gram-negative bacteria identified in samples of modern dental calculus were explored using the name approach.

Gram-negative species constitute approximately two-thirds of bacterial proteins identified in ancient dental calculus from people buried on St Helena (Fig. 9.10a). In calculus from living people this proportion is reversed, with 76% percent of bacterial proteins derived from Gram-positive bacteria (Fig. 9.10b). The most significant factor which appears to be resulting in this difference is the high number of proteins deriving from *Corynebacterium* spp. and *Streptococcu*s spp. (both Gram-positive) in modern dental calculus.

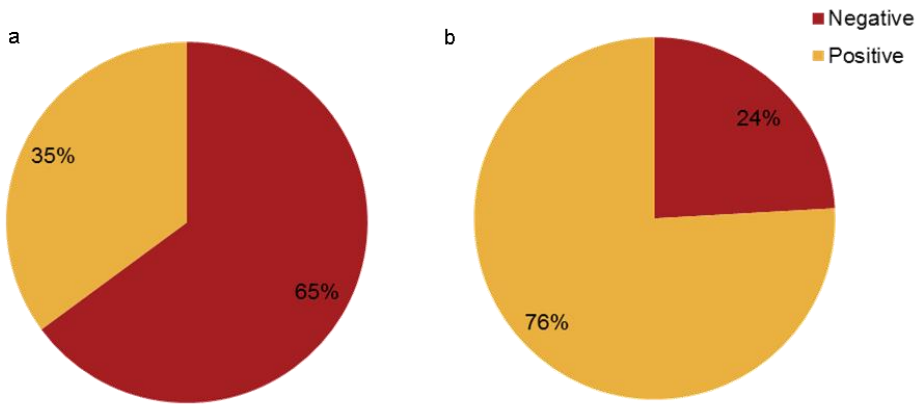

Figure 9.10. Proportion of Gram-negative and Gram positive bacteria identified in a) ancient dental calculus and b) modern dental calculus from Warinner, Rodrigues, et al. (2014).

These results do not support the above hypothesis that Gram-positive bacteria may preferentially survive over Gram-negative species owing to an additional stability from their

cellular structure. However, this observation may not solely be the result of taphonomic processes and could derive from differences in biofilm structure. We do not yet have an understanding of the composition of plaque biofilms in historic populations, so it may be problematic to assume that oral biofilms in the past and the present had similar bacterial compositions. In addition, these modern samples derive from Europe, and the archaeological samples derive from Africa. Thus, these observations could be due to the particular bacterial compositions unique to this population, or could be a result of differences in oral health.

Adler et al. (2013), using ancient DNA analysis in dental calculus from a range of time periods, observed that Gram-negative species were less abundant in ancient samples in dental calculus compared to modern ones and that Gram-negative species were less frequently observed in older samples. The authors ascribe this observation to a taphonomic bias, resulting in the exclusion of Gram-negative taxa in subsequent bacterial composition analysis. It is interesting that proteomic data from this study does not corroborate these observations, or that of Schuenemann et al. (2013) and Donoghue and Spigelman (2006). Further work exploring the nature of the extracellular matrix in dental calculus and the variability of bacterial biofilms across archaeological populations may help to untangle this trend.

Bacterial Morphologies

To explore whether the particular bacterial morphological forms may be influencing protein survival, the number of proteins observed in each individual was compared with the kinds of morphologies observed. This exploration was based on the hypothesis that bacteria with more surface area would have more sites for mineralisation and thus may be preferentially preserved. Bacterial morphologies were ascribed to each identified bacteria by searching available literature, and the number proteins derived from each of these morphologies across

all samples was counted. The most common forms observed were rod and rod-related shapes, followed by cocci morphologies (Fig. 9.11), a reflection of the diverse range of bacterial morphologies observed in mature plaque biofilms (Rickard et al. 2003; Zijnge et al. 2013). In comparing samples with high and low numbers of bacterial proteins, there appeared to be no trend in the kinds of bacterial forms identified in well preserved or poorly preserved samples.

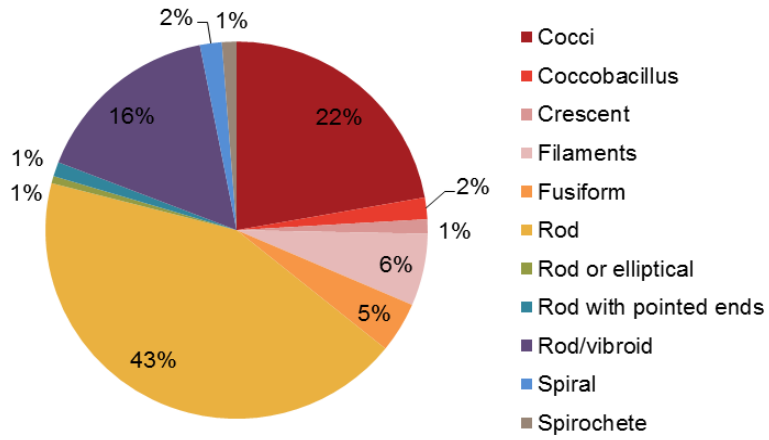

Figure 9.11. Bacterial morphologies observed in ancient dental calculus

9.4.2 Preservation of Proteins in Dental Calculus

Although the survival of collagen (Collins et al. 1995; Holmes et al. 2005; Dobberstein et al. 2009; Buckley and Collins, 2011) and osteocalcin (Collins et al. 2000; Smith et al. 2005; Buckley et al. 2008), the two most abundant bone proteins, have been extensively explored, the survival of other proteins from archaeological material are less well understood. Until recently, this may be in part due to limitations in isolating and identifying less abundant NCPs. However, with tandem mass spectrometry, and the ability to access the proteome from archaeological materials, we have the opportunity to explore this survival (e.g. Wadsworth and Buckley 2014; Buckley and Wadsworth 2014). In a similar mechanism to collagen (Collins et

al. 2002), it may be the case that particular non-collagenous proteins (NCPs) survive because of their binding to calcium phosphate minerals (Wadsworth and Buckley 2014). In this study, a number of proteins identified in dentine are documented to bind to hydroxyapatite, such as lumican, biglycan, secreted phosphoprotein 24, alpha-2-HS-glycoprotein, serum albumin, thrombospondin-1 and Osteomodulin (Zhou 2007; Jágr et al. 2012). The binding of these proteins to hydroxyapatite plays a role in tissue construction and support, so the nature of the function of these proteins may also be the reason they survive archaeologically.

As well as binding to mineral, is it possible that binding of NCPs to collagen, the most stable extracellular protein, may assist with protein survival. One family of proteins where this is documented to occur is small leucine-rich proteoglycans (SLRP) (Iozzo 1999). In this diverse family, proteins contain a structural motif characterised by leucines in conserved positions (Hocking, Shinomura, and McQuillan 1998). In the proteome of dentine we do indeed see proteins with the leucine-rich repeat motif, such as asporin, biglycan, lumican, osteoadherin/osteomodulin, mimecan (Kalamajski et al. 2009). These proteins all bind to collagen in assistance with the assembly of the extracellular matrix. The preservation of SLRP proteins was also documented by Wadsworth and Buckley (2014). In addition, fibronectin and thrombospondin -1 also interact with collagen, binding to extracellular matrix proteins. Buckley and Wadsworth (2014) also assessed the nature of proteins which survive in the bone proteome, observing that NCPs survive when they are mineral-bound, the result of entrapment during bone remodelling.

Explaining protein survival in ancient dental calculus is more complex. In dental calculus the mechanism of biomineralisation is more complicated, as calculus mineral is comprised of at least four crystal types of calcium phosphate, which alter as the mineralised biofilm matures (Roberts-Harry and Clerehugh 2000). While calculus is not a living human tissue like dentine,

similar mechanisms of organic-inorganic interactions may be present. For example, salivary proteins are documented to bind to enamel and hydroxyapatite (Lamkin, Arancillo and Oppenheim 1996; Humphrey and Williamson, 2001), during the formation of acquired enamel pellicle. Given the variety of crystal forms observed in dental calculus, exploring crystallinity and biomineralisation in ancient dental calculus may be one way to explore the mechanism of protein survival. In bone, for example, it has been found that increasing crystallinity of hydroxyapatite correlates with a decrease in osteocalcin preservation (Tuross et al. 1989; Collins et al. 2000; Smith et al. 2005).

Cellular Locations of Human and Bacterial Proteins

The cellular location of each protein extracted from dentine and dental calculus was also assessed to examine whether particular parts of the cell might be preferentially preserved. In terms of bacteria, one might hypothesise that proteins derived from the cell membrane might preferentially survive owing to their robust structure. An analysis of protein cellular locations, defined by Gene Ontologies, revealed that the majority of proteins identified in both calculus and dentine are secreted proteins (Fig. 9.12). In dentine, with a proteome solely from the human host, this is likely to be reflective of the dominance of proteins comprising the extracellular matrix. In dental calculus, the dominance of secreted proteins is probably due to the high abundance of immune proteins, which are secreted from the cell in order to circulate. In addition, secreted proteins from bacteria make up an integral part of the biofilm matrix. For example, proteomic analysis of *Streptococcus pneumoniae* revealed over 200 secreted proteins (Cho et al 2012). Thus, the parts of cells which do survive may be more reflective of the nature of the underlying proteome, rather than any variation in robustness within the cell. In future research, it may be worthwhile exploring whether proteins identified in ancient proteomes are also found in the 'long-lived proteome', proteins which have slow cellular turn-

overs (Toyama et al. 2013), in order to examine whether stability during cell life influences protein survival in the archaeological record.

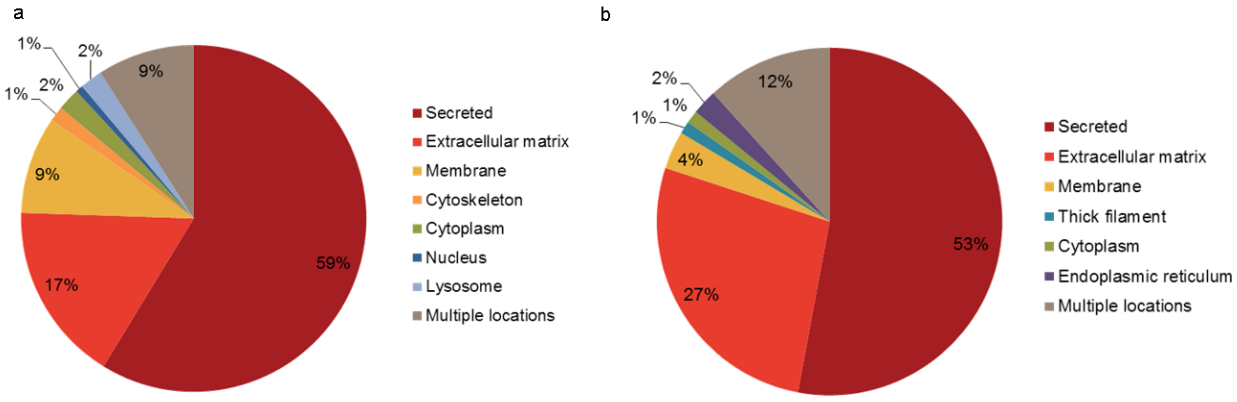

Figure 9.12. Cellular location of proteins extracted from a) calculus, and b) dentine.

9.4.3 Relationship between DNA and Protein Preservation

Ancient proteins may offer an alternative approach when ancient DNA analysis is unsuccessful (e.g. Welker et al. 2015). However, the nature of this survival is still unclear, as discussed above, as is the relationship between the preservation of these two biomolecules. Previous studies have attempted to explore the relationship between DNA and protein preservation, in particular with the notion of using the preservation of protein as a proxy for the preservation of DNA (e.g. Poinar and Stankiewicz 1999). Götherström et al. (2002) observed the strong relationship between hydroxyapatite crystallinity and the amount of collagen. However, Ottoni et al. (2009) examined the relationship between the survival of collagen fibrils and the presence of mtDNA in cattle bones, finding that DNA preservation was variable in spite of good collagen preservation. Similarly, Schwarz et al. (2009) found that there was no clear correlation between collagen content and DNA yield in samples of mammoth bones. In addition, Buckley et al. (2008) found that osteocalcin was less preserved

than mtDNA, despite the hypothesis that because osteocalcin is associated with hydroxyapatite it would demonstrate long-term stability in the archaeological record (Collins et al. 2000). Using proteomic approaches, the relationship between the preservation of the proteome and the preservation of DNA can begin to be explored. Orlando et al. (2013) used the presence of 73 proteins in 780 kyr BP horse bones to infer that DNA would be well preserved in the same sample. Despite this inference, the relationship between the preservation of NCP's and DNA has not been quantified or explored, given the novelty of the technique.

Using data generated from this study, yields of DNA and protein extracted from the same samples of dental calculus were compared (Fig. 9.13). However, no trend or relationship was observed between the normalised DNA yield (nanograms of DNA per milligram of dental calculus) and protein (nanograms of protein per milligram of dental calculus) extracted from the same sample.

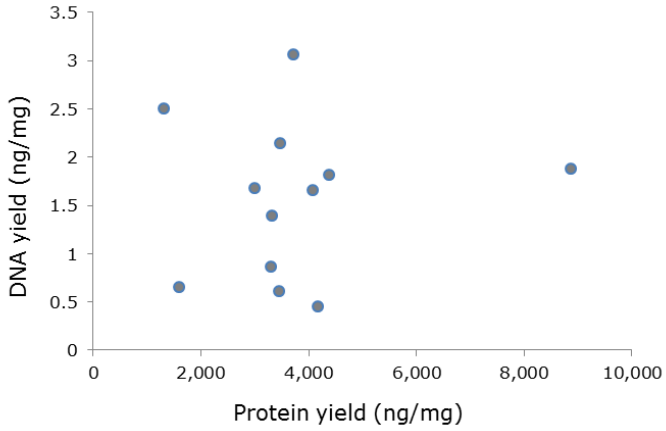

Figure 9.13. Relationship between yields of DNA and proteins in ancient dental calculus.

Consequently, we compared the normalised DNA yield and the *number* of proteins extracted from the same samples of ancient dental calculus (Fig. 9.14). Using a 2-tailed chi-squared test

no statistically significant relationship was identified between the number of proteins identified and DNA yield in samples of dental calculus.

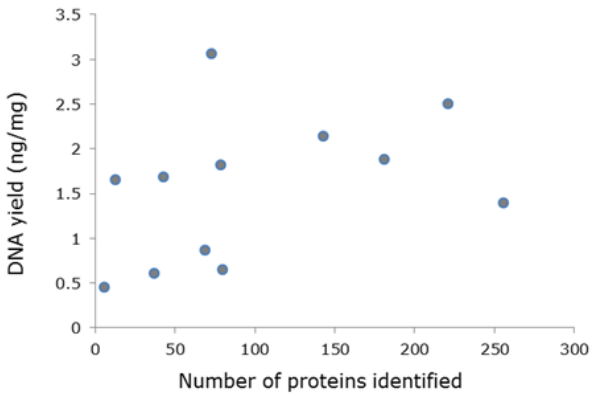

Figure 9.14. Relationship between normalised yields of DNA and the number of proteins in dental calculus.

No relationships were observed between either the protein yield or the number of proteins identified with DNA yield in dental calculus. Thus, the relationship between these two molecules in dental calculus still remains uncertain.

9.5 Conclusion

This study demonstrates that ancient metaproteomics is an effective tool by which to identify bacteria in the archaeological record, an approach which may be particularly applicable for studying populations which derive from tropical environments, where a lack of biomolecular preservation typically inhibits such analyses. This study was able to identify a range of commensal and pathogenic bacterial species deriving from the oral environment in dental calculus, demonstrating that a taxonomic and functional profile is preserved in this tissue in the archaeological record. In addition, these results indicate that the human proteome of dental

calculus and dentine is preserved in these archaeological dental tissues. However, the applications of this observation are yet to be fully realised. In addition, whether these proteomes are an accurate reflection of disease processes occurring at the time of calculus formation, or whether the profile is skewed by taphonomy, remains to be discovered. This study has also begun to explore the mechanisms of protein survival in these archaeological materials, speculating that, like previous studies have suggested, this may be due to protein-mineral interactions. In terms of bacterial preservation this study has observed that proteins derived from Gram-negative bacteria are more prevalent than Gram-positive, in contrast to previous observations using ancient DNA. However, to determine whether this is due to taphonomic processes or is a reflection of variation in the oral biofilm requires further investigation.

Chapter 10: Osteological and Biomolecular Analysis of Oral Pathologies of 19th Century Re-Captive Africans from St Helena

One of the most significant outcomes of Chapter 9 was the identification of possible oral health indicators through the metaproteomic analysis of ancient dental calculus from individuals buried on the South Atlantic island of St Helena. This chapter examines the osteological evidence for dental disease in this population and explores the relationship between biomolecular and osteological indicators of oral health. In particular, it explores how cultural dental modifications found in this population, the largest assemblage of African dental modification to be found archaeologically, may have influenced the oral health of this population.

Abstract

In the mid-19th century over 26,000 "liberated Africans" were received onto the small, South Atlantic island of St Helena, "liberated" by the British Royal Navy as part of their campaign to abolish the transatlantic slave trade. Recent excavations have revealed the skeletal remains of 325 victims of this trade, representing individuals who died as a result of their forced transportation across the Atlantic. In this study we have analysed the relationship between the prevalence of cultural dental modifications and oral pathologies observed in this population. In addition, to further understand the aetiology of these pathologies, we have applied shotgun protein analysis to deposits of dental calculus, revealing some of the specific oral bacteria responsible for these infections. This study demonstrates that when pulp cavities are exposed

through dental modification localised oral pathologies may become prevalent. In addition, we demonstrate that using biomolecular approaches in conjunction with palaeopathological observation it is possible to gain a deeper insight into disease identification and aetiology, although the relationship between osteological and biomolecular disease identification is complex and requires further investigation.

10.1 Introduction

In the 19th century 3.5 million African people were forced across the Atlantic in enslavement, despite the growing movements of abolitionism on both sides of the Atlantic (Emory University 2008). Following the British abolition of the transatlantic slave trade in 1807, Britain established the Royal Navy 'West African Squadron', initially to intercept illegal British traders and later to intercept foreign vessels, a campaign facilitated by a number of bilateral treaties (Lloyd 2012). These treaties were enforced through the trial of captured vessels at a Vice-Admiralty or Mixed Commission Court stationed around the Atlantic (Fig. 10.1). If a vessel was found guilty of illegal trading the ship was sold and any enslaved people on board "liberated". It is estimated that 90,000 enslaved Africans were "liberated" through this campaign between 1836 and 1866, representing about 5% of people embarked for slavery (Huzzey 2012).

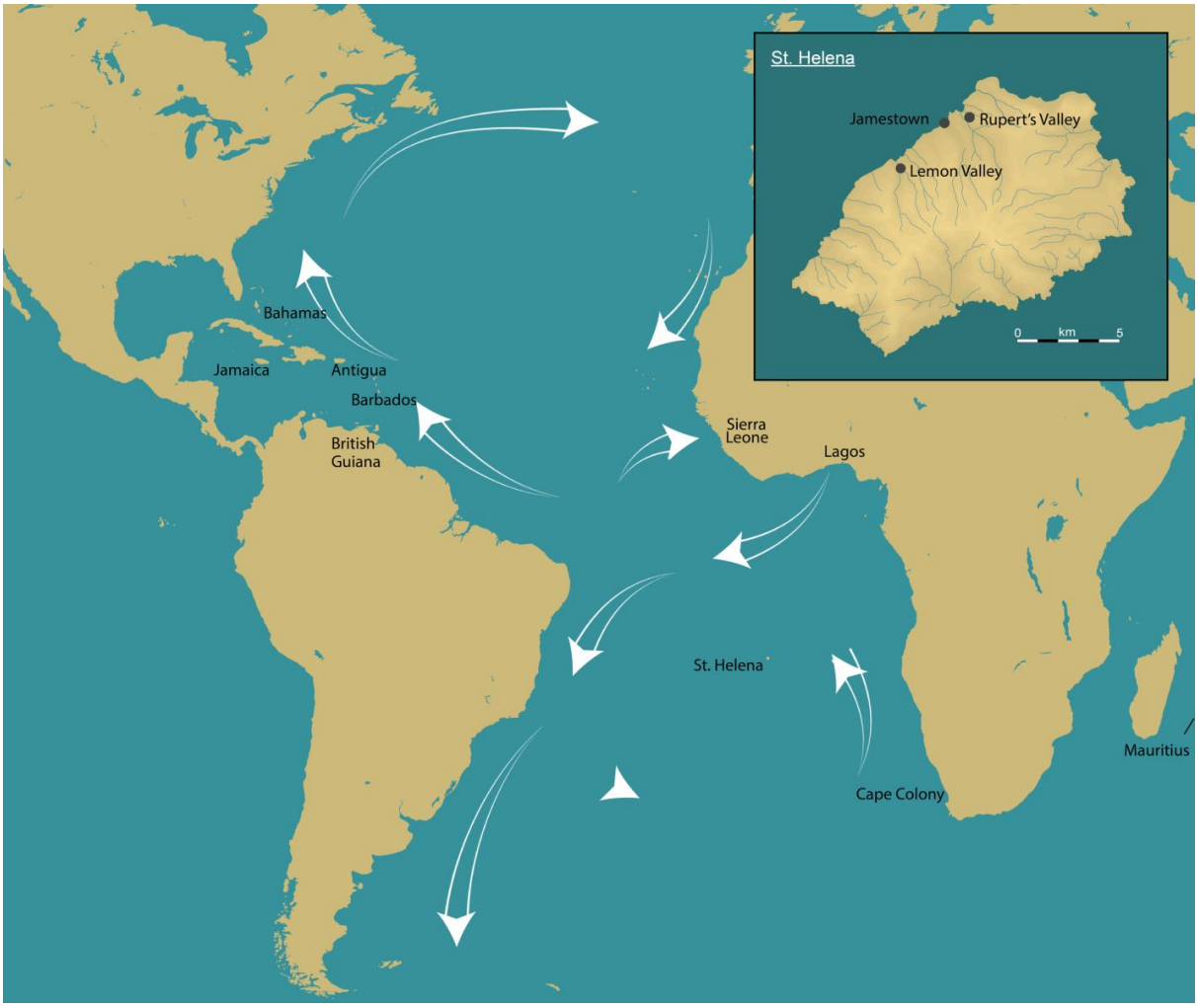

Figure 10.1. Location of St Helena and Vice-Admiralty Courts in the Atlantic. Arrows indicate oceanic currents. Adapted from Huzzey (2012).

The history of St Helena, a remote island in the South Atlantic Ocean (Fig. 10.1), is intertwined with these last decades of the transatlantic slave trade. Discovered by the Portuguese uninhabited in 1502, St Helena came under British influence through the East India Company in 1659, and after the abolition of the East India Company monopolies St Helena became an official Crown Colony in 1834. Owing to the favourable currents between the West African coast and the island, the island was an efficient option for the landing of captured vessels and for provisioning ships present in this region (Fig. 10.1). Between 1840 and 1872 St Helena acted as a Vice-Admiralty Court, during which time a 'Liberated African

Establishment' was established in Rupert's Valley (after an initial phase at Lemon Valley) to treat and temporarily house African people "liberated" from the captured slave ships. Historical sources indicate that up to 26,000 people passed through the island and, judging by rates of mortality, over 8,000 people may be buried on or around St Helena (Pearson et al. 2011). The geographic origin of these individuals is not known but historical records indicate that most ships departed from ports in West Central Africa. The survivors, in the vast majority of cases, did continue a trajectory to the Americas as indentured labourers in British colonies, financed by private individuals or American colonies.

In 2007 and 2008, as part of a commercial development on the island, excavations in Rupert's Valley revealed a skeletal assemblage associated with the 'Liberated African Establishment'. The skeletal material excavated in Rupert's Valley has been previously discussed (Witkin 2011) but is briefly summarised here. A total of 325 articulated skeletons were uncovered, in a dense arrangement of usually shallow graves. Two charnel pits of disarticulated bone were also uncovered. The population was very young, with 54% aged less than 18 years old, and over 30% between 7 and 12 years old, consistent with historical accounts of the increasing number of enslaved children during the era of British abolition (Lovejoy 2006). Of the adults who were able to be sexed (n=160), 84% were male.

One of the most striking features of this assemblage was the high prevalence of cultural dental modifications, present in 115 individuals (35%) (Witkin 2011). An example is shown in Figure 10.2b. To my knowledge, this assemblage represents the largest complete skeletal assemblage of African people with culturally modified teeth uncovered in an archaeological context. Dental modification has been previously observed in archaeological populations of enslaved Africans (Handler, Corruccini, and Mutaw 1982; Cox and Sealy 1997; Tiesler 2002; Blakey and Rankin-Hill 2004; Schroeder, Haviser, and Price 2014), and in individuals of

African descent from the 15-17th centuries (Wasterlain, Neves, and Ferreira 2015). Individuals with tooth modification found in American contexts are often assumed to be first generation Africans (Stewart and Groome 1968; Handler, Corruccini, and Mutaw 1982; Handler 1994; Blakey 1998; Tiesler 2002), an assumption that was confirmed for three individuals buried on Sint Maartin by strontium isotope analysis (Schroeder, Haviser, and Price 2014) and DNA analysis (Schroeder et al. 2015). However, the prevalence, nature and meaning of the practice in the past is not well understood.

Figure 10.2. Examples of a) extensive deposits of dental calculus, b) cultural dental modifications and c) caries observed in individuals from St Helena. All photographs by Andrew Pearson.

Osteological investigations of the remains of enslaved people during the era of transatlantic slavery have indicated widespread malnutrition and physical stress as a result of the conditions of enslavement (Rathbun 1987; Okumura 2011; Shuler 2011; Handler 2006a; Handler 2006b). Oral health also appears to have been poor (Corruccini et al. 1982; Owsley et al. 1987; Corruccini et al. 1987; Okumura 2011), with the exception of the human remains found at Pretos Novos in Brazil (Cook et al. 2015). Whilst bioarchaeological studies of enslaved people have been completed in North America (e.g. Martin, Magennis, and Rose 1987; Rathbun 1987; Owsley et al. 1987; Blakey 1998; Angel et al. 1987), the Caribbean (e.g. Corruccini et al. 1982; Khudabux 1989; Armstrong and Fleischman 2003) and South America (e.g. Cook et

al. 2015; Bastos et al. 2010), excavated skeletal remains from the African continent from this period are few and, to my knowledge, only from South Africa (Sealy et al 1993; Appleby et al. 2012). In addition, the excavated remains of first-generation enslaved people are also rare or can be difficult to identify in American assemblages (Handler 1994). Thus, compared with osteological studies focussed on the Americas, substantially less is known about the health of people enslaved prior to and during their transportation across the Atlantic.

This study explores the oral health of this population by using biomolecular and osteological indicators, and explores the link between cultural modification practices observed in this population and the prevalence of oral pathologies. This will enable a better understanding of the health of enslaved people prior to and during forced transportation. In addition, by using a two-fold approach, this provides an opportunity to explore the relationship between osteological and biomolecular approaches to understanding oral disease.

10.2 Materials and Methods

10.2.1 Analysis of Skeletal Material

Human remains were excavated in 2008 as part of a commercial development. Skeletons were recorded in situ, and then lifted for further osteological analysis. At the time of excavation, the skeletal assemblage was analysed in terms of oral pathologies using established palaeopathological methods which have been described in Witkin (2011). Caries and abscesses were recorded using standards outlined in Lukacs (1989), dental calculus deposits were recorded using Brothwell (1981) and periodontitis was recorded using the approach described in Ogden (2007). In 2012 the assemblage was examined in more detail in terms of

dental modifications, casts of the modification were made and samples of dental calculus were obtained.

Using data collected from both time periods (2008 and 2012), this study examines the relationship between dental modification, caries, abscesses and periodontitis. Data analyses were performed using SPSS version 21.0 (IBM Corp., Armonk, NY). A Pearson's chi-squared test was used to test the independence of each of these pair-wise relationships, based on categories of presence/absence. A probability of $p < 0.05$ was deemed statistically significant.

10.2.2 Proteomic Analysis of Dental Calculus

One notable feature of the oral pathologies of this population was extensive deposits of dental calculus (e.g. Fig. 10.2). Ninety-seven percent of individuals over one year old had dental calculus, with deposits observed on deciduous and permanent teeth. Samples of dental calculus were removed using a dental scaler and place in 2.0 mL Eppendorf tubes. Dental calculus was not removed from any central incisors which had cultural dental modifications. Tryptic peptides were extracted using a filter-aided sample preparation method modified for ancient dental calculus previously described Warinner, Rodrigues, et al. (2014). Analysis was performed on a Q Exactive tandem mass spectrometer located at the Mass Spectrometry Laboratory of the Target Discovery Institute at the University of Oxford, as previously described in Warinner, Hendy, et al. (2014).

Spectra were converted to Mascot generic format using Proteowizard version 3.0.4743 using the 200 most intense peaks in each MS/MS spectrum. MS/MS ion database searching was performed using Mascot (Matrix Science), against the UniProt database (2014) (541762 sequences; searches) and Human Oral Microbiome Database (Chen et al. 2010) (3643231

sequences). Searches were performed against a decoy database to generate false discovery rates (FDR). Search parameters included carbamidomethylation as a fixed modification and acetylation (protein N-term), deamidation of glutamine and asparagine, glutamine to pyroglutamate, methionine oxidation and hydroxylation of proline as variable modifications, based on modification patterns observed in Cappellini et al. (2012) and Warinner, Rodrigues, et al. (2014). Peptide tolerance was 10 ppm with semi-trypsin as the enzyme with up to two missed cleavages. MS/MS ion tolerance was 0.07 Da.

Mascot generated results were filtered to 1% peptide FDR. When there were insufficient protein identifications to make a meaningful 1% FDR calculation, the lowest FDR possible was chosen (Appendix 1, Table 1). When the number of peptides matching to a protein was less than five each of these peptides was searched using BLASTp (Altschul et al. 1990) against all non-redundant protein sequences (consisting of all translated sequences from GenBank and all sequences from Refseq, PDB, SwissProt, PIR and PRF) to confirm a unique match. In some cases identification down to species level was not possible due to the identification of conserved domains. If the peptide could be identified to at least the level of genus the protein was retained in the analysis.

We took precautions to minimise contamination of ancient samples with modern proteins. All protein extractions were performed in a laboratory dedicated to ancient biomolecules. We included blank extractions with every batch of protein extractions. Nitrile gloves were worn to avoid contamination with latex.

10.3 Results

10.3.1 Identification of Oral Pathologies

A range of oral pathologies were observed in the population excavated from St Helena, including dental calculus deposits (96.7%), caries (19.5%), antemortem tooth loss (12.5%) periodontitis (13.6%) and abscesses (24.2%) (Witkin 2011). All frequencies are reported as the percentage of individuals with one or more affected teeth. Congenital abnormalities were also observed, including supernumerary teeth (7 individuals), hyperdontia (2 individuals), and malformed "peg-shaped" teeth (8 individuals), and rotation greater than 45° (7 individuals). These frequencies, along with age and sex distributions, have been documented in Witkin (2011).

Prior to investigating the relationships between dental modification and oral pathologies the skeletal records for each individual were assessed regarding osteological preservation. Firstly, if the alveolar bone on both the maxillary and mandibular was degraded to completely prevent pathological identification, the individual was discounted from analysis associated with these regions. Secondly, individuals where the cranium was absent were clearly discounted, and thirdly, neonates or infants with no, or erupting, teeth were discounted, although erupted deciduous teeth were included.

10.3.2 Dental Modification and Oral Pathologies

The relationship between dental modification and abscesses, abscesses on the central incisors, caries and periodontitis was assessed by using a Pearson's chi-squared test for independence (Table 10.1). In this assessment we removed all individuals where it was unknown whether that individual had modified teeth (i.e. if front teeth were absent either postmortem or

antemortem). Infection resulting from dental modification may have indeed caused antemortem tooth loss on the central incisors, but as this is only speculative, these individuals were removed. In addition, as above, if the presence or absence of a particular pathological indicator could not be determined (due to alveolar preservation, fragmentation or missing crania) these individuals were discounted.

A chi-squared test was performed to determine if there is a statistically significant correlation between **dental modification** and the **presence of abscesses**. Of all individuals with abscesses (n=40), 83% also had dental modification and a statistically significant correlation between the presence of abscesses and the presence of dental modification was observed ($\chi^2 = 34.59$, $p < 0.001$) (Table 10.1). In addition, we observed a statistically significant correlation ($\chi^2 = 35.00$, $p < 0.001$) between the presence of **dental modification** and presence of **abscesses on the central incisors** (Table 10.1). To explore this further, we examined whether the presence of pulp or dentine exposure as a result of dental modification correlated with the prevalence of abscesses on the central incisors. Although this was not statistically viable owing to the small sample size, we did observe that of the 24 individuals who had both dental modification and abscesses on the central incisors, 12 has pulp exposure and two had dentine exposure.

We also assessed the relationship between the presence of **dental modification** and the presence of osteologically identified **periodontitis**. Of individuals with observable periodontitis (41), 24 also had dental modification (58.5%). There was no statistically significant correlation between the presence of periodontitis and the presence of dental modification ($\chi^2 = 6.03$, $p = 0.14$). Similarly, there was no statistically significant correlation between the presence of **dental modification** and **caries** ($\chi^2 = 0.57$, $p = 0.45$) (Table 10.1). This is possibly due to the fact that caries are dominant in molars, teeth which were not

modified. A potential bias should be noted here, in that caries may have formed prior to the modification, meaning that carious teeth on these teeth would go unobserved.

	Abscesses		**Abscesses on the central incisors**		**Caries**		**Periodontitis**	
	No Abscesses	*Abscesses*	*No Abscesses*	*Abscesses*	*No Caries*	*Caries*	*No PD*	*PD*
No dental modification	137	7	12	1	134	41	125	17
Dental modification	66	33	75	24	90	22	76	24
Chi-Squared test, p-value	34.59, $p < 0.001$		35.00, $p < 0.001$		0.57, 0.45		6.03, 0.14	

Table 10.1. Summary of correlations between dental modification and abscesses, abscesses on the central incisors, caries and periodontal disease. Counts only include individuals where it was possible to observe the presence or absence of each of these pathologies. PD = Periodontitis.

In addition, we also compared the relationships between pathologies, to assess whether abscesses, caries and periodontal disease occurred independently of one another. We observed that the presence of **periodontal disease** and **abscesses** appear to be correlated ($\chi^2 = 28.01$, $p < 0.001$), as well as **periodontal disease** and **caries** ($\chi^2 = 4.24$, $p < 0.05$). There is no statistically significant correlation between the presence of caries and abscesses.

10.3.3 Biomolecular Evidence

As an additional line of evidence for exploring oral disease, and in order to explore the aetiology of these oral infections, we applied shotgun proteomics to dental calculus from 20 individuals. Within this group, 6 had osteological evidence of periodontitis, 5 had osteological evidence of abscesses, and 2 had caries (Table 10.2 and 10.3).

Individual	Age	Sex
430	30 - 35	Possible Male
218	25 - 28	Possible Male
474	35 - 40	Possible Male
414	28 - 32	Male
421	8 - 12	Unknown
212	10 - 12	Unknown
343	35 - 40	Male
419	22 - 25	Female
359	24 - 26	Male
237	25 - 29	Male
319	12 - 14	Unknown
276	23 - 25	Male
306	27 - 31	Male
389	22 - 24	Male
268	11 - 13	Unknown
219	26 - 35	Male
423	35 - 39	Male
459	30 - 35	Male
449	25 - 32	Male
358	30 - 39	Male

Table 10.2. Summary of individuals selected for proteomic analysis of dental calculus.

Using shotgun proteomics, 44 different bacteria associated with the oral cavity were identified across all individuals. These included 16 bacteria observed to be associated with oral diseases and 20 recorded to be present in the oral cavity as commensal species (the oral microbiome). The remainder (8) could only be identified to the genus level and therefore could not be assigned as either pathogenic or commensal. Note that the specificity of assignment could fall as a consequence of increased genomic sequencing.

Individual	Oral and Respiratory Pathologies	No. of bacterial species (No. of proteins)	Pathogenic bacteria
430	Caries, periodontitis, antemortem tooth loss	5(6)	*Filifactor alocis*
218	None observed*	1(1)	None
474	Periodontitis, maxillary sinusitis	10(12)	*Filifactor alocis, Porphyromonas gingivalis*
414	Abscesses on culturally modified teeth	5(5)	*Filifactor alocis, Porphyromonas gingivalis*
421	None observed *	1(1)	*Filifactor alocis*
212	None observed (Poor preservation)	8(8)	*Actinomyces graevenitzii, Filifactor alocis, Leptotrichia buccalis*
343	Periodontitis	7(8)	*Atopobium parvulum, Filifactor alocis, Treponema sp.*
419	No oral pathologies observed, but lesion on palatine process *	8(10)	*Alloprevotella tannerae, Filifactor alocis*
359	Abscesses	19(34)	*Actinomyces graevenitzii, Campylobacter showae, Filifactor alocis, Prevotella oris, Tannerella forsythia, Streptcoccus spp.*
237	Caries, Abscesses	2(2)	*Filifactor alocis*
319	None observed	0(0)	None
276	Periodontitis, abscesses, maxillary sinusitis	3(3)	None
306	Periodontitis	6(9)	*Filifactor alocis, Porphyromonas gingivalis*
389	None observed	8(15)	*Actinomyces graevenitzii, Filifactor alocis, Porphyromonas gingivalis, Tannerella forsythia*
268	None observed *	0(0)	None
219	Abscesses on culturally modified teeth	4(4)	*Actinomyces graevenitzii*
423	None observed	4(4)	*Tannerella forsythia*
459	Abscesses	0(0)	None
449	None observed	0(0)	None
358	None observed	28(56)	*Actinomyces graevenitzii, Campylobacter showae, Eubacterium yurii, Filifactor alocis, Lactobacillus sp. Porphyromonas gingivalis, Pseudoramibacter alactolyticus, Selenomonas sp. , Streptococcus mutans, Tannerella forsythia, Treponema denticola*

Table 10.3. Summary of osteological evidence of oral disease and pathogenic bacteria identified in each individual analysed. Individuals marked with an asterisk displayed poor osteological preservation of the teeth and alveolar bone (Witkin 2011).

Proteomic analysis of dental calculus identified a number of pathogenic bacteria implicated in dental disease. *Filifactor alocis*, which is implicated in periodontal disease (Schlafer et al. 2010; Aruni et al. 2015; Chen et al. 2015), was the most prevalent oral pathogen (in 12 of 20 individuals). Members of the "red complex" *(Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola),* which are also highly implicated in advanced periodontal disease (Holt and Ebersole 2005) were also identified, the most common being *P. gingivalis*, found in 5 individuals.

A number of bacteria which have been isolated from abscesses were recovered, including *Streptococci*, *Prevotella*, *Fusobacteria* and *Synergistetes* (Dahlén 2002; Gomes et al. 2004; Robertson and Smith 2009; Siqueira and Rôças 2009*)*. *Streptococcus* and *Lactobacillus*, bacteria which are implicated in caries owing to their ability to produce weak organic acids as a result of carbohydrate metabolism (Selwitz, Ismail, and Pitts 2007) were identified in two individuals.

Although the dataset is small, we explored the relationship between the presence of oral pathogens and dental modification, or the osteological evidence of oral diseases (periodontitis, caries, abscesses). First, we explored whether the presence of pathogenic bacteria was related to the presence of dental modification. Proteomic results were classified into two broad classes "Pathogens identified" or "Pathogens not identified". Dental modification could not be validly assessed for individual 414, owing to antemortem tooth loss of the central maxillary incisors. Using a Fisher's exact test no statistically significant correlation was observed between the presence of dental modification and the presence of pathogenic bacteria.

Secondly, the relationship between the presence of pathogenic bacteria and the presence of osteologically identified oral disease was assessed. Four individuals (218, 268, 419 and 421)

were not sufficiently preserved to enable a valid assessment about the presence of periodontits or abscesses so were excluded from the analysis (Table 10.3). Using a Fisher's exact test, no statistically significant correlation was observed between the presence of pathogenic bacteria and osteologically identified caries, abscesses or periodontitis. However, it should be stressed that the sample size here is small (n=16).

To further explore the relationship between biomolecular and osteological indicators of dental disease, we looked specifically at individuals who had evidence of bacterial species which constitute the "red complex" bacteria. Of individuals who had at least one member of the complex (one of *P. gingivalis, T. forsythia, T. denticola*) only two of seven also had osteological evidence of the disease. Of individuals in this dataset who had periodontitis (6), only two had at least one member of the red complex. Similarly, of the two individuals with caries, cariogenic bacteria was not identified in analysis of their dental calculus.

Given the apparent independence of osteological and proteomic markers of oral health, we explored whether osteological preservation may be a confounding factor in the identification of bacteria. Osteological preservation of the teeth, and maxillary and mandibular bone was classed into five categories; very poor, poor, average, good or very good based on the degradation of the alveolar bone as well as the presence and condition of the teeth (Witkin 2011). As demonstrated in Figure 10.3, there was no observable correlation between the number of proteins (Fig. 10.3a), number of bacterial proteins (Fig. 10.3b), or number of bacterial species (Fig. 10.3c) and osteological preservation.

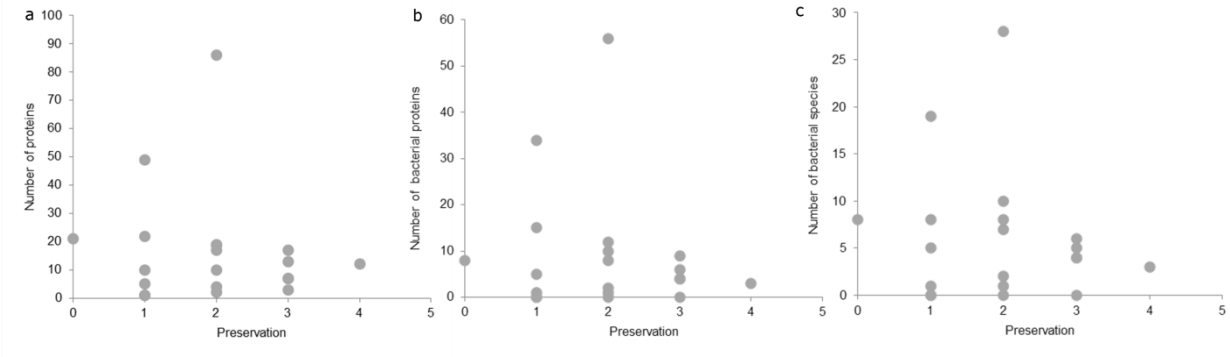

Figure 10.3. Proteomic results as a function of the osteological preservation of the teeth, maxillary and mandibular bone, showing a) the number of proteins, b) the number of bacterial proteins, and c) the number of bacterial species, from 20 individuals from St Helena. Osteological preservation was classified into five groups; 1, very poor; 2, poor; 3, average; 4, good or 5, very good.

10.4 Discussion

Individuals buried at St Helena suffered from large deposits of dental calculus, dental caries, abscesses and periodontitis, as has been previously documented (Witkin 2011). Historical accounts pertaining to St Helena also indicate poor oral health, which may have been exacerbated by the effects of dehydration. Dr Rawlins, the medical officer stationed on St Helena between 1840 and 1843, observed what he described as "bad mouths", although the specific details of this observation are unknown (Colonial Office, Great Britain 1850, pg. 92). Similarly, Dr Vowells, the colonial surgeon on St Helena, described the how dehydration effected the mouth, *"... the face being enormously swollen, the lips everted and, as well as the tongue and lining membrane of the cheeks, covered with sloughs, the teeth with sordes, and a constant flow of saliva from the mouth"* (Colonial Office, Great Britain 1850, pg. 75).

Analyses of the oral health of enslaved populations in the Caribbean and North America have noted the presence of similar pathologies (Rathbun 1987; Okumura 2011; Corruccini et al. 1982; Angel et al. 1987; Mack et al. 2004; Nystrom 2013; Owsley et al. 1987). Comparing

enslaved and 19th century African-American populations in the Caribbean and North America to St Helena, in some cases caries and abscesses in the St Helena population appear to be less prevalent (Table 10.4).

Site	Caries	AMTL	Periodontitis	Abscesses
St Helena (Witkin 2011)	19.50%	12.50%	13.60%	24.20%
NYABG (Mack et al. 2004)	77.70%	-	-	-
FABC (Angel et al. 1987; Nystrom 2013)	93.80%	78.20%	-	73.60%
Urban New Orleans (Owsley et al. 1987)	25%	-	-	31.6%*
Pretos Novos (Cook et al. 2015)	15.60%	6.25%	37.50%	-
Plantation Waterloo, Suriname* (Okumura 2011)	~50%	50%	-	-
Newton* (Handler and Corruccini 1983; Corruccini et al. 1982)	20% (occlusal) 57% (interproximal)	51%	"Virtually all"	0%

Table 10.4. Comparison of St Helena oral health with a selection of Caribbean and North American enslaved and free African-American sites, showing the frequency of affected individuals. All frequencies are the number of affected individuals, apart from Owsley et al. (1987), who record the frequency of tooth sockets affect, not the frequency of individuals affected. AMTL = Ante-Mortem Tooth Loss, NYAGB = New York African Burial Ground, FABC = First African Baptist Church.

The population at St Helena is unique in that it is the only excavated cemetery, to the author's knowledge, of individuals who have died as a result of their transportation as part of transatlantic slavery. A comparable population may be Pretos Novos, Rio de Janeiro, a cemetery containing the remains of newly arrived enslaved Africans between 1824 and 1830 (Cook et al. 2015). Cook et al. (2015) suggest that the use of chewing sticks may have resulted in the relatively low level of dental calculus and other pathologies in this population (although periodontal bone loss was noted in 12 of 32 individuals). The frequencies of oral pathologies

present in St Helena appear to be more similar to Pretos Novos than enslaved populations in the Caribbean and North America. Thus, this could suggest that oral health may have deteriorated once individuals were living in the Americas, possibly owing to the consumption of a carbohydrate rich diet in plantations (Handler and Corruccini 1983), although the pattern among 19th century enslaved and African-American populations appears to be complex (Nystrom 2013).

10.4.1 Dental Modification and Oral Pathologies

Despite the observation of cultural dental modifications in skeletal populations of enslaved Africans (Handler 1994; Cox and Sealy 1997; Mack et al. 2004; Tiesler 2002; Schroeder, Haviser, and Price 2014), the relationship between dental modification and oral health in African archaeological populations is poorly understood. In this study, the presence of cultural dental modifications correlates with the presence of abscesses, particularly on the central incisors. In these latter cases, we observe that most (14 of 24) also have pulp or dentine exposure as a result of the modification. This suggests, not unexpectedly, that dental modification may result in the introduction of bacteria into the tooth structure and periodontal tissues, where it can proliferate and result in abscesses (Dahlen 2002). Indeed, alveolar abscesses may be underrepresented skeletally in this population, because abscesses could be draining through the exposed pulp cavity of the modification. However, we did not find any relationship between dental modification and caries or periodontitis, which could suggest that when dental modification did result in infection, the infection was localised.

Other archaeological studies of modified teeth have also observed patterns of poor oral health. For example, Versiani et al. (2011) observed that penetration of the pulp chamber by inset

stones in a Mayan population may have resulted in calcification of the root canal. Reichart et al. (2008) proposed that alveolar bone pathologies may have been caused by dental modification practices in a collection of 33 individuals from 20th century Cameroon. Molnar (2008) observed a relationship between extra-masticatory wear and tilting, where the exposed pulp cavity may have been the site for periapical lesions (where an infection develops in the space between the tooth apex and bone). Our results support the findings that dental modification correlates with localised infections.

10.4.2 Biomolecular Evidence and Proteomic Analysis of Dental Calculus

Shotgun proteomics of dental calculus has revealed a consortium of oral bacteria in this population (Table 10.2). Of these bacteria *Filifactor alocis* and members of the "red complex" have been proposed in particular as diagnostic markers for periodontal disease (Aruni et al. 2015; Holt and Ebersole 2005). In 10 individuals these bacteria were identified, but osteological indicators of periodontitis were not evident. The presence of these bacteria in the absence of osteological indicators could be evidence of periodontal disease which has not yet affected the bone tissue, such as gingivitis. Similarly, the relationship between osteological evidence of caries and the proteomic detection of caries-causing bacteria was not straightforward. In two individuals displaying caries (237 and 430) none of the acid-producing species implicated in caries were detected (Selwitz, Ismail, and Pitts 2007). However, *Streptococcus* was identified in two other individuals, with one of these also having proteins derived from *Lactobacillus.*

It is possible that this lack of identification of cariogenic bacteria in individuals displaying caries could be due to sampling biases. If caries are highly localised then it could be the case

that cariogenic bacteria may only be detected by sampling calculus directly from a carious tooth, which did not occur in these cases. More generally, given that the mouth contains a diverse range of bacterial niches, in future it would be worthwhile sampling dental calculus which is as close as possible to lesions (without compromising palaeopathological observation) in order to facilitate the identification of disease-causing processes which might be highly localised. Similarly, dental calculus samples were not taken from the central incisors in order to prevent damage, meaning that it may be more difficult to identify bacteria which are associated with these modifications.

In this dataset the presence of pathogenic bacteria does not correlate with the osteological markers of their associated diseases. Several factors may be behind this; a) this is *prima facie* evidence of the osteological paradox in the identification of periodontal disease, b) microbial pathogenesis in the past may not be the same as it is reported in modern, clinical literature, or c) there is a major, confounding preservation bias. Each of these aspects will be discussed in turn.

The osteological paradox explores some of the ways that the identification of disease from skeletal remains may bias our understanding of patterns of past health (Wood et al. 1992). A significant part of this paradox is that a disease which affected the person during life may not leave observable traces on the skeleton, preventing the diagnosis of that disease. In terms of oral diseases we could be seeing, in the case of caries, an early onset of caries formation or, in the case of periodontal disease, a disease process which has not yet skeletally manifested. Proteomic approaches may be one way to explore this disease progression. The adoption of proteomic approaches, as opposed to metagenomic approaches, may be one way to identify disease progression rather than the carriage of disease-causing bacteria, because it is possible to identify proteins which are associated with virulence. For example, in three individuals

(474, 358, 306) we identify major virulence factors of *P. gingivalis*, lys-gingipain and fimbrial proteins (Travis et al. 1997; Lamont and Jenkinson 2000), which suggests an active infection of this bacteria at the time of calculus formation.

Microbial pathogenesis may not be the same in archaeological and modern populations, but we are dependent on modern, clinical data in order to understand this pathogenesis. A metagenomic or metaproteomic identification of disease is typically based on the following scenario; a) the identification of proteins or DNA by a shotgun approach, resulting in b) the identification of a bacteria; c) research on this bacteria in the clinical literature leading to d) the observation that it is implicated in a disease, suggesting e) that the individual was afflicted by that disease, or at least carried the bacteria. However, in applying modern, clinical data on microbial pathogenesis to the past we are making the assumption that this pathogenesis was the same in the past as it is now. For example, we could be identifying bacteria which are classified as commensal in modern literature, but were in fact pathogenic in the archaeological population. If our aim is to understand how disease may have changed through time, or even to identify past disease, is this really a useful assumption? In addition, next-generation sequencing has resulted in the identification of unculturable microbes and, with this, an understanding of the enormous microbial complexity that constitutes the oral environment (Wade 2013b). Given this understanding, it is debatable whether the identification of specific species is even a useful marker of disease. It may be the case that the dysbiosis of this oral microbiome may be more important than the virulence of individual species (Hajishengallis and Lamont 2012).

Although biomolecular preservation will undoubtedly play a role in the identification of disease-causing bacteria, this study has observed that biomolecular preservation (based on the total number of proteins, the number of bacterial proteins and the number of bacterial species)

and osteological preservation do not correlate. This could be reflective of dental calculus acting as closed system, where calculus is more resistant to mineral dissolution than the surrounding bone (Warinner, Speller, and Collins 2015). Alternatively, this could be reflective of different mechanisms of preservation (Collins et al. 2002). In order to make valid comparisons between individuals and populations, the effect of biomolecular preservation needs to be explored in future work.

10.5 Conclusion

This study examined how cultural dental modifications may have affected oral health, and found a correlation between the presence of dental modification and the presence of abscesses, suggesting that the exposure or weakening of the tooth structure may have facilitated bacterial infections. However, there was no correlation between the presence of caries or periodontitis with dental modification suggesting that when infections did occur, they may have been localised. In comparison to the population buried at St Helena, contemporaneous enslaved and free African-American populations in the Americas may have suffered from poorer oral health, possibly owing to the consumption of a carbohydrate rich, cariogenic diet. In combination with evidence of relatively good oral health of first-generation enslaved Africans from Pretos Novos, this may suggest that oral health during the Middle Passage (and prior to transportation) may have deteriorated further once populations were living in the Americas.

Through the shotgun proteomic analysis of dental calculus from the same population, this study has identified a consortium of bacteria which are implicated in periodontal diseases. However, there was no apparent trend between biomolecular and osteological indicators of oral infection. We speculate that this may be due to the observation of periodontal diseases which have not yet become skeletally manifesting. Alternatively, this could be due to a lack of

understanding about past microbial pathogenesis or patterns of microbial and proteomic degradation.

Chapter 11: Discussion and Summary of Work

This chapter will summarise the work presented in this PhD (9.1) and outline how this work achieved the aims proposed in Chapter 1 (9.2). Following this, an assessment of the research design will be made, and some of the technical challenges raised by this research will be highlighted (9.3). Subsequently, the broader significance to archaeology as a discipline, as well as the implications of this research outside the field, will be explored (9.4). The strengths and limitations of dental calculus as an archaeological resource will be focussed on specifically (9.5). To conclude, future work will be outlined based on the outcomes of this research (9.6).

11.1 Summary of Work

This thesis examined how shotgun proteomics can be applied to identifying and characterising ancient proteins, with a focus on the analysis of ancient dental calculus as a biomolecular reservoir for understanding ancient diseases and diets. This approach was then to understanding disease and diet during the transatlantic slave trade.

After contextualising the methodological approach **(Chapter 2)** and outlining the historical background and case study **(Chapter 3)**, **Chapter 4** proposed that dental calculus may offer a non-intrusive but valuable resource for understanding disease during the era of transatlantic slavery.

Chapter 5 compared several tandem mass spectrometers in order to characterise their performance with archaeological proteomes. This research found that the most recently

developed spectrometers were able to generate the largest number of protein identifications and, more broadly, revealed that different instrumental strategies have a large influence over downstream analysis and archaeological interpretations.

Chapter 6 attempted to identify proteins derived from *Mycobacterium tuberculosis* in mummified lung tissue already tested positive using ancient DNA. This research was unable to detect *M. tuberculosis*, but was able to tentatively detect proteins specific to *Mycobacterium tuberculosis* complex bacteria. The results of this study were then used to outline some of the challenges involved in the proteomic detection of *M. tuberculosis*. This research demonstrates the value of accessing sequence information derived from ancient proteins and highlights the importance of revisiting previously "successful" techniques with new methodologies.

Like the preceding chapter, **Chapter 7** also highlighted the importance of revisiting previously reported successes. Chapter 7 continued with methodological explorations of ancient proteomic approaches to disease characterisation, building on the work of Ross Kendall (2014) by examining how protein degradation prevented the detection of immunoreactive antibodies (immunoglobulin G). Using shotgun proteomics on samples of ancient dentine, this research was able to demonstrate that immunoglobulin proteins (hypothesised to be a method for detecting malarial infection) are unlikely to survive in archaeological bone and dentine, even in samples with a relatively rich proteome.

Chapter 8 then explored the complex proteome of ancient dental calculus and how this reservoir could be used to understand ancient diets. This study applied shotgun proteomics to 98 samples of dental calculus from across Eurasia and West Africa. In collaboration with Christina Warinner (Oklahoma University) and Camilla Speller (University of York), this analysis discovered that the milk protein, β-lactoglobulin, survives in dental calculus and is a novel, species-specific biomarker of milk consumption. This has established the first

biomarker of milk consumption which can be directly tied to an individual, as well as a biomarker which may be used as a proxy for lactose consumption. This has far-reaching applications for understanding the origin, spread and nature of dairy consumption, and provides a method by which these practices can be linked to the emergence of lactase persistence, the genetic trait which enables the consumption of lactose into adulthood.

Chapter 9 applied a metaproteomic analysis of dental calculus to individuals buried on the South Atlantic island of St Helena. This chapter characterised two dental tissues (dentine and dental calculus) and found that the functional profile of these two tissues is preserved in archaeological samples. These results also found that dental calculus preserves the composition and function of an oral biofilm and may have potential in characterising host responses to infections. Numerous bacterial proteins could be characterised, including members of the microbiome and oral pathogenic species. As a resource for understanding disease, this chapter discovered that analysis of dental calculus is likely to be the most useful in detecting the aetiology of oral diseases. However, results also demonstrated that biomolecular survival in dental calculus is not yet well-understood.

Using results generated in Chapter 9, **Chapter 10** explored the link between biomolecular and osteological indicators of oral health, as well as the connection between cultural modified teeth and oral health. This chapter found that there was a relationship between cultural dental modifications and localised infections. Proteomic data could be used to identify periodontal disease in individuals where there was no osteological evidence for this infection or when skeletal material was too damaged for observations to be made. However, there appeared to be no correlation between osteological and proteomic evidence for periodontal disease, suggesting that more work is needed to understand biomolecular methods of detecting this disease in skeletal populations.

11.2 Achievement of Aims

This thesis has **applied, explored and developed the use of shotgun proteomics for characterising disease in the archaeological record (Aim 1)** by determining the ideal instrumental strategies for characterising complex, archaeological proteomes (Chapter 5), by highlighting the methodological and bioinformatic challenges in identifying proteins from *Mycobacterium tuberculosis* complex bacteria (Chapter 6), and by using sequence information and patterns of post-translational modifications to understand how protein degradation hampers the detection of antibodies (Chapter 7). Collectively, the key outputs of this study are that instrumental and bioinformatic strategies have a large downstream effect on generating protein identifications, and that the ability to determine sequence information via tandem mass spectrometry enables the identification of bacterial and human derived proteins, but also allows the degradation of such proteins to be characterised so that their usefulness and authenticity can be validated.

Exploring a more complex archaeological resource, this thesis has **developed the biomolecular analysis of ancient dental calculus as a tool for understanding ancient disease and diet (Aim 2)**. This research demonstrates that dental calculus can harbour archaeologically significant dietary related proteins, chiefly, the milk protein β-lactoglobulin (Chapter 8). However, the detection of other proteins derived from the diet has so far been more limited. Building on the work of Warinner, Rodrigues, et al. (2014) this study also revealed that commensal and pathogenic bacteria can be identified through a metaproteomic analysis of dental calculus (Chapter 9), although its most useful application is likely to be in understanding oral disease.

The final aim of this work was to apply the methodological approaches explored in earlier chapters to the **19th century transatlantic slave trade,** in order to gain insight into diseases prevalent during this time and to reveal individual biographies of health, disease and diets. This research has revealed that individuals were exposed to pathogenic bacteria documented to cause disease in the oral cavity, but no diseases particular to, or the direct results of, transatlantic slavery were uncovered. Overall, while the methodological developments explored in earlier chapters are highly significant for future research, their application to address specific questions on the transatlantic slave trade has been more limited. A number of improvements and further research to develop this last aim has been outlined in section 11.6.

11.3 Assessment of Research Design and Challenges

11.3.1 Research Design

This PhD took an explorative approach to investigate the usefulness and applicability of shotgun proteomics to archaeological samples. In part, this approach was taken due to the nature of the technique employed; the fact that tandem mass spectrometry has the ability to sequence every available protein in one analysis. This approach meant that the research was less hypothesis driven and more about assessing the capabilities and potential of the technique.

For an assessment of the transatlantic slave trade this PhD relied on one case study. A number of factors justify the use of a sole case study. As outlined throughout the thesis, this represents the only excavated cemetery of people who died on board or just after disembarking captured slave ships and one of only a few excavated cemeteries of enslaved Africans. Secondly, the timing of this excavation meant that the remains of these people were available and accessible at the start of the PhD. Thirdly, it was also the primary case study of three other EUROTAST

PhD fellows, and hence it was an opportunity to work on the same population using different approaches.

11.3.2 Research Design within the EUROTAST Network

The EUROTAST network focuses on exploring new approaches to understanding the history, legacy and genetics of the transatlantic slave trade. One group within EUROTAST focussed on the St Helena assemblage and applied multiple techniques (analysis of ancient DNA analysis, strontium isotopes and patterns of dental modification) to uncover the origin, health, diet and cultural practices of this population. This is a unique opportunity to explore in-depth biographies of individuals, where the story of a few may illuminate the period as a whole.

Despite this goal of the network, there were challenges in integrating this research together. Variability in biomolecular preservation meant that it was rare to have a sample in which all methods (proteomic and genomic analysis of dental calculus and genomic analysis of human DNA) produced successful results. Samples which yielded successful results for analysis of human DNA were identified only in early 2015. Subsequently, for these individuals where calculus samples were available (four individuals) proteomic extraction was performed. However, these data were too recently generated to be able to be included in this current work. Successful integration of the results of these projects will only be possible after all the individual research projects have collected and analysed their data. Integration with research into dental modification was easier to achieve because much of this data was already available in Witkin (2011) and has been implemented in Chapter 10.

11.3.3 Technical Challenges

Like any piece of research this PhD had its challenges, both practical and theoretical. The following section will outline some of the technical challenges experienced during this PhD.

Ancient DNA Analysis

In exploring dental calculus, a primary focus was on the proteomic analysis of this resource, with a secondary focus on ancient DNA (aDNA). Recent work (Adler et al. 2013; Warinner, Rodrigues, et al. 2014) has indicated that dental calculus is an exceptional reservoir of aDNA. Given this observation it was hypothesised that dental calculus would represent a rich source of aDNA from samples originating from tropical or subtropical regions. However, genomic approaches to dental calculus from St Helena were met with debilitating challenges, such as low DNA yields and challenges in attaining successful amplification of Illumina libraries. The DNA work completed as part of this PhD has been outlined in detail in Appendices 2 and 3, and is not included in the main text in order to not detract from the focus on proteomic analysis.

Initial work on dental calculus by Adler et al. (2013) and Warinner, Rodrigues, et al. (2014), as well as other studies involved in characterising ancient microbial systems (Ubaldi et al. 1998; Cano et al. 2000; Luciani et al. 2006; Rollo et al. 2007; Tito et al. 2012), have suggested that targeting the 16s rRNA gene is an efficient way of characterising bacterial profiles. In characterising modern microbial systems this approach is routine and widely adopted (Claesson et al. 2012; Ou et al. 2013; Schnorr et al. 2014; Obregon-Tito et al. 2015). The initial approach taken for DNA analysis in this PhD was to characterise the microbial composition of dental calculus using this targeted approach. However, during the course of this research it was discovered that this approach is biased towards species of certain taxa, which depends on the size of the V3 region (where taxa with short V3 regions are preferentially amplified). These results are explored in Appendix 3. It is now clear that a

shotgun sequencing approach would be better suited for this analysis, a method which will be explored in future work.

Proteomic Analysis

Proteomic approaches also presented challenges, which was not unexpected given the novelty application to archaeological samples. Results from this study demonstrate that the choice of instrument makes a substantial difference in downstream results, so too will extraction and separation methods. Due to time constraints, different extraction methods could not be explored in a systematic way in this research. Preliminary experiments comparing the (filter-aided) FASP method used here with a (gel-aided) GASP alternative (Fischer and Kessler 2014), suggests that the latter is generally more appropriate to the small, degraded mixtures which characterise ancient samples, and would be a route to further study.

The most significant challenge encountered with ancient proteomics in this study was exploring the best method for bioinformatic analysis of proteomic data. There is no doubt, like extraction and instrumental strategies, that the approach taken for downstream bioinformatics has an effect on generated results. Again, due to time constraints, only a limited number of bioinformatic approaches and programmes were attempted for this work. However, it is worth highlighting specifically here the limitations and challenges of using a database-searching approach for analysing shotgun proteomic data, a problem which is not unique to archaeology but across all disciplines where proteomic and metaproteomic characterisation is sought.

Characterising the metaproteome and proteome requires searching against a reference database which contains protein sequences usually derived from genomic data. This database approach has biases and can make interpreting meaningful identifications difficult. Firstly, the number of sequenced bacterial genomes is grossly under-representative of the number of microorganisms that exist in nature, which clearly poses a problem for characterising

microbial communities, especially from complex environmental samples. Linked to this is an inevitable research bias in favour of the identification of pathogenic organisms, or commercially relevant species. This may result in false positive identifications of closely-related but non-pathogenic species. By analysing the proportion of unidentified spectra (where each spectrum represents a peptide sequence) in samples of dental calculus from St Helena, on average 87% of spectra were unidentified. In part this may be due to the absence of sequences in the reference database. For archaeological material, identifying bacteria is additionally challenging due to bacterial evolution. Modern bacteria may display little genetic resemblance to their ancient counterparts. In addition, we do not yet have even a basic understanding of how the microbiome changes through time, again making this characterisation challenging. In terms of protein databases (compared to genomic databases) proteins often exhibit a high level of homology across species, so assigning those proteins to the right species is challenging.

In addition to these inherent problems with a database-searching approach the *choice* of database can be difficult. A compromise must be made between searching against a database with a lot of reference sequences and long computing time. An additional, related challenge was in determining a suitable approach for confident protein identifications. Searching against large databases increases the false discovery rate (Tanca et al. 2013). However, a false discovery rate was selected as the mechanism by which to control for the confidence of a protein identification as it is comparable between different database searching algorithms. Future work will explore the effect of database size and scoring on protein identifications in archaeological samples.

11.4 Significance, Implications Strengths and Weaknesses of this Research

11.4.1 Identification of Polymicrobial and Oral Diseases

One of the most significant results of this work was the identification of both commensal and pathogenic microorganisms from the oral environment. In terms of palaeomicrobiology this approach is expanding the field as it represents a robust, novel approach to identify microorganisms in the archaeological record. In addition, the ability to identify many bacteria in one analysis, as opposed to isolating one particular species, represents a significant development which is occurring in step with our understanding of the polymicrobial nature of many infections and diseases. In addition, across long time scales proteins are generally more robust than ancient DNA. Hence, using the methods developed in this research we may have the potential to identify ancient bacteria in deeper time than may be possible with ancient DNA.

Although initial work identified microbes from the respiratory and gastrointestinal system (Warinner, Rodrigues, et al. 2014), further analysis of dental calculus in this study has revealed that possibly the most useful application is the study of oral diseases and the oral microbiome. Although this means that dental calculus research may be more limited than first thought, understanding oral diseases is not an insignificant area of inquiry. Periodontal disease and caries represent significant oral health challenges, with almost 100% of adults experiencing cavities (WHO, 2015) and close to 50% of adults over 30 years old experiencing periodontal disease (CDC, 2015). In addition, periodontal disease is linked to a number of non-oral and chronic diseases, such as diabetes (Campus et al. 2005), oral and gastrointestinal cancers (Ahn, Chen, and Hayes 2012) and cardiovascular disease (Kebschull, Demmer, and Papapanou 2010). In particular, *Porphyromonas gingivalis* represents a significant oral pathogen which has been implicated in the development of rheumatoid arthritis due to cross-

reactivity with citrullinated peptides (Mikuls et al. 2014). Understanding the prevalence, aetiology and nature of periodontal diseases in the past would help to explain how this disease may have manifested through time and how it might be linked to cultural or dietary practices.

Some bacteria implicated in non-oral diseases do manifest themselves in the oral cavity, such as *Neisseria gonorrhoeae* and *meningitidis* (Warinner, Rodrigues, et al. 2014), *Treponema pallidum* (Parija 2014), *Mycobacterium leprae* (Martinez et al. 2011) and *Mycobacterium tuberculosis* (Eguchi et al. 2003), although they were not detected in this study. Therefore, whilst dental calculus research might best applied to understanding the ancient oral environment, it is possible that these bacteria could be detected and studied in dental calculus in future work.

11.4.2 Identification of the Oral Microbiome

The significance of identifying the ancient oral microbiome is vast. The Human Microbiome Project has revealed how the microbiomes on and in the human body play critical roles in maintaining host health (Human Microbiome Project Consortium 2012). The oral microbiome has been implicated in maintaining host health and, conversely, the dysbiosis of this microbiome is thought to be implicated in disease progression (Cho and Blaser 2012). Tracking how this microbiome changes through time would give a greater understanding of how we have coevolved with our commensal microbiota and how dietary and cultural practices have contributed to this evolution (Warinner, Speller, and Collins 2015; Warinner et al. 2015).

Given that the oral microbiome is highly individualised, heritable and transmissible (Zaura et al. 2014) and experiences genetic variation due to isolation, drift and founder effects

(Dominguez-Bello and Blaser 2011), could we identify bacterial strains which are vertically or horizontally transmitted in order to identify population groups or understand migrations? For example, this has been explored using *Helicobacter pylori* (Linz et al. 2007; Domínguez-Bello et al. 2008; Moodley et al. 2009), where modern strains of these bacteria can be tied to different population groups. Of more relevance to dental calculus, Caufield et al. (2007; 2009) discovered that plasmid-containing strains of *Streptococcus mutans* (which is vertically transmitted) clustered by geographically defined groups. Similarly, Haubek et al. (2007) identified that the JP2 clone of *Aggregatibacter actinomycetemcomitans* spread from West Africa to the Americas probably as a result of the transatlantic slave trade. Unfortunately, *Aggregatibacter* derived proteins in this study were only specific to the genus level. This is one avenue where genetic approaches would be more applicable than proteomic approaches, given that the evolution of protein sequences is much slower than DNA.

11.4.3 Identification of Salivary Human Proteins

In addition to characterising ancient microbiomes, proteomic analysis of dental calculus may enable the identification of the human oral proteome. The composition of this proteome has been utilised as a diagnostic tool for a range of systemic and latent diseases, such as diabetes (Rao et al. 2009), cystic fibrosis (Minarowski et al. 2008), sclerosis (Baldini et al. 2008), and cancers (Zhang et al. 2013), as it represents a particularly non-invasive method to detect disease (Zhang et al. 2013). As we explore in more detail the ancient human proteome as revealed through dental calculus, it would be useful to advance in step with research in oral proteome diagnostics in order to apply these advances to the past.

For example, the growing concurrent epidemics of obesity and type 2 diabetes represent a significant modern health challenge (Barnett and Kumar 2009). One major question surrounding this epidemic is whether this trend relates to the vast increase in sugar consumption over the past 200 years. One way to explore this could be in the detection of obesity and diabetes in the past. Given that the salivary proteome may reveal species specific markers of type 2 diabetes (Rao et al. 2009), and we know that we can identify at least some of these particular proteins (e.g. leukocyte elastase inhibitor, kallikrein-1, alpha-2-macroglobulin, and alpha-1-antitrypsin), this could represent a method for detecting type-2 diabetes in archaeological samples and for understanding its prevalence through time.

11.4.4 Identification of Food Consumption

Initially in this research, I assumed that dietary proteins would be abundant in dental calculus, given that microscopic identifications of dietary remains have been well-studied in this resource. In fact, biomolecules from bacteria make up the vast majority of this substrate, given that dental calculus is calcified plaque, a biofilm which contains over 200 million bacterial cells per milligram (Socransky and Haffajee 2005). However, the few food-derived proteins which were identified do reveal that a metaproteomic analysis of dental calculus is a valuable method by which to study diets which can be directly tied to individual consumption at a very high resolution.

Understanding broad dietary compositions from skeletal material is commonly achieved through analysis of ^{13}C and ^{15}N stable isotope signatures from bone collagen (Malainey 2010). These signatures typically represent broad dietary compositions diet owing to slow collagen

turnover rates (Hedges et al. 2007), although incremental analysis of dentine facilitates an exploration of childhood and adolescent diet (Beaumont et al. 2013).

While these broad compositions are indeed informative about the broad composition of individual diets, they do not reveal directly the specific foodstuffs that were consumed. Analysis of dental calculus is one way that food consumption can be revealed with a much higher taxonomic resolution than may be possible with isotopic analysis. The extraction of micro-botanical remains from dental calculus is already an established method. However, an alternative approach providing species-specific evidence could come from proteomic and genomic approaches. In individuals from St Helena, peanut (*Arachis hypogaea*) could be identified in two individuals. The whey protein identified in Chapter 8 could be identified to a species-level, allowing the consumption of different animal milks to be discriminated. However, it should be stated that this level of resolution is not always possible. For example, the analysis of individuals from St Helena revealed a plant protein only identifiable to the level of Eudicots, and in Warinner, Rodrigues, et al. (2014) an identified plant protein was only identifiable to the level of Viridiplantae. If species of interest are well represented in a proteomic reference database then this approach could have great potential, complementing other dietary sources.

11.5 Challenges and Limitations of Dental calculus as an Archaeological Resource

Dental calculus clearly represents a rich source of metaproteomic and metagenomic data, a method to characterise a suite of pathogenic and commensal microbiomes and a method to access high-resolution dietary information. However, some of the biases and challenges associated with utilising dental calculus for these applications are highlighted here.

- *The approach is limited to individuals who have dental calculus deposits*

Although dental calculus is ubiquitous in almost all past populations, as a resource for understanding ancient health and diet it is clearly limited to individuals who have dental calculus. Dental calculus deposits are significantly less common before the Neolithic (Arensburg 1996), so this approach becomes more limited in these deeper time scales. However, when dental calculus deposits are present in these much older samples they may be an exceptional reservoir of information, as has already been demonstrated with microfossil analysis (Henry et al. 2011; Hardy et al. 2012). With the rapidly growing interest in dental calculus research, it is likely that these few samples of dental calculus will become highly prized.

- *Large deposits present in archaeological samples are likely to be pathological*

If we are using dental calculus to make a statement about oral health, there is an inherent bias here. An individual who presents deposits of dental calculus is likely to have poorer oral health than one who does not. It is unlikely that analysis of dental calculus would reveal a consortium of only healthy bacteria so this would give bias towards the analysis of diseased mouths. If we are trying to understand the ancient oral microbiome this is a significant challenge because these deposits would be more likely to represent individuals with a dysbiotic microbiome.

In addition, in the analysis of human proteins from dental calculus, to what extent are immune proteins related to oral health or a reflection of a disease progression? Dental calculus represents a bacterial biofilm, so it seems logical that the human proteins observed would be related to the presence of such a biofilm. Like the identification of a healthy consortium of

oral bacteria, it is uncertain whether a “healthy” mouth would be reflected in the dental calculus proteome.

- *Modern and ancient dental calculus may not be directly comparable*

Archaeological deposits of dental calculus are typically much larger (representing more mature deposits) than modern dental calculus, owing to modern dental hygiene practices. This makes comparing modern and ancient dental calculus challenging, as mature deposits of dental calculus may have a different microbial and mineral composition to young deposits. Similarly, comparing ancient dental calculus microbiomes with modern dental plaque is similarly difficult, given the potential biomolecular differences in these two sources. In this study, I chose samples of dental calculus that had enough calculus deposit for both genomic and proteomic approaches, which clearly represents large deposits.

Studying the modern oral environment, let alone the ancient one, is complex because a) the microbiome is only just beginning to be uncovered, b) commensal species may become opportunistic pathogens in compromised individuals, c) pathogen bacteria can be present in a carrier state and have no effect on the host and d) species can act as a community, which is why understanding community-level functioning is an important exploration. Given these challenges, research into ancient dental calculus needs to proceed in step with advances in understanding the modern oral microbiome. Similarly, we are relying on modern dental literature in order to understand whether detected organisms are pathogenic, but, as highlighted in Chapter 10, how do we know the pathogenicity of organisms in the past? For example, at St Helena, despite osteological evidence of caries, *Streptococcus mutans* was observed in only one individual. Similarly, *Porphyromonas gingivalis* was identified in approximately half of the individuals, including in individuals with no evidence of periodontal disease.

- *Taphonomy and preservation may be a confounding factor in the analysis of dental calculus*

This work has demonstrated that there is substantial variability in the number of protein identifications made between individuals. The extent to which taphonomic processes play a role in the identification of the metaproteome is currently unknown, although Chapter 7 in particular highlights the effect that protein degradation can have on methods to characterise ancient disease. Understanding these taphonomic processes is critical to ensure that the proteome observed archaeologically is an accurate reflection of the proteome when the individual was alive. Similarly, to what extent can we understand this polymicrobial nature in the past if we have variable preservation of bacteria?

An analysis of the amount dental calculus used for protein extractions and generated spectral queries reveals that the amount of starting material has (relatively) little influence over the number of identified proteins (Fig. 11.1). This may further indicate that the preservation of proteins in dental calculus varies substantially and that taphonomy plays a major role in affecting proteomic results.

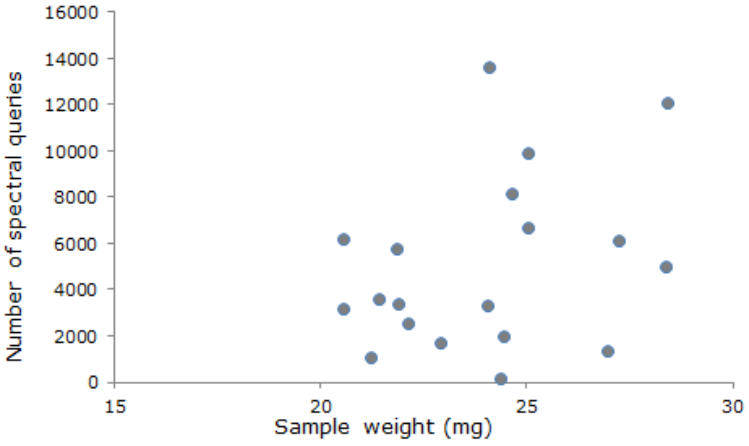

Figure 11.1 Relationship between starting sample weight and number of MS/MS spectral queries per sample of St Helena dental calculus.

- *Calculus is typically a palimpsest of biomolecules accumulated over an undefined time scale*

Dental calculus is accumulative and will be a reflection of the oral environment over the time that calculus has built up on the tooth surface. Understanding the rate of this accumulation is challenging, even in modern contexts, and varies substantially between individuals (White 1997). Thus, it is almost impossible to know whether a possible infection identified in dental calculus represents a single event or an accumulation over a certain time period. The best way to address this with the current methodology may be to select individuals where a recently erupted tooth has accumulated dental calculus. This would narrow the window of this palimpsest because it would only represent an oral environment during the time between that tooth eruption and death of the individual. If dental calculus could be analysed as a longitudinal cross-section, where analysis could take place in layers, this would also help to address this "palimpsest" problem. In addition, such a method would facilitate the identification of the oral environment at the time of death.

11.6 Future Work

Based on the technical challenges (9.3.3) experienced in this work, the implications and significance of the research (9.4), and the challenges of dental calculus research (9.5), I have identified several key areas for future work.

11.6.1 Understanding Protein Survival and Degradation

As been highlighted in Chapter 9 and above, to be able to adopt proteomics and metaproteomics to understand cellular and community-level functions, it is vital to know whether ancient proteins identified in archaeological samples are a true reflection of the living

tissue. Exploring and clarifying variability in the proteomic analysis of archaeological samples is crucial for further research. The fact that the similar proteomes are often observed in modern and ancient dental calculus, as well as modern and ancient dentine, is reassuring that a representative proteome is preserved, but clearly there are also taphonomic influences that are not yet well understood.

One way to explore this would be to analyse deposits of modern calculus to understand the composition of the proteome during calculus formation and during its deposition in the archaeological record. Degradation experiments, using both modern dental calculus and synthetic biofilms, could be performed to characterise how the proteome changes through time. To further understand this mechanism, it would be interesting to compare protein survival in different dental and skeletal tissues (calculus, bone, dentine, cementum), to compare the biomolecular richness of these dental tissues. In this study, only two samples were selected for dentine extraction, limited by sampling permissions and removal of skeletal material from St Helena. It would also be interesting to explore this in parallel with DNA characterisation, to clarify the preservation of both ancient DNA and ancient proteins in these dental tissues. Similarly, teasing apart the relationship between aDNA and ancient proteins could be explored in more detail, given that no relationship could be discerned in Chapter 9.

Similarly, in dental calculus we do not yet understand how the interaction of mineral and molecular phases may influence biomolecular survival. Scanning electron microscopy analysis conducted for this research has revealed some of the variation in crystal forms in ancient dental calculus (Figure 11.2), but understanding what impact this has on biomolecular survival is unknown. Analysis by Raman spectroscopy may be one way to characterise mineral phases present in dental calculus (Warinner, Rodrigues, et al. 2014).

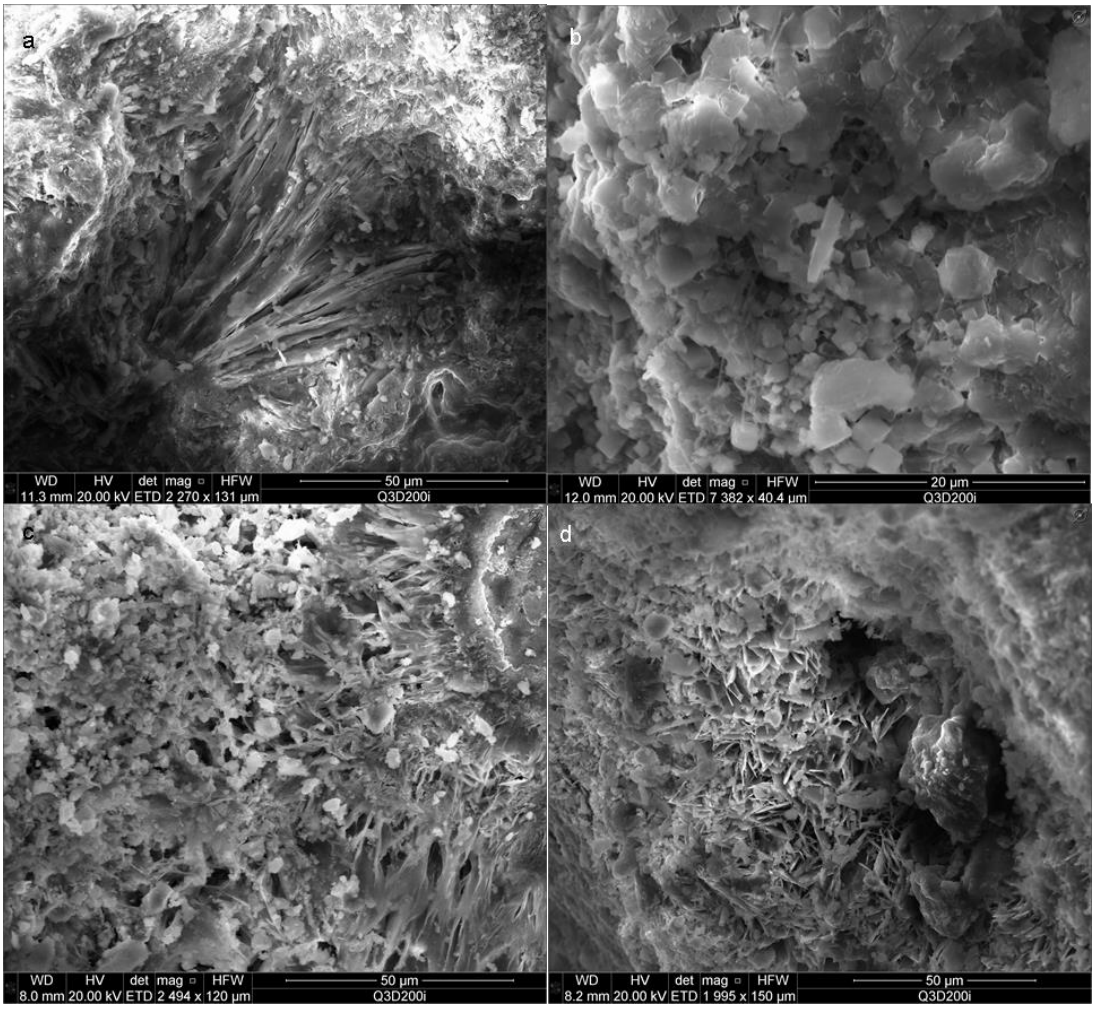

Figure 11.2 Examples of crystal forms present in samples of dental calculus from St Helena.

Another way to explore this mineral-protein interaction could be to identify peptides which commonly survive, and assess whether they have any properties which may facilitate or be linked to mineral binding. For example, in β-lactoglobulin regions of this protein appear to survive preferentially to others (Chapter 8). An exploration of post-translational modifications would be additionally informative in order to understand patterns of degradation.

Even if we do not fully understand the reason behind the survival of the metaproteome in dental calculus, it would at least be useful to develop a screening tool which would enable

only well-preserved samples to go forward for proteomic analysis. Two such tools were briefly explored in this PhD. The first used MALDI TOF, an inexpensive and rapid mass spectrometry-based technique used in ZooMS (Buckley et al. 2009), which can reveal in a broad sense the abundance of extracted peptides. However, no discernable trends were observed between MALDI spectra and the yield or number of identified proteins in samples of ancient dental calculus. The second screening method attempted involved demineralising a small subsample of dental calculus with EDTA and recording the concentration of this extract using a Qubit fluorometer. However, similarly no relationship between protein yields detected by this screening method, the protein yields detected in the extraction, or the number of peptides or proteins identified could be observed. Further work is needed to develop a screening tool.

11.6.2 Bioinformatic Approaches for Ancient Proteomics

Given the challenges of bioinformatic analysis outlined in section 11.3.3, a comparison of commercially available tools for analysing the proteome and metaproteome would be useful. A number of steps within this analysis could be explored, including the evaluation of a) spectral matching programmes (e.g. Mascot, SEQUEST, X!Tandem), b) methods by which to validate matches (e.g. different probability scoring methods, FDR), c) the most useful and appropriate databases (e.g. the Human Oral Microbiome Database, SwissProt or a customised database), and d) tools that enable comparisons between the proteome of different individuals or samples, such as the MetaProteomeAnalyzer (Muth et al. 2015).

11.6.3 Shotgun Proteomics on Other Samples

Given the survival of non-collagenous proteins in dentine and the survival of human proteins and bacteria in dental calculus, proteins extracted from other skeletal tissues could also be informative. For example, identification of human proteins might be one way to uncover healing or active lesions or the early onset of pathologies affecting the skeletal. Shotgun proteomics could also be applied to other calcified tissues, such as kidney stones or calcified pleura (Donoghue et al. 1998) to understand their aetiology (e.g. Bona et al. 2014). Outside of the skeleton, other sample types may be informative about past diseases and diets. For example, mineralised limescale, present in many parts of the world, could be informative about microorganisms that may have been present in ancient waterways.

11.6.4 The Transatlantic Slave Trade

A significant limitation with the approach explored for this PhD was the lack of information which informs directly on the transatlantic slave trade. Although these results are contributing to understanding the health of individuals during this time, there was little identified evidence of disease directly related to this period. Further explorations with DNA-based approaches may reveal further information. Although challenges were experienced owing to low yields, recent developments in capture-based methods (e.g. Schroeder et al. 2015) could assist with this analysis. In addition, a targeted approach for the identification of particular infectious diseases (such as those outlined in Chapter 3) may be a better method for isolating particular organisms of interest. In addition, DNA-based approaches may enable a better understanding the spread and evolution of these particular microorganisms during this era.

Analysis of populations from the American and African continents may enable an understanding of the contemporaneous differences in the disease environment between these two connected continents. Similarly, understanding infectious disease prior to and following European contact in West and West Central Africa is largely unknown and could be an area for future research. Analysis of strontium isotopes in bone and teeth has been successfully applied to understanding individual migrations in the transatlantic slave trade (Schroeder et al. 2009; Schroeder, Haviser, and Price 2014). Preliminary experiments indicate that dental calculus may also preserve an endogenous signature of strontium isotopes. Using dental calculus, migration over a relatively short time scale could be tracked and, with the right sampling strategy, could be particularly informative about movements during the last period of life.

11.7 Conclusion

This research has explored the potential of shotgun proteomics for characterising disease and diet in the archaeological record, revealing that it can be a powerful tool for both these applications. In the analysis of dental calculus, this study has demonstrated that high-sensitivity tandem mass spectrometry is able to characterise complex ancient metaproteomes with sufficient resolution in order to identify ancient bacteria, dietary remains and the proteome expressed by the human host. More specifically, this research demonstrates that the metaproteomic analysis of dental calculus may have particular application in understanding oral health. Although this research makes substantial contributions to the development of ancient proteomics, this research found that using this approach to understand diseases directly relevant to the transatlantic slave was more limited. In future work it will be vital to explore the effect of taphonomic processes on ancient proteomes and clarify the mechanism of

biomolecular survival in dental calculus. Overall, this research has contributed significantly to palaeomicrobiology, palaeopathology and ancient dietary studies by exploring a method in which oral, polymicrobial diseases might be characterised in past populations, the ancient oral microbiome may be identified and explored, and high-resolution dietary information can be directly extracted from the mouths of past individuals.

Appendix 1: Protein Identifications and Additional Sample Information

Table A1.1. Proteins identified in samples of dental calculus from St Helena (Chapter 9).

Species	Protein	Mascot score
Calculus		
Individual 212 MS/MS ID JH1; FDR 3.17%; Normalised protein yield 2528.97 ng/mg		
Actinomyces graevenitzii	Glyceraldehyde-3-phosphate dehydrogenase	72
Actinomyces sp. oral taxon 448	Glyceraldehyde-3-phosphate dehydrogenase	85
Actinomyces spp.	Fructose-bisphosphate aldolase	69
Filifactor alocis	Hypothetical protein	64
Homo sapiens	Keratin, type I cytoskeletal 9	127
Homo sapiens	Serum albumin	150
Homo sapiens	Protein S100-A8	81
Homo sapiens	Collagen alpha-1(I) chain	79
Homo sapiens	Keratin, type II cytoskeletal 1	76
Homo sapiens	Alpha-1-antitrypsin	74
Homo sapiens	Prolactin-inducible protein	71
Homo sapiens	Antithrombin-III	68
Homo sapiens	Neutrophil defensin 1	64
Homo sapiens	Protein S100-A9	60
Homo sapiens	Lysozyme C	54
Homo sapiens	C-reactive protein	45
Homo sapiens	Collagen alpha-2(I) chain	40
Homo sapiens	Complement C3	67
Leptotrichia buccalis	Flagellar motor protein	62
Selenomonas spp.	Flagellin	98
Synergistetes spp.	S-layer homology domain	68
Synergistetes bacterium SGP1	Flagellin and related hook-associated protein	62
Sus scrofa	Trypsin	70
Individual 218 MS/MS ID JH2; FDR 3.45%; Normalised protein yield 3466.96 ng/mg		
Homo sapiens	Keratin, type I cytoskeletal 9	176

Homo sapiens	Keratin, type II cytoskeletal 1	142
Homo sapiens	Collagen alpha-2(I) chain	129
Homo sapiens	Collagen alpha-1(I) chain	111
Neisseria elongata	N-terminal cleavage protein	101
Homo sapiens	Alpha-1-antitrypsin	80
Homo sapiens	Keratin, type I cytoskeletal 10	79
Homo sapiens	Keratin, type II cytoskeletal 2 epidermal	37

Individual 237 MS/MS ID JH3; FDR 3.92%; Normalised protein yield 3130.11 ng/mg

Filifactor alocis	Hypothetical protein	73
Homo sapiens	Keratin, type II cytoskeletal 1	343
Homo sapiens	Collagen alpha-2(I) chain	122
Homo sapiens	Collagen alpha-1(I) chain	87
Homo sapiens	Alpha-1-antichymotrypsin	74
Homo sapiens	Keratin, type I cytoskeletal 9	70
Homo sapiens	Keratin, type II cytoskeletal 2 epidermal	56
Homo sapiens	Prolactin-inducible protein	55
Homo sapiens	Zymogen granule protein 16 homolog B	33
Homo sapiens	Eukaryotic peptide chain release factor subunit 1	44
Saccharomycetaceae	60S ribosomal protein L27-A	48
Synergistetes bacterium SGP1	ATP synthase subunit C	44

Individual 343 MS/MS ID JH4; FDR 4.50%; Normalised protein yield 4386.89 ng/mg

Actinomyces sp. oral taxon 448	Glyceraldehyde-3-phosphate dehydrogenase	72
Atopobium parvulum	Molecular chaperone GroEL	104
Bacteroidetes oral taxon 274	Hemagglutinin protein HagA	113
Desulfatibacillum	Molecular chaperone GroEL	59
Filifactor alocis	Hypothetical protein	111
Homo sapiens	Alpha-1-antitrypsin	726
Homo sapiens	Keratin, type II cytoskeletal 1	129
Homo sapiens	Keratin, type I cytoskeletal 9	113
Homo sapiens	Serpin B10	109
Homo sapiens	Alpha-1-antichymotrypsin	86
Homo sapiens	Antithrombin-III	82
Homo sapiens	Prolactin-inducible protein	71
Homo sapiens	Serum albumin	65
Homo sapiens	Collagen alpha-1(I) chain	64
Homo sapiens	Lysozyme C	51
Synergistetes spp.	S-layer homology domain	55
Synergistetes bacterium SGP1	ATP synthase subunit C	129

Synergistetes bacterium SGP1	Flagellin and related hook-associated protein	116
Treponema spp.	long-chain fatty acid--CoA ligase	83

Individual 359 MS/MS ID JH5; FDR 1.77%; Normalised protein yield 3318.67 ng/mg

Actinomyces graevenitzii	Glyceraldehyde-3-phosphate dehydrogenase	74
Actinomyces oral taxon 448	Enolase	65
Actinomyces sp. oral taxon 448	Glyceraldehyde-3-phosphate dehydrogenase	76
Actinomyces spp.	Phosphoglycerate kinase	78
Actinomyces spp.	L-lactate dehydrogenase	61
Arachis hypogaea	Galactose-binding lectin	76
Bacteroidetes oral taxon 274	RagA protein	65
Campylobacter showae	Major outer membrane protein	57
Capnocytophaga spp.	Phosphoenolpyruvate carboxykinase	128
Corynebacterium matruchotii	Enolase	114
Corynebacterium matruchotii	Lactate dehydrogenase	89
Corynebacterium matruchotii	Fructose-bisphosphate aldolase	69
Enterococcus spp.	L-lactate dehydrogenase 1	48
Filifactor alocis	Hypothetical protein	78
Homo sapiens	Keratin, type II cytoskeletal 1	149
Homo sapiens	Keratin, type I cytoskeletal 9	126
Homo sapiens	Collagen alpha-2(I) chain	92
Homo sapiens	Collagen alpha-1(I) chain	87
Homo sapiens	Keratin, type I cytoskeletal 10	82
Homo sapiens	Serum albumin	80
Homo sapiens	Lysozyme C	81
Homo sapiens	Protein S100-A8	74
Homo sapiens	Alpha-1-antitrypsin	73
Homo sapiens	Ig lambda-2 chain C region or Ig lambda-3 chain C regions	73
Homo sapiens	Prolactin-inducible protein	72
Homo sapiens	Protein S100-A9	65
Homo sapiens	Neutrophil defensin 1 or 3	64
Homo sapiens	Eosinophil cationic protein	52
Homo sapiens	UPF0762 protein C6orf58	48
Homo sapiens	Zymogen granule protein 16 homolog B	38
Lautropia mirabilis	Enolase	81
Lautropia mirabilis	Triosephosphate isomerase	71

Lautropia mirabilis	Hypothetical protein	76
Lautropia mirabilis	Hypothetical protein	69
Lautropia mirabilis	Hypothetical protein	68
Lautropia mirabilis	Lactate dehydrogenase	65
Neisseria elongata	N-terminal cleavage protein	122
Neisseria elongata	Membrane protein	107
Neisseria sicca	Major outer membrane protein P.IB	58
Neisseria sp. oral taxon 014	Membrane protein	92
Neisseria spp.	Hypothetical protein	77
Neisseria spp.	Membrane protein	60
Prevotella oris	Hypothetical protein	76
Prevotella spp.	Hypothetical protein	73
Streptococcus spp.	Enolase	69
Streptococcus spp.	60 kDa chaperonin	58
Streptococcus spp.	30S ribosomal protein S10	56
Streptococcus spp.	Transcriptional regulator	55
Synergistetes bacterium SGP1	Flagellin and related hook-assocated protein	67
Synergistetes bacterium SGP1	ATP synthase subunit C	65
Tannerella forsythia	Hypothetical protein	60
Sus scrofa	Trypsin	78

Individual 421 MS/MS ID JH6; FDR 18.18%; Normalised protein yield 1867.05 ng/mg

Homo sapiens	Alpha-1-antichymotrypsin	77
Homo sapiens	Alpha-1-antitrypsin	58
Synergistetes bacterium SGP1	S-layer homology domain	50

Individual 430 MS/MS ID JH7; FDR 0.94%; Normalised protein yield 1597.34 ng/mg

Filifactor alocis	Hypothetical protein	192
Homo sapiens	Keratin, type II cytoskeletal 1	438
Homo sapiens	Keratin, type I cytoskeletal 10	166
Homo sapiens	Keratin, type I cytoskeletal 9	132
Homo sapiens	Alpha-1-antitrypsin	80
Homo sapiens	Alpha-1-antichymotrypsin	79
Homo sapiens	Serum albumin	62
Homo sapiens	Protein S100-A9	60
Homo sapiens	Lysozyme C	48
Homo sapiens	Collagen alpha-1(I) chain	40
Methylovorus spp.	60 kDa chaperonin	61
Neisseria mucosa	Calcium-binding protein	55
Neisseria sicca	Major outer membrane protein P.IB	54
Prevotella spp.	Hypothetical protein	105

Synergistetes bacterium SGP1	Flagellin and related hook-associated proteins	347
Synergistetes bacterium SGP1	ATP synthase subunit C	60

Individual 474 MS/MS ID JH8; FDR 1.75%; Normalised protein yield 3475.44 ng/mg

Actinomyces spp.	Glyceraldehyde-3-phosphate dehydrogenase	79
Aggregatibacter spp.	Peptidoglycan-associated lipoprotein, putative	71
Campylobacter showae/rectus	Membrane protein	72
Corynebacterium matruchotii	Enolase	99
Filifactor alocis	Hypothetical protein	98
Homo sapiens	Alpha-1-antitrypsin	380
Homo sapiens	Keratin, type I cytoskeletal 10	285
Homo sapiens	Kallikrein-1	151
Homo sapiens	Keratin, type II cytoskeletal 1	129
Homo sapiens	Myeloperoxidase	83
Homo sapiens	Antithrombin-III	73
Homo sapiens	Serum albumin	72
Homo sapiens	Alpha-1-antichymotrypsin	52
Lachnospiraceae bacterium oral taxon 082	Hypothetical protein	87
Porphyromonas gingivalis	Major fimbrial subunit protein type-1	69
Porphyromonas gingivalis	Lys-gingipain	83
Pyramidobacter piscolens	TPP-dependent acetoin dehydrogenase complex, E1 protein subunit beta	70
Synergistetes bacterium SGP1	Flagellin and related hook-associated protein	987
Synergistetes bacterium SGP1	ABC-type Co2+ transport system, periplasmic component	81
Synergistetes bacterium SGP1	ATP synthase subunit C	81

Individual 219 MS/MS ID JH10; FDR 1.09%; Normalised protein yield 3008.76 ng/mg

Actinomyces graevenitzii	Glyceraldehyde-3-phosphate dehydrogenase	57
Actinomyces sp. oral taxon 448	Glyceraldehyde-3-phosphate dehydrogenase	79
Flavobacterium sp. ACAM 123	Hypothetical protein	43
Homo sapiens	Collagen alpha-2(I) chain	1663
Homo sapiens	Collagen alpha-1(I) chain	1252

Homo sapiens	Keratin, type I cytoskeletal 9	117
Homo sapiens	Serum albumin	79
Synergistetes bacterium SGP1	Flagellin and related hook-associated protein	92
Sus scrofa	Trypsin	67

Individual 268 MS/MS ID JH11; FDR 11.10%; Normalised protein yield 4,176.47 ng/mg

Homo sapiens	Alpha-1-antitrypsin	80
Sus scrofa	Trypsin	123
Fusobacterium spp.	Carboxysome structural protein	100
Homo sapiens	Alpha-1-antitrypsin	224
Homo sapiens	Keratin, type I cytoskeletal 10	148
Homo sapiens	Alpha-1-antichymotrypsin	140
Homo sapiens	UPF0762 protein	84
Homo sapiens	Lipocalin-1	69
Homo sapiens	Protein S100-A9	65
Homo sapiens	Serum albumin	64
Homo sapiens	Collagen alpha-1(I) chain	61
Homo sapiens	Lysozyme C	55
Homo sapiens	Prolactin-inducible protein	50
Neisseria spp.	Membrane protein	54
Synergistetes bacterium SGP1	Flagellin and related hook-associated protein	152

Individual 306 MS/MS ID JH13; FDR 1.41%; Normalised protein yield 3188.41 ng/mg

Actinomyces sp. oral taxon 448	Glyceraldehyde-3-phosphate dehydrogenase	68
Actinomyces spp.	Phosphotransferase	88
Actinomyces spp.	Glyceraldehyde-3-phosphate dehydrogenase	84
Actinomyces spp.	Sugar ABC transporter ATO-binding protein	48
Corynebacterium matruchotii	Enolase	89
Corynebacterium matruchotii	Hypothetical protein	59
Filifactor alocis	Hypothetical protein	86
Homo sapiens	Collagen alpha-2(I) chain	183
Homo sapiens	Keratin, type II cytoskeletal 1	159
Homo sapiens	Collagen alpha-1(I) chain	154
Homo sapiens	Lysozyme C	126
Homo sapiens	Alpha-1-antitrypsin	73
Homo sapiens	Keratin, type I cytoskeletal 10	40
Homo sapiens	Zymogen granule protein 16 homolog B	39
Homo sapiens	Antileukoproteinase	366
Neisseria spp.	Hypothetical protein	62
Porphyromonas gingivalis	Major fimbrial subunit protein type-2	46

Prevotella spp.	Hypothetical protein	67
Individual 319 MS/MS ID JH14; FDR 14.29%; Normalised protein yield 2313.40 ng/mg		
Homo sapiens	Alpha-1-antitrypsin	43
Homo sapiens	Keratin, type I cytoskeletal 9	55
Individual 389 MS/MS ID JH15; FDR 1.56%; Normalised protein yield 8883.35 ng/mg		
Actinomyces sp. oral taxon 448	Heat-shock protein Hsp20	64
Actinomyces sp. oral taxon 448	L-lactate dehydrogenase	58
Actinomyces graevenitzii	Phosphoglucomutase	71
Actinomyces graevenitzii	glyceraldehyde-3-phosphate dehydrogenase	70
Actinomyces graevenitzii	Phosphoenolpyruvate carboxykinase	67
Actinomyces sp. oral taxon 448	glyceraldehyde-3-phosphate dehydrogenase	92
Actinomyces spp.	Molecular chaperone GroEL	117
Corynebacterium matruchotii	Enolase	121
Filifactor alocis	Hypothetical protein	99
Homo sapiens	Alpha-1-antitrypsin	224
Homo sapiens	Serum albumin	167
Homo sapiens	Keratin, type I cytoskeletal 9	136
Homo sapiens	Keratin, type II cytoskeletal 1	113
Homo sapiens	Ig gamma-1 chain C region	100
Homo sapiens	Antithrombin-III	72
Homo sapiens	Myeloperoxidase	68
Homo sapiens	Lysozyme C	61
Porphyromonas gingivalis	Hemagglutinin A	183
Synergistetes bacterium SGP1	Flagellin and related hook-associated protein	409
Synergistetes bacterium SGP1	Glycine/sarcosine/betaine reductase component B subunits	138
Synergistetes bacterium SGP1	ABC-type Co2+ transport system, periplasmic component	57
Synergistetes bacterium SGP1	S-layer homology domain	52
Tannerella forsythia	Hypothetical protein	78
Individual 419 MS/MS ID JH16; FDR 1.99%; Normalised protein yield 1319.96 ng/mg		
Actinomyces spp.	Surface-anchored membrane protein	75
Alloprevotella tannerae	ATP synthase subunit C	97

Filifactor alocis	Hypothetical protein	100
Homo sapiens	Keratin, type I cytoskeletal 10	592
Homo sapiens	Collagen alpha-2(I) chain	483
Homo sapiens	Collagen alpha-1(I) chain	358
Homo sapiens	Keratin, type II cytoskeletal 1	265
Homo sapiens	Alpha-1-antitrypsin	146
Homo sapiens	Keratin, type I cytoskeletal 9	141
Homo sapiens	Serum albumin	79
Homo sapiens	Neutrophil defensin 1 or 3	77
Homo sapiens	Protein S100-A9	75
Homo sapiens	Ameloblastin	74
Kingella denitrificans	Hypothetical protein	55
Lautropia mirabilis	Hypothetical protein	99
Neisseria sicca	Outer membrane insertion C-signal	66
Neisseria sicca	Calcium-binding protein	80
Neisseria sicca	Major outer membrane protein P.IB	69
Stenotrophomonas maltophilia	TonB-dependent receptor	50
Synergistetes bacterium SGP1	ATP synthase subunit C	64
Veillonella spp.	Hemagglutinin	67
Sus scrofa	Trypsin	79

Individual 449 MS/MS ID JH17; FDR 10.53%; Normalised protein yield 4084.06 ng/mg

Homo sapiens	Collagen alpha-2(I) chain	179
Homo sapiens	Collagen alpha-1(I) chain	87
Homo sapiens	Antithrombin-III	42

Individual 414 MS/MS ID JH19; FDR 1.20%; Normalised protein yield 815.88 ng/mg

Homo sapiens	Collagen alpha-1(I) chain	370
Homo sapiens	Collagen alpha-2(I) chain	245
Homo sapiens	Keratin, type II cytoskeletal 1	232
Homo sapiens	Keratin, type I cytoskeletal 9	150
Homo sapiens	Keratin, type I cytoskeletal 14	122
Homo sapiens	Keratin, type II cytoskeletal 6A	116
Porphyromonas gingivalis	Zinc carboxypeptidase	95
Kingella denitrificans	hypothetical protein	78
Filifactor alocis	hypothetical protein	67
Corynebacterium matruchotii	Enolase	59
Fusobacterium sp. oral taxon 370	Hep/Hag repeat protein	58
Sus scrofa	Trypsin	80

Individual 423 MS/MS ID JH20; FDR 4.76%; Normalised protein yield 3731.13 ng/mg

Actinomyces sp. oral taxon 448	Glyceraldehyde-3-phosphate dehydrogenase	81
Filifactor alocis	Hypothetical protein	63
Homo sapiens	Keratin, type I cytoskeletal 9	173
Homo sapiens	Keratin, type II cytoskeletal 1	103
Homo sapiens	Serum albumin	73
Homo sapiens	Collagen alpha-1(I) chain	57
Synergistetes bacterium SGP1	Flagellin and related hook-associated protein	57
Tannerella forsythia	Hypothetical protein	54
Individual 459 MS/MS ID JH21; FDR 25%; Normalised protein yield 6861.45 ng/mg		
Homo sapiens	Alpha-1-antitrypsin	42
Homo sapiens	Collagen alpha-1(I) chain	42
Individual 358 MS/MS ID 8786; FDR 1.87%; Normalised protein yield 3722.22 ng/mg		
Actinomyces graevenitzii	Phosphoenolpyruvate carboxykinase	56
Actinomyces sp. oral taxon 448	Glyceraldehyde-3-phosphate dehydrogenase	151
Actinomyces spp.	Sugar ABC transporter ATP-binding protein	200
Actinomyces spp.	Triosephosphate isomerase	86
Actinomyces spp.	ribosome recycling factor	76
Actinomyces spp.	L-lactate dehydrogenase	135
Actinomyces spp.	Enolase	133
Actinomyces spp.	Phosphoglycerate kinase	94
Actinomyces spp.	Malate dehydrogenase	81
Actinomyces spp.	Pyruvate kinase	70
Actinomyces spp.	Phosphoenolpyruvate carboxykinase	66
Actinomyces spp.	Pyrophosphate--fructose-6-phosphate 1-phosphotransferase	61
Actinomyces spp.	Type 2 fimbrial subunit	56
Actinomyces spp.	Formate acetyltransferase	54
Arachis hypogaea	Conglutin-7	114
Arachis hypogaea	Conglutin	90
Bos taurus	Keratin, type II cuticular Hb1	59
Campylobacter showae	Major outer membrane protein	75
Capnocytophaga spp.	Phosphoenolpyruvate carboxykinase	67
Cardiobacterium hominis	Hypothetical protein	54
Cardiobacterium valvarum	Porin	48
Corynebacterium matruchotii	Peptidase	85
Corynebacterium matruchotii	Phosphopyruvate hydratase	51

Corynebacterium matruchotii	Glyceraldehyde-3-phosphate dehydrogenase, type I	81
Corynebacterium matruchotii	Hypothetical protein	79
Eikenella corrodens	Hypothetical protein	54
Eubacterium yurii	Peptidase	54
Filifactor alocis	Hypothetical protein	88
Homo sapiens	Alpha-1-antichymotrypsin	525
Homo sapiens	Alpha-1-antitrypsin	1214
Homo sapiens	Antithrombin-III	309
Homo sapiens	Bactericidal permeability-increasing protein	67
Homo sapiens	C-reactive protein	70
Homo sapiens	Collagen alpha-1(I) chain	459
Homo sapiens	Complement C3	67
Homo sapiens	Glyceraldehyde-3-phosphate dehydrogenase	85
Homo sapiens	Hornerin	77
Homo sapiens	Ig alpha-2 chain C region	54
Homo sapiens	Ig gamma-1 chain C region	51
Homo sapiens	Ig kappa chain C region	69
Homo sapiens	Ig lambda-2/3 chain C regions	76
Homo sapiens	Kallikrein-1	68
Homo sapiens	Keratin, type I cytoskeletal 10	282
Homo sapiens	Keratin, type I cytoskeletal 9	158
Homo sapiens	Keratin, type II cytoskeletal 1	229
Homo sapiens	Keratin, type II cytoskeletal 2 epidermal	135
Homo sapiens	Leukocyte elastase inhibitor	100
Homo sapiens	Lysozyme C	120
Homo sapiens	Myeloperoxidase	127
Homo sapiens	Neutrophil defensin 3	132
Homo sapiens	Peptidoglycan recognition protein 1	50
Homo sapiens	Prolactin-inducible protein	207
Homo sapiens	Prosaposin	77
Homo sapiens	Protein S100-A8	142
Homo sapiens	Protein S100-A9	333
Homo sapiens	Resistin	355
Homo sapiens	Serpin B6	68
Homo sapiens	Serum albumin	774
Homo sapiens	UPF0762 protein C6orf58	121
Homo sapiens	Zinc-alpha-2-glycoprotein	61
Homo sapiens	Zymogen granule protein 16 homolog B	62
Lactobacillus spp.	Phosphoglucomutase	82
Lautropia mirabilis	Nitric oxide reductase	87
Lautropia mirabilis	Fructose-bisphosphate aldolase	86
Lautropia mirabilis	Nitrite reductase	58
Lautropia mirabilis	Glyceraldehyde-3-phosphate dehydrogenase	52

Lautropia mirabilis	50S ribosomal protein L18	48
Lautropia mirabilis	Gram-negative porin	92
Lautropia mirabilis ATCC 51599	Gram-negative porin	71
Neisseria elongata	Porin	83
Neisseria spp.	Porin	87
Neisseria sp. oral taxon 014 str. F0314	Putative major outer membrane protein P.IA	78
Ovis aries	Keratin, type I microfibrillar 48 kDa, component 8C-1	90
Porphyromonas gingivalis	Lys-gingipain	182
Porphyromonas gingivalis	Major fimbrial subunit protein type-1	83
Porphyromonas gingivalis	Pg-II fimbriae a/hypothetical protein/cell surface protein precursor	69
Pseudoramibacter alactolyticus	Propanediol utilization protein	68
Salinispora spp.	60 kDa chaperonin 1	116
Selenomonas spp.	S-layer protein	68
Shuttleworthia spp.	Lactate dehydrogenase	74
Streptococcus mutans PKUSS-LG01	Fructose-1,6-biphosphate aldolase	59
Streptococcus spp.	Enolase	89
Streptococcus spp.	Hypothetical protein	78
Synergistetes bacterium SGP1	Hypothetical protein SY1_18680	77
Synergistetes bacterium SGP1	ATP synthase subunit C	79
Synergistetes bacterium SGP1	Hypothetical protein SY1_18680	80
Synergistetes bacterium SGP1	Flagellin and related hook-associated protein	247
Tannerella forsythia	Hypothetical protein	94
Tannerella forsythia	Surface layer protein B	61
Tannerella forsythia	Hypothetical protein	57
Tannerella forsythia	Outer membrane protein 40	57
Treponema denticola	Hypothetical protein	53
Veillonella spp.	Hypothetical protein	56
Veillonella spp.	S-layer protein	131
Sus scrofa	Trypsin	255

Dentine

Individual 430 MS/MS ID 10806; FDR 0.97%; Normalised protein yield 5016.18 ng/mg

Gluconacetobacter diazotrophicus	Ribonuclease P protein component	98
Homo sapiens	Alpha-2-HS-glycoprotein	2355
Homo sapiens	Alpha-2-macroglobulin	46
Homo sapiens	Amyloid beta A4 protein	45
Homo sapiens	Antithrombin-III	69
Homo sapiens	Apolipoprotein A-IV	73
Homo sapiens	Asporin	58
Homo sapiens	Biglycan	798
Homo sapiens	C-type lectin domain family 11 member A	58
Homo sapiens	Clusterin	72
Homo sapiens	Coagulation factor IX	40
Homo sapiens	Coagulation factor X	67
Homo sapiens	Collagen alpha-1(IV) chain	49
Homo sapiens	Collagen alpha-1(VI) chain	64
Homo sapiens	Collagen alpha-1(XXII) chain	66
Homo sapiens	Collagen alpha-2(I) chain	12865
Homo sapiens	Collagen alpha-2(XI)	250
Homo sapiens	Collagen alpha-3(VI) chain	86
Homo sapiens	Collectin-12	47
Homo sapiens	Complement component C9	75
Homo sapiens	Cystatin-C	47
Homo sapiens	Dentin matrix acidic phosphoprotein 1	69
Homo sapiens	Endoplasmin	43
Homo sapiens	Extracellular superoxide dismutase [Cu-Zn]	52
Homo sapiens	Growth arrest-specific protein 6	88
Homo sapiens	Ig gamma-1 chain C region	79
Homo sapiens	Keratin, type I cytoskeletal 10	162
Homo sapiens	Keratin, type I cytoskeletal 9	68
Homo sapiens	Keratin, type II cytoskeletal 1	200
Homo sapiens	Kininogen-1	95
Homo sapiens	Lumican	167
Homo sapiens	Matrix Gla protein	103
Homo sapiens	Matrix metalloproteinase-20	103
Homo sapiens	Mimecan	47
Homo sapiens	Myosin-7	79
Homo sapiens	Osteomodulin	80
Homo sapiens	Periostin	46
Homo sapiens	Pigment epithelium-derived factor	681
Homo sapiens	Prothrombin	82
Homo sapiens	Secreted phosphoprotein 24	77
Homo sapiens	Serum albumin	241
Homo sapiens	Signal peptide, CUB and EGF-like domain-containing protein 3	70
Homo sapiens	Thrombospondin-1	141

Homo sapiens	Transforming growth factor-beta-induced protein ig-h3	66
Homo sapiens	Vitamin K-dependent protein C	65
Homo sapiens	Vitamin K-dependent protein S	65
Homo sapiens	Vitamin K-dependent protein Z	40
Homo sapiens	Vitronectin	212
Sus scrofa	Trypsin	273

Individual 212 MS/MS ID 10809; FDR 0.98%; Normalised protein yield 2305.62 ng/mg

Homo sapiens	Alpha-1-antichymotrypsin	57
Homo sapiens	Alpha-2-HS-glycoprotein	1677
Homo sapiens	Alpha-2-macroglobulin	78
Homo sapiens	Amyloid beta A4 protein	63
Homo sapiens	Antithrombin-III	99
Homo sapiens	Biglycan	239
Homo sapiens	C-type lectin domain family 11 member A	75
Homo sapiens	Clusterin	75
Homo sapiens	Coagulation factor X	98
Homo sapiens	Collagen alpha-1(I) chain	13026
Homo sapiens	Collagen alpha-1(XXII) chain	131
Homo sapiens	Collagen alpha-2(VI) chain	43
Homo sapiens	Collagen alpha-2(XI) chain	292
Homo sapiens	Complement component C9	89
Homo sapiens	Dentin matrix acidic phosphoprotein 1	58
Homo sapiens	Extracellular superoxide dismutase [Cu-Zn]	60
Homo sapiens	Fibroleukin	66
Homo sapiens	Fibronectin	60
Homo sapiens	Histidine-rich glycoprotein	56
Homo sapiens	Ig gamma-1 chain C region	44
Homo sapiens	Insulin-like growth factor-binding protein 1	91
Homo sapiens	Insulin-like growth factor-binding protein 3	74
Homo sapiens	Keratin, type I cytoskeletal 10	234
Homo sapiens	Keratin, type I cytoskeletal 9	132
Homo sapiens	Keratin, type II cytoskeletal 1	200
Homo sapiens	Kininogen-1	92
Homo sapiens	Lumican	78
Homo sapiens	Lysozyme C	68
Homo sapiens	Matrix Gla protein	68
Homo sapiens	Matrix metalloproteinase-20	55
Homo sapiens	Osteomodulin	163
Homo sapiens	Periostin	98
Homo sapiens	Pigment epithelium-derived factor	134
Homo sapiens	Protein disulfide-isomerase	60
Homo sapiens	Protein S100-A7A	62
Homo sapiens	Protein S100-A9	82

Homo sapiens	Prothrombin	153
Homo sapiens	Serum albumin	502
Homo sapiens	Signal peptide, CUB and EGF-like domain-containing protein 3	69
Homo sapiens	SPARC	51
Homo sapiens	Tetranectin	58
Homo sapiens	Thrombospondin-1	198
Homo sapiens	Transforming growth factor beta-1	60
Homo sapiens	Vitamin K-dependent protein S	82
Homo sapiens	Vitamin K-dependent protein Z	64
Homo sapiens	Vitronectin	114
Sus scrofa	Trypsin	239
Anabaena variabilis	Bifunctional protein ThiO/ThiG	45

Soil

Soil 1 FDR 4.65%; Normalised protein yield too low

Homo sapiens	Keratin, type I cytoskeletal 10	252
Sus scrofa	Trypsin	110
Homo sapiens	Keratin, type II cytoskeletal 1	85
Homo sapiens	Keratin, type I cytoskeletal 9	71
Homo sapiens	Dermcidin	51
Homo sapiens	Collagen alpha-1(I) chain	46

Soil 2 FDR 3.45%; Normalised protein yield too low

Homo sapiens	Keratin, type I cytoskeletal 10	173
Sus scrofa	Trypsin	81
Homo sapiens	Keratin, type II cytoskeletal 2 epidermal	73
Bos taurus	Cationic trypsin	69
Homo sapiens	Collagen alpha-1(I) chain	63
Homo sapiens	Keratin, type I cytoskeletal 9	62
Homo sapiens	Keratin, type II cytoskeletal 1	51
Pectobacterium carotovorum	chaperone protein DnaK	46

Negative controls

Negative 1 (Calculus extraction) FDR 25%

Homo sapiens	Alpha-1-antichymotrypsin*	109

Negative 2 (Calculus extraction) FDR N/A

Sus scrofa	Trypsin	99

Negative (Dentine extraction) FDR 1.96%

Homo sapiens	Keratin, type I cytoskeletal 10	213
Homo sapiens	Keratin, type I cytoskeletal 9	124

Homo sapiens	Keratin, type II cytoskeletal 1	84
Homo sapiens	Dermcidin	42
Homo sapiens	Collagen alpha-2(I) chain	60
Homo sapiens	Keratin, type II cuticular Hb5	54
Homo sapiens	Collagen alpha-1(I) chain	52
Ovis aries	Keratin, type I microfibrillar 48 kDa, component 8C-1	44
Sus scrofa	Trypsin	113
Saccharomycetaceae	V-type proton ATPase catalytic subunit A	35

*Protein was retained in analysis of the dental calculus proteome owing to the likelihood that alpha-1-antichymotrypsin is endogenous in this proteome, given its observation in the oral proteome (Sun et al. 2009; Bostanci et al. 2010) and having been previously observed in ancient dental calculus (Warinner, Rodrigues, et al. 2014).

Table A1.2. Osteological information for analysed individuals, from Pearson et al. (2011) and Witkin (2011).

Age	Sex	Stature (cm)	All pathologies	Calculus severity*	Dental Modification	Skeletal Preservation
Individual **430** (group 2458)						
30 - 35	Possible Male	163	Caries, PD, antemortem tooth loss, DJD, SDJD, osteochondritis dissecans, cranial porosity, bowing of femora, NSI	4	None	75-100%
Individual **218** (group 2050)						
25 - 28	Possible Male	152	Ankylosis of DIP joint, lesion of intemedial and distal phalange, spina bifidia, cranial porosity, inflammatory porosity	2-3	Type 2 Simple	75-100%
Individual **474** (group 2524)						
35 - 40	Possible Male	Unknown	PD, DJD, SDJD, maxillary sinusitis, possible anaemia	2	None	75-100%
Individual **414** (group 2431)						
28 - 32	Male	164	Abscesses, fused carpels, cranial lesions	4	None	75-100%
Individual **421** (group 2444)						
8 - 12	Unknown	Unknown	Enamel hypoplasia	3-4	None	75-100%
Individual **212** (group 2025)						
10 - 12	Unknown	Unknown	EH, periosteal reaction on medial tibiae	4	None	25-29%
Individual **343** (group 2282)						
35 - 40	Male	158	PD, EH, NSI, C7 cervical rib, inflammatory porosity on cranium	1-2	Type 1 Simple	75-100%
Individual **419** (group 2443)						
22 - 25	Female	147	EH, cranial porosity, lesion on palatine process, possible oral disease	3-4	None	75-100%

Individual **359** (group 2320)

24 - 26	Male	166	Abscesses, L4 compression trauma, SDJD, type 2 cribra orbitalia, supernumerary mandibular premolars, tooth 15 90° rotated	1-2	Type 1 Simple	75-100%

Individual **237** (group 2076)

25 - 29	Male	172	Caries, abscesses, healed type 3 CO (left orbit), type 2 CO (right orbit), cranial porosity, healed lamellar bone left femur, left tibia damage, enthesophyte on right tibia, non-union of neural arches on L1, healed rickets	4	Type 1 Simple	75-100%

Individual **319** (group 2245)

12 - 14	Unknown	Unknown	None observed	3-4	Type 1 Simple	75-100%

Individual **276** (group 2157)

23 - 25	Male	150	PD, abscesses, SDJD, DJD, CO type 4	2-3	Type 1 Simple	75-100%

Individual **306** (group 2082)

27 - 31	Male	168	PD, DJD, SDJD, ankylosis, possible trauma of T4 neural arch, CO type 4	3-4	None	75-100%

Individual **389** (group 2047)

22 - 24	Male	166	SDJD, DJD, lesions on proximal tibia condyles, scurvy, NSI on tibiae and fibulae, ectocranial porosity	1-2	Type 1 Simple	75-100%

Individual **268** (group 2145)

11 - 13	Unknown	Unknown	None observed	2-3	None	50-75%

Individual **219** (group 2041)

26 - 35	Male	Unknown	Abscesses, DJD, SDJD, CO	2	Type 1 Simple	75-100%

Individual **423** (group 2064)

35 - 39	Male	Unknown	EH, DJD, pseudofracture on left inferior pelvic ramus, osteomalacia, possible rickets, NSI on right tibia,	2-3	Type 6 Complex	75-100%

			osteophytes on phalanx, possible amputation and subsequent infection of distal phalanx			
Individual **459** (group 2509)						
30 - 35	Male	168	Abscesses, rickets, scurvy, porotic hyperostosis, NSI	3	Type 2 Complex	75-100%
Individual **449** (group 2491)						
25 - 32	Male	165	SDJD, possible scurvy, NSI on tibiae, non-union of 1st thoracic vertebrae, cranial porosity	2-3	None	75-100%
Individual **358** (group 2320)						
30 - 39	Male	162	Ankylosis of pedal phalanges, SDJD, DJD, healed NSI, cranial porosity	1-2	Type 1 Simple	75-100%

*graded using Brothwell and Dobney et al. (1987)

Abbreviations:

SDJD: Spinal Degenerative Joint Disease
DJD: Degenerative Joint Disease
PD: Periodontal Disease
EH: Enamel Hypoplasia
NSI: Non-specific Infection
CO: Cribra Orbitalia

Appendix 2: DNA Methods

Although proteomic analysis was the main focus of this PhD ancient DNA analysis was also performed following the success observed by Adler et al. (2013) and Warinner, Rodrigues, et al. (2014). This appendix highlights the work performed for ancient DNA analysis of samples of dental calculus from St Helena, outlining experiments with different extraction methods and preparation of libraries for 16S sequencing. We now realize that a 16S-based approach may be taxonomically biased owing to the degradation of 16S rRNA (Appendix 3). This section has been placed as an appendix as not to detract from the focus on proteomics as part of this PhD and because it is an outline of work completed rather than the presentation of a discrete study. These experiments were performed in conjunction with Camilla Speller, Sarah Fiddyment and Christina Warinner.

Introduction

Analysis of ancient DNA derived from dental calculus has enormous potential for understanding ancient oral microbiome, identifying oral and respiratory bacteria, as well as dietary evidence sourced directly from the oral environment (Adler et al. 2013; Warinner, Rodrigues, et al. 2014; Warinner, Speller, and Collins 2015; Weyrich, Dobney, and Cooper 2015). It represents a unique archaeological resource, both in terms of its documented high DNA yield and, like other ancient microbiomes (Tito et al. 2008; Tito et al. 2012; Warinner et al. 2015), its complexity - containing DNA of bacterial, human and dietary origin. Optimisation of ancient DNA extraction and analysis is necessarily to fully access the wide potential of ancient dental calculus.

Ancient DNA extractions have typically focussed on either a phenol-chloroform based extraction (Hagelberg and Clegg 1991; Leonard, Wayne, and Cooper 2000) or the use of silica particles which immobilise DNA molecules (Höss and Pääbo 1993; Rohland and Hofreiter 2007; Rohland, Siedel, and Hofreiter 2010). Isopropanol (Hänni et al. 1995) and ethanol (Kalmár et al. 2000) based precipitation were also explored. Yang et al. (1998) explored the use of Centricon and QIAquick columns following digestion in EDTA and pK.

In terms of dental calculus analysis, Adler et al. (2013) extracted DNA by initial digestion in lysis buffer (EDTA, SDS and proteinase K), followed by isolated using QIAamp DNA Investigator kit (Qiagen). Warinner, Rodrigues, et al. (2014) compared five different extraction methods, finding that a phenol-chloroform based extraction with an initial digestion in EDTA and pK resulted in the highest DNA yields, ranging from 5.0 - 437.2 ng of DNA per mg of calculus.

This appendix explores a number of methodological approaches and troubleshooting experiments to optimise DNA extraction and analysis from dental calculus from St Helena. Three extraction methods from ancient dental calculus from St Helena were performed in order to compare the performance of each method. In addition, preliminary experiments were conducted in order to understand a) the extent of endogenous DNA loss in silica and Amicon-based binding methods, and b) whether these methods influence extracted DNA fragment lengths.

Excavation and Handling of Human Remains

Human remains were excavated from Rupert's Valley on St Helena 2008 (Pearson et al. 2011). As documented in Witkin (2011), during the excavation remains were dry-brushed and

packaged in polyester wadding for transport. Each individual was packaged in heavy corrugated cardboard boxes with polyester wadding, and smaller elements placed in ventilated zip-lock bags. The storage environment was matched (as feasibly as possible) to the site conditions. Nitrile gloves were worn at all times when handling human remains. Calculus samples were removed from teeth using a dental pick and stored in Eppendorf tubes until extraction was performed.

Comparison of Extraction Methods

We performed two different methods in order to explore their performance with ancient dental calculus. Silica extractions based on Rohland and Hofrieter (2007) were performed at the ancient DNA facilities at the University of York. Phenol-chloroform based extractions based on Warinner, Rodrigues, et al. (2014) were performed at facilities based at Oklahoma University.

Decontamination Procedures

In both the University of York and Oklahoma University, the ancient DNA work area was physically separate from other laboratories to avoid the contamination of extracted ancient DNA with PCR products, and movement between these laboratories is restricted. Researchers wore Tyvex suits, masks, hairnets and gloves when handling ancient samples and performing extractions. Contamination from the burial matrix was minimised by removing the outer layer of the sample with either an EDTA wash or under UV. Negative extraction blanks were performed alongside every batch sample extractions, and negative controls were used and monitored during all amplification steps. Amplifications were performed in triplicate, producing consistent results, before being pooled and purified before sequencing.

Silica-Based Extraction

21 calculus samples were extracted by a silica-based extraction method based on Rohland and Hofreiter (2007). Briefly, this comprises a) decalcification and digestion in EDTA and proteinase K buffer, b) purification through the binding of DNA to silica in the presence of guanidinium thiocyanate, and c) elution of the silica-bound DNA through columns. A detailed extraction procedure is outlined in Box 1.

1. Sample preparation and decontamination

Samples of dental calculus (between 20 and 50mg) were cleaned by vortexing in HPLC water. The sample was then placed under UV for 10 minutes on each side in order to remove surface contamination. Micropestles were used to grind calculus samples to a powder.

2. Decalcification and Digestion

An extraction buffer was prepared with pK (2.0 mg/ml) and EDTA (5 M). The prepared buffer (2 mL) was added to each sample and incubated at 50 °C overnight under rotation.

3. Silica-binding

A binding buffer was prepared from guanidinium thiocyanate, sodium acetate and acetic acid. Samples were centrifuged and supernatant transferred to fresh 15 mL tubes containing 1 mL of binding buffer. Silica (100 μL) was added and agitated in the dark at room temperature for 3 hours. Columns were prepared using MobiCols with a 10uM filter. A washing buffer comprised of NaCl (5 M), Tris (1 M), EDTA (0.45 M), ethanol and water. Samples were spun and supernatant discarded. Binding buffer (400 μL) was added and the silica pellet resuspended and transferred to the column.

4. Column extraction

Washing buffer was added to the columns (two washes of 450 μL) for purification. In a fresh collect collection tube 50 μL of TE was added, distributed evenly over the silica, and spun at maximum speed to elute the DNA from the silica.

Box 1. Silica extraction method based on Rohland and Hofreiter (2007).

Phenol-Chloroform Extraction

Ancient DNA was extracted from 15 samples of dental calculus using a phenol chloroform method described by Warinner, Rodrigues, et al. (2014) in a dedicated ancient DNA laboratory at the Stephenson Research and Technology Centre at the University of Oklahoma. Briefly, this comprises a) decalcification and digestion in EDTA and proteinase K, b) extraction with phenol-chloroform to remove other organic molecules, and c) purification using Qiagen MinElute columns. A detailed extraction procedure is outlined in Box 2.

1. Sample preparation and decontamination

Samples of dental calculus (between 20 and 25 mg) were placed in EDTA (1 mL, 0.5 M) a vortexed for 5 minutes to remove surface contaminates. The samples were centrifuged and the supernatant removed and placed in a fresh Eppendorf tube.

2. Decalcification and Digestion

1 mL of EDTA (0.5 M) was added to the calculus pellet and placed placed on rotating nutator. After 3 hours proteinase K (50 μL) was added and placed back on the nutator overnight. A second batch of Proteinase K (50 μL) was added, placed at 55 °C for 7 hours, then rotated overnight.

3. Phenol-chloroform extraction

Samples were centrifuged and the supernatant transferred to fresh 2mL tubes. 750 μL of phenol:chloroform:isoamyl alcohol was added to each sample, mixed gently for 1 minute, and centrifuged. The aqueous phase was removed transferred to a fresh 2 mL tube and a second batch of phenol chloroform:isoamyl alcohol was added to each sample and the same process repeat. The aqueous phase was then transferred to a fresh 2mL tube and chloroform:isoamyl alcohol was added, mixed gently and centrifuged. The aqueous phase was transferred to a 15 mL tube and 13 mL of PB buffer was added and mixed.

4. Purification

Extract/PB solution was transferred to Zymo columns with an attached MinElute column and spun for 6 minutes. The MinElute column ws removed from the Zymo column, centrifuged and the flow through discarded. The column was washed twice with PE buffer (two washes of 750 μL) and the flow through discarded. After a dry spin, the column was placed in a fresh collection tube and EB buffer (30 μL) was added to the column and incubated for 5 minutes. The column was centrifuged at maximum speed and a second 30uL of EB was added, then spun a maximum speed to elute.

Box 2. Phenol-Chloroform extraction based on Warinner, Rodrigues, et al. (2014).

Comparison of Extraction Methods and Observations

All DNA yields were measured on a Qubit fluorometer high-sensitivity assay. Extractions using the silica-based approach yielded DNA in the range 0.11 to 10.66 ng/mg (average yield 2.69 ng/mg), and extractions from the phenol-chloroform approach yielded from less than 0.454 to 3.211ng/mg (average yield 1.73 ng/mg) (Table A2.1, Fig. A2.1). There appears to be no correlation between DNA yields of samples extracted using both methods. Although the silica-based method produced, on average, greater yields of DNA, there were major inhibition problems when it came to library preparation. Although the phenol chloroform method produced lower DNA yields on average were no problems with amplification inhibition. In comparison to results observed by Warinner, Rodrigues, et al. (2014), the yield of DNA from dental calculus from St Helena was substantially lower (DNA yields from in this study ranged from 5.0 to 437.2 ng/mg).

Sample	Silica-based extraction yield (ng/mg)	Chloroform-based extraction yield (ng/mg)
203	2.00	3.211
417	1.56	-
430	1.40	0.651

218	0.72	0.609
474	1.95	2.138
414	0.11	-
421	3.99	-
212	0.77	-
343	0.64	1.815
419	0.61	2.497
359	3.37	1.393
237	0.68	-
319	1.94	too low
276	4.38	0.865
306	5.59	-
389	2.88	1.879
268	3.62	0.454
219	10.66	1.683
423	5.85	3.061
459	2.71	-
449	1.23	1.655
358	-	2.354

Table A2.1. Comparison of normalised DNA yields (ng of DNA extracted per mg of dental calculus used) for silica and phenol-chloroform based extraction methods.

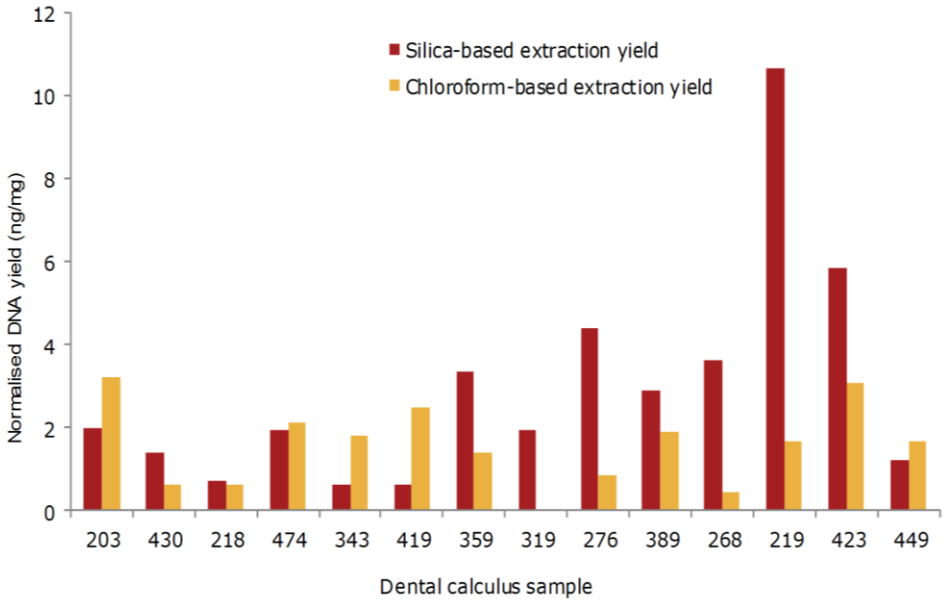

Figure A2.1. Comparison of normalised DNA yields for silica and phenol-chloroform based extraction methods.

Comparison of Silica and Amicon-Based Extractions

An additional experiment was carried out to directly compare silica and Amicon-based Yang et al. (1998) extraction methods. Four samples of dental calculus were digested under the same decontamination and digestion procedures as Box 1. The supernatant from these digestions was then divided between 15 mL tubes containing silica-binding buffer (for extraction in silica columns) and 10 K Amicon filters (Amicon extraction based on Yang et al. (1998) is detailed in Box 3 below). This revealed that of the four samples used in this experiment an Amicon-based extraction had higher DNA yields than a silica-based method (Table A2.2). Like, the phenol-chloroform/silica comparison, there appears to be no correlation between DNA yields of samples extracted using both methods.

1. Sample preparation and decontamination

Samples of dental calculus (between 20 and 25 mg) were rinsed in HPLC water and placed under UV for 20 minutes on each side in order to remove surface contamination. Micropestles were used to grind calculus samples to a powder.

2. Decalcification and Digestion

An extraction buffer was prepared with pK (2.0 mg/ml) and EDTA (5 M). The prepared buffer (2 mL) was added to each sample and incubated at 50 °C overnight under rotation

3. Amicon-based extraction

Samples were centrifuged and the supernatant transferred to 10 K Amicon filters. These were spun at 4400 RPM until less than 100 µL of sample remained in the filter. The DNA should be retained in the filter.

4. Purification

Samples were purified using QIAquick Nucleotide Removal Kits. PB (binding) buffer (500 µL) was resuspended with the sample solution retained in the filter membrane, then all the solution transferred to the column, spun and flow through discarded. PE (washing) buffer was added (two increments, 500 µL then 300 µL) and the flow-through discarded. EB buffer (55 µL) was added to the column, incubated at 65 °C for 1 minute and spun at maximum speed to elute.

Box 3. Extraction method based on Yang et al. (1998).

Individual	*Silica-based (ng/mg)*	*Amicon-based (ng/mg)*
8735	0.298	1.598
8759	0.503	1.670
8808	0.935	4.242
8810	0.671	6.003

Table A2.2. Comparison of normalised DNA yields (ng of DNA extracted per mg of dental calculus used) for silica and Amicon-based extraction methods.

Analysis of Fragment Length

A preliminary experiment was carried in order to explore how the distribution of DNA fragment lengths is affected by different extraction methods. For this experiment we "extracted" a commercially available DNA ladder (O'GeneRuler Low Range DNA Ladder, Ready-to-Use 25-700 bp) using silica-based (Rohland and Hofreiter 2007) and Amicon-based (Yang et al. 1998) extractions methods. In addition, we also included an extraction method based on Dabney et al. (2013), a modified silica-method. For comparison, we also purified the ladder using MinElute columns spun at high speed (13k RPM). Experimental work-flow and results are in Figure A2.2.

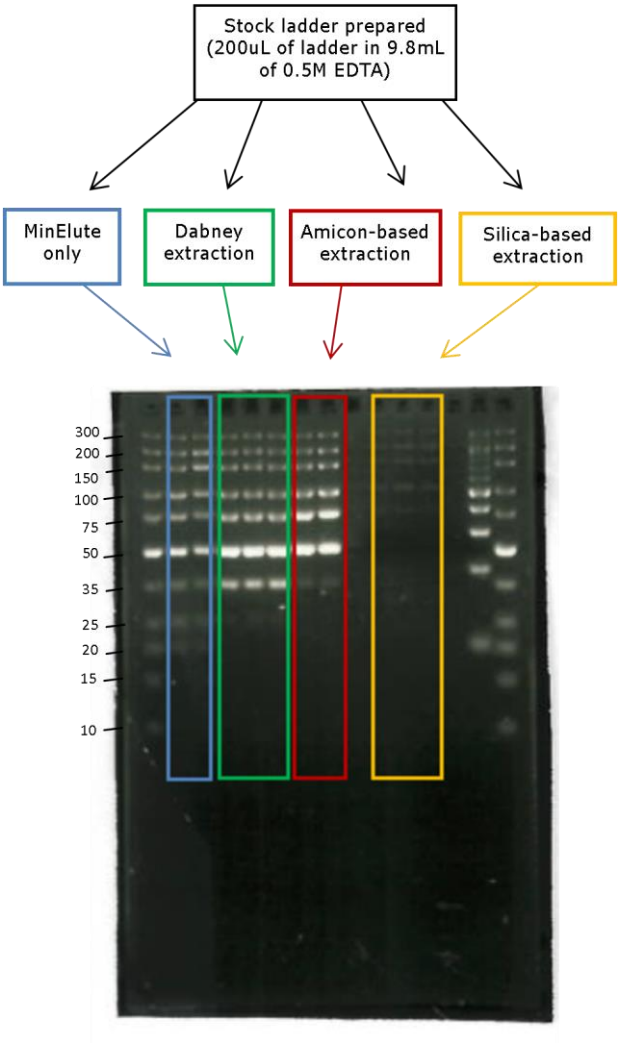

Figure A2.2. Comparison of DNA fragment lengths by different extraction methods.

The Dabney method appears to have extracted fragments down to 20 bp, while the amicon-based method approach appears to have extracted fragments as small as 30 bp with the distributed fragment lengths being longer compared to the Dabney extraction (Fig. A2.2). MinElute spun at full speed, 13k RPM, appear have preserved fragments as short as 15 bp. Thus suggests that a Dabney based extraction may be more capable at extracting shorter DNA fragments. This also suggests that spinning columns at full speed does not dramatically affect the fragment length distribution.

Preparation of 16S Libraries

DNA extracts were prepared for 16S sequencing. Only libraries built from phenol-chloroform extracts were sent for 16S sequencing. The 16S rRNA hypervariable region of the mitochondrial genome was targeted using V3 primers, as performed by both Adler et al. (2013) and Warinner, Rodrigues, et al. (2014). The V3 region was targeted because V5 and V6 primer bias was previously observed by Warinner, Rodrigues, et al. (2014). Library preparation with V4 primers was also tested, but no libraries were successfully amplified with this primer.

Samples extracted using the silica-approach required substantial optimisation owing to possible problems with PCR inhibition. It was found that the most successful amplifications occurred with a dilution of 1:20 creating a concentration of less than 0.05 ng/uL. No libraries built using the Dabney extraction successfully amplified. Successful amplification of 16S libraries occurred with the following library preparation. Each PCR reaction contained 12.75 µl molecular grade water, 5 µl 5x Phusion buffer, 0.25 µL 10 mg/ml BSA, 0.25 µL 25 mM decontaminated dNTPs, 1.25 µl 10 µM primer V3-F, 1.0 µl 10 µM primer V3-R, 0.25 µl Phusion Taq polymerase (0.05 U/µl) and 4.0 µl of DNA template for a total volume of 25 µl. The temperature profile for the reactions included an was activation of the enzyme at 98 °C for 30 seconds, followed by 35 cycles of 98 °C for 15 seconds, 65°C for 20 seconds, 72 °C for 20 seconds, followed by a final 5-minute extension at 72°C**.** Three samples were able to be amplified (268, 423, 430).

Preparation of libraries derived from the phenol-chloroform extracts required some optimisation owing to low DNA yields and the presence of primer-dimers. It was determined that the best results for library amplification were when to up 10 ng/µL of DNA extract was used and primer concentration reduced to a volume of 0.5 µL per reaction. Each PCR reaction

contained 9.25 µl molecular grade water, 5 µl 5x Phusion buffer, 1 µL 2.5 mg/ml BSA, 2.5 µL 10 mM decontaminated dNTPs, 0.5 µl 10 µM forward primer, 1.0 µl 10 µM reverse primer, 0.25 µl Phusion Hot Start II DNA polymerase (2 U/µl) and 1.0 µl of DNA template (5 ng/µl) for a total volume of 20 µl. The temperature profile for the reactions was an initial activation of the enzyme at 98 °C for 30 seconds, followed by 35 cycles of 98°C for 15 seconds, 52 °C for 20 seconds, 72 °C for 20 seconds, followed by a final 5-minute extension at 72 °C**.** Sequencing took place using Illumina MiSeq v2 2x150bp chemistry at the Yale Center for Genome Analysis. Results of this sequencing are discussed in Appendix 3.

Appendix 3: Intrinsic Challenges in Ancient Microbiome Reconstruction using 16S rRNA Gene Amplification

This appendix is included because the extraction and preparation of DNA from St Helena represented significant lab-work completed for this PhD. A form is this paper is published in Scientific Reports (*Scientific Reports* 5, Article number: 16498, doi:10.1038/srep16498), although with changes to keep the referencing style (Chicago, as opposed to Nature) and language (i.e. UK English, as opposed to US English) and formatting in keeping with the thesis as a whole. Supplementary data and associated references are placed at the end of the main text.

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Abstract

To date, characterisation of ancient oral (dental calculus) and gut (coprolite) microbiota has been primarily accomplished through a phylotyping approach involving targeted amplification of one or more variable regions in the 16S rRNA gene. Specifically, the V3 region (*E. coli* 341-534) of this gene has been suggested as an excellent candidate for ancient DNA amplification and microbial community reconstruction. However, in practice this phylotyping approach often produces highly skewed taxonomic frequency data. In this study, we use non-targeted (shotgun metagenomics) sequencing methods to better understand skewed microbial profiles observed in four ancient dental calculus specimens previously analysed by amplicon sequencing. Through comparisons of microbial taxonomic counts from paired amplicon (V3 U341F/534R) and shotgun sequencing datasets, we demonstrate that extensive length polymorphisms in the V3 region are a consistent and major cause of differential amplification leading to taxonomic bias in ancient microbiome reconstructions based on amplicon sequencing. We conclude that systematic amplification bias confounds attempts to accurately reconstruct microbiome taxonomic profiles from 16S rRNA V3 amplicon data generated using universal primers. Because *in silico* analysis indicates that alternative 16S rRNA hypervariable regions will present similar challenges, we advocate for the use of a shotgun metagenomics approach in ancient microbiome reconstructions.

Introduction

The human body harbors an astounding number of microorganisms, collectively known as the *human microbiome* (Lederberg, Keener, and Taylor 2015). The number of these microorganisms (approx. 100 trillion) is estimated to exceed that of the human cells in our bodies (approx.10 trillion) by an order of magnitude (Peterson et al. 2009), and the number of microbial genes in our microbiome (approx. 3,300,000) has been shown to outnumber our own (approx. 22,000) by a factor of more than 150 (Qin et al. 2010). These microbial genes encode a wide range of biological functions including those related to host nutrient acquisition, metabolism and immunity (Hooper, Littman, and Macpherson 2012; LeBlanc et al. 2013; Lee and Mazmanian 2010; Lozupone et al. 2012; Tremaroli and Bäckhed 2012). Moreover, recent comparative studies with non-human primates suggest rapid changes in human microbiota occurred during human evolution (Moeller et al. 2014). Consequently, there is tremendous interest in characterising the evolutionary ecology of the human microbiome through the direct investigation and comparative analysis of both modern and ancient forms (Warinner, Speller, and Collins 2015; Warinner et al. 2015; Weyrich, Dobney, and Cooper 2015).

Recent studies of microbiome variation in humans (Claesson et al. 2012; Obregon-Tito et al. 2015; Ou et al. 2013; Schnorr et al. 2014; Yatsunenko et al. 2012) and non-human primates (Moeller et al. 2014; J. Li et al. 2013; Ochman et al. 2010; Yildirim et al. 2014; Yildirim et al. 2010) have primarily characterised host-associated microorganisms using an amplicon phylotyping approach, in which a targeted variable region of the 16S rRNA gene is amplified by polymerase chain reaction (PCR), deep sequenced using Next Generation Sequencing (NGS) technology, and compared to a reference database of 16S rRNA gene sequences. The 16S rRNA gene encodes the small subunit of prokaryotic ribosomal RNA and contains nine hypervariable regions (V1-V9) separated by ten highly conserved regions (Cox, Cookson, and

Moffatt 2013). It is the most widely used gene in microbial phylotyping (phenetic classification), in part because the 16S rRNA gene is sufficiently conserved across members of the paraphyletic prokaryotic domains Bacteria and Archaea to allow the design of "universal" primers for microbial PCR amplification, yet also sufficiently variable to allow full 16S rRNA sequences to be classified at an approximate species level. The 16S rRNA gene is among the most studied and best characterised genes among prokaryotes, and more than 100,000 full 16S rRNA sequences are available for microbial taxa in publicly accessible databases such as RDP (Wang et al. 2007), SILVA (Quast et al. 2013), and Greengenes (DeSantis et al. 2006).

While it is possible to amplify and sequence the entire 16S rRNA gene (approx. 1540 bp) using conventional cloning and Sanger sequencing, this approach is impractical for high-throughput studies of microbial communities. Alternatively, a shorter target comprising one or more hypervariable regions may be amplified and sequenced on an NGS platform, allowing for high coverage characterisation of hundreds of samples simultaneously. For example, the Earth Microbiome Project (Gilbert, Jansson, and Knight 2014) has developed a standardised and widely used NGS assay for microbiome characterisation that targets the V4 region of the 16S rRNA gene using the universal primers 515F/806R (Caporaso et al. 2012). However, the relatively long length of the V4 region (approx. 292 bp, primer inclusive) makes it impractical for ancient DNA (aDNA) studies given that aDNA is known to be highly fragmented and rarely exceeds 200 bp in length (Sawyer et al. 2012).

Alternatively, ancient microbial studies have targeted other hypervariable regions in the 16S rRNA gene, including V1 (Adler et al. 2013; Luciani et al. 2006; Rollo et al. 2007; Ubaldi et al. 1998), V1-V2 (Rollo et al. 2007; Cano et al. 2000), V3 (Adler et al. 2013; Rollo et al. 2007; Ubaldi et al. 1998; Tito et al. 2012; Warinner, Rodrigues, et al. 2014), V1-V3 (Rollo et al. 2007), V5 (Warinner, Rodrigues, et al. 2014), and V6 (Adler et al. 2013; Warinner, Rodrigues,

et al. 2014). Following both *in silico* and *in vitro* testing of multiple 16S rRNA gene variable region primer pairs, two studies concluded that the V3 region was an optimal target for ancient microbial studies (Adler et al. 2013; Warinner, Rodrigues, et al. 2014) because: (1) it is relatively short (approximately 100 bp shorter than the V4 region); (2) it exhibits high sequence variation, resulting in good taxonomic discrimination; and (3) it is less subject to primer taxonomic bias than other primer pairs when compared to data generated using non-targeted (shotgun) whole metagenome sequencing.

Despite these characteristics, we have recently observed that for many ancient microbiome samples, the microbial community profiles reconstructed from targeted sequencing of the 16S rRNA gene V3 region do not conform to biological expectations and show systematic taxonomic biases, such as exceptionally high frequencies of the human-associated archaeon *Methanobrevibacter*, that cannot be explained by exogenous contamination. Although 16S rRNA amplicon sequencing has been the primary means of characterising ancient microbiome samples since 1998 (Adler et al. 2013; Luciani et al. 2006; Rollo et al. 2007; Ubaldi et al. 1998; Cano et al. 2000; Tito et al. 2012; Warinner, Rodrigues, et al. 2014; Rollo et al. 2000), no study has yet systematically investigated the effect of aDNA fragmentation on the fidelity of amplicon-based ancient microbiome reconstructions. Because of this, it is unclear how to interpret reported differences observed between modern and ancient microbial communities (Adler et al. 2013).

To address this problem, we conducted a series of *in silico* and *in vitro* experiments to explore the role of inherent structural variation in the 16S rRNA gene on downstream microbiome reconstruction from archaeological samples. First, we present and describe microbiome profiles generated by targeted amplicon sequencing of the V3 region for a large collection of archaeological dental calculus samples (n=107). Then, we used non-targeted paired-end

shotgun metagenome sequencing to empirically determine the length distribution of aDNA fragments in a subset of four samples from diverse geographic and temporal contexts. Next, using sequences in the SILVA SSU 111 database, we investigated the V3 region of the 16S rRNA gene for primer bias, amplicon length, and variation in amplicon length, and compared microbiome profiles between amplicon and shotgun metagenome datasets for the four archaeological dental calculus samples. We then estimated the probability of a nucleotide being damaged (λ) assuming a random degradation model and simulated the effect of this damage on taxonomic profiles generated from a hypothetical oral microbiome at different thermal ages. We show that, assuming random degradation, longer targets will be underrepresented in thermally older samples, leading to a perceived shift in V3 target composition. We conclude that extensive length polymorphisms in the V3 region are a major cause of amplification dropout and taxonomic bias in ancient microbiome reconstructions generated by amplicon sequencing. Overamplification of archaeal taxa and altered microbial diversity estimates are predictable artifacts observed in poorly preserved (highly fragmented) but relatively uncontaminated aDNA samples. Finally, we analysed 16S rRNA gene sequences in taxonomically diverse microorganisms in the SILVA SSU 111 16S rRNA database and evaluated the other hypervariable regions on a variety of quality metrics, including predicted primer amplification bias, median amplicon length, and variation in amplicon length. We demonstrate that although amplicon-based 16S rRNA gene sequencing may be a useful high-throughput screening tool for qualitative characterisation of the preservation and contamination burden of ancient microbiome samples, it cannot be used to reliably reconstruct quantitative information regarding microbial diversity or taxonomic frequency in ancient microbial communities.

Results

Taxonomic analysis of 16S rRNA V3 amplicon sequence data generated from dental calculus

To investigate taxonomic profiles generated by amplicon sequencing in temporally and geographically diverse archaeological dental calculus specimens, we selected and analysed samples (n=107) from five sites: Middenbeemster, the Netherlands (n=76); Rupert's Valley, St. Helena (n=15); Anse à la Gourde, Guadeloupe (Caribbean) (n=5); Lavoutte, St. Lucia (Caribbean) (n=5); Tickhill, Yorkshire, UK (n=4); Samdzong, Nepal (n=1); and Camino del Molino, Spain (n=1) (Table 1; Supplementary Table 1). A larger number of samples were profiled from the Dutch Middenbeemster cemetery in order to examine oral microbiome variation within a single site. Following deep sequencing of 16S rRNA gene V3 amplicons on an Illumina MiSeq platform, we assigned sequences to Operational Taxonomic Units (OTUs) at 97% similarity using the closed-reference OTU protocol implemented in QIIME (Caporaso et al. 2010; Kuczynski et al. 2011), and the Greengenes 13.8 database (DeSantis et al. 2006) as a reference (see Methods). We then analysed the resulting taxonomic data using three complementary approaches (Fig 1): (a) by scoring the assigned genera to the categories of "Oral" or "Other", as inferred by the presence or absence of each genus in the Human Oral Microbiome Database (HOMD)(Chen et al. 2010) and tabulating the resulting frequency data; (b) by proportionally assigning the sample data to human microbiome and environmental sources using the Bayesian microbial source tracking program SourceTracker (Knights et al. 2011); and (c) by directly examining the phylum frequency data. A modern dental calculus sample (analysed in duplicate), two laboratory controls (osteologist's hand swab and osteology lab bench swab), and three extraction blanks (non-template negative extraction controls) were also analysed in parallel and are provided for comparison. Median sequencing depths for the dental calculus samples were high: modern (96,157), Middenbeemster (85,478), Tickhill

(68,170), St. Helena (10,941), Samdzong (68,253), Camino del Molino (90,416), Anse à la Gourde (41,271), and Lavoutte (38,229). Lower sequencing depths were achieved for the laboratory controls, in part because of their lower starting biomass: osteologist's hand swabs (20,368 read pairs), osteology lab bench tops (4,617 read pairs). No amplification was observed for the extraction or PCR blanks, but they were nevertheless prepared into libraries. Accordingly, sequencing depth for the three blanks was very low: 17, 190, and 316 read pairs.

Geographic origin	Time Period	Thermal Age λ[a]	Amplicon sequencing (N)[b]	Shotgun sequencing (N)	Shotgun sample ID[c]
Rupert's Valley, St. Helena	1840-1872 CE[f]	0.0030	15	-	-
Middenbeemster, Netherlands	1611-1866 CE[d]	0.0006	76	1	454C[e]
Tickhill, Yorkshire, UK	ca. 1450-1600 CE[i]	0.0021	4	-	-
Anse à la Gourde, Guadeloupe	ca. 975-1395 CE[g]	0.0701	5	1	F1948C
Lavoutte, St. Lucia	ca. 990-1255 CE[h]	0.0863	5	-	-
Samdzong, Nepal	ca. 400-650 CE[j]	0.0045	1	1	37C[k]
Camino del Molino, Spain	ca. 2340-2920 BCE[l]	0.0149	1	1	214C[m]

Table A3.1. Archaeological dental calculus samples analysed in this study. aMean thermal age λ calculated using the JRA 1: PrediCtoR tool hosted on www.thermal-age.eu (Smith et al. 2003). bSee Supplementary Table 1 for full list of analysed samples. cThe suffix "C" was added to the sample name to denote that analysis was performed on calculus. dThe majority of burials date to CE 1829-1866. Cemetery dates were retrieved from Middenbeemster municipal records (Waters-Rist and Hoogland 2013). Sample S454C is from an individual with a recorded death date of CE 1856. eSample ID abbreviated from S454V0963. See Supplementary Table 1. fCemetery dates are based on historical records (Pearson et al. 2011). gBurial dating to the Caribbean Late Ceramic Age (Hoogland, Romon, and Brasselet 2001; Mickleburgh and Others 2013). See Supplementary Table 1 for radiocarbon dates. hBurials

dating to the Caribbean Late Ceramic Age (Hofman et al. 2012). See Supplementary Table 1 for radiocarbon dates. iCemetery dates of death (CE 1412-1532) were retrieved from St. Mary's Church, Tickhill. jShaft tomb burial dating to the Samdzong period (M Aldenderfer and Eng, n.d.; M Aldenderfer 2010; Mark Aldenderfer 2013). See Supplementary Table 1 for radiocarbon dates. kSample name has been abbreviated from 37.UM2010.9. See Supplementary Table 1. lBurial dating to the Chalcolithic period. Time period is inferred from radiocarbon dated associated skeletal material (Lomba-Maurandi et al. 2009). See Supplementary Table 1 for radiocarbon dates. mSample ID abbreviated from CMOL214.

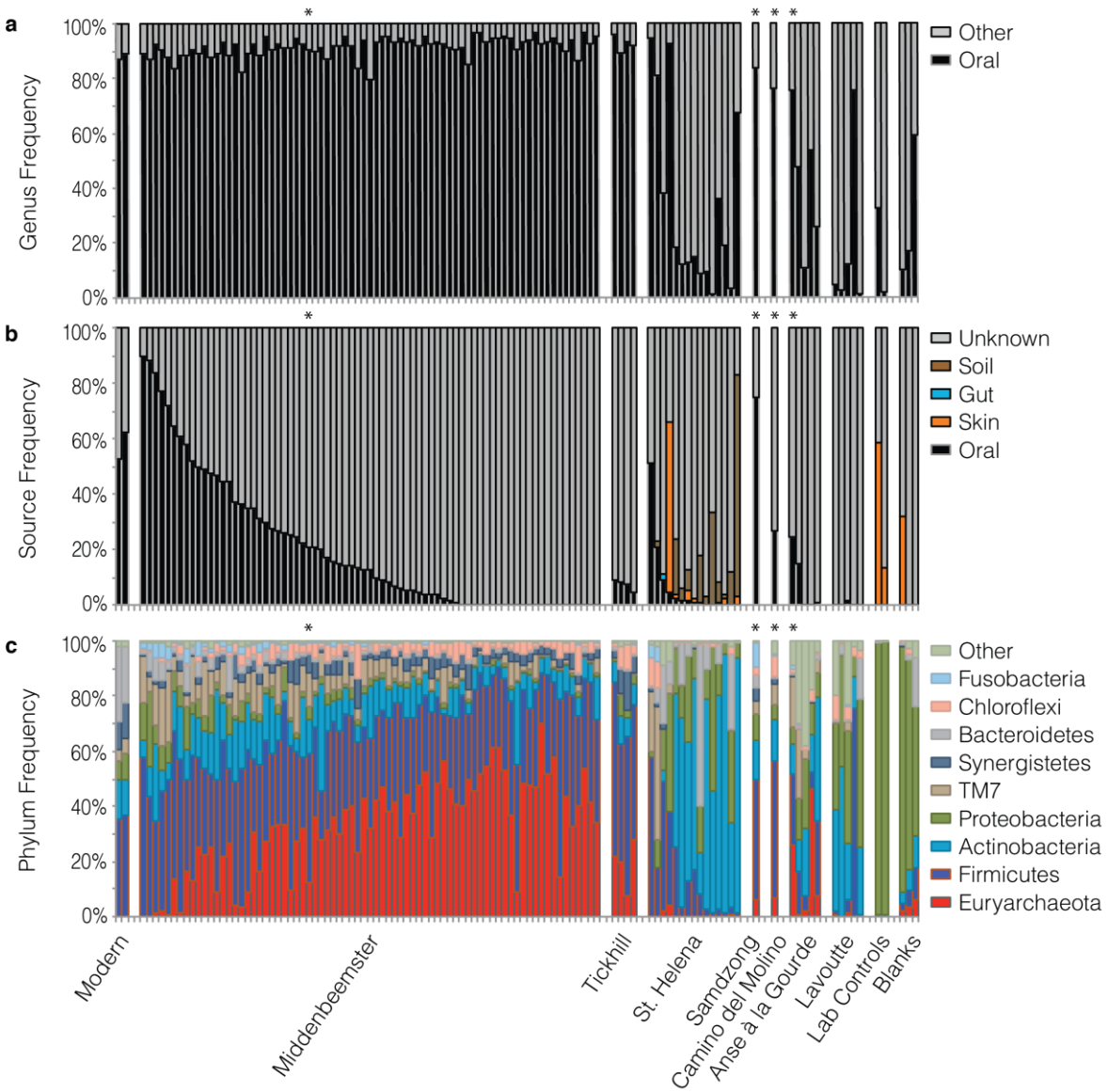

Figure A3.1. Unusual microbiome profiles observed in 16S rRNA gene V3 amplicon data from archaeological dental calculus. Relative abundance charts summarising: (a) Frequency of oral associated genera in dental calculus and control samples. The Dutch, UK, Nepalese, and

Spanish calculus samples show a greater proportion of oral-associated genera compared to the St. Helena and pre-Columbian Caribbean samples. (b) Contribution of oral, gut, and environmental sources to microbiome composition estimated by Bayesian source tracking. The oral microbiome (saliva, supragingival plaque, subgingival plaque) is a major source (>50%) in only a small proportion of archaeological dental calculus samples (10%), and an oral source is not indicated for more than a quarter of samples (26%). Laboratory controls (osteologist hands and osteology lab bench surfaces) and extraction blanks are largely consistent with a skin microbiome source and unassigned contaminants. (c) Frequency of microbial phyla inferred from V3 amplicon sequencing. The taxonomic profile reveals an unusual and non-biological pattern of exceptionally high Euryarchaeota levels in the Dutch, UK, and some Caribbean dental calculus samples. All Euryarchaeota OTUs are assigned to the genus Methanobrevibacter, the only prevalent genus of Archaea in the oral cavity. Methanobrevibacter is typically found at low frequencies (<0.5%) in healthy human dental plaque (Human Microbiome Project Consortium 2012), but in archaeological samples it may reach frequencies >60%, as seen here. Starred samples (*) were also analysed using shotgun metagenome sequencing. Sites are ordered from left to right by increasing thermal age (see Table 1). Fig. 1 data is available in Supplementary Data 1.

The results of the first approach reveal a high proportion of oral-associated genera in the Middenbeemster (median 92%) and Tickhill (median 92%) dental calculus samples, comparable to modern dental calculus (median 88%) (Fig. 1a). The non-oral taxa in the modern dental calculus sample consist almost entirely of *Paludibacter*, a genus of commensal plant bacteria that may be of dietary origin. The Samdzong and Camino del Molino samples likewise exhibit a high proportion of oral associated genera (84% and 76%, respectively), despite their greater age. By contrast, the Caribbean and St. Helena samples are highly variable in composition, and only 3 of the 10 Caribbean samples and 4 of the 15 St. Helena samples contain oral-associated genera at a frequency of >50%. The remaining samples are dominated by soil and environmental taxa, including members of the orders Actinomycetales,

Acidimicrobiales, Nitrospirales, Rhizobiales, Rhodospirillales, Solirubrobacterales, and Xanthomonadales. This pattern is consistent with a high degree of specimen degradation and exogenous soil contamination, a pattern not unexpected for specimens obtained from a tropical environment with a high thermal age(Hofreiter et al. 2014; Smith et al. 2003). The laboratory control samples are dominated by a narrow range of nasal and skin-associated taxa, notably Oxalobacteriaceae and Moraxellaceae, which comprise >80% of the sample. *Acinetobacter* and *Pseudomonas*, two genera associated with both the skin and oral microbiome, were also observed. The extraction blanks include a diverse range of soil, skin, and oral microbe sequences, all found at very low absolute abundance (17-316 sequences).

Applying Bayesian source tracking (Knights et al. 2011) to the same dataset, a stark pattern emerges. Although the SourceTracker results broadly confirm several of the above observations – that the Caribbean dental calculus preservation is generally poor, that the Samdzong and Camino del Molino calculus preservation is better, and that skin microbes have contributed to the composition of the control samples – the Middenbeemster samples exhibit a gradient of oral microbiome contribution that differs sharply from that inferred from constituent genera alone (Fig. 1b). Interestingly, this gradient is inversely correlated with the proportion of reads assigned to the archaeal phylum Euryarchaeota (Spearman's rho = -0.30, $p<0.001$) (Fig. 1c). Because SourceTracker takes into account weighted community composition when inferring source communities, large shifts in the taxonomic frequency of endogenous bacteria can reduce source assignments as strongly as exogenous contamination.

Examining the dental calculus OTU assignments more closely, nearly all of the Euryarchaota sequences (>99.8%) can be assigned to the genus *Methanobrevibacter*. In the oral cavity, *Methanobrevibacter* is a genus with low abundance (<0.01% in our modern control), represented almost exclusively by the taxon *M. oralis*. Thus, the exceptionally high and

variable *Methanobrevibacter* frequencies observed within the archaeological dental calculus, and especially within the Middenbeemster samples (median 33.8%), are unlikely to reflect a biological pattern. Likewise, within Chloroflexi, the vast majority of sequences (>98.5%) can be assigned to a single OTU within Anaerolinaceae. This bacterial family, which is present but not well characterised within the human oral cavity, is also typically found at low abundance (<0.01% in our modern control), but is found at a median frequency of 3% in our archaeological samples.

These observed patterns are difficult to explain on the basis of present evidence. Contamination is an unlikely cause of the taxonomic skew in the Middenbeemster samples, in part because the taxa that are overrepresented are members of the oral microbiome. Alternatively, postmortem microbial overgrowth or biased amplification due to taphonomic alteration are two additional possibilities. To test whether either or both of these factors could be the cause of the taxonomic skew, we selected and analysed a subset of archaeological human dental calculus samples using shotgun metagenome sequencing.

Shotgun characterisation of dental calculus samples

From our initial pool of 107 dental calculus samples, we selected four samples (Table 1) for further analysis using non-targeted shotgun metagenomics: 454C (Middenbeemster), F1948C (Anse à la Gourde), 37C (Samdzong), and 214C (Camino del Molino). The shotgun and amplicon libraries were built from the same original set of aDNA extracts, and the samples were selected from different sites to ensure that the results would not be biased by a specific geographic location or temporal period.

Overall, aDNA yields obtained from these ancient dental calculus samples were high (24.7-60.5 ng/mg), as is now known to be typical for this mineralised biofilm (Warinner, Speller, and Collins 2015; Warinner, Rodrigues, et al. 2014). The extracts were built into Illumina libraries and sequenced on an Illumina HiSeq 2000 NGS platform run in 2x100 PE mode. After filtering 16S rRNA gene sequences from the metagenomics dataset, we assigned taxonomy following the methods described above and compared the results to the amplicon data. Paired comparisons reveal that median *Methanobrevibacter* and *Anaerolineae G-1* levels are consistently lower in the shotgun data (1.6%, 1.3%, respectively) than the amplicon data (9.4%, 2.7%, respectively) for the same samples. In some cases this difference is extreme, as observed for F1948C, where the frequency of *Methanobrevibacter* is 26.1% in the amplicon dataset and 2.2% in the shotgun dataset. Thus, postmortem microbial overgrowth does not explain the taxonomic skew observed in the amplicon data. Next, we considered DNA preservation and possible amplification biases.

DNA fragmentation in ancient dental calculus

In general, aDNA is known to be highly degraded and fragmented (Sawyer et al. 2012; Hofreiter et al. 2014; Pääbo et al. 2004). However, the fragmentation of aDNA within dental calculus is not well explored. To investigate the degree of DNA fragmentation in our samples, we filtered the paired-end reads for 16S rRNA gene sequences by aligning them to the SILVA SSU 111 database. Fragment length distribution for this gene within each sample was then estimated using Picard-tools (http://broadinstitute.github.io/picard/). Median aDNA fragment lengths were relatively short (75-91 bp), as expected for authentic aDNA. We observed no differences in DNA preservation on the basis of cell wall composition (Supplementary Figures 1 and 2), as has been previously hypothesised (Adler et al. 2013) and reported for

Mycobacterium leprae (Schuenemann et al. 2013). Median fragment length was greatest in the youngest sample (454C) and was observed to decrease with the age of the sample (Fig. 2). For all samples, the median aDNA fragment length is significantly shorter than the median required template length for V3 U341F/534R amplification (183 bp), and the lengths of the V3 U341F/534R amplicons fall within the 4th quartile of the dental calculus aDNA distributions, suggesting that amplification is possible from only a minor proportion of the total ancient 16S rRNA gene template molecules (214C, 2.9-7.5%;37C, 3.1-8.2%; F1948C, 2.0-6.6%; 454C, 6.6-13.5%; ranges represent percentage of fragments available for upper and lower limits of V3 region lengths, respectively).

Because the number of aDNA fragments that are sufficiently long for targeted V3 U341F/534R PCR amplification is both low and variable across samples, taxonomic dropout and stochastic effects are expected because not all molecules are equally accessible to dual primer hybridisation. The extent of this amplification bias (differential PCR amplification) is expected to scale with sample complexity. As highly complex systems, microbiomes are particularly vulnerable to this bias (Wintzingerode, Göbel, and Stackebrandt 1997). However, stochastic processes alone cannot account for the consistent taxonomic shifts, such as the overrepresentation of Euryarchaeota observed in our ancient dental calculus samples. One possible explanation for consistent taxonomic shifts resulting from differential PCR amplification is variation in PCR amplicon length, as is evident for V3 U341F/534R (Fig. 2). To test whether inherent amplification biases could be the cause of the taxonomic skew, we examined the V3 region of the 16S rRNA gene in greater detail.

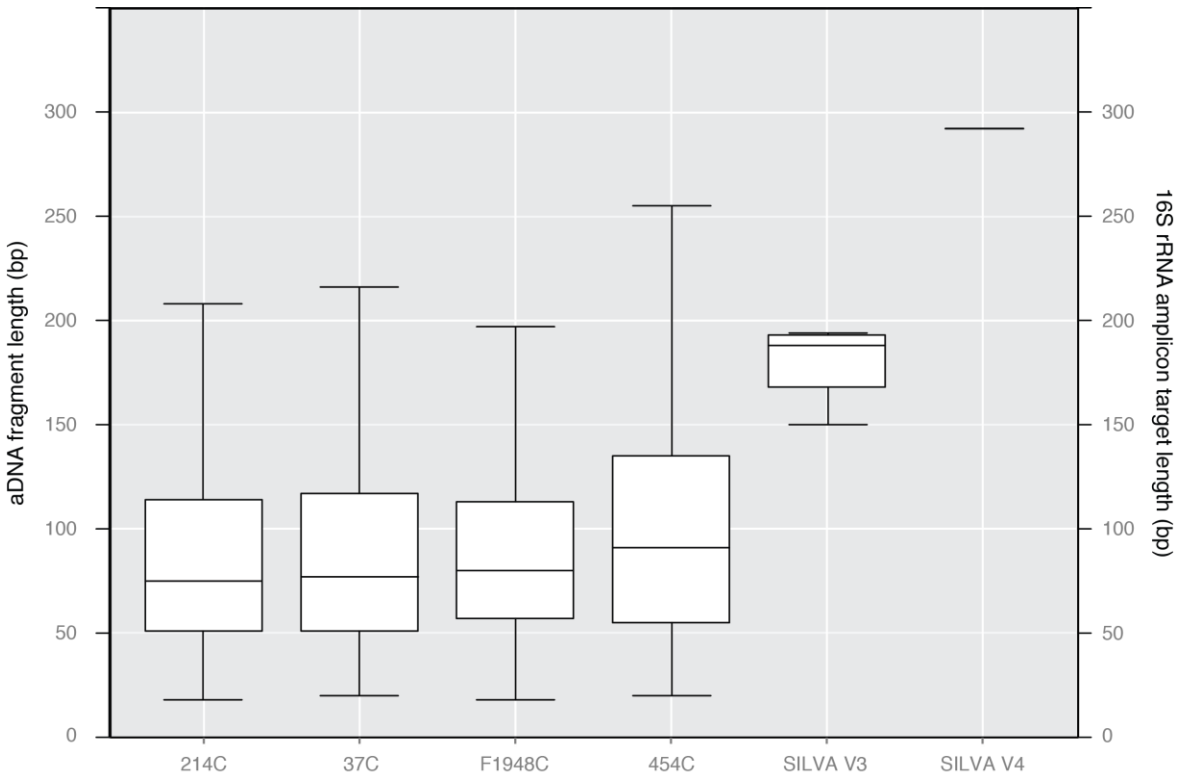

Figure A3.2. Length distribution box plots of aDNA extracted from archaeological dental calculus and calculated V3 and V4 16S rRNA amplicon lengths for microbes in the SILVA SSU 111 database. As expected for aDNA, the genetic material within dental calculus is highly fragmented to median lengths <100 bp: 214C, 75 bp; 37C, 77 bp; F1948C, 80 bp; 454C, 91 bp. This is significantly shorter than the median lengths of the 16S rRNA V3 (183 bp) and V4 (292 bp) amplicon targets. The number of read pairs comprising each box plot are as follows: 214C, 20,355; 37C, 17,962; F1948C, 26,517; 454C, 15,736; SILVA V3, 651,163; SILVA V4, 649,660.

Characteristis of 16S rRNA V3 region

Taxonomic coverage

The V3 region of the 16S rRNA gene (*E. coli* position 341-534bp) has been extensively documented for its ability to distinguish microbial genera through both sequencing and DGGE based studies. To better understand taxonomic coverage exhibited by the V3 region, we used

PrimerProspector (Walters et al. 2011) to evaluate *in silico* amplification of 16S rRNA sequences from the SILVA database using the V3 U341F/534R primer pair. Overall, the primer pair showed approx. 98% recovery for bacterial sequences and approx. 91% recovery for archaeal sequences, distributed over 46 major phyla each with over 100 representative sequences in the database. When limited to phyla commonly found in the human oral cavity, this primer pair has a >97% recovery rate with the exception of Euryarchaeota and Chlamydiae, where the numbers fall to 91% and 71% respectively. However, these primers do amplify *Methanobrevibacter oralis* and *Chlamydophila pneumoniae*, the only known members of Euryarchaeota and Chlamydiae, respectively, in the oral cavity. Thus, the taxonomic skew observed in the V3 amplicon data for Middenbeemster, Samdzong, and Camino del Molino samples cannot be attributed to predicted taxonomic biases in primer binding for the U341F/534R primer pair.

Length polymorphisms in the V3 region

Next, we examined amplicon distribution profiles for the V3 U341F/534R primers. While the V3 region is often described as being <200bp in length (*E. coli*), more specifically, we observe a multimodal distribution in amplicon lengths, differing by as much as 44 bp (150-194 bp). In comparison, the commonly used V4 primer pair 515F/806R has a median amplicon length of 292bp, with a range of 290-295 bp. Because the 16S rRNA gene encodes the small subunit of prokaryotic ribosomal RNA, its sequence reflects the properties of rRNA secondary folding structure (Fig. 3). Here we have highlighted the parts of the 16S rRNA corresponding to the V3 U341F/534R (pink) and V4 515F/806R (blue) gene amplicon targets, which overlap in the 534R and 515F primer binding sites (purple). Within these regions variation occurs through both SNPs and Insertion/Deletions (indels), with the V3 region having both types, and the V4 region having primarily SNPs. Specifically, the V3 region contains two stem-loop structures

exhibiting length polymorphisms that differ across taxonomic clades (Fig. 3a-c, arrows). The shortest stem-loop structures are found in archaea, such as *Methanobrevibacter oralis* (Fig. 3a), while bacteria exhibit high length variability within these structures, as observed for *Corynebacterium diphtheria* (Fig. 3b) and *Streptococcus pyogenes* (Fig. 3c). In contrast, the V4 region is relatively length invariant and exhibits no major structural variation in stem loop structures for these same taxa (Fig. 3e-g). To provide greater resolution of taxonomic associations of the V3 region length polymorphisms, we systematically analysed predicted V3 U341F/534R amplicon lengths at the phylum and genus levels using PrimerProspector and the SILVA SSU 111 database. We selected 31 representative oral genera from nine phyla for analysis and plotted a heatmap of the distribution of the predicted V3 U341F/534R amplicon lengths for all OTUs assigned to these genera (Fig. 4a). Clear taxonomic associations are observed with respect to predicted amplicon length. As previously noted, the archaeon *Methanobrevibacter* has the shortest predicted amplicon length (median 151 bp), followed by the bacteria *Anaerolineae G-1* (median 169 bp) and the bacterial candidate phylum TM7 (median 169 bp). *Treponema* and Proteobacteria have among the longest predicted amplicon length (median >191 bp, except *Campylobacter*). The length of the V3 region varies widely among Actinobacteria, even within the same genus, and several phyla, especially Actinobacteria, Firmicutes, and Bacteroidetes, contain genera with multimodal length distributions. To confirm that these results are not a database artifact, we repeated this exercise using the RDP, NCBI, and Greengenes databases, and found comparable results (Supplementary Fig. 1).

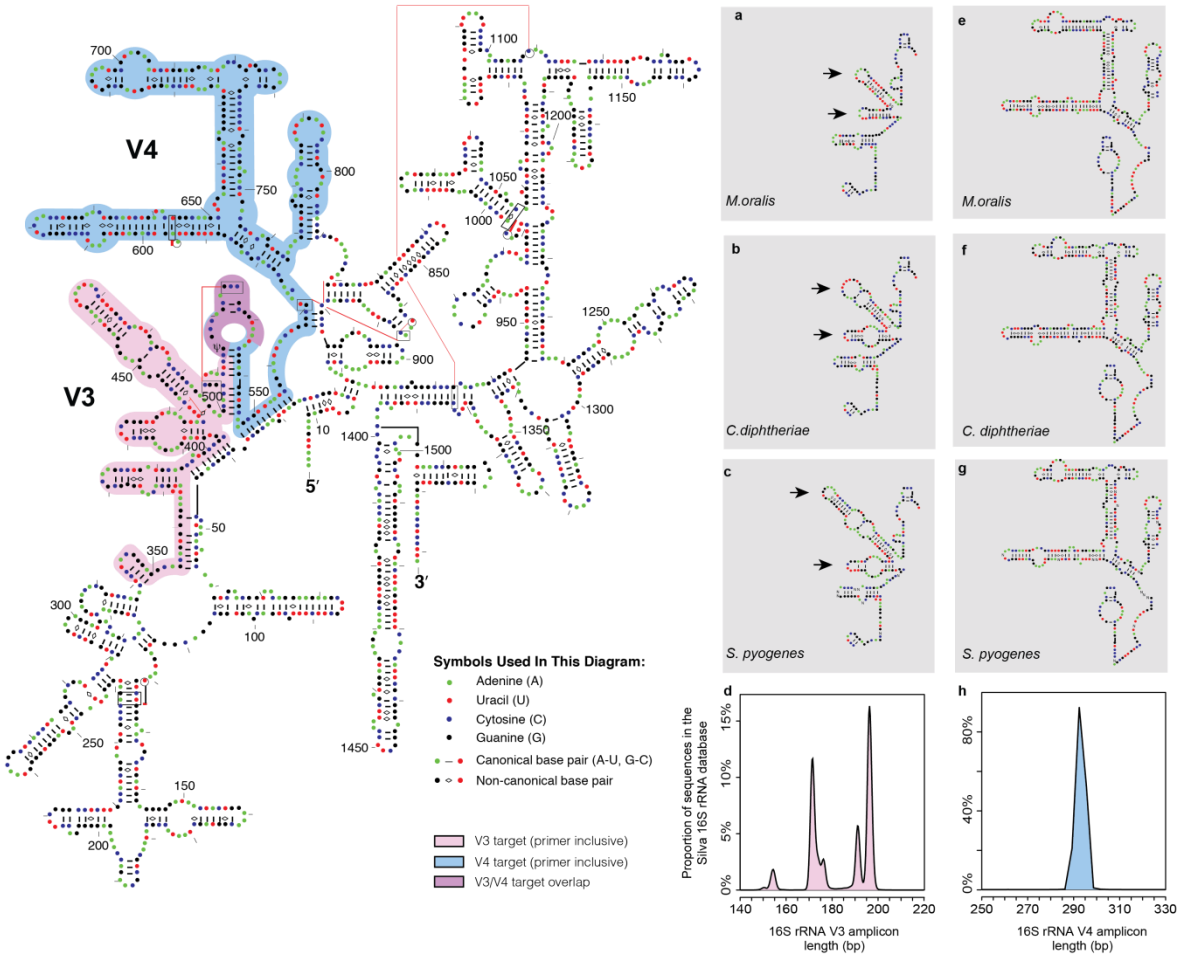

Figure A3.3. Simplified 16S small subunit ribosomal RNA secondary structure. Secondary structure of Escherichia coli (J01695) 16S rRNA (main panel). Amplicon targets (primer inclusive) for the third (V3, 341F/529R) and fourth (V4, 515F/806R) variable regions are highlighted in pink and blue, respectively. Overlapping V3/V4 target sequences are highlighted in purple. Although widely used in ecological studies, the V4 region is impractical for aDNA research because of its long length (approx. 292 bp, primer inclusive). The V3 region is considerably shorter, but comparative sequence analysis (a-d) reveals that the V3 region exhibits extensive length polymorphisms (arrows) in archaeal (e.g., Methanobrevibacter oralis) and bacterial (e.g., Corynebacterium diphtheria, Streptococcus pyogenes) taxa, with predicted V3 amplicon lengths ranging from 150-194 bp when queried against the SILVA SSU 111 16S rRNA database (d). By contrast, the V4 region is relatively length invariant (e-h), ranging from 290-295 bp (h). 16S rRNA secondary structure has been adapted from Comparative RNA Web Site and Project (Cannone et al. 2002).

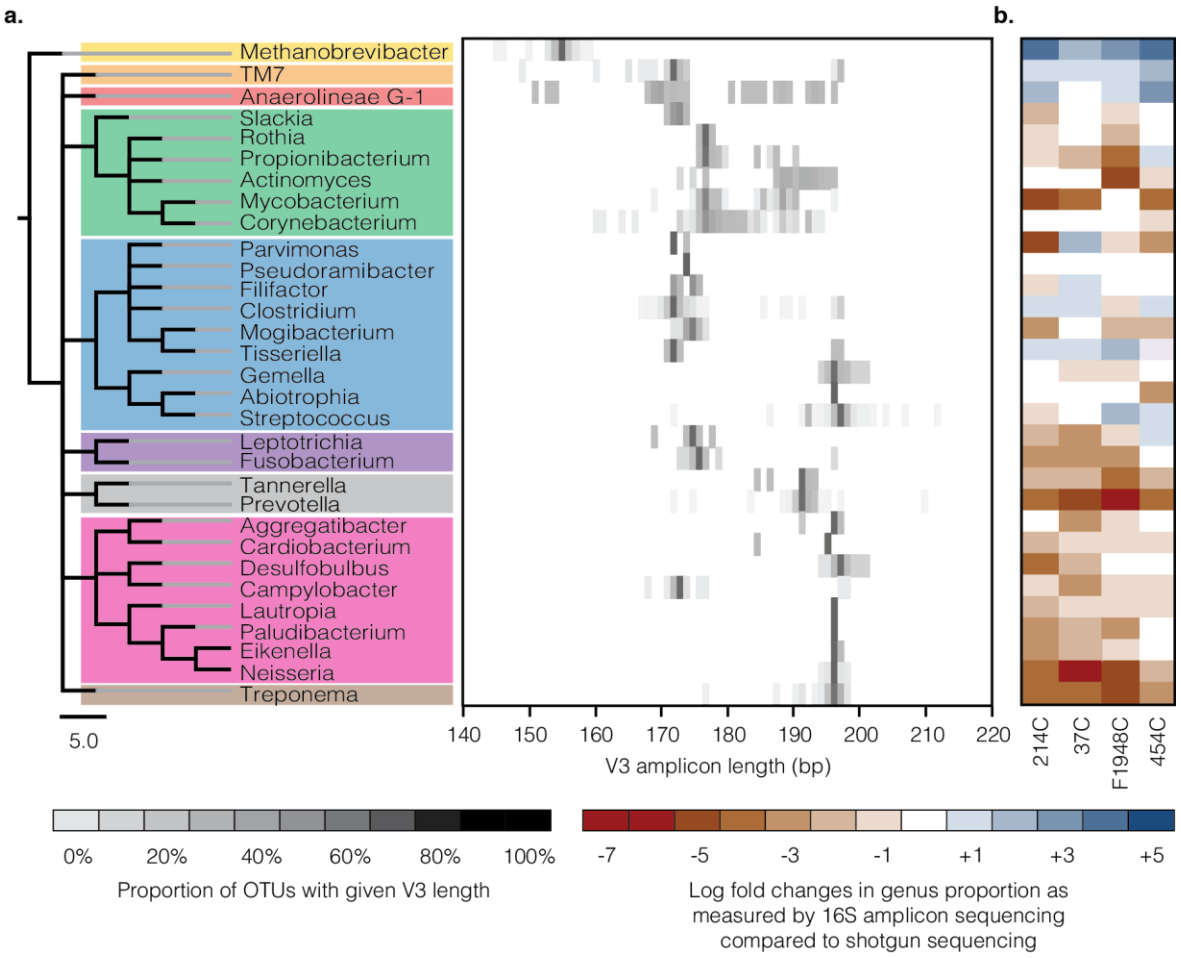

Figure A3.4. Heatmap of 16S rRNA V3 amplicon lengths reveals high variability but broad taxonomic patterns. (a) 16S rRNA V3 sequence data was analysed in silico for 36,634 OTUs belonging to 31 representative oral microbiome genera from 9 major microbial phyla: Euryarchaeota (yellow), TM7 (orange), Chlorflexi (red), Actinobacteria (green), Firmicutes (blue), Fusobacteria (purple), Bacteroidetes (gray), Proteobacteria (pink), Spirochaetes (brown). (b) Log fold changes in genus frequency within archaeological dental calculus when comparing data generated by targeted V3 U341F/534R amplicon sequencing to non-targeted shotgun metagenomics data. Methanobrevibacter, Anaerolinea, and TM7, which have very short predicted V3 U341F/534R amplicon lengths, strongly overamplify compared to frequency data obtained from non-targeted shotgun metagenomic sequencing. Most other taxa underamplify, especially genera with very long predicted V3 U341F/534R amplicon lengths, such as Treponema and members of Proteobacteria, and Bacteroidetes.

Interestingly, unusually high levels of *Methanobrevibacter* are a common feature of the taxonomic skew observed in amplicon based characterisation of dental calculus. This suggests that length differences in the V3 region play a role in this taxonomic skew, with taxa having shorter V3 regions showing better amplification than those with longer V3 regions.

Comparison of taxonomic profiles generated by targeted amplicon and shotgun metagenome sequencing

Given the highly fragmented nature of aDNA, length polymorphisms in the V3 region of the 16S rRNA gene may lead to differential PCR amplification, specifically overamplification of taxa with very short V3 regions and underamplification or taxonomic dropout of taxa with very long V3 regions. To test this hypothesis, we generated and compared taxonomic frequency data from paired V3 U341F/534R amplicon libraries and shotgun metagenome libraries built from the four archaeological dental calculus samples described above. Rarefaction analyses show plateauing for both the amplicon and shotgun metagenome datasets indicating sufficient sampling for analysis (Supplementary Fig. 2). We found that the amplicon datasets exhibit an excess of taxa with very short V3 sequences (e.g., *Methanobrevibacter*, *Anaerolineae G-1,* and TM7), and a deficiency of taxa with very long V3 sequences (e.g., *Treponema*, *Neisseria*, and *Prevotella*) (Fig. 4b). Hence, genera with shorter median fragment lengths, such as *Methanobrevibacter,* are strongly overrepresented using an amplicon sequencing approach, whereas genera with longer median fragment lengths, such as *Treponema*, are strongly underrepresented, with other taxa exhibiting intermediate frequency changes (Fig. 5). In addition to the V3 region length, the magnitude of this bias is likely to depend on both the degree of postmortem aDNA degradation and the original relative abundance of the DNA template in the sample.

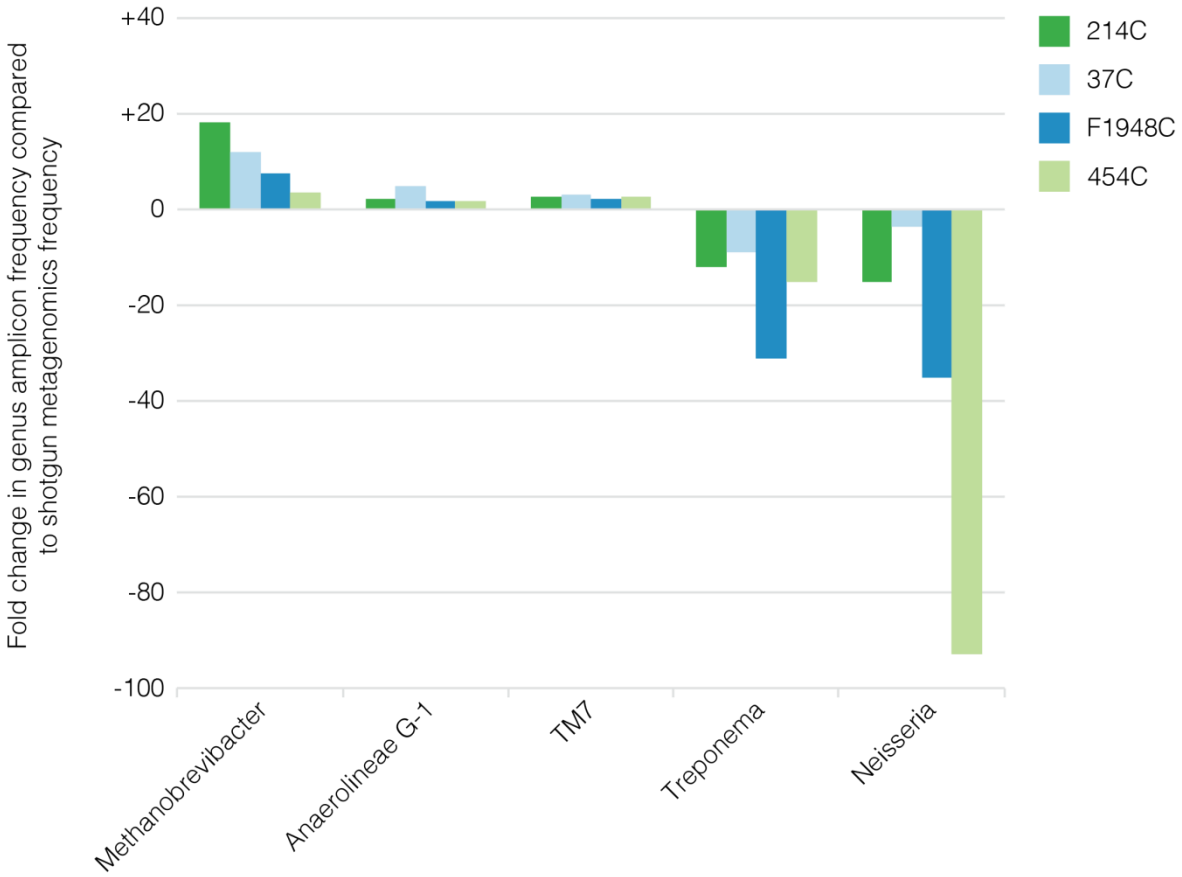

Figure A3.5. Fold changes in taxon frequency between 16S rRNA V3 U341F/534R amplicon and shotgun metagenome data. Genera with relatively short median amplicon lengths (Methanobrevibacter, Anaerolineae G-1, TM7) are overrepresented in the 16S rRNA V3 U341F/534R amplicon dataset, while genera with relatively long median amplicon lengths (Treponema, Neisseria) are strongly underrepresented.

Predicted V3 U341F/534R amplicon lengths of specific oral microbiome taxa of interest

Overall, our comparative data indicate that V3 U341F/534R amplification results in a highly skewed taxonomic profile when applied to archaeological microbiome samples. Nevertheless, because amplicon sequencing can be scaled for high-throughput analysis and is more economical on a per sample basis than shotgun metagenome sequencing, it may still have value as a sample screening tool prior to shotgun metagenome analysis. In this case, it is

important to know which taxa are likely to over- or underamplify, or perhaps dropout altogether. In Table 2 we provide the predicted V3 U341F/534R amplicon lengths for a range of oral microbiome taxa of interest, including prominent commensals and pathogens implicated in caries formation, periodontal disease, respiratory illness, and a variety of acute and chronic systemic diseases. It is worth noting that most pathogenic taxa have very long V3 regions and hence are likely to be underrepresented or absent from V3 U341F/534R amplicon datasets generated from archaeological material. This has important implications for high-throughput sample screening using this approach because low abundance pathogens, such as *Mycobacterium tuberculosis*, are likely to drop out.

Finally, because of the close evolutionary relationship between chloroplasts and cyanobacteria(Green 2011), the chloroplast 16S rRNA gene is also amplified by the V3 U341F/534R primers (170 bp amplicon). Chloroplast sequences have been previously reported in modern dental plaque(Dewhirst et al. 2010), and we have observed them at variable, but generally very low, frequencies in archaeological dental calculus. Although such sequences indicate the presence of chloroplast DNA, and thus possible dietary components, sequence variation within the V3 U341F/534R amplicon is insufficient to resolve taxonomy below the level of Streptophyta, an unranked plant clade that includes all land plants and some green algae.

Oral taxa of interest	16S rRNA V3 amplicon length (bp)	Rank abundance in HOMD
Oral commenals		
Actinomyces naeslundii	190	0.23%
Anaerolineae G-1 sp.[a]	169	<0.01%
Atopobium parvulum	173	0.30%
Campylobacter gracilis	169	1.04%
Corynebacterium matruchotii	189	0.49%

350

Eubacterium saphenum	171	0.37%
Fusobacterium nucleatum	172	1.35%
Methanobrevibacter oralis	151	*
Mogibacterium timidum	171	0.27%
Neisseria mucosa	193	0.33%
Oribacterium sinus	168	0.14%
Parvimonas micra	168	0.68%
Prevotella melaninogenica	188	0.22%
Rothia dentocariosa	173	0.60%
Streptococcus mitis	194	5.66%
TM7 spp.[b]	168	0.24%
Veillonella parvula	194	6.63%
Caries pathogens		
Lactobacillus salivarius	193	0.09%
Streptococcus mutans	194	4.31%
Periodontal pathogens		
Aggregatibacter actinomycetemcomitans	193	0.03%
Filifactor alocis	171	0.31%
Porphyromonas gingivalis	188	0.26%
Tannerella forsythia	188	0.25%
Treponema denticola	194	0.22%
Respiratory pathogens		
Bordetella pertussis	193	**
Corynebacterium diphtheriae	173	**
Haemophilus influenzae	193	0.29%
Streptococcus pneumoniae	194	0.18%
Streptococcus pyogenes	194	*
Systemic pathogens		
Neisseria meningitidis	193	0.17%
Mycobacterium tuberculosis	185	**
Mycobacterium leprae	185	**

Treponema pallidum	194	**
Dietary		
Streptophyta (plant chloroplast)	170	***

Table A3.2. 16S rRNA V3 (U341F/534R) amplicon lengths for oral microbiome taxa of interest. *Rank abundance could not be determined for this taxon because the HOMD primers do not amplify archaeal sequences (Dewhirst et al. 2010). **Taxon below detection limits of molecular cloning in HOMD. ***Chlorplast DNA not included in HOMD rank abundance calculation. aWithin Chlorflexi, the bacterial class Anaerolineae contains several oral taxa that have not yet been formally described, and naming of these taxa is inconsistent across databases. We observed one of these Anaerolineae taxa in both our modern and ancient datasets, and its 16S rRNA sequence is identical to Anaerolineae G-1 (HOMD oral taxon ID 439) and SHD-231 (Greengenes 13). bTM7 is a bacterial candidate phylum without cultured members.

Theoretical modelling of the effect of thermal age on oral microbiome taxonomic frequencies

To explore the diachronic effects of DNA degradation on a single sample, we used thermal age calculations (Hofreiter et al. 2014; Smith et al. 2003) to model predicted taxonomic skew in a hypothetical oral microbiome sample evaluated at multiple thermal ages. We estimated the probability of a nucleotide being damaged (λ) resulting in chain scission assuming a random degradation model for each of the archaeological sites in this study (Table 1). Using this probability term, we then explored the impact that temperature history would have on the relative survival of the V3 regions of the taxa presented in Table 2 for a hypothetical oral microbiome sample averaged from all entries in the HOMD. In this model, the probability of a DNA fragment of size x or greater being present is given by $e^{-\lambda x}$. As such, samples of greater thermal age will exhibit greater fragmentation, and within a sample, longer templates have a higher probability of acquiring damage than shorter templates. By modeling the amplification success of DNA templates within a hypothetical microbiome sample at multiple thermal ages,

we observe a clear pattern whereby specific taxa systematically increase or decrease in frequency simply as a function of aDNA degradation (Fig. 6).

Overall, these results confirm the biases inherent to aDNA microbial community characterisation using V3 region primers. Next, to evaluate the suitability of the other variable regions for aDNA community analysis, we performed extensive *in silico* analyses to examine taxonomic and length biases in these regions.

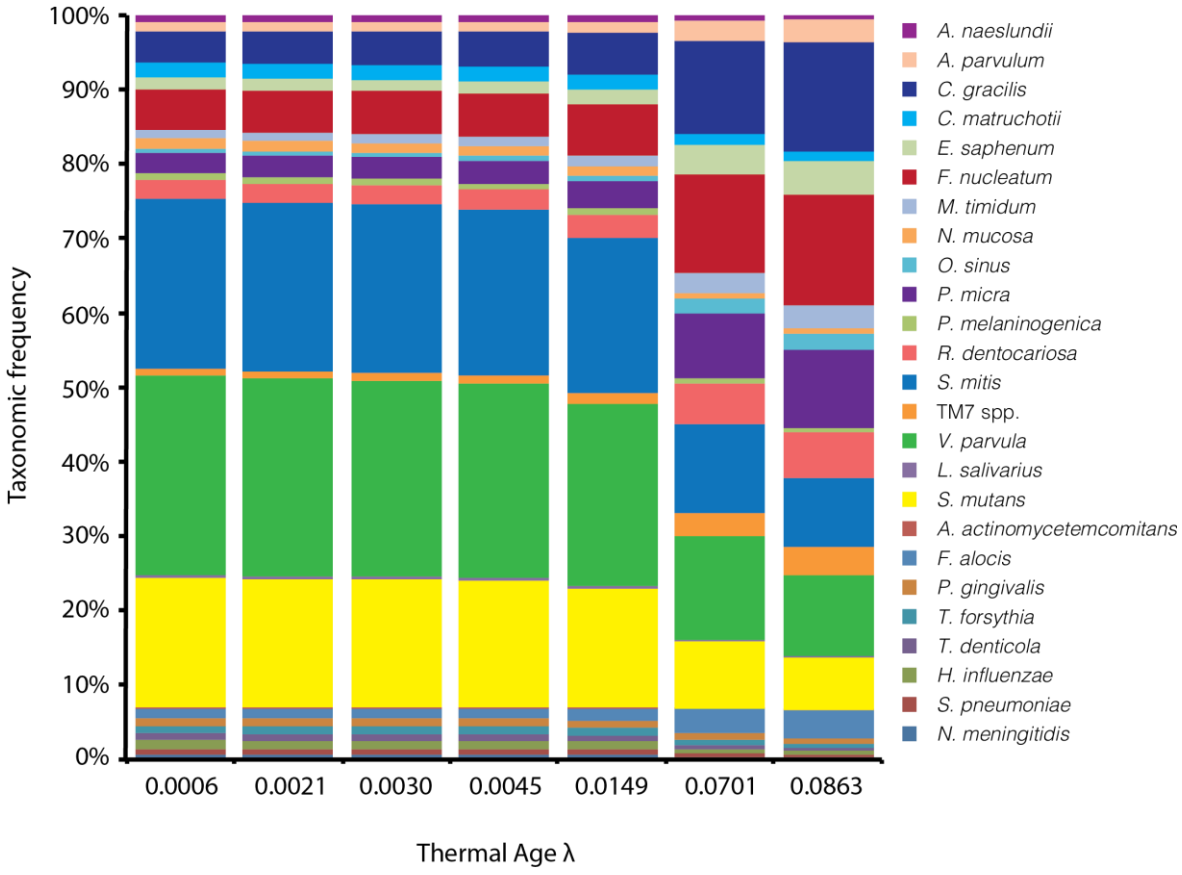

Figure A3.6. Predicted effect of thermal age on reconstructed taxonomic frequencies of selected oral bacteria from V3 U341F/534R amplicon data. Using a random DNA degradation model, the relative abundance of taxa presented in Table 2 is modelled at different thermal ages, corresponding to the thermal ages of the archaeological sites in this study. The probability of chain scission (λ) (Deagle, Eveson, and Jarman 2006; Allentoft et al. 2012)) is

estimated using temperatures estimated from www.thermal-age.eu using previously published kinetic parameters.(Allentoft et al. 2012) Starting taxonomic frequencies were taken from mean HOMD values, and taxa with a frequency of <0.01% in the HOMD are not shown. Together, the taxa shown account for 25% of the HOMD human oral microbiome.

In silico evaluation of 16S rRNA gene and universal primers

When selecting universal 16S rRNA primers, two characteristics are paramount: high amplicon taxonomic resolution and high amplicon taxonomic coverage. With respect to primers that will be applied to aDNA, short amplicon length is also critical given the known fragmented and degraded state of aDNA. To date, a total of eleven different 16S rRNA gene universal primer pairs have been applied in studies of ancient microbiomes. We analysed ten of these primer pairs (all primer pairs generating amplicons <500 bp at 99% CI), as well as four additional primer pairs widely used today in ecological studies, for these metrics *in silico* using PrimerProspector (Walters et al. 2011)and SILVA (Quast et al. 2013), a database containing more than 1.5 million 16S rRNA gene sequence entries (Table 3). Overall, the following results indicate that irrespective of the variable region being targeted, accurate reconstruction of community profiles will be limited dependent on degree of aDNA fragmentation, taxonomic coverage of primers, and degree of length variation of primers.

Amplicon taxonomic resolution

Amplicon resolution here is defined as the degree to which amplified sequences allow the discrimination of distinct taxa (OTUs). Using the SILVA SSU 111 database, clustering of all 16S rRNA sequences at 97% similarity threshold yields a total of 138,462 OTUs, representing the maximum taxonomic resolution of this gene. Individual 16S rRNA gene variable regions

contain only a portion of the total sequence variation, and thus have lower taxonomic resolution. To determine the anticipated taxonomic coverage of each primer pair, predicted amplicons generated from the SILVA SSU 111 database were clustered *de novo* using uclust(Edgar 2010) and a 97% similarity threshold. The V4 515F/806R primers yield the highest taxonomic resolution of the primers analysed in this study (Table 3), with 56,463 predicted OTUs (41% of the total). The V6 926F/1046R primers have the next highest resolution (50,892 OTUs, 37% of total), closely followed by the V3 U341F/534R primers (49,397 OTUs, 36% of total). By comparison, the V1-V2 and V5 primers yield amplicons with only moderate resolution (30-32% and 20-23%, respectively) and V1 taxonomic resolution is very poor (<1%). In fact, one V1 primer pair previously used in ancient microbiome studies (Luciani et al. 2006; Ubaldi et al. 1998) is not predicted by our analysis to yield any amplicons.

16S rRNA gene primer pair position			Amplicon length statistics[a]			Amplicon taxonomic resolution	Amplicon taxonomic coverage by phylum[b]														Primer Reference
Hypervariable region	Primer pair[c]	Position in *E. coli*[d]	Min. amplicon length (primer inclusive)[e]	Max. amplicon length (primer inclusive)[e]	Max.-min. amplicon length	OTUs[f]	Firmicutes	Bacteroidetes	Proteobacteria	Actinobacteria	Spirochaetes	Fusobacteria	Candidate division TM7	Tenericutes	Synergistetes	Candidate division SR1	Chlorobi	Chloroflexi	Euryarchaeota	Chlamydiae	
V1	8F/120R*	8-27; 101-120	140	276	136	773	0.42	0.26	0.39	0.30	0.29	0.04	0.42	0.32	0.25	0.13	0.31	0.31	-	0.03	Adler et al. 2013
V1	29F/98R*	20-38; 107-127	-	-	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Luciani et al. 2006; Ubaldi et al. 1998
V1-V2	27F/338R	8-27; 338-355	314	379	65	41,781	0.40	0.38	0.38	0.32	0.30	0.31	0.41	0.30	0.44	0.39	0.53	0.22	-	0.03	Ravel et al. 2011
V1-V2	27F/342R*	8-27; 342-357	315	380	65	44,144	0.41	0.38	0.38	0.39	0.30	0.31	0.42	0.32	0.44	0.42	0.55	0.32	-	0.04	Rollo et al. 2007; Cano et al. 2000; Rollo et al. 2000
V3	338F/531R*	320-339, 532-551	216	233	17	18,051	0.14	0.38	0.72	0.55	0.10	0.01	0.03	0.02	0.30	-	0.32	0.01	-	0.00	Rollo et al. 2007; Rollo et al. 2000
V3	338F/533R*	338-358; 515-533	190	216	26	29,731	0.65	0.83	0.85	0.80	0.32	0.04	0.83	0.17	0.74	0.20	0.96	0.23	-	0.00	Adler et al. 2013
V3	351F/507R*	338-358; 515-536	174	200	26	46,118	0.99	0.99	0.99	0.96	0.31	0.98	0.96	0.98	0.99	0.98	0.98	0.70	-	0.71	Warinner et al 2014
V3	U341F/534R*	341-357; 517-531	148	192	44	49,397	0.99	0.99	0.99	0.99	0.98	0.99	0.97	0.99	0.99	1.00	0.98	0.98	0.91	0.71	Tito et al. 2012; Baker, Smith, and Cowan 2003; Hansen et al. 1998
V4	515F/806R	515-533; 787-806	290	295	5	56,463	0.98	0.98	0.98	0.90	0.88	0.98	0.95	0.99	0.99	1.00	0.98	0.96	0.97	0.97	Caporaso et al. 2012; Reysenbach, Wickham, and Pace 1994
V5	800F/900R*	783-806; 908-927	144	148	4	27,009	0.97	0.97	0.98	0.98	0.78	0.95	0.08	0.87	0.94	0.08	0.98	0.37	0.00	1.00	Warinner et al. 2014
V5	785F/907R	785-805; 907-926	141	146	5	32,063	0.98	0.98	0.98	0.98	0.99	0.98	0.96	0.98	0.99	0.08	0.99	0.87	0.96	1.00	Lane et al. 1985; Zaura et al. 2009
V6	926F/1046R*	907-927; 1046-1064	152	167	15	50,892	0.99	0.99	0.99	0.99	0.99	0.99	0.80	0.98	0.99	0.01	0.99	0.96	0.18	1.00	Adler et al. 2013
V6	917F/1061R	917-934; 1061-1080	160	172	12	47,332	0.98	0.97	0.98	0.99	0.90	0.96	0.96	0.59	0.99	0.01	0.99	0.98	-	0.99	Crielaard et al. 2011; Keijser et al. 2008
V6	958F/1044R*	941-964; 1052-1071	144	220	76	516	0.92	0.96	0.85	0.33	0.98	0.98	0.35	0.87	0.98	0.01	0.98	0.04	0.00	0.97	Warinner et al. 2014

Table A3.3. 16S rRNA gene primer pair information and in silico amplicon statistics. *Primer pair has been used in previous ancient microbiome studies. aLength statistics obtained from SILVA SSU 111 database. bIn silico estimate using PrimerProspector (Walters et al. 2011) of the proportion of sequences in SILVA SSU 111 database (http://www.arb-silva.de) that the primer pair will amplify per phylum. Phyla are sequentially ordered from left to right by the number of species entries per phylum in the HOMD (Chen et al. 2010). cPrimer pair naming conventions have changed through time and are not consistent across studies. dPosition of primer start and stop coordinates relative to Escherichia coli Genbank accession J01695. Full primer sequences are provided in Supplementary Table 2. eReported for 99% CI of the SILVA SSU 111 database. Outliers, which may include chimeras, are excluded. fOTUs generated per primer pair by de novo clustering of the Silva 111.1 database at 97% similarity. The total number of OTUs generated from full 16S rRNA gene sequences is 138,462.

Amplicon taxonomic coverage

Amplicon taxonomic coverage is here defined as the proportion of species-level taxonomic entries for each phylum in the SILVA SSU 111 16S rRNA database that are predicted to amplify using a given primer pair. The 16S rRNA gene contains numerous point mutations and insertion-deletion sites. Although most of these sequence variants are concentrated within the hypervariable regions, some are found within the "conserved" regions as well. As a result, it is not possible to design truly universal primers for the 16S rRNA gene (Baker, Smith, and Cowan 2003). Nevertheless, certain primer pairs are "more universal" than others and can amplify a greater diversity and proportion of microbial phyla than others. Among the primers analysed in this study, the V3 U341F/534R primers have the highest predicted taxonomic coverage for the 14 most important oral microbial phyla (Table 3). As discussed above, these primers are predicted to amplify >97% of the taxa in these phyla, except for Euryarchaeota (91%) and Chlamydiae (71%). Notably, however, the V3 U341F/534R primers do amplify *Methanobrevibacter oralis* and *Chlamydophila pneumoniae*, the only characterised members

of Euryarchaeota and Chlamydiae, respectively, in the oral cavity. Thus, the V3 U341F/534R primers have very high predicted taxonomic coverage for the oral microbiome. The V4 515F/806R, V5 785F/907R, and V6 926F/1046R primer pairs also yield relatively good overall taxonomic coverage but exhibit poor amplification for one or more phyla. In the case of the V4 515F/806R primers, this includes the relatively important phyla Actinobacteria (90%) and Spirochaetes (80%). The V5 785F/907R primers poorly amplify minor oral taxa in the phyla Chloroflexi (8%) and Candidate division SR1 (87%), and the V6 926F/1046R primers show poor coverage for Candidate division TM7 (80%), Candidate division SR1 (1%), and Euryarchaeota (18%; and do not amplify *Methanobrevibacter*). Worst of all are the V1 and V1-V2 primers, which are predicted to yield low taxonomic coverage (<50%) for nearly all oral phyla.

Amplicon length

Amplicon length is here defined as the full length of the 16S rRNA amplicon, including primers. Primer inclusion is important because amplification requires a DNA target of a length sufficient to include the entire targeted region of interest and primer binding sites. For modern DNA, this is rarely an issue, but for aDNA, which is typically highly degraded and fragmented, only a small fraction of the total aDNA extract may be of sufficient length to allow amplification. Median lengths of aDNA reported from bone are typically less than 100 bp (Hofreiter et al. 2014), and because of this primer pairs targeting regions >200 bp are rarely effective in ancient DNA studies. The 16S rRNA gene variable regions differ in length, and with a total amplicon length up to 295 bp, the V4 region is the longest of the individual variable regions (Table 3). Likewise, the V1 (up to 276 bp) and combined V1-V2 (up to 380 bp) regions are similarly long. For the remaining variable regions, the V3 U341F/534R, V5

515F/806R, and V6 926F/1046R primers yield the shortest amplicons, 150-194 bp, 141-146 bp, and 152-167 bp, respectively. While amplicons of this size fall within the range of those that have been successfully amplified from mitochondrial (Krüttli et al. 2014) and microbiome (Adler et al. 2013; Tito et al. 2012; Warinner, Rodrigues, et al. 2014) aDNA in the past, they greatly exceed the median length of typical aDNA fragments.

Discussion

Amplicon deep sequencing of one or more 16S rRNA gene hypervariable regions is a highly economical method for high-throughput taxonomic characterisation of the human microbiome. However, this approach requires high quality and well-preserved DNA in order to prevent differential PCR amplification and consequent biases in downstream taxonomic analyses. As such, the method has limited applicability for genetic investigations of ancient human microbiomes, where the aDNA is known to be highly degraded and fragmented. For example, we find that median DNA fragment lengths within archaeological dental calculus are less than half (41-50%) the required template length for amplifying the commonly targeted 16S rRNA V3 region. Moreover, this problem is further exacerbated when the targeted hypervariable region contains extensive length polymorphisms, as is true for the V1, V2, and V3 regions. In such cases, the effects of differential PCR amplification are not random, but rather are biased toward taxa with the shortest amplicon lengths. In the case of the universal V3 U341F/534R primers, archaeological specimens tend to produce taxonomic profiles with unusually high frequencies of *Methanobrevibacter*, and to a lesser extent Anaerolinaceae and TM7, all taxa with very short V3 sequences. At the same time, these taxonomic profiles typically have unusually low frequencies of taxa with very long V3 sequences, such as *Treponema*, *Neisseria*, and *Prevotella*. Because *Methanobrevibacter* is the oral taxon with the shortest V3

region (17 bp shorter than the shortest bacterial sequence), it may reach extreme frequencies in some ancient microbiome datasets, even exceeding 60% of the total sequences (e.g., Fig. 1c), a taphonomic artifact we term the "Archaea effect". Such high frequencies of *Methanobrevibacter* are not observed in corresponding shotgun metagenome datasets (Supplementary Table 3), further confirming that it is an artifact specific to targeted PCR amplification. We have observed that the Archaea effect is characteristic of ancient microbiome taxonomic profiles produced using V3 U341F/534R universal primers from samples with a high degree of endogenous DNA fragmentation, but low exogenous contamination. Other V3 primers, such as 338F/531R, 338F/533R, and 351F/507R do not typically generate this effect because they are less universal and contain primer sequence mismatches that strongly disfavor amplification of Archaea. Nevertheless, the remaining length based amplification biases documented in other taxa remain applicable.

Although the 16S rRNA gene V3 region is the focus of this study, other hypervariable regions are subject to similar challenges. The V1 and V2 regions exhibit even greater length polymorphisms than the V3 region, and the V4 region, although relatively length invariant, is simply too long (>290 bp) for efficient amplification of aDNA. The V5 785F/907R primer pair performs well on a number of metrics: it has very good predicted taxonomic coverage and is relatively short (144-148 bp) with little amplicon length variation; however, it also contains relatively low information and resulting amplicons have only 57% and 64% of the OTU resolution as the V4 515F/806R and V3 U341F/534R primers, respectively. Perhaps the most promising alternative to the V3 U341F/534R primers is the V6 926F/1046R primer pair; however, it too has drawbacks. The V6 926F/1046R target is relatively short (152-167 bp) with only moderate sequence length variation, and it is predicted to produce amplicons with high taxonomic resolution. However, it has low taxonomic coverage for the bacterial candidate phyla TM7 and SR1, and it is not predicted to amplify the archaeal genus
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Methanobrevibacter. Hypervariable regions V7, V8, and V9, which are not analysed in this study, are rarely targeted for phylotyping because of their low information content and poorly conserved primer-binding sites. There are no truly universal 16S rRNA primers, and especially with respect to ancient DNA studies, all phylotyping approaches involving targeted PCR amplification are likely to be inefficient and consequently at high risk for taxonomic bias as a function of aDNA taphonomy. As such, this greatly limits the utility of this approach in geographic and temporal comparative studies (Adler et al. 2013), and even in studies focusing on a single place and time, as observed for the Middenbeemster samples in this study. The results of this study also have implications for other amplicon-based approaches, such as direct PCR or qPCR of species-specific targets. Species-specific targeted PCR assays have been developed for a number of oral taxa, especially pathogens, and have been used to detect the presence/absence and relative abundance of these bacteria in ancient dental calculus from different temporal periods spanning the Mesolithic to medieval periods (Adler et al. 2013; de La Fuente, Flores, and Moraga 2013). However, in each case, detection of these taxa depends on the successful amplification of relatively long PCR targets (114 to 179 bp), and thus it is intuitive that taxonomic dropout will increase in samples of higher thermal age simply because they are likely to be more fragmented. For example, the recent reported failure to detect *Streptococcus mutans* in Neolithic and Mesolithic samples compared to Bronze Age samples (Adler et al. 2013) cannot be attributed to a true biological absence of this species without first accounting for the possibility that the aDNA in these samples is more highly fragmented, resulting in PCR-dropout. Moreover, attempting to control for this factor through co-amplification of other taxa is problematic when the comparative taxon is (1) present at a higher relative frequency, and/or (2) when primer specificity for the comparative taxon is low, and thus non-specific amplification is possible.

Additional evidence that PCR dropout may skew temporal datasets is evident in a recent study of South American dental calculus samples (de La Fuente, Flores, and Moraga 2013). The age of the samples ranged from the recent past (ca. 1960-1970) to more than 4,000 years ago, and among the taxa targeted was *Streptococcus gordonii*. This taxon is a highly prevalent and abundant member of the dental plaque microbiome. In the Human Microbiome Project healthy cohort (Human Microbiome Project Consortium 2012), it is present in 100% of the 104 dental plaque samples, and it is characterised as a common inhabitant of the oral cavity with an observed relative abundance of 1.4% in the HOMD (Chen et al. 2010). Among the ancient South American dental calculus samples, more than half of the samples (22/38) yielded no amplification for any of the bacterial targets, and of those that did amplify at least one target, *S. gordonii* was observed in only 75% of the samples. As discussed by the authors of the study, DNA degradation has likely limited PCR amplification in this sample set. Thus, while the detection of an ancient microbe within a sample can be taken as evidence of its presence, the failure to detect an ancient microbe cannot be used to confirm its absence.

While the disadvantages of 16S rRNA V3 length polymorphisms in ancient microbial phylotyping are clear, there are also potential advantages. First, because modern microbial contaminants are expected to preferentially amplify over highly degraded and fragmented aDNA, analysis of V3 amplicons should provide a conservative method for qualitatively estimating the relative proportion of endogenous DNA sequences within an ancient microbial sample. Second, amplification bias towards ancient taxa with shorter V3 regions provides information about the approximate size of the amplifiable ancient DNA molecules in the sample. This information can be useful for planning downstream shotgun metagenome analyses, including both ancient sample selection and selection of appropriate sequencing chemistry.

It is clear that quantitative characterisation of oral microbiome taxa using either universal or species-specific targeted PCR is problematic for archaeological microbiome samples. Stochastic taxonomic skew resulting from differential PCR amplification is expected for degraded and highly fragmented aDNA in general, and non-random bias toward shorter amplicons is expected for PCR targets containing length polymorphisms. With respect to the 16S rRNA gene, we conclude that extensive length polymorphisms in the V3 region are an important cause of amplification dropout and taxonomic bias in ancient microbiome reconstructions based on this hypervariable region. When using the universal V3 U341F/534R primers, such reconstructions may contain archaeal frequencies in excess of 60%, a clearly non-biological pattern attributable to taphonomy. Such systematic amplification bias confounds attempts to accurately reconstruct microbiome taxonomic profiles from 16S rRNA gene amplicon data. Thus, amplicon phylotyping, the most commonly used method for comparative microbiome characterisation in living humans and primates, as well as microbial ecology studies in general, cannot be applied to ancient samples in a simple and straightforward manner.

Given the highly degraded and fragmented nature of aDNA and the associated difficulties o amplicon phylotyping, shotgun metagenomics presents a viable alternative for ancient microbiome characterisation. Shotgun metagenome sequencing is a powerful molecular approach made possible by massively parallel NGS that allows complex microbiomes to be comprehensively sampled and characterised for microbial structure and diversity. Unlike amplicon-based approaches, shotgun metagenomics is not compromised by short DNA fragment lengths, and in fact it operates with high efficiency on DNA templates of the size range typical of aDNA. Additionally, because it is a non-targeted approach, it is not affected by loci-specific length polymorphisms. While microbial community reconstruction from

shotgun metagenome data is robust to many of the taphonomic challenges posed by ancient DNA, it is susceptible to other known biases, including variations in GC content (Aird et al. 2011; Ross et al. 2013) and genome length (Beszteri et al. 2010; Nayfach and Pollard 2015). However, these biases are not specific to ancient DNA, but rather are inherent to current library construction and NGS sequencing methods. Thus, while shotgun metagenome sequencing provides a promising alternative to amplicon sequencing for ancient microbiome samples, one must continue to evaluate these sources of bias when interpreting metagenomic datasets.

In conclusion, while amplicon-based approaches may be useful for qualitatively assessing sample preservation and contamination, they are vulnerable to taphonomic artifacts and are not appropriate for the reconstruction of ancient microbial communities. Instead, we recommend generating community taxonomic data using shotgun metagenome sequencing for ancient microbiome characterisation.

Methods

Samples

Dental calculus for targeted sequencing was obtained from human skeletal remains (n=107) originating from: Middenbeemster, the Netherlands (n=76); Rupert's Valley, St. Helena (n=15); Anse à la Gourde, Guadeloupe (n=5); Lavoutte, St. Lucia (n=5); Tickhill, Yorkshire, UK (n=4); Samdzong, Nepal (n=1); and Camino del Molino, Spain (n=1). A subset of these samples (n=4) was also selected for non-targeted sequencing (Table 1). Dental calculus was sampled according to previously described methods (Warinner, Rodrigues, et al. 2014).

DNA extraction

All samples except those from Tickhill were extracted in a dedicated ancient DNA laboratory at the Laboratories of Molecular Anthropology and Microbiome Research (LMAMR) in Norman, Oklahoma, U.S.A. The Tickhill samples were extracted in a dedicated ancient DNA laboratory at the University of York. Both labs operate in accordance with established contamination control precautions and workflows. Non-template extraction controls and reagent blanks were processed in parallel to screen for modern contamination during laboratory procedures. The positive pressure Class 7 ancient DNA clean rooms are physically separated from all laboratories in which PCR is performed. Full body Tyvek suits, masks and gloves were worn to prevent contamination. Buffers and reagents were decontaminated using published protocols (Champlot et al. 2010). For all samples except Tickhill, DNA extraction was performed as described by Warinner and colleagues (Warinner, Rodrigues, et al. 2014) (Extraction Method A, preceded by EDTA decontamination). In brief, 10-20 mg of dental calculus were agitated in 1 mL 0.5M EDTA for 15 minutes to remove surface contaminants. The decontaminated dental calculus was then decalcified in a solution of 0.45M EDTA and 10% proteinase K (Qiagen, the Netherlands) at 55°C for 8-12 hours and then at room temperature for 5 days. Following centrifugation, the supernatant was extracted for DNA by phenol:chloroform:isoamyl alcohol (25:24:1) extraction. The extracted DNA was isolated by silica purification (Dabney et al. 2013) and quantified using a Qubit fluorometer. The Tickhill samples were extracted according to the protocol of Rohland and colleagues (Rohland, Siedel, and Hofreiter 2010) and also quantified using a Qubit fluorometer (Supplementary Table 1).

16S rRNA amplicon Illumina library preparation and sequencing

Approximately 5 ng of ancient DNA was used to build each targeted library at the LMAMR and at York. Samples, negative extraction controls, and reagent blanks were PCR-amplified using primer constructs containing the universal 16S rRNA V3 region U341F/534R primers

and Illumina adapter sequences. Golay barcodes were also included in each reverse primer to allow for sample pooling (Caporaso et al. 2012). Each PCR reaction contained 9.25 µl molecular grade water, 5 µl 5x Phusion buffer, 1 µL 2.5 mg/ml BSA, 2.5 µL 10 mM decontaminated dNTPs, 0.5 µl 10 µM primer 341F, 1.0 µl 10 µM primer 534 R, 0.25 µl Phusion Hot Start II DNA polymerase (2 U/µl) and 1.0 µl of DNA template (5 ng/µl) for a total volume of 20 µl. The temperature profile for the reactions included an initial activation of the enzyme at 98°C for 30 seconds, followed by 35 cycles of 98°C for 15 seconds, 52°C for 20 seconds, 72°C for 20 seconds, followed by a final 5-minute extension at 72°C**.** PCR products were then visualised on 2% agarose gel. For each sample, the PCR products of three successful amplifications were pooled. The pools were then combined and purified using a Qiagen MinElute column. The resulting pooled DNA was quantified using a NanoDrop spectrophotometer, and size selection of the amplicons was performed using a PippinPrep. Prior to sequencing, the amplicon size distribution and successful removal of dimer peaks was confirmed using a Bioanalyser High Sensitivity DNA assay. All libraries except for those from Tickhill were sequenced using Illumina MiSeq v2 2x150bp chemistry at the Yale Center for Genome Analysis. The Tickhill samples sequenced using Illumina2x250 bp chemistry at the Wellcome Trust Sanger Institute. Sequencing depths are provided in Supplementary Table 5.

Shotgun metagenomic Illumina library preparation and sequencing

Approximately 100 ng of ancient DNA was built into each Illumina shotgun library at the Center for GeoGenetics, Copenhagen, Denmark using the NEBNext DNA Library Prep Master Set (E6070) and blunt-end modified Illumina adapters (Meyer and Kircher 2010). The protocol followed the manufacturer's instructions with minor modifications. Nebulization was skipped. End-repair was performed in 50 µl reactions with 30 µl of DNA extract. The end-repair cocktail was incubated for 20 min at 12°C and 15 min at 37°C and purified using

Qiagen MinElute silica spin columns following the manufacturer's instructions and eluted in 30 µl. After end-repair, Illumina-specific adapters (Meyer and Kircher 2010) were ligated to end-repaired DNA in 50 µl reactions. The reaction was incubated for 15 min at 20°C and purified using Qiagen QiaQuick columns before elution in 30 µl EB. The adapter fill-in reaction was performed in a final volume of 50µl and incubated for 20 min at 37°C followed by 20 min at 80°C to inactivate the Bst polymerase. Libraries were amplified and indexed in a 50 µl PCR reaction, using 15 µl of library template, 25 µl of a 2x KAPA U+ master mix, 5.5 µl H2O, 1.5 µl DMSO, 1 µl BSA (20 mg/ml), and 1 µl each of a forward and reverse indexing primer (10 µM). Thermocycling conditions were 5 min at 98°C, followed by 10-12 cycles of 15 sec at 98°C, 20 sec at 60°C, and 20 sec at 72°C, and a final 1 min elongation step at 72°C. Amplified libraries were purified using Agencourt AMPure XP beads, and eluted in 30µl EB. The size distribution of the full Illumina-compatible construct was estimated using an Agilent Bioanalyser. Libraries were pooled in equimolar amounts sequenced using v2 2x100 bp chemistry on a single lane of the Illumina HiSeq 2000. Sequencing depths are provided in Supplementary Table 5. 16S rRNA reads represent 0.2% of total shotgun sequencing reads.

16S rRNA gene amplicon data analysis

Read pairs were quality filtered, trimmed, and merged to reconstruct the full V3 region using 'PEAR' (Zhang et al. 2014). Briefly, sequences with ambiguous bases ('N') were removed, and reads were quality filtered to remove bases with a Phred score <30. These merged read pairs were demultiplexed in QIIME, followed by closed-reference OTU assignment using 'uclust'(Edgar 2010). A sequence similarity threshold of 97% was used to assign reads to OTUs against the Greengenes 13.8 database (DeSantis et al. 2006) as a reference. The resulting OTU tables were not rarefied (in order to retain the low read count control samples), and summarised at the taxonomic levels of phylum and genus. Oral-associated genera were

determined based on presence or absence in the HOMD. Bayesian microbial source tracking was performed using SourceTracker (Knights et al. 2011), with the inclusion of three classes of soil, gut, skin and oral samples as sources. The published datasets used as sources in this analysis are provided in Supplementary Table 4. Read statistics are summarised in Supplementary Table 5. Data for Fig. 1 is available in Supplementary Data 1.

Shotgun metagenomic data analysis

Illumina adapters were removed from paired end reads using 'Cutadapt' (Martin 2011). Trimmed reads were then processed using 'Sickle' (Joshi and Fass 2011) to trim low quality bases (Phred score <30), remove reads shorter than 25bp, and remove reads with ambiguous bases. The resulting forward and reverse read pairs were reordered using custom Perl scripts. Read pairs were aligned locally (no soft clipping of ends) against the SILVA SSU 111 reference dataset using Bowtie 2 (Langmead and Salzberg 2012) (--no-unal --local). Resulting alignment files were processed using 'samtools' (Li et al. 2009), followed by Picard-tools to generate fragment-length statistics (default parameters). Additionally, reads mapping to the 16S rRNA gene were recovered from the alignment files, and assigned to OTUs following the closed-reference OTU protocol in QIIME v.1.8 using default settings and Greengenes 13.8 as the reference database, similar to the V3 amplicon dataset. Read statistics are summarised in Supplementary Table 5. In addition to 16S read analysis, genus level taxonomic summaries (Supplementary Table 6) were also generated from phylogenetically informative single copy marker loci as previously described (Sunagawa et al. 2013).

Ancient DNA damage profiles were explored by mapping shotgun metagenomic reads to reference genomes for *Streptococcus gordonii* (gram +ve) and *Lautropia mirabilis* (gram –ve), two common oral bacteria found among all the four ancient calculus samples. Read mapping

was performed using Bowtie2 (Langmead and Salzberg 2012) (--no-unal –local), followed by removal of PCR and optical duplicates using Picard-tools.

The resulting alignment file was used to generate fragment-length distributions (Supplementary Fig. 1) using Picard-tools, and to characterise DNA damage patterns using mapDamage (v2.0) (Jónsson et al. 2013) (Supplementary Fig. 2).

In silico analysis 16S rRNA gene V3 and V4 regions

16S rRNA secondary structures for *E. coli*, *M. oralis*, *C. diphtheria*, and *S. pyogenes* were retrieved from the Comparative RNA Web Site and Project (http://www.rna.icmb.utexas.edu). To investigate the V3 and V4 regions of the 16S rRNA gene, the SILVA SSU 111 database was queried using PrimerProspector v 1.0.146 and the primers U341F/534R (V3) and 515F/806R (V4), to retrieve the corresponding sequences. Length distribution statistics were generated for the extracted V3 and V4 region sequences using the R statistical package, with limits of length variation determined using 99% confidence intervals (to reduce impact of chimeras and mispriming). Sequence length distribution was also represented as density plots, generated in R. To ensure the multimodal distribution of V3 is not a feature unique to the SILVA SSU 111 database, *in silico* amplicons were also generated for the V3 region using the SILVA SSU 115, Greengenes 13.8, NCBI's 16S collection (last updated 7/30/2013), and the RDP 11.3 databases (Supplementary Fig. 3). Sequence length distribution was represented as density plots as described above. To further investigate V3 length variation at the genus level, we selected 31 representative oral genera from 9 major microbial phyla from the SILVA SSU 111 dataset and used the R statistical package to plot the length distribution of OTUs assigned to each genus in a heatmap. The genera are organised into a cladogram according to NCBI taxonomy. Log fold changes in observed frequency between V3 amplicon and shotgun

metagenome datasets from archaeological dental calculus samples was calculated for these genera.

Thermal age modelling

Thermal age modelling was performed using the JRA 1: PrediCtoR tool hosted on www.thermalage.edu. We used as our starting oral microbiome community HOMD frequency data for a select group of oral taxa provided in Table 2. Thermal ages were determined for the seven archaeological sites included in this study (Table 1) and applied to this model. Model parameters for estimating the probability of a nucleotide being damaged (λ) included archaeological site age, longitude, latitude, altitude, sediment type, and effective burial temperature. We calculated the probability of a DNA fragment of size x or greater being present as $e^{-\lambda x}$. We applied this probability to the frequency of each taxon in our starting community and predicted resulting taxon frequency for the seven thermal age λ values.

In silico analysis of primers

The position of the primer start and stop coordinates are reported relative to *Escherichia coli* (Genbank accession J01695). *In silico* amplicons for each primer pair were generated using PrimerProspector 1.0.1(Walters et al. 2011) and the SILVA SSU 111 (Quast et al. 2013) and Greengenes 13.8 (DeSantis et al. 2006) databases as references. The resulting amplicons were analysed for length variation, taxonomic resolution and taxonomic coverage. Sequence length variation was analysed using the R statistical package. Taxonomic resolution was inferred from the number of OTUs generated through *de novo* clustering of the amplicons at 97% sequence similarity using 'uclust' (Edgar 2010) as implemented in QIIME v.1.838 was used to analyse the taxonomic resolution. The number of OTUs generated from the amplicons was compared to the total number of OTUs generated from full-length 16S rRNA gene sequences

(138,462 OTUs). Taxonomic coverage was retrieved from the results of PrimerProspector (Walters et al. 2011).

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Author Contributions Statement

C.W., K.S., and H.S. designed the research. K.Z., A.O., J.H., C.S., and H.S. performed the experiments. K.Z., A.M., K.S., B.B., E.Z., G.T., S.M., and C.W. analysed the data. C.H., M.H., A.W.R., D.W., D.C.S.G, M.A., M.J.C., and C.M.L. provided materials and resources.

K.Z., C.W., A.M., and K.S. wrote the manuscript text, with input and review from the other co-authors.

Additional Information

Accession codes: Genetic data have been deposited in the NCBI Short Read Archive (SRA) under the project accession PRJNA278036. Sample accession IDs are provided in Supplementary Table 5.

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