Processes of Industrialisation Influencing the Human Oral Microbiome

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Contents

Abstract

Oral disease affects an estimated half of all people globally—the most common of any noncommunicable disease to be contracted throughout an individual's lifetime. Yet, despite numerous technological and scientific developments of the past century, the prevalence of oral disease continues to increase alongside urbanisation and industrialised lifestyles; a major public health problem marked with inequalities and ethnic disparities. Critically, oral health is a key indicator of overall systemic health, and thus, the study of the human oral microbial communities has tangible outcomes that can improve—both oral and systemic—health and well-being.

Evidence supports a mutually beneficial relationship between humans and their microbiome (*i.e.* the microorganisms and their genomic content, living on and within the human body), evident by the reliance of human physiological function upon the synergistic interactions with their microbes. Most oral diseases typically stem from a 'microbial imbalance', where the disruption of oral microbial ecology no longer supports a symbiotic or mutually cooperating microbiome optimal for human health. In this thesis, I investigate, inform, and improve upon our understanding of the human oral microbiome. I focus predominately on the processes of industrialisation—principally, the consequential alterations to human sociocultural and environmental factors—that are known to influence the microbiome and augment oral disease risk, and by extension, impact human systemic health.

Within this thesis, I synthesise our current understanding of and the research pertaining to the human microbiome, advocating for the inclusion of human-microbiome co-evolutionary history within public health and biomedical research. This is especially important regarding the health inequalities impacting Indigenous populations globally, wherein evolutionary life history may underscore contemporary population health. Inclusivity of Indigenous populations within human microbiome research is needed in order to better understand the influence of industrial processes upon the microbiome, especially in regard to human health and disease. I analyse the salivary microbial community of Aboriginal Australian and Torres Strait Islander children, one of the first studies to investigate whole oral community changes in response to oral health treatments. Finally, I sought to examine the historical impact of Industrial Revolution on the European oral microbiome, using novel paleomicrobio-

logical methods that grant access to the preserved microbial communities of calcified dental plaque (calculus). By analysing the methodological bias of taphonomy—the biochemical processes of fossilisation—upon calculus microbiomes, I was able to illuminate the ecological alterations of the human oral microbiome, consequent of 200 years of industrial development, that has cultivated the contemporary European oral microbial composition.

My thesis contributes to oral health research by providing context and perspective of evolutionary medicine, with the application of evolutionary history, to oral microbiome research to the realm of contemporary public health. Further, I identify promising and prospective areas for future oral and systemic health research through the investigation of historical Industrialisation and its impacts on the human oral microbiome. The genomic understanding of past and present microbial ecological communities can offer more precise inferences of prevailing sociocultural and environmental forces regarding the risks, contributions, and development of oral disease. I hope, in the endeavour to progress our understanding of the human oral microbiome, this work furthers innovation and technical understanding to improve global population health.

Signed Statement

I, Emily Skelly, certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Introduction

The human microbiome and the evolutionary history of human-associated microorganisms

The rapid expansion of research into human microbiota—the communities of microorganisms that live on and within the human body—of the 21st century has dramatically changed how we define and comprehend human health, disease, and the interconnection of human biology and the environment. From the acquisition of the microbes moments after birth, the establishment of our microbial community during the post-natal period is essential for the correct morphological and functional development of the human immune system [1]. The contribution of gut microbiota and their derived compounds (nutrients or metabolites) is critical for immune system signalling, epithelium homoeostasis, and developmental cell programming [2, 3]. Moreover, microbial signalling is vital in the regulation of energy homoeostasis, fermentation, metabolism, and nutrient utilisation [4, 5]. Studies also implicate the microbiota in signalling mechanisms crucial for normal brain development and subsequent behavioural functions [6, 7], and have found links between the gut microbiota to mental illness and neuro-degenerative disorders, through gastrointestinal-brain communication (commonly referred to as the gut-brain axis) [8]. Overall, the fundamental functionality of the human microbiota underline the importance of these microbial communities within the host systemic health and disease.

Until the development of molecular tools, microbiome research was limited to the minority of bacterial taxa that could be grown within a laboratory (*i.e.* cultured). Even today, research typically focuses on the bacterial communities that dominate the microbial consortium [9, 10]. Much of what we understand about human microbiota is owed to both the advances in and cost-reduction of high-throughput sequencing technology. However, the significance of microbiota within the realm of human health was first deliberated in the 1960s by microbiologist René J. Dubos (1901–1982) [11]. Dubos' experimental research observed the interactions between microbiota and lifestyle factors (such as nutrition, social interactions, and stress) in germ-free and specific-pathogen-free mice [12]; perceptivity beyond the contemporary thinking of his time. Dubos spent much of his later career discussing interconnections of anthropological and biopsychosocial variables, which often focused on the epidemiological impacts of technologically-focused and environmentally-disconnected lifestyles [11]. Today, this disconnect is commonly discussed with respect to human and microbial co-evolutionary relationships.

Our understanding of the co-evolutionary relationship between microbes and

humans is still in its infancy, but there is an inferred understanding that the dependence of human physiology upon their microbiota implies a long co-evolutionary history [13, 14, 15]. Understanding the processes that lead to microorganisms evolving and adapting to a human host, or insights into the evolution of human biological dependence upon microbial functionality, has the capacity to improve human health with conceivable microbiota-assisted medical treatments, personalized therapies, or disease-preventative medication. The first step towards this future potential is a broadening our comprehension of the evolutionary history between human and microbial co-adaption, and those factors that have contributed to such co-dependence.

The current state of the human microbiome—the amalgamation of the ecosystem, with all microorganisms and their genetic material, present within the defined environment—is largely considered beneficial, until the microbial community is altered [16]. Changes to microbial ecology can affect the functionality of the microbiome, and equally that, loss of necessary functional properties can induce changes to the microbial composition, and these changes can induce disease. The gut microbiome, for example, has been linked to a variety of chronic conditions, such as obesity [17, 18], irritable bowel syndrome [19], inflammatory bowel disease [20, 21], colon cancer [22], rheumatoid arthritis [23], and surprisingly, even associated with mental health disorders, such as depression and schizophrenia [24, 25]. The complexity of microbial interactions with human health makes it difficult to unravel causality, but the breadth of such interconnections suggest microbial relevance in physiological disease susceptibility [26]. This susceptibility is linked to the rapid cultural and technological developments of contemporary industrialised human lifestyles (by which the definition is still Eurocentric), for which many factors are at odds with the long evolutionary history shared between humans and their microbiota [16, 27].

Numerous hypotheses have been brought forth by epidemiologists, medical doctors, microbiologists, anthropologists, and even ecologists, to explain the link between human-microbe co-evolution and so-called 'Western diseases' (*i.e.* chronic non-communicable diseases associated with industrial development [28]). The famed 'hygiene hypothesis' first arose in 1989, proposing the increased hygiene practices, smaller family sizes, and antibiotic usage were limiting the early-life exposure to environmental microbes needed to help build a comprehensive immune system, causing the increased prevalence of allergies [29]. Similarly, the 'old friends hypothesis' argues that limited contact with the environment has promoted a loss of commensal microorganisms ('old friends') required for the induction and regulation of mucosal immunity [30]. More specifically, the 'diet hypothesis' condemns the industrialisation of food production and consumption; increased food processing and the reduced consumption of fibrous plant products is leading to a deficiency in microbial by-products (from the fermentation of these fibres), which are essential for numer-

ous gut metabolic functions and gastrointestinal maintenance [5]. Notably, all these hypotheses share a common thread of thought; the recent transition to the industrialised lifestyle has induced a loss of commensal microorganisms (*i.e.* a unidirectional relationship where organism benefits and the other is neutrally impacted). This loss has structured a microbiome (whenever due to hygiene practices, reduced environmental contact, or dietary-caused extinction) that is discordant with the evolutionarily physiological co-dependence. In order to understand how and why commensal microorganisms have been lost, it is essential to study the temporal relationship between humans and microbes to identify those evolutionary drivers that shape and define the microbiome.

The study of the evolutionary history of human microbiomes has adhered to primarily to two methods. The first—the phylogenetic approach—uses bioinformatic techniques to track protein-coding regions of bacterial genomes, assessing diversification and congruence between humans and microbiota phylogeny [15]. This technique has been used to identify changes in microbial diversity from the divergence of apes and human ancestors, through hominid evolution and cospeciation of microorganisms with human physiology [31]. While this phylogenetic estimate provides evidence for reduced microbial diversity through human evolutionary history, it does not provide details of this ancestral microbiome composition. The second, and more informative, approach uses ancient DNA to reconstruct the microbial communities preserved in ancient samples. This method is able to produce real-time snapshots of the ancestral microbiome, allowing for direct comparisons between the human microbiome composition and function throughout evolutionary history.

DNA from dental calculus reconstructs the ancient oral microbiome

Ancient DNA (aDNA) is genomic material extracted from archaeological or palaeontological remains, which can be recovered from a broad range of materials, such as mummified tissues, preserved medical or museum specimens, bones, seeds, hair, and even ice or permafrost cores [32, 33]. The study of aDNA presents many difficulties and challenges, stemming from the continuous deterioration of DNA molecules following an organism's death; post-mortem modification can destabilise, degrade, and destroy DNA structures [34, 32]. The preservation of aDNA overtime is therefore highly dependent upon the environment in which it was deposited; DNA biochemistry favours low temperatures, dry localised climates, and high salt concentrations [33]. Even with optimum conditions, difficulties in survival translate to difficulties in aDNA retrieval; DNA degradation, caused by spontaneous hydrolysis and oxidation,

is characterised by breakages in the sugar-phosphate backbone, nucleotide modifications, baseless sites, and intermolecular cross-linkages [35]. Such DNA damage patterns will interfere with PCR amplification, blocking DNA polymerase or causing incorrect nucleotide insertions [34]. Technical difficulties and DNA damage make ancient DNA research extremely vulnerable false positives from exogenous contamination from the environment, humans, reagents, and PCR reactions [32, 33]. Contamination is the unwanted DNA molecules from external sources (*e.g.* laboratory reagents, modern human DNA, or even other samples in the processing batch) that can confound the sample source DNA [36]. Accordingly, aDNA research requires thorough authentication. Cooper and Poinar (2000) noted "criteria of authenticity" to validate good research practice within the aDNA field [37]. Now, nearly 20 years after Cooper and Poinar's publication, authentication is commonly dependent upon computational verification of 'true' aDNA by studying the damage patterns within high-throughput sequencing reads. For example, mapDamage2.0 program quantifies estimates of damage parameters expected of aDNA sequences by statistical modelling of the expected deamination patterns [38]. Despite the difficulties, aDNA remains advantageous in providing insight into the coevolution of microorganisms and humans. The ability to sample discrete temporal and spatial locations, throughout historical cultural, social, and environmental transitions provides direct biological evidence of past microbial ecology in 'real time'.

From the detection of the pathogenic microorganism *Mycobacterium tuberculosis* back in the 1990s using PCR techniques from ancient skeletal remains, the study of ancient microorganisms—paleomicrobiology—flourished with the development of molecular techniques [39]. The field of paleomicrobiology chiefly looks to resolve diagnostics, epidemiology, and the evolution of past pathogens using targeted sequencing. But with the advent of high-throughput sequencing technology granting access to ancient microbial communities, paleomicrobiology now encompasses the study of ancient microbiomes. In the examination of ancient human microbiota, several different samples have been utilised, including mummified human remains [40], historical medical specimens [41], and microbial deposits in bone (derived from seepage from decomposition) [42]. The difficulties of contamination and aDNA authenticity are twofold in the realm of microbiome metagenomics. Contamination with modern microbial DNA can inundate the endogenous damaged and fragmented aDNA, with sequencing technology unable to discriminate between modern or ancient DNA. Reagent and laboratory contamination notably impacts contemporary microbiome analyses [43, 44], but this becomes especially problematic in aDNA research wherein ancient samples are low in both biomass and abundance. Much like the criteria of aDNA research, there are a number of recommended practices in extracting ancient microbiota from samples: importantly, specialised clean laboratories and practices,

positive and negative sampling controls, and downstream contamination analyses [45]. Computational analyses are often used to track contaminant DNA from exogenous sources and cross-contamination (unintentional sample-to-sample transfer during labortory processing), and assess the level and types of contaminants. For example, the Bayesian model SourceTracker estimates the proportion and origin of contamination based on sequenced biological samples and their respective laboratory controls [46]. The more recently developed R package decontam calculates the statistical probability of whether a microorganism is contaminant based on its prevalence within laboratory controls versus biological samples [47]. These approaches, both in the laboratory and bioinformatically, offer a means to minimise and distinguish exogenous contamination from the ancient microbial sample of interest. However, the burden of contamination can also depend upon the type of sample from which microbial aDNA is extracted from.

Within paleomicrobiology, there are two prevailing sources of ancient microbiome samples that are considered analogous to living human microbiota: fossilised faecal material (coprolites) representative of the gut microbiome, or calcified dental plaque (also known as tartar, or dental calculus) that depicts the oral microbiome. Coprolites maintain biological information of the ancestral gut microbiome [48, 49]. Analysis of the gut microbiome can provide direct evidence of dietary information—what was eaten and how it was consumed—in addition to representing broader dietary lifestyles, where certain behaviours drive the composition and structure of the gut microbiome [50, 51]. Coprolites can also be a good indicator of gastrointestinal health, symptomatic of digestion and metabolic capability, both from dietary input and microbial taxonomic and gene composition [51, 49]. However, there are number of disadvantages to using coprolites for ancient microbiome research. Firstly, faeces are highly biologically active and normally begin to rapidly decompose after deposition, making the immediate environment critical for biomolecular survival [52]. Furthermore, even if the environmental conditions happen to be optimal for faecal fossilisation, coprolites are customarily found in communal latrine areas or middens (*i.e.* rubbish pits) dissociated from any specific individual, and necessitating any recovered microbial information to be interpreted on a host-population level [49]. Finally, as an open system, gastrointestinal contents or excreted faecal matter are incredibly susceptible to environmental microbes, creating difficulties in distinguishing environmental contamination from true biological signal [48]. Many of the issues regarding coprolite prevalence, survival, and contamination are reasons why dental calculus is a superior source of ancient microbiomes.

Human dental calculus is formed by the presence of calcium and phosphate salt in saliva depositing into dental plaque, which mineralises the plaque into a cement-like (both in terms of physical hardness and adherence strength) form [53]. Microor-

ganisms that survive on the surface of teeth use specific cell-to-cell recognition and adherence partnerships to bind both to the tooth surface and to one another, forming an extracellular matrix known as a biofilm, or dental plaque [54, 55]. As the human oral cavity is a gateway into the human body, this open system—and the microorganisms that inhabit it—are continuously exposed to exogenous microorganisms and environmental compounds. The mineralisation processes of dental plaque occur throughout an individual's lifetime, such that dental calculus not only fossilises the oral microbial community but can trap food particles or transient microorganisms (such as bacterial pathogens, viruses or fungi species), potentially providing evidence of individual pathological data and personal dietary information [49, 56]. The mineralised structure and formation of calculus safeguards the endogenous microbiome from the contamination pitfalls experienced by coprolites. The calcified matrix protects microbial aDNA from external contamination even after host decomposition situated in a contaminate-filled, post-mortem environment [57]. Additionally, the presence of dental calculus in the archaeological record is far more abundant than coprolites, as calculus is ubiquitous in all post-agricultural societies, and documented within the hominid archaeological record as far back as *Australopithecus* species, two million years ago [58, 59]. More often than not, dental calculus is found attached to human remains, thus has the advantage of providing additional anthropological data to the study of the ancient oral microbiome [60].

The oral microbiome preserved within dental calculus is a small representation of the diverse community of microorganisms inhabiting the human mouth. The physiochemical properties of the oral cavity drive compositional differences of the microbial communities inhabiting different niches, in which the tongue, teeth, and different tissue surfaces (mucosa, palate, and gingiva) all harbour distinctive microbial communities [61]. Despite the oral cavity's external interactions with the environment, the microbiota of the mouth is one of the more conserved microbial communities across the human body (both within and between individuals), with relative ecological stability over time [62]. It has been suggested that this homogenous conservation is likely due to the transient availability of food, limiting the dietary influence on the microbial community (with the exception of dietary sugars). Saliva and gingival crevicular fluid (*i.e.* serum exudate carrying an immune response for the prevention of tissue inflammation to oral bacteria) are therefore considered the primary nutrient source for oral microorganisms [63, 64]. This relationship advocates for the tight interconnection between the oral microbiome composition to systemic health, to which the immune system and salivary biochemical elements are intrinsically linked. The relationship is recapitulated in the association between dental health and systemic health; poor oral health has been linked to cardiovascular disease [65], type 2 diabetes mellitus [66], and inflammatory disorders, such as osteoporosis and

rheumatoid arthritis [67, 68].

Oral disease is largely caused by the breakdown of microbial homeostatic mechanisms, altering the microbial community composition in a manner that is detrimental for oral health [69]. For example, dental decay is consequent of an ecological imbalance in the plaque microbial community, with increased consumption of simple carbohydrate sugars, shifting the ecosystem towards a more aciduric and acidogenic functionality [70]. Those microorganisms that can rapidly metabolise dietary sugars into acid and thrive in acidic conditions will out-compete acid-sensitive microbiota [69]. Yet, much of our current understanding of oral health and disease is based on culturing work, omitting the substantial proportion of bacterial taxa that cannot be cultivated, let alone, the viral, archaeal, fungal components. This produces a distorted comprehension of microbial composition associated with oral disease, misguiding development of oral therapies to erroneously target singular 'pathogenic' species, without anticipating the repercussions of the ecological impact [71, 72]. This is particularly problematic in that half of the global human population is currently affected by oral disease [73]. Thus a key component in addressing oral health and disease is by gaining knowledge of the ecological community as a whole, investigating the ecological interactions and understanding the alterations to the ecosystem [74]. By studying the ancient human oral microbiome through dental calculus, I look to advance our understanding of such evolutionary forces on the ecology of the oral microbiome and the evolution of microbial stasis.

Despite its relative novelty, research into the ancient microbiome using dental calculus has already contributed to a greater understanding of the human microbiome evolutionary history, and by proxy, the health and environmental experience of the host, with an unprecedented level of detail. One of the first publications to use dental calculus found evidence that the agricultural revolution—the successive dietary transition from hunting-and-gathering lifestyles to farming—impacted the composition the human oral microbiome [75]. The authors argued that this transition to an agricultural-based lifestyle, lead to an increased abundance of disease-associated microorganisms [75]. Moreover, the perpetuation of this lifestyle saw a significant increase in the abundance of dental decay-associated (*i.e.* acidogenic and aciduric) microorganisms post-agriculture, observed from the European medieval period (900–1600CE) into contemporary populations [75]. Discernibly, the contemporary human oral microbiome appears less diverse than our ancestors' communities, an ecological predicament associated with low resilience and productivity [76, 77]. In fact, against the evidence of oral microbiota's dependence upon salivary proteins, metagenomic analyses of ancient hominid *Homo neanderthalensis* (Neanderthal) dental calculus revealed distinct microbial composition according to meat-eating behaviour [78]. Presumed low-meat eating or meat-free diets of Neanderthals constructed a mi-

crobial composition resembling that of the wild forager-gathering chimpanzee oral microbiome, whereas putative meat-eating individuals had a microbiome relative to ancient hunter-gatherer *Homo sapiens* [78]. Both groups were ecologically distinct from ancient agriculturalists or contemporary populations [78]. However, what these longitudinal transects do not give credence to are the cultural and environmental changes that occur in parallel with dietary transitions. Using dental calculus collected from individuals living in the city of London, England, from the medieval to post-medieval period (1066–1853 CE), Farrer *et al.* (2018) was able to detect significant links to microbiota structure and systemic health (inferred from osteological evidence) that likely reflect the physiological impact of socioeconomic status upon an individual's microbiome [79]. These ancient metagenomic studies have provides insights into past cultural, environmental, and social changes throughout the evolutionary history of the human microbiome, associating microbial compositional changes with human health, and embodying what little is known about the origins and evolutionary history of the human oral microbiome.

The processes of Industrialisation

To enrich our comprehension of oral microbial evolutionary history, conducive to the understanding of contemporary oral health and disease, it is perhaps more valuable to assess recent evolutionary alterations. The Industrial Revolution was arguably the greatest—and most recent—cultural, environmental, and social change to occur in human history. Originating in Great Britain at the beginning in the 18^{th} century, the Industrial Revolution epoch is defined by the rapid technological transition to automated manufacturing of everyday common products, which were previously made by human hands [80]. The ensuing process of industrialisation encompasses the social consequences following such economic development, such as urbanisation or progressive social services, and thus, the term 'industrialisation' embodies both economic progress and the subsequent social change [81]. These new methods of production—and the novel commodities developed alongside them—transformed industrial structures, led to the development of new social classes, advanced processes of transportation, migration and urbanisation, and the inadvertently and irreversibly altering the Earth's environment [80]. Together, these changes culminate to the contemporary industrialised societies that we associate with increased noncommunicable, chronic diseases rates and detrimental alterations to the human microbiome.

Within this thesis, I focus on three main components hypothesised to have the greatest impact upon the human microbiome, evident by contemporary research: diet, culture, and environment. While this obvious simplification of entwined com-

ponents may understate the complexity of relationships between socio-cultural and physiological changes, this compartmentalisation facilitates investigations of microbial change associated with industrialisation. The underlying principles of consequential microbial alterations with diet, culture, and the environment is discussed in detail within **Chapter 1**; nonetheless, it is important to reiterate that much of what is theorised to change the human microbiome is based on contemporary research and understanding. Microbiome research has shown that not only an individual's genetics and biology (including physical age and biological sex) [10, 82, 83] but their associated lifestyle behaviours can impact their microbial composition; factors such as physical activity [84], social interaction [85], varying ratios of dietary macronutrients [50, 86], or sleep patterns [87]. The potential for these lifestyle factors to contribute to the composition and structure of the microbiome indicates that human behaviours and interactions play an important role in shaping the functionality of the microbiome [88]. Therefore, individuals experiencing the greatest lifestyle alterations—dietary, culturally, or environmentally—during and throughout the Industrial Revolution, theoretically would have experienced the greatest ensuing microbial alterations.

In the late 17$^{\text{th}}$ century, the increased sophistication of agricultural techniques and subsequent greater food production instigated a population boom, seemingly the trigger to the processes leading up to the Industrial Revolution [80]. Up until then, most pre-Industrial Europeans were primarily consuming, and wholly dependent upon cereal crops, mainly in the form of barley, rye, oats, and wheat [89]. But with industrialisation, mechanisation of transportation (increasing trade capability) and agricultural production supplied markets with affordable, formerly luxury food goods, such as animal proteins, fruits, and vegetables [89, 80]. The increased dispersal of wealth among the masses, which provided access to previously limited dietary goods, was driven by the increasing number of workshops, factories, mills, and mines requiring a ready supply of mobile and cheap labour [80]. Consequently, the peasantry class was no longer tied to a manorial system—which forced dependency of a peasant to their land or to their lord who owned land—and sought employment wherever they could find it [80]. This distribution of wealth was fundamental in the cultural changes of industrialisation, with the establishment a new socioeconomic division, the middle class, caused by subsequent differing of lifestyles between employers and the workforce [90]. Socioeconomic class dictated access to resources, convoluted the gendered division of labour, and mandated behaviour, social interactions, and living standards [91, 80, 90].

While contact with a circumscribed environment was governed by social class, the processes of industrialisation also radically transformed this environment. Factories and workshops were built in cities for the advantage of accessible transportation

and a higher concentration of people, precipitating the massive migration from rural to urban areas [80]. Rapid urbanisation was often outpaced by the volume of rural migrants, and housing conditions were overcrowded, poorly constructed, and lacked basic sanitary facilities [90]. In early industrialised cities, the lack of sanitation extended into the public sphere, with garbage and bodily waste discarded in the streets with inadequate sewage and waste facilities [80]. Industrial working conditions were no better; manufacturing processes produced noxious or toxic by-products and chemicals polluted the air, with little thought given to ventilation, let alone to human health [90]. It was not just rapid urbanisation that impacted the environment; industrialisation inflicted irreversible damage to the planet with the extraction of elements from the earth (particularly coal and iron ore, the two main components of industry) and industrial outputs of heavy metals began polluting the air and water systems [80]. Industrialisation was a turning point in the human relationship with the environment, a legacy that endured beyond the Industrial Revolution era [80].

From an ecological perspective, we understand that all these changes, from diversification of nutrients to environmental contamination, would have induced changes to the human microbiome through the alteration of ecological pressures upon the physiological niche [14]. For example, reduced dietary fibre consumption within murine models induced progressive loss in gut microbial diversity, correlating with a loss of function potential of enzymatic degradation of complex carbohydrates, in that the host-driven alterations effectively selected for microbial community with the greatest low-fibre metabolic potential [92, 93]. Conceivably, there was no greater alteration than what was experienced by individuals during 'The Age of Imperialism'; from around 1760s, Europe began the process of annexing, influencing, and colonising other countries around the globe [94, 95]. While the discovery of new lands and the formation of colonies began long before the Industrial Revolution, it was the industrial production and economic growth that increasingly drove imperialism and colonialism for the acquisition and control of resources [96, 95]. Within this thesis, I define colonialism and imperialism as forms of intergroup domination, in which a culturally heterogeneous group exerts power over another, culturally differentiated, society [94]. However, the crucial difference between colonialism and imperialism is the presence or absence, respectively, of permanent settlers migrating from the dominating power to the colony [94]. For example, Australia and New Zealand were colonised, in that a number of Europeans migrated from European countries to these colonies, whereas countries such as India, Zimbabwe (Rhodesia), and Hong Kong Island of China, were imperialised, *i.e.* dominated but not extensively settled by European migrants [94]. Colonies across the world were created and settled for different political and economic reasons, inviting different socioeconomic resettle-

ments, formed under distinctly different moral, cultural, and legal circumstances [97]. Relating to our three overarching components of microbial alterations, culture and diet in the colonies deferred to and was defined by the environment. Geographic differences of new countries and continents not only meant contact with an entirely new consortium of microorganisms [98], but the adaptation of culture and diet to the available environmental resources dictated alterations to the environmental factors that originally constructed their microbiome [99, 97, 100].

However, the process of industrialisation throughout the 18th and 19th centuries did not end with the Industrial Revolution; the final stages of industrial advancement—known as the 'Great Acceleration'—began in the mid-20th century, after World War II [101]. This post-1950s epoch is characterised by the very rapid population growth, alongside intense resource consumption, energy use and pollutant output [101]. Subsequently causing global changes in biodiversity, methanogenesis, carbon dioxide production, oceanic acidity, climate, and nutrient cycles [101]. These ecological disturbances are not felt solely at macroecological-levels; increased outputs of heavy metal pollutants and waste chemicals will have long-term consequences upon environmental microbial communities, likely impacting human microbial development [102]. Much of this ecological damage inflicted upon the Earth is linked to the increased urbanisation; more than half of the Earth's estimated 7.5 billion people live in cities, a dramatic increase from the five per cent of the 700 million populace in pre-industrial 1750 [103]. However, unlike the urban hubs of the early industrial era, public health moved to the forefront of city structure and design during the early 20th century, with the establishment of sanitation, sewage systems, clean water, and improved housing [103].

Parallel to improvements in structural public health, the corresponding medical advances radicalised hygiene, childbirth, and antibiotic treatments. For example, surgical developments and decreased maternal mortality rate lead to a dramatic increase in caesarean births, which were rare prior to the 1950s, and are now as common as one in three birthing events [102]. The practice of caesarean births has been shown to impact the maturation and inheritance of the human microbiome, suspected to influence later-life disease risk and susceptibility [104, 105]. Equally so, the adoption of artificial baby formula, another practice which was objectively non-existent prior to the 18th century, has shown to stimulate changes in the infant gut microbiome [106, 107]. The medical and scientific advancements of the late Industrial era pushed human disease ecology into the "second epidemiological transition"; dominance of acute, chronic, and noncommunicable disease [108]. While this epidemiological disease model links hygiene and antibiotics to a loss of microbial diversity associated with an increased rate of acute and chronic diseases (*e.g.* the aforementioned 'hygiene hypothesis'), this model also incorporates the industriali-

sation and commercialisation of food [108, 109].

Diets of the Great Acceleration skewed the balance of three major macronutrients—carbohydrates, fats, and proteins—as behaviour concerning food and food preparation changed with urbanisation [110]. Urban populations are entirely dependent upon imported resources, raising the demand for surplus food production and precipitating the integration of national and international levels of food distribution, and subsequently a need for durability (*e.g.* processing or added preservatives) through transportation [103, 110]. For instance, sugar consumption and the periodic problems in producing regions drove breakthrough manufacturing techniques for the exploitation of cheap food surplus, leading to the production of high-fructose corn syrup in the 1970s [111]. Within 30 years, high-fructose corn syrup consumption rose to a high of 64.8 lbs (29.4 kg) per capita in the United States alone, engendering alterations to the gut microbiome associated with inflammation and metabolic disorders [112, 113]. Urban and technological developments from the onset of the Great Acceleration have driven the adaptations and changes in both human behaviour and environments that are linked to our altered microbial composition and functionality [16]. However, contemporary analyses isolating specific factors are confounded by many concurrent factors of industrialisation and their subsequent historical microbial alterations; research is missing a definitive understanding of prior microbial compositions and how they were transformed. Understanding our microbial heritage positions medical and public health researchers to better grasp the evolutionary dissociation between human hosts and their microbiota in order to substantiate preventative measures and medical treatments for microbiome-associated diseases [26].

Thesis overview

In this thesis, I explore the use of the oral microbiome and dental calculus to gain greater insights into the impact of industrialisation processes upon human microbiome and systemic health. The sociocultural and environmental alterations of the past 200 years of human evolutionary history have transformed the relationship between human hosts and their microbial symbionts in a manner that it is now hypothesised to have adversely affected human health. Nevertheless, the processes of industrialisation are still occurring to this day, globally, at different rates, in different populations, with different outcomes. Thus, a greater understanding of these historical those sociocultural and environmental changes were in the past, and what their consequential impacts were on the human microbiome, has pertinent application in contemporary public health. Together, the following manuscripts work towards the conceptualisation of the human microbiome in the realms of public health and

dental research, by reconstructing the oral microbial ecologies within contemporary and historic microbiomes of individuals undergoing and/or experiencing industrial processes.

Chapter 1: *Consequences of Colonialism: A microbial perspective to contemporary Indigenous health*

Within this paper, I hypothesise the historical impact of European colonialism—specifically focusing upon the three overarching components of diet, culture, and environment—altered the microbiome of the Indigenous population, with repercussions contributing to contemporary Indigenous health. Colonialism was one of the most antagonistic alterations that occurred throughout the Industrial Revolution, yet for some Indigenous populations, these changes were often experienced rapidly, over a shorter period, and with greater intensity, than that of the colonial settlers. Incorporating microbial evolutionary history into our understanding of human health proposes an explanation for the additional 'unknown' risk factor that contributes to the health disparity between Indigenous and non-Indigenous populations.

Chapter 2: *Incorporating microbial evolutionary history into Indigenous public health*

This opinion piece is a concise reiteration of **Chapter 1**, aimed at dissemination among medical professionals and public health researchers. **Chapter 2** emphasises the importance of microbial evolutionary history as an important consideration for understanding the human microbiome in contemporary health research, especially in regard to efforts in closing the global health disparity between Indigenous populations and their non-Indigenous counterparts.

Chapter 3: *Salivary Microbiome Response to Caries Preventative Treatment in Australian Indigenous Children*

One of the failings of contemporary microbiome research is the lack of consideration, and accordingly, the lack of understanding, of ethnic and geographic population differences within the human microbiome. This ascertainment bias (wherein industrialised European populations dominate microbiome investigations) is not only detrimental to understanding past and present microbial relationships but moreover limits the understanding of microbial contributions to human health and disease. In this research chapter, I investigate the salivary microbiota of Indigenous Australian children undergoing a novel oral health treatment for dental decay. This treatment

was designed and tested based on dental research from industrialised individuals of predominantly European descent. By investigating the impact of industrialised medicine upon non-European populations, I endeavour to improve our comprehension of oral health treatments upon oral microbiota ecology.

Chapter 4: *Impacts of Storage Methods Over Time on Reconstructing Dental Calculus Microbial Communities*

In the emerging field of paleo-microbiome research, dental calculus is the superior material in the investigation of ancient and historic human oral microbiota and their evolutionary changes through time. However, what has been ignored up until now is the impact of decay and preservation processes (*i.e.* taphonomy) upon microbial communities stored within dental calculus. In **Chapter 4**, I assess the impact of two long-term storage conditions on the oral microbiome reconstructed from dental calculus samples. This research contributes to a greater understanding of the taphonomic processes within dental calculus, illuminating the potential biases in the reconstruction of ancient oral microbiomes.

Chapter 5: *Ancient DNA from dental calculus tracks microbial changes with the Industrial Revolution*

In one of the largest dental calculus meta-analyses to date, I investigate the historical changes to the oral microbiome throughout the Industrial Revolution and the Great Acceleration. Through the reconstruction of the oral microbiome of multiple European individuals across varying geographic locations, I am able to ascertain particular sociocultural changes that contribute to the differences in the oral microbial community through time. With the incorporation of expected taphonomic biases revealed in **Chapter 4**, I am able to extricate the evolutionary history of the European oral microbiome, identifying the compositional changes between historic populations and their contemporary counterparts.

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Chapter 1

Consequences of colonialism: A microbial perspective to contemporary Indigenous health

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THEORY AND SYNTHESIS

Consequences of colonialism: A microbial perspective to contemporary Indigenous health

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Nearly all Indigenous populations today suffer from worse health than their non-Indigenous counterparts, and despite interventions against known factors, this health "gap" has not improved. The human microbiome—the beneficial, diverse microbial communities that live on and within the human body—is a crucial component in developing and maintaining normal physiological health. Disrupting this ecosystem has repercussions for microbial functionality, and thus, human health. In this article, we propose that modern-day Indigenous population health may suffer from disrupted microbial ecosystems as a consequence of historical colonialism. Colonialism may have interrupted the established relationships between the environment, traditional lifeways, and microbiomes, altering the Indigenous microbiome with detrimental health consequences.

KEYWORDS

dysbiosis, Indigenous peoples, microbiome, public health, social-cultural change

1 | INTRODUCTION

The development of cheap and fast high-throughput sequencing techniques has illuminated the many roles the human microbiota performs in human health. The term "microbiota" refers to microorganisms inhabiting a specific environment; these microbes—bacteria, fungi, viruses, and archaea—along with the microbiota's genetic material and environmental products, comprise the "microbiome" (Marchesi & Ravel, 2015). The human microbiome is essential for vital life functions within the human body, contributing to nutrient absorption and provisions of energy (Brestoff & Artis, 2013; Kau, Ahern, Griffin, Goodman, & Gordon, 2011; Tilg & Kaser, 2011), to processes, such as the normal development of the immune system (Gensollen, Iyer, Kasper, & Blumberg, 2016; Mazmanian, Liu, Tzianabos, & Kasper, 2005), and providing a barrier against pathogen invasion (Bäumler & Sperandio, 2016; Cameron & Sperandio, 2015; Hooper, Littman, & Macpherson, 2012). Such a high degree of physiological dependence on the microbiome suggests a long co-evolutionary history between human hosts and their microbiota (Zilber-Rosenberg & Rosenberg, 2008). Despite these important findings, the functional capacity of these microbes and how these functions contribute to human health are not well understood, along with the factors that shape and develop these communities and their functions within the body. Existing work has shown that diet (David, Maurice, et al., 2014; Zimmer et al., 2012), antibiotics (Modi, Collins, & Relman, 2014), medical treatment (Le Bastard et al., 2018), and disease (Duvallet, Gibbons, Gurry, Irizarry, & Alm, 2017) can impact and modify human microbial communities. Thus, lifestyle and environmental changes altering the original microbe–host co-evolutionary systems are likely to have major impacts on microbial functionality.

As a result, a prominent area of microbiome research focuses on the impact of urban or industrialized lifestyle factors on the microbiome and human health. Several hypotheses (e.g., the "hygiene hypothesis" (Strachan, 1989; Wold, 1998) or the "old friends hypothesis" (Guarner et al., 2006; Harper & Armelagos, 2013)) have tried to mechanistically explain how industrialization may have altered the human microbiome. Recent research emphasizes how two critical factors—the post-Industrial diet (e.g., low in fiber, high in fat and sugar) and so-called "Western medicine"—have transformed the human microbial ecosystem into a state of "dysbiosis": a disruption of the normal and healthy dynamic equilibrium, that is maladapted for human health (Brestoff & Artis, 2013; Frei, Lauener, Crameri, &

O'Mahony, 2012; Kau et al., 2011). This post-Industrial diet originated around the 1870s with flour-milling technology pioneering the production of refined low-fiber grain, a durable staple food commodity (Winson, 2013). Today, wide-spread consumption of fiber-depleted grains is associated with reduced microbial diversity, modified metabolic pathways, and altered bacterial gene expressions (Cordain et al., 2005; Turnbaugh et al., 2009). These microbial changes are likely due to the decreased microbial digestion and fermentation of complex plant polysaccharides, which produce fatty acids (such as butyrate or propionate) hypothesized to be critical immunoregulators (Maslowski & Mackay, 2011; Sonnenburg & Sonnenburg, 2014). Similarly, the pervasive use of antibiotics, starting in the early twentieth century, has been shown to disrupt the human microbiome, especially early in life during critical periods of immune system and microbiome development (Blaser, 2016; Larson, 2007). The use of antibiotics diminishes the diversity of gut microbiota, altering the trajectory and maturation of the gut microbiome, and consequently, leads to metabolic perturbation and abnormal immunological development (Bokulich et al., 2016; Cho et al., 2012; Cox et al., 2014). While the long-term microbial repercussions of antibiotic usage are clear in some studies (Jakobsson et al., 2010; Jernberg, Löfmark, Edlund, & Jansson, 2007; Wipperman et al., 2017), there are still numerous confounding factors and unknown variables (e.g., the microbial structure prior to disturbance (Raymond et al., 2016)) that can influence the dysbiotic consequences. Further research is needed to fully disentangle and identify significant factors of industrialized lifestyles that alter the microbiome.

Microbial dysbiosis is not exclusive to the lifestyle changes in contemporary industrialized societies and urban environments. Equally dramatic sociocultural changes have occurred throughout human history and over much longer evolutionary time periods. Of these, the changes inflicted globally on Indigenous populations during the Colonial Period are potentially some of the most drastic and rapid. This article will explore how historical colonialism may have altered Indigenous microbiomes, and subsequently, Indigenous health. First, we discuss the health disparity between Indigenous and non-Indigenous populations and the microbiome-linked diseases that underpin this disparity. Next, we review the co-evolutionary nature of the human microbiome and why disrupting this relationship could have lasting implications for health. Last, we explore the potential impacts on Indigenous microbiomes during the Colonial Period by providing key examples where diet, environment, and lifestyle were altered irreversibly. In this article, we explore how microbiome alterations may be a unique mechanism that underlies the significant health disparity suffered by Indigenous populations worldwide.

2 | INDIGENOUS POPULATION HEALTH

Despite global cultural and historical differences, evidence shows that the majority of Indigenous people world-wide have poorer health than their non-Indigenous counterparts (Anderson et al., 2016). However, the assessment of human health is complicated by multiple determinants enmeshed with socioeconomic, environmental, biological, policy-making (including public health services), and personal behaviors (AIHW, 2010; King, Smith, & Gracey, 2009; Woodward & Kawachi, 2000). Measures of health are further complicated by the entanglement of interconnected causal pathways which can attribute or influence health (Leon & Walt, 2000). The concept of "Indigenous" also convolutes matters; defining Indigenous status, or what constitutes indigeneity, within specific settings can confound measurements and insights into population health (Kuper, 2005; Stephens, Porter, Nettleton, & Willis, 2006). However, accurately measuring health and monitoring these determinants are critical to the development and sustainability of public health measures to prevent disease and promote health within Indigenous populations (AIHW, 2010, p. 201; Stephens et al., 2006). With an estimated 370 million Indigenous peoples worldwide, it is critical that accurate assessments of global Indigenous health are undertaken, and despite the difficulties, all the various health determinants are explored to improve overall well-being (Hall & Patrinos, 2012).

Defining the term Indigenous is the first step in assessing Indigenous health. "Indigenous" is typically used with recourse to the first recorded inhabitants in a nation or area at the time of European contact, especially where there is a clear distinction between the Indigenous population and the colonial settlers (e.g., Australia, New Zealand, Canada, and the United States) (Anderson et al., 2006; Montenegro & Stephens, 2006; Stephens et al., 2006). In other parts of the world, this distinction is less clear when the colonial history and Indigenous status is obscured by ethnic or intrapopulation domination, serial conquests, or imperialism (Ohenjo et al., 2006; Stephens et al., 2006). For example, over 100,000 years of colonial history in South Africa convoluted with the apartheid, civil wars, intrapopulation domination, and ethnic genocide have formed a very complex platform for identifying indigeneity (Ohenjo et al., 2006). Therefore, self-identification is commonly the most prominent means for inclusion within Indigenous definitions, followed by community acceptance: most governments now include these definitions in national censuses (Stephens et al., 2006). As the nature of population health data often relies on systematic analysis of government census data, the discussion and accuracy of global Indigenous population health is affected by the use and nature of accepted Indigenous status (Stephens et al., 2006).

With the use of large-scale census data, Anderson et al. (2016) was able to conduct one of the first global Indigenous population health studies. However, social and health information was only available from 23 of the total 90 countries, representing only half of the total estimated global Indigenous populations (Anderson et al., 2016; Gill et al., 2006). Despite this limited and incomplete data set, common themes in Indigenous health still emerged; lower life expectancies, higher infant, child, and maternal mortality rates, greater infectious and chronic disease loads, increased levels of malnutrition, and escalating poor mental health, substance abuse, and structural violence were all higher in Indigenous populations in comparison to their non-Indigenous counterparts, (Anderson et al., 2016; Gracey & King, 2009; King et al., 2009; Valeggia & Snodgrass, 2015).

Of all the troubling themes in Indigenous health, the higher rates of infectious disease than their non-Indigenous counterparts is most notable (Butler et al., 2001; Carville et al., 2007; Gracey & King, 2009; Montenegro & Stephens, 2006; Ohenjo et al., 2006). While numerous socioeconomic, geographic, and health-related factors influence the

intensity, severity, and frequency of infection, Indigenous populations are discernibly more vulnerable to infectious diseases than their non-Indigenous counterparts (Butler et al., 2001; Gracey & King, 2009). The impact of colonization and accompanying introduction of novel pathogens to new continents is well known; so-called "virgin soil" epidemics decimated multiple Indigenous populations who had no immune defense to these unfamiliar pathogens (Crosby, 1976; Kunitz, 1996). However, the risks of such epidemics continues today with both the vulnerability of Indigenous populations to infection and the repercussions of globalization on isolated Indigenous tribes, bringing them into proximity with unfamiliar infections (Hurtado et al., 2005; Valeggia & Snodgrass, 2015).

While chronic diseases are largely burdensome within industrialized societies, these diseases appear to have a greater debilitating effect on health and mortality of Indigenous populations (Gracey & King, 2009; King et al., 2009; Marmot, Friel, Bell, Houweling, & Taylor, 2008). For example, the prevalence of diabetes is three to five times higher in Aboriginal Australians and Torres Strait Islander populations relative to Australia's non-Indigenous population (Australian Bureau of Statistics, 2013). In Aboriginal Canadians, while diabetes prevalence in an age-standardized population was similar to non-Aboriginals, diabetes prevalence in Aboriginal children was far greater than their non-Aboriginal counterparts (e.g., 20-fold higher in Aboriginal children in Manitoba, Canada) (Amed et al., 2010; Public Health Agency of Canada, 2011). Notable chronic diseases within Indigenous populations, especially cardiovascular disease and diabetes, are often attributed to the impacts of urbanization and industrialization, which have emerged more recently for the majority of Indigenous populations compared to their non-Indigenous counterparts (Gracey, 2014; Gracey & King, 2009; Popkin, 1999). Today, chronic health problems and risks associated with urbanization are especially felt within remote and rural Indigenous communities, usually concomitant with the loss of ancestral land, depletion, or dispossession of traditional resources, or the overall the abandonment of traditional lifestyles, which impacts dietary composition, physical activity, and psycho-emotional health (Kirmayer, Brass, & Tait, 2000; Kirmayer, Dandeneau, Marshall, Phillips, & Williamson, 2011; Kuhnlein, Receveur, Soueida, & Egeland, 2004; Snodgrass, 2013; Valeggia & Snodgrass, 2015). Chronic diseases are a worldwide health problem in which preventable risk factors are heightened by environmental and social change; it is an epidemic that is only worsening, for which Indigenous populations are disproportionately suffering (Anderson et al., 2016; Gracey & King, 2009; Strong, Mathers, Leeder, & Beaglehole, 2005).

The limited public health data available on Indigenous health largely preclude our understanding of the underlying causes of the gap between Indigenous and non-Indigenous populations. Many of these disparities are entrenched within social inequalities; poor health is aggravated by low socioeconomic standing and social marginalization (Evans & Kantrowitz, 2002; Frohlich & Potvin, 2008; Woodward & Kawachi, 2000). Yet, despite efforts of government programs engaged in closing the health gap and providing strategies and programs administering clinical services and health education, the Indigenous health disparity has shown little improvement, and in some cases, worsened (Marmot et al., 2008; Mitrou et al., 2014). Strikingly, some studies even suggest that the health of Indigenous populations is worse than that of other populations of similar socioeconomic standing (Valeggia & Snodgrass, 2015; Williams, Mohammed, Leavell, & Collins, 2010). Therefore, while socioeconomics is a vital component in the discussion of population health, the limited progress in bridging the socioeconomic gap to improve Indigenous health disparities calls for an exploration of all potential contributors to health and disease.

3 | THE HUMAN MICROBIOME

The number of microbes hosted by a human body rivals the number of human cells of that individual, and the microbial genomic capabilities outnumber the human genome 100:1 (Sender, Fuchs, & Milo, 2016; The Human Microbiome Jumpstart Reference Strains Consortium, 2010; Yang, Xie, Li, & Wei, 2009). Human-associated microbes are predominantly bacteria (estimates between 88% and 99%) (Qin et al., 2010; Xie et al., 2010; Zhernakova et al., 2016); therefore, microbiome research typically focuses on the bacterial communities that constitute the microbiome. Human-associated microbes are often described as beneficial or "commensal"; that is, a biological relationship between humans and the microorganisms for which their interactions are typically either benign (of neither detriment nor benefit) or symbiotic (with mutual benefit) (Blaser & Falkow, 2009; Brucker & Bordenstein, 2012). Until the development of molecular tools, research was limited to the minority of bacteria taxa that could be grown within a laboratory (i.e., cultured). Now with culture-independent and high-throughput DNA sequencing technology, the study of microorganisms has moved past single isolates into community-based analyses, which serve as the foundation of the human microbiome research.

The human microbiome is initially established during an infant's postnatal period and is essential for the correct morphological and functional development of their immune system (Gensollen et al., 2016; Mazmanian et al., 2005). The human microbiome continues to develop over the first 3 years of life and eventually becomes largely partitioned into five major sites across the human body: the oral cavity, respiratory tract, gastrointestinal tract, skin, and vaginal sites. Each of these body sites has specific environmental conditions that form distinct microbial communities. This intrapersonal variation in the microbiome is characteristic of both environmental and physical factors, such as temperature, pH, and available nutrients, that influence which microorganisms can inhabit a particular niche (Costello et al., 2009; Fisher, Mora, & Walczak, 2017; The Human Microbiome Project Consortium, 2012). Despite these diverse site differences, these communities across the human body are interrelated (Costello, Stagaman, Dethlefsen, Bohannan, & Relman, 2012); alterations in a single microbial community can impact other communities across the body. In rheumatoid arthritis patients, Zhang et al. (2015) found that both the oral and gut microbiomes were in an associated state of dysbiosis compared to healthy individuals. The concordance of oral and gut microbiomes was reiterated when these same patients were treated with anti-inflammatory disease-modifying antirheumatic drugs; both oral and gut microbiome dysbiosis were partially relieved (Zhang et al., 2015). Hence, site-specific microbiomes are not disconnected from one another.

Understanding microbial ecosystems and their functions, networks, and development is fundamental for health research, as the functions of the human microbiome are imperative for human physiological well-being and development. For example, the microbiome and microbial-derived compounds (nutrients or metabolites) in the gut contribute to the education of the immune system, influence epithelium homeostasis, and guide developmental cell programming (Aidy, Hooiveld, Tremaroli, Bäckhed, & Kleerebezem, 2013; Brestoff & Artis, 2013; Hooper et al., 2012; Kau et al., 2011; Maslowski & Mackay, 2011). The gut microbiome is also vital in the regulation of energy homeostasis, fermentation, metabolism, and nutrient utilization (Brestoff & Artis, 2013; Cheesman & Guillemin, 2007; Sonnenburg & Sonnenburg, 2014; Tremaroli & Bäckhed, 2012) and is crucial to develop the signaling mechanisms required for normal brain development, the hypothalamic–pituitary–adrenal axis programming, central nervous system function, and subsequent behavioral functions (e.g., stress reactivity) (Cryan & Dinan, 2012; J. A. Foster & McVey Neufeld, 2013; Heijtz et al., 2011). There is surmounting evidence for the role of the microbiome in normal physiological development, yet there is much to be explored regarding the effect of microbiome compositional change or variation.

Intra- and interpersonal variation within the human microbiome is driven by numerous, sometimes linked factors, including host genetics and physiology (Blekhman et al., 2015; Bonder et al., 2016; Mariat et al., 2009; Yatsunenko et al., 2012), and lifestyle factors, such as, physical activity (Clarke et al., 2014), medication (Blaser, 2014; Modi et al., 2014), diet (David, Maurice, et al., 2014; Zimmer et al., 2012), and interactions with the physical environment (Broussard & Devkota, 2016; David, Materna, et al., 2014). Human genetics and physiological differences shape microbial communities in the human body through aforementioned abiotic factors and biotic components, such as host-to-microbes interactions that control microbial inhabitants; environmental compartmentalization through epithelial barriers; or microbial monitoring through Toll-like receptor proteins (Rakoff-Nahoum, Paglino, Eslami-Varzaneh, Edberg, & Medzhitov, 2004; Slack et al., 2009; The Human Microbiome Project Consortium, 2012). These host factors have matured through selection pressures on the host genome for a beneficial (or neutral) microbiome (K. R. Foster, Schluter, Coyte, & Rakoff-Nahoum, 2017; Ley, Peterson, & Gordon, 2006) and are most commonly immune-related functions (Blekhman et al., 2015; Bonder et al., 2016; Zhernakova et al., 2016). However, the contribution of human genetics in microbial heritability (i.e., the variation of microbial composition attributable to human genetics) is only estimated between 1.9% and 8.1%, suggesting that lifestyle and environmental factors largely drive intra- and interpersonal variations (Rothschild et al., 2018). For example, diet has been shown to be a major driving force in microbiome diversity (Falony et al., 2016). Dietary research has typically concentrated on variations in macronutrient consumption; high-fat and high-sugar versus low-fat and high-fiber diets embody the main differences between industrialized societies and traditional hunter-gatherer ones (Obregon-Tito et al., 2015; Rampelli et al., 2015; Schnorr et al., 2014). Yet, these diet-induced changes of the microbiome have shown a range of plasticity, from repetitive reversible dysbiosis (Davenport et al., 2014; David, Maurice, et al., 2014; Turnbaugh, Backhed, Fulton, & Gordon, 2008) to unrecoverable microbial species extinctions and permanent transitions (Sonnenburg et al., 2016). These irresolute results point to a hysteresis of the gut microbiome, wherein the state of complex microbial system is dependent upon historical exposures, not just the current circumstances (Carmody et al., 2015; Griffin et al., 2017). Other factors, such as sociality, may play smaller roles in guiding microbiome diversity, but are no less important (Lax et al., 2014). For example, household sharing contributes to microbial similarities between family members (Rothschild et al., 2018; Song et al., 2013), with shared environments driving analogous microbial compositions and functionality (Chu et al., 2017; Korpela et al., 2018; Rothschild et al., 2018).

Collective studies on the factors that shape the composition and structure of the microbiome community highlight how population level differences in microbiota can arise; genetic factors, alongside lifestyle and environmental exposures, both early and later in life, each play key roles (Dehingia et al., 2015; Strickland, Lauber, Fierer, & Bradford, 2009). As there is little evidence of a core microbiome across individuals—as yet, no single taxon has been found universally shared across all humans—this, therefore, limits the current theoretical framework in understanding how compositional differences impact the microbial functions in different human populations (Shade & Handelsman, 2012). Thus, the significance of external factors on the microbiome composition and structure must be explored to fully understand how changes in microbial function may subsequently impact human physiology and health (McFall-Ngai et al., 2013), especially within unique human populations.

Dysbiosis, or alteration of the microbiome in a negative capacity to support disease, has already been linked to nearly all chronic diseases, such as cardiovascular health (Ettinger, MacDonald, Reid, & Burton, 2014), cancer (Ou et al., 2013; Sears & Garrett, 2014), respiratory diseases (Fujimura et al., 2014; Riedler et al., 2001; Ruokolainen et al., 2015), obesity (Ley, Turnbaugh, Klein, & Gordon, 2006; Tilg & Kaser, 2011; Turnbaugh et al., 2008), and diabetes (Qin et al., 2012), as well as mental illness (for example, schizophrenia (Liu et al., 2014) and depression (J. A. Foster & McVey Neufeld, 2013)), immunity disorders (Kau et al., 2011; Mathis & Benoist, 2011; Nikoopour & Singh, 2014; Zhang et al., 2015), and the rise in allergies and asthma prevalence (Armelagos & Barnes, 1999; Haahtela et al., 2013). However, these findings have been largely conducted in populations of European descent, which have all undergone similar sociocultural changes over time. These findings bias the predictive accuracy of microbiome related diseases in non-European populations (Lewis, Obregón-Tito, Tito, Foster, & Spicer, 2012). Alterations to microbiomes in other populations may lead to different diseases or different manifestations of disease in separate human populations. For example, some ethnic populations have greater risk factors for disease than others, even accounting for socioeconomic status (Ward et al., 2004); while this can sometimes be attributed to genetics, the concomitant contributions of the microbiome remain unexplored.

4 | CO-EVOLUTION OF HUMANS AND THE MICROBIOME

Several features of the human microbiome imply that humans and their microbes are co-evolved and have co-adapted; these microbes

are (1) specifically conserved within human hosts, (2) persistent through generations of familial inheritance, and (3) defined by environmental exposures and lifestyle factors (Blaser & Falkow, 2009; Zilber-Rosenberg & Rosenberg, 2008). This co-evolutionary relationship is mutually dependent; humans cannot live without their microbiome any more than human-established microbes can survive without a human host. Indeed, the human microbiome is so crucially beneficial to physiological health that the microbiome and human genome may be considered a "human supraorganism" (Turnbaugh et al., 2007). Through the analysis of three predominant gut taxa and their evolutionary relationships, Moeller et al. (2016) traced the evolutionary diversification from modern ape species and modern humans and found these specific bacterial species were maintained throughout hominid evolution (microbial divergence dated to 15 million years ago from gorilla-hominid split), suggesting that this symbiotic association that has persisted over evolutionary time. While the composition and structure of the microbiome have developed in response to external environmental factors, it is also importantly influenced by its evolutionary history, which has shaped and constructed its present structure.

Human evolutionary history indicates that groups of human populations diverged and remained isolated from one another for thousands of years, imprinting geographical signatures on the human and mitochondrial genomes (Rosenberg et al., 2002). Human populations in the Americas, Australia, and the Pacific Islands remained isolated by oceans (Bonatto & Salzano, 1997; Duggan et al., 2014; Tobler et al., 2017). Likewise, populations throughout Europe, Asia, and Africa—while not geographically disconnected—inhabited distinct territories for tens of thousands of years (Barbujani & Sokal, 1990; Melton, Clifford, Martinson, Batzer, & Stoneking, 1998; Tishkoff et al., 2007). Thus, the microbiomes associated with each isolated human population have genomes that are divergent from any other population (e.g., *Helicobacter pylori* (Falush et al., 2003; Wirth, Meyer, & Achtman, 2005)). Research into contemporary populations' microbial differences has shown that these different geographical and sociocultural populations maintain distinct microbial community configurations and diverse functional potential (Rampelli et al., 2015; Yatsunenko et al., 2012). For example, the Indigenous ethnic group of hunter-gatherers, the Hadza, living in north-central Tanzania have a microbiome that is compositionally unique from both urban/industrialized individuals and to that of other hunter-gatherer groups (Dehingia et al., 2015). The Hadza microbiome has distinguishable and unique metabolic functions that are adapted to the consumption of complex polysaccharides (Rampelli et al., 2015), including the unusual presence of *Treponema* bacterium in healthy Hadza gut. The gut *Treponema* strain provide a beneficial metabolic role in carbohydrate digestion, challenging the common perception of *Treponema* as solely a pathogenic microorganism (Obregon-Tito et al., 2015). Human adaptation to a unique physical and cultural environments over evolutionary time suggests that the microbiome similarly adapts to that environment and is therefore likely shaped by the available dietary resources, established human customs and behaviors, and the physical climate and environment.

Understanding the potential health consequences arising from changes in dissimilar Indigenous microbiomes requires an understanding of how these different microbiomes had previously adapted throughout their evolutionary life history, and how severely these co-evolutionary processes between the microbiome and host were disrupted. The majority of Indigenous populations globally have experienced extreme and rapid lifestyle changes throughout their recent evolutionary history, when many of their non-Indigenous counterparts did not. These recent changes were constituted through historical colonialism—one of the most influential sociocultural transitions throughout human history.

5 | COLONIALISM AND THE IMPACTS UPON THE HUMAN MICROBIOME

Colonialism, within this article, is defined as a form of intergroup domination (i.e., between culturally heterogeneous societies) where a substantial number of settlers permanently migrated to a colony from a colonizing power (Horvath, 1972). There were differing motives for long-term or permanent changes during colonialism (e.g., exploration, the conquest of nations, or riches) that often determined the subsequent interactions with native populations and their land, hence the nature of the colonial transitions manifested in a variety of different ways. However, there are numerous shared processes that occurred cross-culturally; colonialism transformed Indigenous populations' dietary lifeways (i.e., the cultural behaviors or customs surrounding diet, including particular foods consumed), adjusted their social networks and behaviors, and impacted their physiological health. These changes occurred rapidly, prompting drastic adaptations within a single individual's lifetime, and collectively demanded both humans and their microbes to adapt (Whittaker, 1972; Zilber-Rosenberg & Rosenberg, 2008). We will explore three overarching transformative changes that colonists often enforced upon Indigenous populations, directly or indirectly, which have been documented in current research to significantly impact the human microbiome. Specifically, through colonialism, Indigenous populations experienced (1) pronounced changes to their established dietary lifeways, (2) rapid adjustments in behaviors, rituals, and social dynamics, and ultimately, and (3) were introduced to novel, destructive agents of infectious disease. While it can be challenging to discuss these interconnected factors exclusively, the following examples of combined historical documentation and recent corroborating microbial research support our hypothesis: Indigenous populations underwent alterations to their microbiomes because of the lasting lifeway changes inflicted upon them during the Colonial Period.

5.1 | Postcontact modifications to dietary lifeways

European colonists reduced Indigenous access to resources required for diverse subsistence farming, indirectly or directly eliminated traditional dietary sources, and often demanded tributes for missionaries and government administrators, which impacted both socioeconomic status and the food available for consumption (Earle, 2010; Klaus & Tam, 2010; Larsen, 1994; Nunn & Qian, 2010). Frequently, Indigenous agriculture was also fully replaced by European crops to maintain a traditional European diet, or for exportation and trade (Franke, 1987).

Novel additions to dietary lifeways were more often an indirect consequence of global trade networks created by the dominant colonizing power (i.e., the importation of European food stuffs, such as wheat, wine, olive oil, and livestock; (Earle, 2010)). In South America, ethnohistoric evidence suggest colonists emphasized the proliferation of specific crops for trade, giving priority to foods, such as tomatoes or cacao, for exportation back to Europe (Nunn & Qian, 2010). Food was also a tool used in "civilizing" Indigenous populations; eating European foods was thought to make them more like the colonizers (Earle, 2010).

The impact of diet upon the gut microbiome is one of the better studied areas in contemporary microbiome research, as alterations to diet have the greatest potential for therapeutic self-regulation of microbiome-associated conditions (Brown, DeCoffe, Molcan, & Gibson, 2012; Cotillard et al., 2013; Ercolini et al., 2015). More specifically, one of the largest areas of dietary research relates to the consumption of microbiota-accessible carbohydrates (MACs), defined as carbohydrates which the human host is unable to digest and absorb nutrients without the prior metabolism by members of their gut microbiome (Sonnenburg & Sonnenburg, 2014). MAC intake has been linked to greater microbial diversity, broader carbohydrate metabolic capabilities (Rampelli et al., 2015), short-chain fatty acid production (Campbell, Fahey, & Wolf, 1997), and increased clinical markers for health (Sonnenburg & Sonnenburg, 2014). Research looking at "humanized" gut microbiome in mouse models (i.e., a previously germ-free mouse colonized by human fecal microbes) showed that a low-MAC diet induces microbial extinction, successively reducing the microbial diversity of the gut over multiple generations (Sonnenburg et al., 2016). Although this loss could be recovered if a high-MAC diet was reintroduced within a single generation, the damage was irreversible and microbial diversity never returned to its original state after several generations (Sonnenburg et al., 2016). While the underlying mechanisms of the link between microbial diversity and health are still unknown, increased species diversity within a community is thought to develop greater ecosystem stability, promote sharing of resources, and lower host invasibility, thus supporting greater metabolic and colonic health (Cardinale, Palmer, & Collins, 2002; Cotillard et al., 2013; Gonzalez et al., 2011; Tilman, 2004).

A population in the small town of Mórrope, Peru, provides a definitive example of dietary change and a case study to examine the impact of colonialism on Indigenous Andean foodways (Klaus & Tam, 2010). Anthropologists, Klaus and Tam (2010), used both regional ethnohistoric evidence and skeletal remains from both late pre- and postcontact periods to examine changes in diet and health. After the Spanish colonization, the people of Mórrope became increasingly reliant on starchy carbohydrate consumption, as evident by increased prevalence of dental caries and tooth loss (due to poor oral health) and heightened accumulation of calculus (symptomatic of greater plaque progression, which can extend to additional oral problems) (Hillson, 1996; Klaus & Tam, 2010). It was suggested that the elevated consumption of starchy carbohydrates would have helped buffer against malnutrition from restricted access to traditional food sources, after being resettled in a resource-poor area due to European exploitation of arable land for cash crops (Franke, 1987; Klaus & Tam, 2010). However, a carbohydrate-based diet not only stimulates oral disease, but also leads to growth retardation and impaired skeletal development from nutrient deficiency (Larsen, 1995). The metabolic stress within the Mórrope postcontact population was great enough to leave skeletal lesions, such as cribra orbitalia and porotic hyperostosis (i.e., localized areas of spongy porous bone tissue caused by anemia) (Klaus & Tam, 2010).

From Sonnenburg et al. (2016), it could be inferred that the people of Mórrope would have experienced microbial extinctions over several generations caused by a reliance on starchy carbohydrates and limited access to complex carbohydrates (i.e., a low-MAC diet). Ancient DNA research in ancient European populations also suggests that the switch to starchy carbohydrates had marked impacts on composition of the microbiome (Adler et al., 2013; Weyrich et al., 2017). However, carbohydrates are not the sole cause of alterations in microbial ecosystems. Many additional dietary modifications have been shown to induce changes in the gut microbiome composition and function, such as the switch from a plant-based diets and to that of animals (David, Maurice, et al., 2014; Zimmer et al., 2012), seasonal dietary variation (Davenport et al., 2014; Zhang et al., 2014), and consumption of fermented products (Veiga et al., 2014). Probable unexplored consequences include individuals consuming a novel introduced dietary source for which they have little to no evolutionary experience, or inversely, consequent adaptation to the indefinite removal of a dietary food source.

5.2 | Influence of colonialism on social structures and behaviors

Historically, the enforcement of "European ways" on Indigenous populations represents one of the most direct cases of sociocultural change, established through colonial settlers and governing authorities, most commonly in the form of missionization (Earle, 2010; Larsen, 1994; Van Buren, 2009). "Missionization" is the process of Christian proselytism, and its corresponding acculturation programs instituted at formal bases, known as "missions" (Van Buren, 2009). The consequences of missionization varied regionally; however, it almost always resulted in significant and cumulative changes to Indigenous lifeways. For example, the historical colony "New Spain" enforced Indigenous acculturation through the *reducción* (Van Buren, 2009). As part of this process, Indigenous populations were forced from their villages and homes and were bound to reside within mission centers (Larsen, 1994). The spatial organization of missions imposed close living conditions on diverse multiethnic populations, with no organizational attention to linguistic barriers or tribal animosities, which fractured families and impeded traditional courtship customs and practices (Panich & Schneider, 2015; Van Buren, 2009). Even in the absence of aggressive missionization, exposure to European customs and behaviors prompted far-reaching cultural adaptations.

Cultural alterations in behavior or customs are the most erratic and variable of any postcontact colonial change, and therefore, impacts of any Indigenous sociocultural behavioral alteration should be explored within the local background and history of the Indigenous–colonist relationship. However, this makes the exploration of microbial alterations difficult; accordingly, this article focuses on

how the transmission of microbes may have been impacted by sociocultural changes. As the human microbiome is inherited by social transmission, then matures throughout growth and development by the surrounding environment (especially through contact between household members), differences in kinship structures and social networks will impact the vertical transmission of microorganisms between individuals (Moeller, Foerster, et al., 2016; Tung et al., 2015; Yatsunenko et al., 2012). Microbiome research has shown, despite direct maternal microbial exposure at birth, fathers share as many microbial similarities with their children, as does the mother (Yatsunenko et al., 2012). While not yet explored in humans, social interactions and relationships within a community of baboons imprinted explicit patterns of exchange within their microbiome, highlighting the importance of social interactions in structure and composition of the microbiome (Tung et al., 2015). This research suggests that the differences in cultural behavior and social networks impact microbial dispersal and transmission routes in defining microbiome structure and community development (Martínez et al., 2015). Whether the colonists goal was to exterminate, assimilate, or remain in relative equilibrium with the Indigenous population (neither extermination nor assimilation), changes certainly occurred to Indigenous kinship structures, social networks, and cultural lifestyle alterations (Horvath, 1972).

The breakdown of the historic Hawaiian Kapu system is a good example of microbial change through sociocultural restructuring. The Kapu system dictated Hawaiian daily life through religious rules and regulations, governing social stratification, the interactions between social classes, and gender roles and relationships (Else, 2004). However, the acceptance of the European cash economy led to the breakdown of traditional subsistence farming, directly impacting and eroding the relationships between social classes (Else, 2004; Friedman, 1985). The deterioration of the Kapu system lead to greater enduring cultural changes, such as economic distributions of food encouraging the immigration of foreigner laborers or the adoption of the colonial religion, as a result of missionaries and subsequent establishment of missions, or the creation of a mercantile economy, inducing the revaluation of sex for commerce (Buck, 2010; Else, 2004).

As social networks influence microbial transfer between individuals, changes within social networks can introduce new microbes from foreign exposures, or restrict contact with Indigenous microbes (i.e., the missions adjusting the social dynamics and accessible contact between individuals will have altered the transmission of microbes between the members of an Indigenous community and simultaneously introduced colonist microorganisms). Sociocultural behavior adaptations can potentially introduce new sources and recipients of foreign microbes, but changes to cultural customs or behaviors can equally restrict or assist access to microbes from certain individuals or groups. The breakdown of the Kapu and the introduction of the cash economy changed cultural ideals regarding divisions of labor, emphasizing the colonist values of females within the domestic spheres and males within the public spheres, which created differential group access to unique microbial sources (Van Buren, 2009). The gendered roles in food preparation and consumption within Hadza society contributed microbial differences between males and females; thus, it is likely that historical gendered-based microbial differences could be detected, perhaps playing a role in health (Schnorr et al., 2014). On a larger scale, it is likely that the Hawiian microbiome would have integrated some level of commensal microorganisms from a European-adapted microbiome, through increased interactions with colonists. Furthermore, contact between individuals within the society itself would have changed (e.g., differences in caretaking and caregiving, socially acceptable sexual liberties, interactions through occupation), which could impact microbial inheritance of the next generation.

5.3 | Introduction of infectious disease

Unquestionably, the most devastating effect of colonialism was the introduction of novel pathogens. Globally, Indigenous populations were decimated by epidemics of infectious diseases introduced by colonists; some of the hardest hit areas lost up to 90% of their population (Cook, 1998; Kunitz, 1996; Zubrow, 1990). In the Americas, no specific case study can be reliably ascertained because the speed by which the pathogenic agents spread and obliterated the Indigenous population outran European ethnohistorical records, leaving only indirect archaeological evidence, such as specific demographic patterns in mortuary samples (Hutchinson & Mitchem, 2001; Milner, 1980). Despite inadequate information, it is presumed that the Indigenous population had no "immunological memory" of the introduced diseases from the "Old World" and that the malignance of these pathogens was due to the separate evolutionary histories between the continents (Crosby, 1976; Ramenofsky, Wilbur, & Stone, 2003). The evident introduction of novel pathogenic microorganisms simultaneously proposes the introduction of nonpathogenic microorganisms, supporting evidence of changes to the microbiome and immune profiles of Indigenous populations.

Research has implicated the microbiome in the development and education of the immune system in infancy, but the microbiome also plays a role in pathogen resistance through "bacterial interference" or "colonialization resistance" (Brook, 1999). Bacterial inference refers to antagonistic and competitive relationships between bacterial species, in which bacteria have developed mechanisms to interfere with the capability of other bacteria to colonize and survive alongside them (Buffie et al., 2015; Falagas, Rafailidis, & Makris, 2008). There are a number of mechanisms of bacterial interference; principally, nutrient rivalry or host–cell binding site competition, where the endemic human microbes outnumbered and outcompeted invading microorganisms (Reid, Howard, & Gan, 2001) Another aspect of bacterial interference is the capacity of endemic microbes to produce antagonistic compounds, such as bacteriocins, (i.e., toxic proteins produced by bacteria that inhibit the growth of, or even kill, other bacteria, without causing harm to themselves) or simple molecules, like hydrogen peroxide or lactic acid, to change the microenvironment and deter invader establishment (Brook, 1999). Some research has shown that dysbiotic perturbations to the microbiome can weaken the effects of colonization resistance, leaving the host susceptible to pathogen invasion (Bäumler & Sperandio, 2016; Brown et al., 2012). The impact is cumulative; the establishment of a pathogen can exacerbate dysbiosis and disrupt microbial functionality, negatively influencing host physiology, immunity, and susceptibility to infectious disease (Kau et al., 2011; Lu et al., 2013). Pathogens can also induce apparent

competition, utilizing host immune response to preferentially displace or alter the host microbiome for its own benefit in such that the dysbiotic microbiota act as a pathogenic community (Hajishengallis, Darveau, & Curtis, 2012; Sears & Pardoll, 2011).

Infectious disease would have directly altered the microbiome, but the consequential human depopulation would have also altered human population structures, both genetically and socially, further impacting microbial transmission to surviving generations. While there is little agreement on the timing of depopulation, the size of precolonial Indigenous populations, or the overall mortality rates, there is a shared consensus on the indirect impacts of disease on the Indigenous population; high mortality and morbidity would have disturbed subsistence activities and the labor force, reduced political influence, and forced social reorganization (Cook, 1998; Dobyns, 1966; Milner, 1980; Snow & Lanphear, 1988; Zubrow, 1990). Survivors of one community decimated by disease often resettled among different communities, contributing to the spread of disease and influencing horizontal microbial transmission among different communities (Warrick, 2003). It is hard to predict the variety of indirect repercussions depopulation had on Indigenous life, let alone the subsequent impact upon their microbiomes. A case in point, albeit with very little available archaeological evidence, is the suggestion that depopulation of South America resulted in the loss of domesticated crop diversity (Clement, 1999). The reduction in labor force would have reduced the number of horticulturalists to maintain widespread minor crops, and a loss in dietary diversity would have induced a loss in microbial diversity, potentially instigating dysbiosis, and thus, further increasing pathogen susceptibility (Clement, 1999; Ley, Peterson, et al., 2006). Under colonialism, Indigenous populations likely encountered novel pathogens at an alarming rate, while simultaneously enduring the impacts of dietary change and/or malnutrition, socioeconomic restriction, and both psychological and biological stress. All of which are factors that have been described in contemporary research as instigators of microbial dysbiosis (Bailey et al., 2011; Brown et al., 2012; David, Maurice, et al., 2014; De Palma, Collins, Bercik, & Verdu, 2014).

DISCUSSION

Colonialism represents one of the greatest and swiftest historical sociocultural adaptations throughout human evolutionary history. Through anthropological and archaeological evidence, it is evident that the process of colonialism was detrimental to the traditional lifestyles and health of the Indigenous populations. Moreover, it is evident that the ensuing rapid lifestyle changes that Indigenous populations endured would have likely altered their microbiomes. Explorations of the unintentional alterations to the microbiome throughout progressive industrialization have shown that modifications to the composition and structure of the microbiome can be detrimental to human health. However, our fundamental understanding of contemporary microbiome alterations requires recognition of the current ascertainment bias; the majority of microbiome studies examine populations of European descent, who live industrialized lifestyles (Lewis et al., 2012; Warinner & Lewis, 2015). The little existing research on different ethnic populations has shown that there are taxonomic, compositional, and functional differences in the microbiomes of different human populations (Anwesh et al., 2016; Martínez et al., 2015; Ozga et al., 2016; Rampelli et al., 2015; Yatsunenko et al., 2012; Zhang et al., 2014). Therefore, it cannot be assumed that the same instigator will equally impact different microbiomes; dysbiosis may take different forms, provoking various disease responses. Researchers have shown that rheumatoid arthritis patients' disease-associated dysbiosis was compositionally similar across all patients, but the "stabilization" of the microbiome after taking rheumatoid arthritis drugs of each patient concluded with compositionally disparate recoveries (Zhang et al., 2015). The impact of alterations to different microbiomes (especially across different populations) has not been explored with regard to the subsequent co-evolutionary histories of populations, and therefore the burden upon health.

The rapid transition into a disadvantageous lifestyle, inflicted upon Indigenous populations throughout colonialism, would have selected for the best microbiome for survival through the detrimental transition, or rather a microbiome most suitably adapted for the novel lifestyle (Ley, Peterson, et al., 2006; Wilson, 1997; Zilber-Rosenberg & Rosenberg, 2008). However, the microbial functional repercussions of these alterations may not necessarily be the best adaptations for human physiological health. Recent investigations suggest that genetic predisposition to disease is contingent upon the composition and function of the microbiome (Bonder et al., 2016; Knights et al., 2014). Thus, the dysbiosis of the ecologically adapted functional microbiome could trigger adverse immunological and metabolic genetic phenotypes (Bonder et al., 2016). Furthermore, human genetics were altered during the Colonial Period. Ancestry admixture has shown a strong link between population-specific alleles and host genetic factors that mediate immunity and pathogen-resistance (Lindo et al., 2016; Rishishwar et al., 2015); as previously discussed, the greatest genetic influence on the human microbiome stems from immune-related factors. The disruption to the Indigenous microbiome, induced by colonialism, altered the stable co-evolutionary relationship that was predetermined by genetic background and cultural history.

While the effects of colonialism are still being felt today, especially among Indigenous populations, our current understanding of microbial kinship patterns implies that alterations to the microbiome could be passed onto future generations and may not ever be restored to their original state (Ley, Peterson, et al., 2006; Sonnenburg et al., 2016). While the long-term repercussions of microbial change over successive generations are not fully understood, there are a number of mechanisms that can propagate and participate in transgenerational inheritance of microbiome alterations. Primarily, there is selective maternal transmission of specific bacterial strains to young infants (Chu et al., 2017; Korpela et al., 2018). The origin of some specific species can be traced back to the mother, and they remain consistent and stable during and throughout infant development, implying a selective advantage in familial microbial inheritance and an adaption of some symbiotic bacterial species to have evolved vertical transmission dependence (Duranti et al., 2017; Korpela et al., 2018). However, while caregivers transfer microbes to the infant microbial community throughout their development, recent evidence does suggest that environmental drivers are more critical for the maturation of microbiome composition (Chu et al., 2017). Therefore, shared environments

(e.g., family household) will promote microbial sharing through sociality; transgenerational inheritance occurs within nuclear family units sharing familial microbes (Bokulich et al., 2016). This means that community dysbiosis can also be "inherited" in a non-traditional sense; if the fetus or neonate are exposed to maternal dysbiosis during this critical developmental window, the infant "inherits" a dysbiotic microbial state, although not necessarily the same dysbiotic state as their mother (Miyoshi et al., 2017; Mulligan & Friedman, 2017). The dysbiosis experienced by Indigenous populations today may not represent the dysbiosis directly caused by the events of colonization, but instead, is the downstream remnant of historical perturbations that define the hysteretic microbiome.

In suggesting the colonial transition was detrimental to contemporary Indigenous health, we introduce the paradox of contemporary colonists, whose ancestors immigrated to novel lands and experienced changes to their own diets, lifestyles, and contact with novel diseases, but have consistently better health than their Indigenous counterparts. However, the perturbations to the colonial microbiome, and the consequential impact on their health, would be different. It is possible that the microbial disruption felt by colonists was less drastic than what was experienced by Indigenous populations; colonists were able to maintain some microbial stability through cultural lifestyle (for example, preservation of familiar dietary sources, such as wheat or milk, or sustained familial ties maintaining familial microbes; Earle, 2010; Phillips, 2009). As long as the colonists were able to maintain some cultural stability, the largest demarcating factor between Indigenous and non-Indigenous populations during the colonial transition is the fact that Indigenous populations were not able to reestablish precolonial lifestyles and traditions, i.e. the environmental factors that underpin the origin of their microbiomes. On the other hand, perhaps the co-evolutionary history between European populations and their microbiomes through ancestral perturbations of the Neolithic Revolution and earlier population transformations provides greater resilience or adaption to change within new environments (Adler et al., 2013; Mathieson et al., 2018; Olalde et al., 2018). Understanding the impacts of disruptive change on both the Indigenous populations and their colonial counterparts will be critical in illuminating microbial ecosystem functions to improve human health.

To be clear, highlighting a microbial role in Indigenous health does not negate the significance of the role of socioeconomics in the Indigenous health disparity. There is evidence that indicates socioeconomic status impacts the composition of the microbiome (Belstrøm et al., 2014; Chong et al., 2015); hence, socioeconomic status may be exacerbating the influence of the microbial evolutionary history on Indigenous health. In proposing an underlying microbial element in Indigenous health disparities, we offer a potential explanation for an additional "unknown" risk factor that contributes to the discrepancy in health between Indigenous peoples and their non-Indigenous counterparts. Effective reduction of any disease prevalence requires a consideration of all determinants involved (Findley, Williams, Grice, & Bonham, 2016). Factors involved in disease risk—social, behavioral, biological, economic, and environmental—are also involved in the structuring of the microbiome; thus, a greater understanding of the symbiotic microbiome–human relationship will aid public health efforts within Indigenous communities to improve population health.

In the implementation of such microbial investigations, researchers need to go beyond global health programs, and look toward community engagement and translating microbiome research into something malleable for health care providers or public health policies (O'Doherty, Virani, & Wilcox, 2016; Valeggia & Snodgrass, 2015). Most notably, these inquiries require the inclusion of Indigenous communities, especially in regards to therapeutic benefits (Lewis et al., 2012). Partnerships between researchers and Indigenous communities can provide opportunities for locals to gain first-hand experience regarding specific factors contributing to illness and disease, to learn preventative techniques in health care, and to understand health-related skills and management (Gracey, 2014). Importantly, allowing community control over both their own health care and research, including sharing experimental data, allows efficient research processes to assist in developing tangible beneficial community outcomes (James et al., 2014; Sankaranarayanan et al., 2015). Research efforts need to be cognizant in ethics of care frameworks, to be aware of the potential challenges in research practices that may do disservice to Indigenous communities, and give attention to the relationship between researchers and Indigenous communities (Held, 2006; Sharp & Foster, 2007; Taylor & Guerin, 2010). While these potential issues may be community-specific, additional challenges can stem from interpretation of these ethical guidelines. For example, difficulties can arise in the ability to disentangle group interests from individual concerns, identifying whom is able to provide community representation, and furthermore, whether this representative is able to present the range of community perspectives (M. W. Foster & Sharp, 2000; Sharp & Foster, 2007). The global health inequalities between the Indigenous populations and their non-Indigenous counterparts demand greater efforts in tracking the health of Indigenous communities. Failure to note the impact of Indigenous identity within microbiome research is not a neutral stance, but risks hiding existing inequalities or neglecting communities (Kirmayer & Brass, 2016). Studying the microbiomes of Indigenous peoples involves recognition of specific local, cultural, and historical contexts (Kirmayer & Brass, 2016).

While we propose colonialism as a key agent for microbial dysbiosis, it is equally likely for microbial dysbiosis to be an independent variable of the consequential physiological and psychological changes endured by Indigenous peoples throughout colonialism. In other words, was dysbiosis of the microbiome caused by the alterations in diet, introduction of novel microorganisms, and adjustments to cultural lifestyles, or did microbial dysbiosis arise in parallel to the nutritional disease, infectious diseases, and psychological trauma caused by colonialism? Both scenarios are plausible. Furthermore, both scenarios have significant ramifications for Indigenous health. Elucidating the cause of dysbiosis enables diagnosis and treatment of dysbiotic-related pathology, for it is therapeutically important to discern whether remediating dysbiosis will cure disease or merely provide palliative remedy. To delineate between cause and effect, Frank et al. (2011) suggest three modes of investigation: observation, experimentation, and modelling. First, large-scale surveys of both microbial composition and functionality must be integrated alongside screening human genotypes and their molecular phenotypes, which can provide associations between microbial profiles and genetic predispositions

(Frank et al., 2011). Second, there needs to be experimental support for the contribution of dysbiosis to disease (e.g., double-blind, randomized controlled experiments involving both the normalization of dysbiotic profiles in individuals with disease and inducing dysbiosis in healthy individuals), and last, it is necessary to be able to model, experimentally demonstrate, and analyze these relationships computationally and statistically (Frank et al., 2011). Realistically, the determination of colonialism's impact on modern-day Indigenous health will not be straightforward, as these cause or effect scenarios are not mutually exclusive. Until the cause of dysbiosis can be explained, perhaps insight can be instead gained by studying the historical populations of the past and investigating their microbial changes through colonialism in real time.

We may be able to reconstruct and examine the historic changes in Indigenous microbiota using ancient DNA research; microbial DNA from the past can be extracted from archaeological or paleontological remains and provide a direct assessment of the evolutionary history of ancient microorganisms and microbiomes (De La Fuente, Flores, & Moraga, 2013; Willerslev & Cooper, 2005). Ancient DNA extracted from dental calculus has already been used to ascertain oral microbiomes of ancient populations, providing direct biological evidence of microbiome-related changes linked to alterations in lifeway, diet, and environment (Warinner, Hendy, et al., 2014; Adler et al., 2013; Weyrich et al., 2017). In this case, ancient microbial DNA could be used to reconstruct the ancient oral microbiomes of pre- and postcolonial individuals, allowing researchers to directly analyze alterations to the microbiome community composition, structure, and function throughout the colonial transition. While contemporary research is concentrated on the gut microbiome, the preservation of the ancient oral microbiome in dental calculus (calcified dental plaque) is superior to fossilized feces (source of ancient gut microbiome) in protecting microbial DNA from exogenous DNA, contamination, and the postmortem environment (Warinner, Rodrigues, et al., 2014; Weyrich, Dobney, & Cooper, 2015). The interconnection of the microbial niches on the human body suggest that if significant changes within the oral microbiome occurred, this would also indicate transformations in the gut community (Said et al., 2013; Zhang et al., 2015). By reconstructing the microbial profile of ancient populations, we can detect microorganisms that have evolved exclusively within specific populations and environments, track the introduction of novel microorganisms, and distinguish those microorganisms that adapted and adjusted to the alternative environment introduced with colonialism. Furthermore, we can identify which microorganisms persisted into subsequent generations, and how they function to assist in modern human health or disease. As the long-term effects of alterations to the microbiome are presently unknown, it is important to evaluate the capacity for these ancient and historic transitions to impact modern-day human population health, especially where it is detriment. Through the reconstruction of ancestral microbiomes, we can gain a greater comprehension of microbiome and host interactions, strengthening the foundation of microbiome research to be used in contributing to the improvement of Indigenous health.

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Chapter 2

Incorporating microbial evolutionary history into Indigenous public health

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Principal Author

Name of Principal Author (Candidate)	Emily Skelly		
Contribution to the Paper	Conceptualised, researched literature, and wrote the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

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

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

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

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

Name of Co-Author	Laura S. Weyrich		
Contribution to the Paper	Conceptualised, contributed and edited the manuscript		
Signature		Date	17 04 2019

Incorporating microbial evolutionary history into Indigenous public health

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2.1 Main text:

Our increased understanding of the vast microbial communities living on and within the human body—the microbiota—and their contributions to human physiology and health are reshaping how we assess public health issues. A key public health crisis—the global health inequality between Indigenous populations and their non-Indigenous counterparts [1]— desperately needs a reassessment in the context of recent microbiota research. Higher infant mortality rates, lower life expectancies, and a growing burden of 'lifestyle diseases' (*e.g.* obesity, cardiovascular disease, type 2 diabetes mellitus, and renal disease) occur in Indigenous people at rates significantly higher than local non-Indigenous populations [2]; diseases which correspond with alterations to gut microbiota communities, at least in non-Indigenous populations. While Indigenous microbiota remain understudied [3], recent evidence also suggests that the co-evolutionary history of microbiota and their host may play key roles in identifying novel causes of disease that plague Indigenous peoples [4]. In this respect, the current health disparity in numerous different Indigenous populations is perhaps symptomatic of recent shared historical disturbances to their microbiota; as such, human colonialism may have interrupted the co-evolutionary synergy between microbe and human [5].

Millennia of coadaptation between microbiota and the human body have led to evolved co-dependence. Our microbial communities are responsible for numerous basic physiological functions, including food digestion, metabolic regulation, immune education and propagation, and protection against invading pathogens [6, 7]. The acquisition of initial microbial communities is dependent upon both familial inheritance and social contact [8], and continues to be shaped throughout early life

development, consistently challenged by medical, dietary, social, and lifestyle factors throughout a lifetime—factors that are specialised to one's cultural background and heritage. These processes create microbiota specific to certain environments, explicit to isolated cultures and geographic segregation. Past populations maintained unique microbiota compared to the relatively ameliorated and familiarised lifestyles present across Industrialised societies today.

The disruption caused by European contact, expansion, and Colonial practices drastically reshaped Indigenous lifestyles, diet, disease exposure, and environment. Radical changes to such factors should be expected to leave significant and profound consequences upon an Indigenous individual's microbiota, potentially interrupting the microbial functional processes that are requisite for healthy human physiology [9]. Furthermore, we should expect that concomitant changes also occurred within microbial functional capabilities, result in adaptations under new conditions. Alterations to an established microbiome could undermine microbial survival and alter selection pressures to favour new environmental or dietary inputs [10]. Most importantly, our current understanding of microbial kinship patterns implies that potential repercussions of the microbiota alterations could be hereditary and passed along to future generations of Indigenous peoples.

The current bias of microbiome research to Industrialised populations has clouded our understanding of the links between the microbiota and human health. Including the microbiota of ethnically diverse populations, and critically, the inclusion of evolutionary history into microbial health research, will enlighten the capability of medical interventions to manipulate microbiota for human health. These investigations should also look towards community engagement and the translation of microbiota data into a culturally-appropriate options to improve Indigenous health through health care providers, public health policies, or the community at large [11]. While the evolutionary history of Indigenous populations' microbiota may explain some 'unknown' risk factors in the health disparities between Indigenous peoples and their non-Indigenous counterparts, additionally identifying factors linked to disease risk—social, behavioural, biological, economic, and environmental—involved in the structuring microbiota, provide a greater understanding of Indigenous populations' microbiota to aid current public health efforts.

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Chapter 3

Salivary Microbiota Response to Caries Preventative Treatment in Australian Indigenous Children

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Overall percentage (%)	65		
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By signing the Statement of Authorship, each author certifies that:

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

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

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Contribution to the Paper	Obtained funding. Designed microbiome study. Contributed to data analysis and interpretations. Edited and contributed to manuscript.		
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Contribution to the Paper	Dental examiner and sample collection. Assisted in the interpretation of data and provided editorial guidance in the manuscript.		
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Contribution to the Paper	CIA for NHMRC project. Designed dental study, headed and managed project, contributed to all field work and sample collection, to data management and dental data analyses. Contributed and edited manuscript.		
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Salivary Microbiota Response to Caries Preventative Intervention in Australian Indigenous Children

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3.1 Abstract

Aboriginal and Torres Strait Islander children have disproportionately poorer oral health than non-Indigenous Australian children, especially those living in rural communities. A once-annual caries preventative treatment (Intervention) was offered to schoolchildren of the Northern Peninsula Area, Queensland; a rural population predominantly of Aboriginal Australian and Torres Strait Islander descent. After only two consecutive years, the Intervention was seen to significantly improved the rate of dental decay. Here, we examine the salivary oral microbiota of these children to understand the ecological mechanisms behind this improvement in clinical outcome. Saliva samples from children (mean age = 10 ± 2.96 years old; n = 104) were used to reconstruct bacterial community composition and taxonomic abundance, with high-throughput sequencing of the V4 region of bacterial 16S ribosomal RNA gene. The salivary microbial community distinguished between children receiving the Intervention from those who did not, with lower taxonomic diversity and abundance (Shannon index, Bray-Curtis; $p < 0.05$). In children, both with and without the treatment, the oral microbial communities were associated with presence and severity of carious lesions existing at the time of saliva collection. The relative abundance of *Lactobacillus salivarius*, *Lactobacillus reuteri*, *Lactobacillus gasseri*, *Prevotella multisaccharivorax*, *Parascardovia denticolens*, and *Mitsuokella* species HMT 131 were significantly increased in children with severe caries, especially within children who did not receive Intervention treatment. This is the first study to describe the oral microbiota from Aboriginal Australian and Torres Strait

Islander peoples, simultaneously providing insight into microbial associations with dental decay and the microbial ecological response to treatment. Further studies are required for the understanding of how such caries-preventative therapy induces these microbial ecological shifts and what the microbial functional repercussions of such alterations are in the long-term, to improve upon oral health disparities within Australia.

3.2 Introduction

Aboriginal and Torres Strait Islander people (hereinafter also respectfully referred to as "Indigenous") make up 2.8% of Australia's population but suffer approximately 2.3 times the disease burden than that of non-Indigenous Australians [1, 2]. Of this burden, 64% can be attributed to chronic disease conditions: the primary contributors include cardiovascular diseases (12%), cancer (9%), and respiratory diseases (8%) [2]. Often overlooked, but certainly contributing to this disease burden, is the impact of poor oral health, for which dental decay is the most common affliction in children [2, 3]. In fact, Indigenous Australian children have, on average, twice the number of decayed or missing teeth than non-Indigenous children, and adolescents suffer from 2.7 times the rate of dental decay than similarly aged non-Indigenous Australians [4]. This not only precedes long-term systemic health problems, but manifests pain and discomfort, causing difficulties in chewing and potential malnutrition, generating sleep disturbance, behavioural problems, a lack of concentration and cooperation—all factors that can hinder learning, quality of life, and overall well-being in young children [5, 6]. Despite the importance of good oral health, the current trajectory appears to be worsening in Indigenous Australian populations [7], especially within rural communities that lack access to regular dental care. Ease of access to dental services significantly impacts the rate of dental decay, as rural or remote Indigenous Australian children have poorer oral health relative to their urban counterparts [4]. As such, a 2004 oral health survey of the Northern Peninsula Area (NPA), Queensland, found the dental decay rate of 6- and 12-year-old children to be double that of the state average, and more than four times greater than that of the average Australian child overall [8].

In order to combat this oral health gap, a novel dental caries preventative intervention was designed by Lalloo *et al.* [9] to decrease and/or slow the incidence of decay, with a focus on children living in remote-rural communities. This novel preventative approach was designed to be sustainable and cost-effective, using a combination of three common dental interventions all within a single-annual visit [9]. Initially, an oral antiseptic povidone-iodine (PVP-iodine) tropical treatment is applied to all tooth surfaces. PVP-iodine has been shown to interfere directly with the binding ability of mutans streptococci to the tooth surface, as well as having broad antimicrobial activity [10, 11]. Next, pit and fissure sealants are applied to the occlusal surfaces of posterior teeth, preventing decay and/or the development of incipient lesions [12]. Finally, fluoride varnish is applied to all tooth surfaces, strengthening the enamel structure and promoting remineralisation, which is especially important in remote communities that lack water fluoridation [13, 14]. This preventative strategy was implemented in the communities of the NPA region, Far

North Queensland, which is located over 1,000 kilometres north of the nearest city (Cairns, population ∼146,000). After two consecutive applications, Lalloo *et al.* reported this preventative intervention to have significantly improved oral health, resulting in a 29.3% decrease in caries incidence relative to children who did not receive treatment [15].

Despite the reduction in dental decay, the mechanisms that underpin this observation are not entirely understood. Caries are a multifactorial disease, dependent on both the microbial community inhabiting the mouth (*i.e.* the microbiota) and abiotic environmental conditions of the mouth. While dietary carbohydrate fermentation reduces the salivary pH to induce the demineralisation process of the enamel [16], the 'ecological plaque hypothesis' suggests that demineralisation begins with a disruption to the 'balanced' (*i.e.* in dynamic equilibrium) oral microbial ecosystem of the mouth, which pressures selection for 'pathogenic' microorganisms with aciduric and acidogenic properties [17]. Thus, by examining the microorganisms present in saliva—the most accessible, non-invasive, and child-friendly sampling strategy—we gain insight into the microbial community that may contributes to dental decay, or indicative of the number and severity of carious lesions. [18]. Furthermore, the microbial load of saliva has been shown to be reflective of the microbial response to therapeutic modulations and treatments, thus providing an avenue to explore the impact of this novel preventive intervention on the microbial ecology [19, 20].

Current evidence suggests that oral microbiota are distinct across populations, wherein geographic locations and/or ethnic identities predicate specific microbial communities [21]. Unfortunately, there is little evidence on how population-specific microbiota contribute to health and disease, despite early evidence to suggest that certain Indigenous populations may have increased or decreased disease susceptibility because of their microbial community composition. For example, the analysis of dental plaque from Canadian First Nation children showed unique microbial abundances of cariogenic organisms in severe early childhood caries, and conversely, caries-free children were abundant in microbes not previously associated with oral health [22]. Similar research has not yet been conducted in Indigenous Australian children. However, preliminary work studying the oral microbiota from dental calculus of Aboriginal Australian adults showed a distinctive microbial community from that found in non-Indigenous Australians, despite their shared periodontal disease state [23]. Such research highlights the importance of exploring both the microbial differences between ethnic groups, and how these specific microbial signatures may drive disease susceptibility.

Here, for the first time, we describe the salivary microbiota of Aboriginal and Torres Strait Islander children, who participated in a three-year-long trial of a caries preventative intervention programme. Using bacterial 16S ribosomal RNA (rRNA)

amplicon sequencing, we investigate the impact of this novel preventative treatment on the salivary microbial community and explore the microbiota associated with dental decay development in this rural Indigenous Australian population.

3.3 Methods and Materials

Ethics statement

Ethics approval was granted by the Griffith University Human Research Ethics Committee (GU Ref No: DOH/05/15/HREC); the Far North Queensland (FNQ) Human Research Ethics Committee (FNQ HREC/15QCH/39-970); the Department of Education and Training (Queensland Government) to approach participants at the schools; and the Torres and Cape Hospital and Health Service for Site Specific Approval. All surveys were conducted with the full understanding and written consent of parents/guardians of children from the three school campuses in the NPA of FNQ.

Study population and design

All children attending school in the NPA (two primary schools and one secondary) were invited to participate in this longitudinal caries preventive programme. Due to both the inclusivity and discretionary design of this programme, the number of participating children varied each year. Participants consented to the overall study and received dental care, and all participants could additionally accept or refuse the caries preventative treatment (herein referred to as the 'Intervention'), which included dental therapy, placing of fissure sealants on suitable posterior teeth, swabbing dentition with povidone-iodine, and the application of fluoride varnish [9]. Children who opted out of the Intervention due to cultural or logistical reasons acted as a natural untreated 'control group' (herein referred to as the 'Control' group), receiving the same examinations, but not the three-step Intervention. Of the 177 children who participated in the 2017 study year, only children who attended all three years of the study (2015, 2016, 2017) were included in this analysis (n = 104; Intervention n = 69, Control n = 35). As saliva was taken prior to treatment application, Intervention children had received two consecutive treatments as of 2017 (SI Table 1).

Prior to the application of the Intervention, the research team (consisting of dentists and/or oral health therapists) undertook a detailed head, neck, and dental clinical examination, alongside a questionnaire on basic demography (age and gender), residential history (exposure to fluoridated drinking water), and perceptions surrounding general and oral health (such as oral health behaviours, attitudes, and knowledge, dental visits, and dietary information). Dental caries experience

was recorded using the International Caries Detection and Assessment System [24]. Saliva samples were collected prior to the treatment of existing oral health problems, and the Intervention was applied after any required restorative treatments were completed.

Sample collection

Stimulated saliva samples were collected at the initial epidemiological examinations, by chewing on paraffin wax for five minutes and dribbling into a sterile cup; the expectorated volume was recorded. 2 mL of saliva was transferred into an OMNIgene•Oral OM-501 collection tube (DNA Genotek). Samples were stored at room temperature, until transfer to The University of Adelaide, where samples were frozen at –20°C until extraction, according to the manufacturer's instructions.

For control of potential airborne microbial contamination, samples of the air (n = 11) were collected through opening blank OMNIgene•Oral OM-501 collection tubes in the dental examination room for at least a minute, both at the start and end of a day of salivary collection. Air control samples were transported and stored along with saliva samples.

DNA extraction, amplification, and sequencing

Saliva samples were extracted in a dedicated clean facility for microbiome research at the University of Adelaide. Standard personal laboratory equipment included a laboratory coat, surgical facemask, shoe covers, and two layers of gloves (to allow frequent glove changes without skin exposure). All surfaces are cleaned prior to laboratory work with Decon 90 (Decon Laboratories Limited) and KlerAlcohol 70% v/v Isopropyl Alcohol (EcoLab Life Sciences). All extractions were prepared and completed in still-air cabinets, which were cleaned with a 2% bleach (NaClO) solution, and UV-treated for 30 minutes prior to beginning any work.

200 μL of saliva was incubated at 50°C for an hour prior to extraction. The total genomic bacterial DNA was extracted using the Roche High Pure PCR Template Preparation Kit (Roche Life Sciences), following the manufacturer's instructions. Two sample blank controls (as known as extraction blank controls; EBCs) were included for each extraction batch (two EBCs per 22 saliva samples). All samples were amplified in triplicate alongside an additional PCR no-template control, using barcoded primers specific to the V4 region of the 16S rRNA gene, primer 515F (5'-GTGCCAGCMGCCGCGGTAA – 3') and 806R (5'-GGACTACHVHHHTWTCT AAT-3') [25]. Each PCR reaction contained: 18.05 μL sterile H20, 1 μL of DNA extract, 0.25 μL of Hi-Fi taq (Life Technologies), 2.5 μL of 10X Hi-Fi reaction buffer (Life Technologies), 1 μL MgSO4 (50 mM), 0.2 μL dNTPs (100 mM), and 1

μL each of the forward and reverse primers (10 mM). Samples were amplified under the following conditions: 95°C for 6 minutes; 38 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 90 seconds; and final step, 60°C for 10 minutes.

PCR triplicate products were pooled (to a final volume of 75 μL) and visualised by electrophoresis on a 2.5% agarose gel to check for size and quality of representative sample. Samples were prepared for high throughput sequencing by quantification on a fluorometer using a High Sensitivity dsDNA reagent kit (Qubit 2.0, Life Technologies), and pooled at equimolar concentrations for a normalized 5 nmol/L, before purification using AMPure cleanup (Ampure, Agencourt Bioscience). DNA sequencing was completed across two MiSeq runs using 150bp paired end chemistry (Illumina) at Australian Genome Research Facility Ltd. in Adelaide, Australia.

Data processing and analysis

Raw Illumina BCL files were processed through BCL2fastq (v. 1.8.4; Illumina) to produce three fastq files (forward, barcodes, and reverse sequences). Metagenomic data was then processed using the open-source QIIME2 platform (v. 2018.8) [26]. Raw multiplexed paired-end fastq files were imported and demultiplexed using barcodes, then denoised using the Deblur algorithm QIIME2 plugin [27]. Sequences were truncated to 120 bp based on the median quality score. One saliva sample was removed from downstream analysis due to extremely low sequencing depth of 68 sequences (Sample ID: Bam17.200), leaving 103 samples for downstream analysis. In the remaining samples, MAFFT [28] was called in QIIME2 to create a masked sequence alignment, removing highly variable positions. 16S rRNA sequences were assigned to taxonomic groups using the Greengenes (v. 13.8) [29], Human Oral Microbiome Database (HOMD; v. 15.1) [30] and ribosomal database SILVA (132 release) [31]; taxa names reported in text were chosen based on specificity and assignment confidence.

All statistical analyses were performed using QIIME1 (v. 1.9.1) [26]. Alpha diversity metrics were computed using Shannon, observed species, and Chao1 indices at rarefaction depth of 19,255 sequences (the lowest sequencing depth of any sample), with significant group differences determined by nonparametric t-test. Beta diversity analysis was completed using Bray-Curtis dissimilarity and binary Jaccard. Anosim (analysis of similarities) and adonis permutational multivariate analysis of variance were used to test significant differences in Bray-Curtis dissimilarities and binary Jaccard values across sample groups. Taxonomic group differences were determined using Kruskal-Wallis nonparametric ANOVA. All significant differences were assessed using FDR corrected p-values < 0.05.

3.4 Results

3.4.1 Authentic oral microbial community recovered from saliva

16S rRNA gene amplicons from all samples (biological samples and controls; n = 146) after data trimming and quality filtering produced a total of 6,991,276 sequences. The saliva samples (n = 103) produced an average of 65,678 sequences (SD = 51,278, range 19,255–551,410; 96.76% of total sequences). Blank control samples (extraction blank controls (EBCs), PCR negatives, and air filter controls; n = 43) contributed to a total of 226,401 sequences (3.24% of total sequences), with an average of 5265 sequences per sample (SD = 9821.62, range 18–49,953). All sequences clustered into 1,221 features (the QIIME2 term for sub-operational taxonomic units or amplicon sequence variants).

Saliva samples shared a total 1,056 features, with 165 features unique to the EBC samples. Blank control samples shared 280 overlapping features with salivary samples; likely due to reagent contamination and/or cross-contamination [32, 33]. Blank controls predominantly contained Proteobacteria (mean relative abundance 46% of total sequences), Firmicutes (25%), Actinobacteria (15%), Bacteroidetes (6%), Fusobacteria (1%), Cyanobacteria (1%), and Chloroflexi (1%). There were 17 assigned genera with a mean relative abundance greater than (1%), and the top 5 dominating genera, *Staphylococcus* (mean relative abundance of 10.3% of total sequences), *Acinetobacter* (7.7%), *Pseudomonas* (7%),*Novosphingobium* (6.2%), and *Micrococcus* (5.8 %) are all known laboratory contaminants (Figure 1) [33].

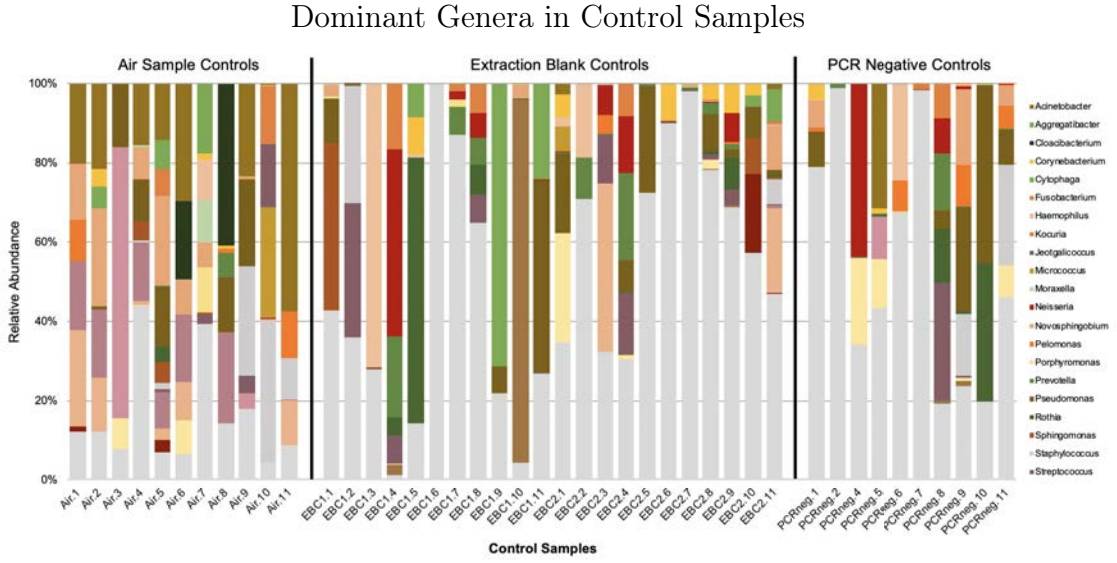

Figure 1: **Dominant genera of control samples.** Each bar represents a single sample; all genera contributing more than 1% of total sequences are coloured, showing the variation of taxonomy and contamination content within the control samples. Controls have an average sequencing depth of 5265 (ranging from 18–49,953 sequences).

A total of 14 phyla, 23 classes, 42 orders, 76 families, 119 genera, and 1,166 features were detected from 103 saliva samples. The most abundant phyla were Proteobacteria (average 29% of total sequences), Bacteroidetes (26%), Firmicutes (25%), Actinobacteria (11%), Fusobacteria (8%), and Spirochetes (1%). From the total of 119 genera detected, 15 genera dominated with a mean relative abundance >1% of the total sequences, with a total average contribution of 88.9% of sequences: *Prevotella* (19.3%), *Neisseria* (13.1%), *Haemophilus* (12.5%), *Streptococcus* (9.2%), *Rothia* (7.4%), *Veillonella* (5.1%), *Fusobacterium* (4.6%), unclassified genera of family Gemellaceae (3.5%), *Actinomyces* (2.4%), *Granulicatella* (2.4%), *Porphyromonas* (2.4%), unverified Prevotella (2.3%), *Leptotrichia* (2.2%), *Aggregatibacter* (1.5%) and *Oribacterium* (1%) (Figure 2).

3.4.2 Age, dentition, and gender did not drive significant variation in salivary microbiota

Previous salivary research identified microbial differences in saliva associated with age and dentition [34, 35]. Therefore, we tested the impact of dentition (mixed dentition-permanent dominant (n = 31), vs mixed dentition-deciduous dominant (n = 29), vs all-permanent dentition (n = 43)), age group (ages 6–8 (n = 33), vs ages 9–13 (n = 54), vs ages 14–17 (n = 16)), as well as sex (male (n = 38) vs female (n = 65)) (Table 1) on microbial community composition and structure, as measured by alpha and beta diversity metrics. We found no support for significant compositional differences between any demographic groups (Shannon, observed species, Chao1, $p > 0.05$, t (range) = -1.53–1.82).

Moreover, there was no support for age or sex contributing to microbial community variation as confirmed by Bray-Curtis and binary Jaccard metrics (adonis, $p > 0.1$, R^2 (range) = 0.009–0.022; anosim, $p > 0.05$, R (range) = 0–0.051). Binary Jaccard diversity found variation to be driven significantly by dentition groups (adonis, $p = 0.045$, $R^2 = 0.025$), but these groups were not significantly different from one another (anosim, $p = 0.155$, $R = 0.022$). This suggests that while dentition may describe the variation of unique features within the microbial composition,it does not significantly differentiate structure. Overall, these results suggest demographic factors are unlikely to be driving microbial diversity within this population.

	Demographics and Oral Health			
Groups	**Age ($\bar{x}$ years ± SD)**	**Gender (Male/Female, n (%))**	**Caries (Active/Free, n (%))**	**Dentition (Mixed/All Permanent, n (%))**
Intervention	10.43 ± 2.95	27/42 (39/61)	38/31 (55/45)	38/31 (55/45)
Control	9.6 ± 2.75	11/23 (32/68)	29/5 (85/15)	22/12 (65/35)
Total	10 ± 2.96	38/65 (37/63)	67/36 (67/35)	60/43 (58/42)

Table 1. Sample demographics by treatment group, (N = 103)

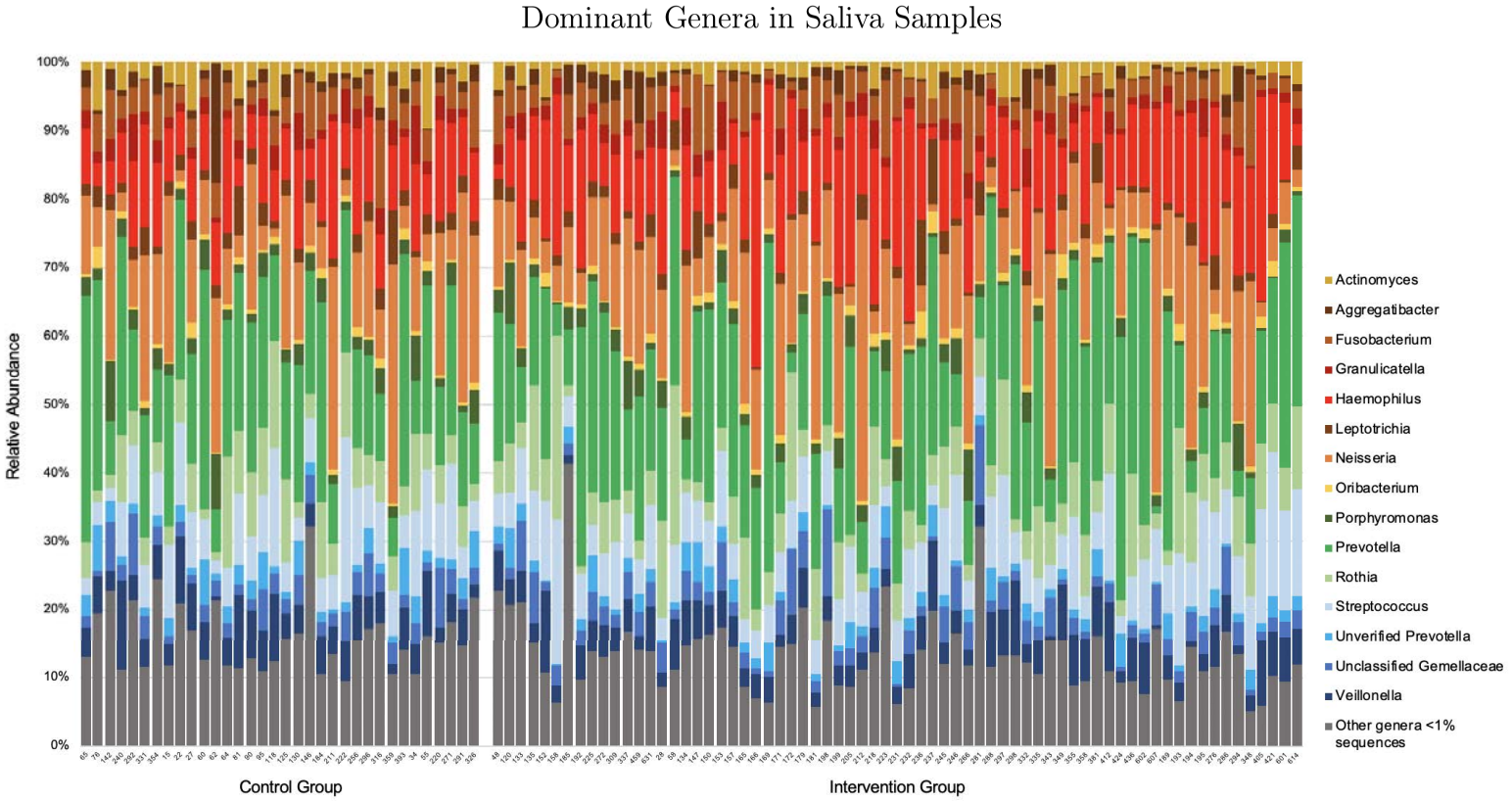

Figure 2: **Relative abundance of the dominant genera (1% of total sequences) of saliva samples, sorted by treatment group.** Each bar represents an individual salivary sample; microbial composition is similar between samples despite treatment group

3.4.3 Intervention decreases microbial diversity

To investigate the impact of the Intervention on the salivary microbial community, we analysed the microbial diversity of the Control and Intervention group samples, as calculated using Shannon, observed species, and Chao1 index metrics, compared using a nonparametric t-test (Table 2). Saliva samples of the Control group contained significantly higher microbial diversity (Shannon, observed species, Chao1, $p < 0.05$, $t = 2.77$–3.5), illustrating a reduction in both the taxonomic diversity and richness in children who received the Intervention. However, this difference in diversity did not significantly change the microbial community composition. While the presence or absence of microbial variation could be explained by treatment (Control vs Intervention; binary Jaccard, adonis, $p = 0.007$, $R^2 = 0.016$), the overall community composition was not significantly dissimilar between treatment groups (Bray-Curtis, adonis, $p = 0.05$, $R^2 = 0.018$; Bray-Curtis anosim, $p = 0.70$, $R = -0.019$; Jaccard anosim, $p = 0.70$, $R = -0.019$). Overall, this suggests that while the Intervention impacts the microbial diversity, the Intervention has minimal impacts on the overall microbial ecology.

	Alpha Diversity Metrics			
Metric	**Control group (mean ± SD)**	**Intervention group (mean ± SD)**	**T statistic**	**P-value**
Chao1	249.5 ± 46.1	222.2 ± 47.0	2.77	0.009
Observed species	227.2 ± 41.1	199.7 ± 45.1	2.96	0.004
Shannon	5.2 ± 0.4	4.8 ± 0.5	3.50	0.001

Table 2. **Alpha Diversity of Intervention and Control Groups.** Significance ($p < 0.05$) calculated at QIIME2 feature level, p-values are FDR corrected.

To examine whether the Intervention adversely impacted 'pathogenic' microorganisms, we tested significant associations of microbes between the Intervention group and the Control group with Kruskal-Wallis (Table 3). This was calculated at the feature level, then assigned in QIIME2 to three different reference databases (Greengenes, Human Oral Microbiome Database, and SILVA) to achieve best possible species identification. Three species were detected with significantly greater abundance within the Control group: *Lactobacillus salivarius* ($p = 0.04$, $t = 15.42$), Unassigned *Selenomonas* ($p = 0.04$, $t = 14.85$) and *Actinomyces* sp. HMT 896 ($p = 0.04$, $t = 14.78$). The decrease in the decay-associated *L. salivarius* suggests that the Intervention may have an impact on microbes associated with dental decay present at the time of sampling.

3.4.4 Presence or absence of caries is not associated with microbiota

Given previous work identifying signals of dental decay in salivary microbial communities [36, 37, 38], we initially tested the presence or absence of dental decay using the merged code ICDAS system, without accounting for Intervention participation [39]. We found no significant differences in the microbial diversity between all children who were caries-free (CF; ICDAS scores of 0–2, *i.e.* showing no obvious sign of local enamel breakdown) vs caries-active (CA; ICDAS scores = 3–6), using any alpha diversity metric (Shannon, observed species, Chao1; $p > 0.4$, t (range) = 0.72–0.81). Further examination also revealed no significant differences in the overall composition between the two groups (Bray-Curtis anosim, $p = 0.13$, $R = 0.04$; Jaccard anosim, $p = 0.10$, $R = 0.04$). Although significant variation was detected in the microbial abundance associated with the presence or absence of caries using Bray-Curtis (adonis; $p = 0.028$, $R^2 = 0.019$), binary Jaccard did not support differences in microbial variation, as it was not determined by the presence or absence of unique species (adonis; $p = 0.09$, $R^2 = 0.012$). Overall, these results suggest very little difference between the microbial communities associated with presence or absence of dental decay in these children without accounting for the participation of the Intervention programme.

Sequence	Greengenes (v 13.8) output		SILVA (132 release) output		HOMD (v. 15.1) output		Test-Statistic	P value	FDR P-value	Control (mean)	Intervention (mean)
	Assignment	Assigned Confidence	Assignment	Assigned Confidence	Assignment	Assigned Confidence					
TACGTAGGTGGCAAGCGTTGTCCGGATTTATT GGGCGTAAAGGGAACGCAGGCGGTCTTTTAA GTCTGATGTGAAAGCCTTCGGCTTAACCGGA GTAGTGCATTGGAAACTGGAAGACTT	*Lactobacillus salivarius*	0.94629286	*Lactobacillus salivarius*	0.9997102	*Lactobacillus salivarius*	0.99999484	15.42	8.58E-05	0.04258	5.42	0.59
TACGTAGGTGGCGAGCGTTGTCCGGAATCAT TGGGCGTAAAGGGAGCGCAGGCGGGCATGT AAGTCTTTCTTAAAAGTTCGGGGCTCAACCCC GTGATGGGAAAGAAACTATATGTCTTG	Unassigned Selenomonas	0.99999821	*Selenomonas* uncultured bacterium	0.82363492	Unassigned *Selenomonas*	0.99747897	14.85	0.000116	0.04258	5.79	2.59
TACGTAGGGCGCGAGCGTTGTCCGGAATTAT TGGGCGTAAAGGGCTTGTAGGCGGCCGGCC GCGTCCGTCGTGAAATCCTTCGGCTCAACCG GGGGCTTGCGGTGGGTACGGGTCGGCTG	Unidentified *Actinomyces* sp.	0.99969328	*Actinomyces* unidentified	0.93119961	*Actinomyces* sp. HMT 896	0.90713142	14.78	0.000121	0.04258	1.76	0.25

Table 3. **Kruskal-Wallis group significance calculated at QIIME2 feature-level.** Differences in the mean relative abundance of sequences between Intervention and Control groups. QIIME2 feature IDs were assigned to using the Greengenes (v. 13.8) [29], Human Oral Microbiome Database (HOMD; v. 15.1) [30] and ribosomal database SILVA (132 release) [31] databases. Significance was determined by FDR corrected p-value < 0.05.

Sequence	Greengenes (v 13.8) output		SILVA (132 release) output		HOMD (v. 15.1) output		Test-Statistic	P value	FDR P-value	Caries Severity		
	Assignment	Assigned Confidence	Assignment	Assigned Confidence	Assignment	Assigned Confidence				ICDAS 0–2	ICDAS 3–4	ICDAS 5–6
TACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGC GTAAAGCGAGCGCAGGCGGTTGCTTAGGTCTGATG TGAAAGCCTTCGGCTTAACCGAAGAAGTGCATCGGA AACCGGGGCGACTT	*Lactobacillus reuteri*	0.982440212	*Lactobacillus reuteri*	0.976459196	Unassigned *Lactobacillus*	0.999999999	27.16	1.26E-06	0.00067	0	0	4.24
TACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGC GTAAAGGGAACGCAGGCGGTCTTTTAAGTCTGATGT GAAAGCCTTCGGCTTAACCGGAGTAGTGCATTGGAA ACTGGAAGACTT	*Lactobacillus salivarius*	0.946292856	*Lactobacillus salivarius*	0.999710196	*Lactobacillus salivarius*	0.999994842	27.16	1.27E-06	0.00067	0	0	6.62
TACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGC GTAAAGCGAGTGCAGGCGGTTCAATAAGTCTGATGT GAAAGCCTTCGGCTCAACCGGAGAATTGCATCAGAA ACTGTTGAACTT	Unidentified *Lactobacillus* sp.	0.975094238	*Lactobacillus gasseri*	0.896556283	*Lactobacillus gasseri*	0.953617554	24.53	4.72E-06	0.00166	0	0.27	9.38
TACGGAAGGTTCTGGTGTTATCCGGATTTATTGGGT TTAAAGGGAGCGCAGGCTGTCAGATAAGCGTGTTGT GAAATGCGGTTGCTCAACATCCGCACTGCAGCGCG AACTGTCTGACTT	Unidentified *Prevotella* sp.	0.83803847	*Prevotella* unidentified	0.997566587	*Prevotella multisaccharivorax*	0.999999804	19.00	7.48E-05	0.01475	0	0.12	43.85
TACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGC GTAAAGGGAGCGCAGGCGGTCAGGAAAGTCTGGAG TAAAAGGCTATGGCTCAACCATAGTGTGCTCTGGAA ACTGTCTGACTTG	Unassigned *Streptococcus*	0.880074047	*Streptococcus mutans*	0.999999363	*Streptococcus mutans*	0.999999993	18.94	7.72E-05	0.01475	7.72	32.79	81.74
TACGTAGGGTGCAAGCGTTGTCCGGATTTATTGGGC GTAAAGGGCTCGTAGGCGGTTCGTCGCGTCTGGTG TGAAAGCCCGCTGCTTAACGGTGGGTTTGCGTTGGA TACGGGCGGGCTT	Unidentified *Scardovia* sp.	0.989812418	Unassigned sp. Bifidobacteriaceae	0.988635794	*Parascardovia denticolens*	0.968289857	18.77	8.40E-05	0.01475	0.06	0.49	6.23
TACGTAGGTGGCGAGCGTTGTCCGGAATCATTGGG CGTAAAGGGAGCGCAGGCGGGCATGCAAGTCCATC TTAAAAGCGCGGGGCCCAACCCCGCGAGGGGATG GAAACTGCGTGCCTTG	Unidentified sp. of Veillonellaceae	0.878288966	Veillonellaceae uncultured	0.993500987	*Mitsuokella* HMT 131	0.999999985	18.46	9.78E-05	0.01475	0.06	0.06	5.62
TACGGAAGGTTCTGGTGTTATCCGGATTTATTGGGT TTAAAGGGAGCGCAGGCTGTCAGATAAGCGTGTTGT GAAATGCGGTTGCTCAACATCCGCACTGCAGCGCG AACTGTTTGACTT	Unassigned *Prevotella*	0.843514429	*Prevotella* unidentified	0.999193496	*Prevotella multisaccharivorax*	0.999999988	16.44	0.00027	0.03553	0.14	1.85	23
CACGTAAGGGGCGAGCGTTGTTCGGAATTATTGGGC GTAAAGGGTATGCAGGCGGTTAGATAAGCCCGGTGT GAAATGCTCAAGCTTAACTTGAGAACTGCACTAGGTA CTGTCTAACTT	Unidentified Treponema sp.	0.715773014	Unassigned Treponema	0.950848411	Unassigned Treponema	0.998944839	15.90	0.00035	0.04141	3.53	2	0.27

Table 5. **Kruskal-Wallis Group Significance calculated at QIIME2 feature-level.** Differences in the mean relative abundance of sequences between Intervention and Control groups. QIIME2 feature IDs were assigned to using the Greengenes (v. 13.8) [29], Human Oral Microbiome Database (HOMD; v. 15.1) [30] and ribosomal database SILVA (132 release) [31] databases. Significance was determined by FDR corrected p-value < 0.05

3.4.5 Intervention differentially influences microbiota according to decay status

At the time of sampling, 85% of children in the Control group had carious lesions, relative to 55% of children in the Intervention group (Table 1). Therefore, to determine the impact of treatment accounting for dental decay, we initially examined the differences in diversity and composition of CF children to determine how the Intervention treatment impacted the microbial community of children without dental decay. We found no significant differences in microbial diversity between therapeutic groups (CF Intervention group (n = 31) vs CF Control group (n = 5); Shannon, observed species, Chao1, p = 1, t (range) = -0.80—1). Furthermore, Intervention appears not to have impacted the overall microbial community structure or variation in CF children (Bray-Curtis adonis, p = 0.54, R^2 = 0.026, binary Jaccard adonis, p = 0.92, R^2 = 0.022; Bray-Curtis anosim, p = 0.9, R = -0.15; binary Jaccard anosim, p = 0.95, R = -0.16). These results suggest that the Intervention, despite the use of broad-spectrum antimicrobials, did not appear to affect the diversity or composition of the salivary microbial community in children with good oral health *ab initio*.

Next, we compared all children with active dental decay (CA; ICDAS score 3–6) between treatment groups. Microbial diversity of CA Intervention children was significantly lower than the microbial diversity of the CA Control group (CA Intervention (n = 38) vs. CA Control (n = 29); Shannon, p = 0.006, t = 3.41; observed species, p = 0.024, t = 3.04; Chao1, p = 0.048, t = 2.70). This suggests that the decay-associated microbial diversity is different between therapeutic groups. Nevertheless, significant differences in microbial composition were not generally explained by caries presence (binary Jaccard anosim, p = 0.279, R = 0.02; Bray-Curtis adonis, p = 0.06, R^2 = 0.024; Bray-Curtis anosim, p = 0.377, R = 0.005), although some variation in microbial composition could be induced by the presence or absence of unique species (binary Jaccard adonis, p = 0.017, R^2 = 0.02). Generally, these results suggest despite impact of Intervention treatment upon community diversity, the overall community composition still supported the development of dental decay. However, the Intervention only impacted the microbial diversity of children with active decay, which may be characteristic of the preventative treatment mechanisms acting upon pathogenic oral microbiota.

Caries severity	Intervention group, n (%)	Control group, n (%)	Total, n (%)
None (ICDAS score 0–2)	31 (45%)	5 (15%)	36 (35%)
Low (ICDAS score 3–4)	22 (32%)	11 (32%)	33 (32%)
High (ICDAS score 5–6)	16 (23%)	18 (53%)	34 (33%)

Table 4. Sample distribution of caries severity by therapeutic group, based on the merged International Caries Detection and Assessment System (ICDAS) score

3.4.6 Severity of dental decay impacts microbial composition

Given the minimal impact of the presence or absence of carious lesions on oral microbial diversity, we looked to examine how caries severity influences the oral microbiota—regardless of Intervention participation—by grouping children into three levels of decay (Table 4): None (ICDAS score of 0—2; n = 36), Moderate (ICDAS 3–4; n = 33), and Severe (ICDAS 5–6; n = 34). No differences in microbial diversity were detected between the varying levels of decay (Shannon, observed species, Chao1; p = 1, t (range) = -1.01–0.5). However, significant differences in microbial community structure were identified using binary Jaccard (adonis, p = 0.032, R^2 = 0.025; anosim, p = 0.02, R = 0.034), but not Bray-Curtis (adonis, p = 0.13, R^2 = 0.026; anosim, p = 0.138, R = 0.014), indicating that these community differences are perhaps driven by the presence or absence of unique species.

We looked to identify the microbial species that may underpin the differences in composition between the three groups of varying decay. Seven species with a significantly greater relative abundance corresponding with increasing caries severity were detected using Kruskal-Wallis (Table 5; $p < 0.036$, $t > 16.44$): three *Lactobacillus* species, *Prevotella multisaccharivorax*, *Streptococcus mutans*, *Parascardovia denticolens*, and *Mitsuokella* HMT 131. Interestingly, we were also able to detect an increase of an unassigned *Treponema* species associated with decrease in dental decay (p = 0.04, t = 15.9), suggesting a relationship to oral health. Overall, it appears the detection of several key 'pathogenic' taxa within this population is dependent upon caries severity.

	Greengenes (v 13.8) output		SILVA (132 release) output		HOMD (v. 15.1) output					Control			Intervention		
Sequence	Assignment	Assigned Confidence	Assignment	Assigned Confidence	Assignment	Assigned Confidence	Test-Statistic	P value	FDR P-value	ICDAS 0–2	ICDAS 3–4	ICDAS 5–6	ICDAS 0–2	ICDAS 3–4	ICDAS 5–6
TACGTAGGTGGCAAGCGTTGT CCGGATTTATTGGGCGTAAAG GGAACGCAGGCGGTCTTTTAA GTCTGATGTGAAAGCCTTCGG CTTAACCGGAGTAGTGCATTG GAAACTGGAAGACTT	*Lactobacillus salivarius*	0.946292856	*Lactobacillus salivarius*	0.999710196	*Lactobacillus salivarius*	0.999994842	42.35	5.00E-08	5.28E-05	0	0	10.22	0	0	2.56
TACGTAGGGTGCGAGCGTTGT CCGGAATTACTGGGCGTAAAG AGCTCGTAGGTGGTTTGTCGC GTCGTCTGTGAAATACCAATG CTTAACGTTGGTCGTGCAGGC GATACGGGCATTACT	Unassigned *Corynebacterium*	0.977614648	Unassigned *Corynebacterium*	0.812810013	Unassigned *Corynebacterium*	0.999530024	39.58	1.81E-07	9.56E-05	1.4	0	0	0	0	0
TACGTAGGTGGCAAGCGTTAT CCGGATTTATTGGGCGTAAAG CGAGCGCAGGCGGTTGCTTA GGTCTGATGTGAAAGCCTTCG GCTTAACCGAAGAAGTGCATC GGAAACCGGGCGACTT	*Lactobacillus reuteri*	0.982440212	*Lactobacillus reuteri*	0.976459196	Unassigned *Lactobacillus*	0.999999999	35.49	1.20E-06	0.00034	0	0	6.44	0	0	1.75
TACGGAAGGTTCTGGTGTTAT CCGGATTTATTGGGTTTAAAG GGAGCGCAGGCTGTCAGATA AGCGTGTTGTGAAATGCGGTT GCTCAACATCCGCACTGCAGC GCGAACTGTCTGACTT	Unidentified *Prevotella* sp.	0.83803847	Unidentified *Prevotella* sp.	0.997566587	*Prevotella multisaccharivorax*	0.999999804	35.35	1.28E-06	0.00034	0	0	20.94	0	0.18	69.63
TACGGAAGGTTCTGGTGTTAT CCGGATTTATTGGGTTTAAAG GGAGCGCAGGCTGTCAGATA AGCGTGTTGTGAAATGCGGTT GCTCAACATCCGCACTGCAGC GCGAACTGTTTGACTT	Unassigned *Prevotella*	0.843514429	Unidentified *Prevotella* sp.	0.999193496	*Prevotella multisaccharivorax*	0.999999988	31.17	8.67E-06	0.00183	0	0.18	33.61	0.16	2.68	11.06
TACGTAGGTGGCGAGCGTTGT CCGGAATCATTGGGCGTAAAG GGAGCGCAGGCGGGCATGCA AGTCCATCTTAAAAGCGCGGG GCCCAACCCCGCGAGGGGAT GGAAACTGCGTGCCTTG	Unidentified sp. of Veillonellaceae	0.878288966	Veillonellaceae uncultured	0.993500987	Mitsuokella HMT 131	0.999999985	29.22	2.10E-05	0.00370	0	0	5.5	0.06	0.09	5.75
TACGTAGGTGGCAAGCGTTGT CCGGATTTATTGGGCGTAAAG CGAGTGCAGGCGGTTCAATAA GTCTGATGTGAAAGCCTTCGG CTCAACCGGAGAATTGCATCA GAAACTGTTGAACTT	Unidentified *Lactobacillus* sp.	0.975094238	*Lactobacillus gasseri*	0.896556283	*Lactobacillus gasseri*	0.953617554	25.95	9.13E-05	0.01377	0	0.36	12.44	0	0.23	5.94
TACGTATGTCGCAAGCGTTAT CCGGAATTATTGGGCATAAAG GGCATCTAGGCGGCCAGACA AGTCTGGGGTGAAAACTTGTG GCTCAACCGCAAGCCTGCCCT GGAAACTGTTTGGCTA	Unidentified *Leptotrichia* sp.	0.999999833	*Leptotrichia* uncultured bacterium	0.845070546	*Leptotrichia* sp. HMT 225	0.808648489	25.59	0.00011	0.01416	0	1.18	0	0	0	0

Table 6. **Kruskal-Wallis Group Significance calculated at QIIME2 feature-level.** Differences in the mean relative abundance of sequences between decay severity within Intervention and Control groups. QIIME2 feature IDs were assigned to using the Greengenes (v. 13.8) [29], Human Oral Microbiome Database (HOMD; v. 15.1) [30] and ribosomal database SILVA (132 release) [31] databases. Significance was determined by FDR corrected p-value < 0.05

3.4.7 Intervention differentially impacts taxa associated with severe caries

Lastly, we explored whether the Intervention impacted these specific microorganisms linked to decay severity inclusive of Intervention participation. To do so, we further partitioned the caries severity groups based on therapeutic group: Intervention None (n = 31), vs Intervention Moderate (n = 22), vs Intervention Severe (n = 16), vs Control None (n = 5), Control Moderate (n = 11), vs Control Severe (n = 18). We were able to detect six of the seven taxa prior associated with caries severity regardless of Intervention, using Kruskal Wallis: three *Lactobacillus* species, *P. multisaccharivorax*, *P. denticolens*, and *Mitsuokella* HMT 131 (Table 6; $p < 0.013$, $t > 25.95$). *S. mutans* was no longer significantly detected when accounting for treatment groups ($p = 0.06$, $t = 21.48$), suggesting the mean relative abundance of *S. mutans* is not impacted by the Intervention therapy.

Chiefly, the three *Lactobacillus* species were detected at lower relative abundance (at each level of decay) within the Intervention group compared to the Control group (Table 4; $p < 0.013$, $t > 25.95$). Yet, some species increased in relative abundance within the Intervention group (*e.g.* total assigned *P. multisaccharivorax*, $p < 0.002$, $t > 31.17$). *Parascardovia denticolens* and *Mitsuokella* HMT 131 show minimal differences between Intervention or Control groups, but maintained increasing relative abundance correlated with caries severity ($p < 0.04$, $t > 23.04$). Further, some taxa were only detected within the Control group (*Corynebacterium* and *Leptotrichia* HMT 225, Kruskal Wallis, $p < 0.014$, $t > 25.59$), potentially indicative of the reduced microbial diversity within the Intervention treatment. These results highlight the specificity of the Intervention on particular bacterial taxa, namely *Lactobacillus* species, and present a number of salivary biomarkers that are representative of increasing decay in our population of Indigenous Australian children.

3.5 Discussion

We examined the salivary microbial community from Aboriginal and Torres Strait Islander children of remote NPA communities, Far North Queensland, in response to both caries and the novel caries preventative Intervention programme. To our knowledge, this is the first study to investigate the oral microbiota of Indigenous Australian, and the first to review the impact to the salivary microbial community of children after undergoing a longitudinal oral health therapy. After characterising the salivary microbiota, we were able to show that the Intervention decreased the microbial diversity but did not significantly impact the overall microbial composition. In addition, we were able to detect microbial signals of dental decay in saliva; notably,

we identified seven species associated with extensive carious lesions: *Lactobacillus salivarus*, *L.reuteri*, *L. gasseri, Streptococcus mutans*, *Prevotella multisaccharivorax*, *Parascardovia denticolens*, and *Mitsuokella* HMT 131.

As this study is the first to look at the whole-community changes to the microbiota in response to a caries preventative treatment, there is limited literature currently available to interpret whole-community changes in the mouth linked to oral treatments. While the topical disinfectant, fissure sealants, and fluoride varnish are accepted as decay preventative measures, their implementation has been promoted only after studying the direct impact of the treatment on mutans streptococci and other limited caries-associated species *in vitro* [10], or by subsequent visual scoring of carious lesions to measure caries increment [11, 12]. Our results show that children who received the Intervention experienced a loss in species diversity (*i.e.* richness), which is typically indicative of an ecological disturbance—a discrete event causing the loss of microorganisms and an alteration to community structure [40]. Research has shown antimicrobial treatments to reduce microbial diversity on the human oral microbiota in the short-term, usually linked with the depletion of one or several specific taxa [41]. However, antimicrobial insults on the salivary microbiota appear transient, and there is a near complete recovery of the microbial community over time [20, 42]. For children of the NPA, the repeated annual application of the Intervention appears to be driving a more permanent change and/or an incomplete recovery of the initial microbial community state.

While conventional ecological theory suggests lower species diversity may reduce resilience to ecological instability or invading pathogens [43], this may not be pertinent to oral health, where greater microbial diversity has been observed with oral disease, compared to that of orally healthy individuals [36, 37, 44]. This observation is supported by our results, where decreased microbial diversity was detected with Intervention treatment, and despite the presence of dental decay, was overall linked to improvements in oral health within this population [9]. This might suggest that decreased microbial diversity induced by the Intervention is symptomatic of preventative mechanisms supporting oral health. We hypothesise that the Intervention modifies the microbial ecology towards a state supportive of oral health; but the processes by which this occurs cannot be elucidated by the study of microbiota alone. The Intervention may be directly impacting microbial community function or indirectly impacting the environmental variables that define microbial ecology [43]. Future work looking at the functional potential of the microbial ecology may illuminate the underlying agents of this ecological state. Understanding the impact of these treatments on the overall microbial ecology of the mouth is critical for understanding the long-term implications, benefits, or risks, associated with novel dental therapies. Longitudinal tracking of the possible downstream effects from an

initial ecological shift has often been disregarded in oral health research and needs to be included in studies moving forward.

Using saliva, we detected several bacterial species associated with severe dental decay in Aboriginal and Torres Strait Islander children. While oral microbes in a planktonic state are not usually regarded as direct causal agents of dental decay, understanding specific bacteria associated with poor oral health can facilitate prevention and treatment, especially against those acidogenic and acidophilic species that are more likely to contribute to the caries process. In this study, species *L. salivarus*, *L.reuteri*, *L. gasseri*, *S. mutans*, *P. multisaccharivorax*, *P. denticolens*, and *Mitsuokella* HMT 131 were all significantly increased within the salivary microbiota of children with severe carious lesions. *S. mutans* was initially detected in association with increasing caries severity, but was no longer significant after accounting for Intervention participation. Our results could suggest the limited impact of the Intervention upon the relative abundance of *S. mutans* in saliva. Alternatively, since the initial detection of *S. mutans* within the severe decay group (regardless of treatment) was at very low relative abundance, the genomic results are perhaps symptomatic of the generalised low prevalence of the *Streptococcus* genus within this population. *Streptococcus* genus only contributed to 9.2% of the average relative abundance within our Aboriginal and Torres Strait Islander population compared to multiple reports of *Streptococcus* abundance contributing to more than 20% of the saliva microbial community [34, 36, 37].

L. salivarius has been previously detected in saliva of individuals with progressive carious lesions within multiple populations [45, 46, 47]. *Lactobacillus* species are hypothesized to supervene the formation of the carious lesion, supporting downstream enamel demineralisation by more acidogenic species, such as *S. mutans* [48]. As such, it is surprising that *L. reuteri* has been suggested as a probiotic agent against the formation of carious lesions, identified in the dental plaque of people with good oral health [49], for our results do not support its beneficial association. The *Lactobacillus* species' functional repertoire (*i.e.* the ability to thrive in a low pH environment and produce lactic acids [50]) suggests that its presence supports the development of carious lesions. Similarly, the increased levels of *Mitsuokella* HMT 131, *P. denticolens*, *P. multisaccharivorax* in saliva from children with extensive caries are likely symptomatic of the acidic, relatively anaerobic oral environment. *P. denticolens* has previously been detected in association with caries from salivary microbiota [46, 51].

While *Mitsuokella* HMT 131 has not previously been associated with dental caries, it has been found in other anaerobic environments, such as the subgingival plaque of periodontitis and dental root canals [52, 53]. Similarly, *P. multisaccharivorax* both has been previously associated with a wide-range of oral diseases (including

severe early-childhood caries, root caries, and periodontal disease), as it is an obligate anaerobic species [54, 55]. Nevertheless, detection of caries-associated microorganisms in this population suggests that the salivary microbiota can be indicative of caries advancement. By identifying biomarkers for dental decay, we may also gain greater insights into not only predicting caries development, but also additional oral or systemic diseases. For example, while *P. multisaccharivorax* is associated with severe caries in these children, its links to periodontal disease further suggest that additional, longitudinal work should explore the relationships between these microorganisms and the increased risk for developing periodontal disease in Aboriginal and Torres Strait Islander people, both in childhood and later in life [56, 2].

Despite both the cost-effectiveness and ease of large-batch processing provided by 16S rRNA sequencing, the approach has its limitations. First, there are known biases in using the 16S gene for identifying microbial species. We used the V4 region of the 16S rRNA and protocols used in the Human Microbiome Project (HMP) [25], shown to have one of the highest species assignment accuracy [57]. Yet, the discrepancies between different variable regions and the different protocols used in other salivary studies limit our ability to compare across populations. Second, sequencing with 16S rRNA also restricts the ability to describe increased or decreased “abundance" of a particular microbial species associated with oral health or disease, as detection can be influenced by the number of 16S rRNA operon copies present in a particular bacterial genome [58]. Thus, only relative abundance can be discussed, which may not reflect the true biological ecosystem [59], although we used both the use of normalization and nonparametric Kruskal Wallis test to circumvent some of these issues [59].

Lastly, the choice of reference database will influence the taxonomic assignment (as seen in Tables 3,5,6). While Greengenes database was popularised by the HMP, unfortunately, it has not been updated since May 2013 and is quickly becoming outdated. The HOMD database is also problematic; although it can more accurately classify microorganisms present in the oral environment (of predominantly urban-industrialised populations), it impedes assignments to any species not previously identified in the oral environment. This latter impediment likely masks potentially novel species found in understudied populations of various cultural and environmental niches [24], as well as concealing potential contaminant taxa in oral samples. SILVA database has the opposite dilemma, wherein its assignment to various environmental niches is accurate, it has less specificity for oral taxa. We attempted to mitigate these ascertainment biases through the use of multiple databases for taxonomic identification. While shotgun sequencing techniques will mitigate some of these issues, further exploring the microbiota in underrepresented populations is still a key issue for the future dental research [60].

Our study is the first characterisation of the salivary microbiota of Aboriginal and Torres Strait Islander children. A number of studies have already identified population- and/or ethnic-based differences in oral microbiota [61, 62]. Since cultural and environmental factors driving salivary microbial community variation can dominate familial or hereditary signals [62, 63, 34], the coevolutionary history of microbiota and their host may be confounded or influenced by current cultural and environmental practices today [64]. Such as the processes of industrialisation, evident in Indigenous Australian populations. Prior to the 1980s, Indigenous Australians were noted for having considerably better oral health than their non-Indigenous counterparts [65]. It is hypothesised that this cultural transition to an 'industrialised' lifestyle—especially dietary impact of increased sugar intake and other readily-fermentable carbohydrates—in addition to socio-economic risk factors [66], may have induced an oral microbial community detrimental to oral health [4]. However, studies have shown that accounting for socio-economics variables still does not explain the disparity in oral health that exists between Indigenous Australians and their non-Indigenous counterparts [67]. Assessing the contributions of microbiota to Indigenous Australian health and disease may require an understanding of what their microbial communities were prior to impacts of sociocultural processes, such as colonialism and industrialisation [64]. At the most basic level, future research is immediately needed to explore if unique oral microbial communities exist in Indigenous Australians compared to those of non-Indigenous descent, and determine if these communities contribute to poorer oral health in Aboriginal and Torres Strait Islander Australians compared to non-Indigenous people: such studies should be recognized in the efforts to diminish the oral health gap.

3.6 Concluding remarks

In conclusion, the Intervention markedly improved the rate of dental decay of NPA children, in association with an ecological disturbance to the microbial community that is atypical of health elsewhere in the body. In addition, we identified six unsuspected biomarkers for severe caries in this population of Aboriginal and Torres Strait Islander children. This study demonstrates the use of non-invasive saliva collection to assess the links between the oral microbiota, dental disease and caries preventative therapy, providing key information to assist in the development of such oral health interventions and to assess longitudinal outcomes of caries prevention programmes, especially within Indigenous populations. This research highlights the need for further microbiome research in children and adults of underrepresented populations across the globe.

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3.8 Author contributions

NWJ designed the Intervention study and participated, alongside KK, in all field work and sample collection, data management and clinical interpretations. NWJ, ES, and LSW designed the microbiome study. ES performed lab work. ES, KK and LSW led analysis and interpretation. ES wrote the initial draft, which was edited and contributed to by all authors.

The authors declare that they have no competing or conflicts of interest.

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Chapter 4

Impacts of Storage Methods Over Time on Reconstructing Dental Calculus Microbial Communities

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

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Impacts of Storage Methods Over Time on Reconstructing Dental Calculus Microbial Communities

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4.1 Abstract

The study of ancient human-associated microbial communities provides an unprecedented opportunity to assess evolutionary and anthropological questions, which have substantial implications for medical research. Calcified dental plaque (calculus) has quickly become the palaeomicrobiological material of choice, as the pre-mortem calcification process protects endogenous DNA from post-mortem environmental conditions. However, the impact of taphonomy (*i.e.* the processes of decay and preservation) on the reconstruction of ancient microbial communities—especially preserved within dental calculus—remains largely unexplored. Here, we analysed metagenomic shotgun sequences acquired from modern dental calculus samples, stored long-term (>2 years) at room temperature (n = 6)—indicative of typical storage conditions for ancient dental calculus samples in museum collections—to both fresh samples (n = 18) and those stored long-term at -20°C (n = 6). There were significant differences in the microbial diversity of dental calculus samples stored long-term at room temperature (Shannon, Kruskal-Wallis, p = 0.004, H = 10.87), notably impacting *Fusobacterium* (p = 0.033, t = 10.84), *Leptotrichia* (p = 0.033, t = 10.01), and *Selenomonas* (p = 0.045, t = 8.67) genera, with greater phyla-level alterations to Fusobacteria (p = 0.002, t = 13.02) and Bacteroidetes (p = 0.008, t = 9.04). Long-term storage at -20°C had greater congruence to freshly extracted samples (p > 0.05), but there were still a significant difference in abundance of phyla Fusobacteria (Kruskal-Wallis, p = 0.048, t = 7.14) and Actinobacteria (p = 0.048, t = 6.08). Overall, phylum Fusobacteria appeared to be greatly impacted by long-term storage regardless of temperature. Analysis of microbial reconstruction with 16S rRNA amplicon sequencing found a lesser impact of long-term storage; only room-temperature storage impacting relative abundance of phylum Firmicutes (Kruskal-Wallis, p =

0.02, t = 9.80). Our study is the first to explore the taphonomic impact of microbial community reconstruction over time within modern dental calculus samples, identifying the biases that will impact both contemporary and ancient dental calculus research, which need to be considered when translating paleomicrobiological records for modern human health.

4.2 Introduction

A new era of palaeomicrobiology commenced with the breakthrough of high-throughput sequencing technology producing millions of DNA sequences in parallel, allowing access to whole ancient microbial communities. Studies have already retrieved ancient microbiota (*i.e.* human-associated microorganisms) from mummified human remains [1], historical medical specimens [2], fossilised faecal remains (coprolites), and microbial deposits in bone (deriving from seepage from decomposition) [3]. However, the most reputable source of palaeomicrobiological material is that of calcified dental plaque, known as dental calculus or tartar. Dental plaque is a microbial biofilm, formed by the specialised signalling mechanisms and adherence partnerships between human oral microorganisms to one another and to the enamel tooth surface [4]. The salivary and cervical fluids naturally deposit calcium and phosphate salts into the biofilm, petrifying it into dental calculus [5]. The ability to extract micorbial DNA from mineralised calculus has been already been exploited within a number of studies: such as, paleoepidemiological explorations [6], the examination of bacterial genome evolution through time [7], employed within ancient human dietary analyses [6, 7], as well as monitoring the evolutionary history of microbiota communities through time [8].

There are numerous benefits in using dental calculus for the analysis of ancient microbiota through time. Firstly, as the mineralisation process occurs prior to the cessation of the host, building up through an individual's lifetime, the structure and formation of dental calculus aids in safeguarding the endogenous microbiota from host decomposition and post-mortem environmental contamination; which other ancient microbiome samples (*e.g.* coprolites) for prone [9, 10, 5]. Additionally, the presence of dental calculus in the archaeological record is far more abundant than any other ancient microbiome sample; calculus commonly found on human teeth of most pre-agricultural societies, but is ubiquitously in nearly all post-agricultural populations [11]. Since dental calculus is often found alongside human remains, additional anthropological data specific to the host can also further aid in the study of ancient microbial communities [12].

The application of ancient dental calculus in the field of palaeomicrobiology is still relatively new, but its analytical power in evolutionary and anthropological research is tremendous, already providing insights from the past that have important implications for contemporary public health and medical research. However, the significance of these conclusions is dependent upon the ability to compare microbial communities from ancient to modern dental calculus microbial communities. Like most ancient DNA studies, paleomicrobiology of dental calculus has a number of biases that need to be accounted for. For example, the ratio of guanine-cytosine

bases within a microbial genome has been shown to impact ancient DNA preservation or damage [13], or the length of DNA fragments within ancient samples [7, 14], have both been shown to bias taxonomic reconstruction. While the field of ancient dental calculus research acknowledges the potential biases of taphonomy (*i.e.* the processes of decay and preservation that impact the microorganisms in fossilisation), how this bias may impact microbial community reconstruction, or comparisons to modern dental calculus samples, is not yet understood. Conventionally, modern dental calculus samples are removed from the mouth of a living individual, often intermixed with fresh dental plaque, and then are typically stored at -20°C or below until time of DNA extraction. In contrast, ancient dental calculus specimens are typically associated with remains that have persisted in the environment, from decades to millennia, and then are stored at room temperature in archaeological collections (*e.g.* museums), sometimes for decades. As a result, the field of paleomicrobiology has typically avoided direct whole community comparisons between modern and ancient samples all together, despite the value that those comparisons could possess [7].

Within modern microbial research, the investigation of sample storage conditions on microbial community profiles have been tested upon human faecal material [15, 16, 17], vaginal samples [18], skin [19], dental plaque [20], and environmental soil samples [21]. In general, these studies have demonstrated an accumulation of compositional alterations positively associated with the amount of time stored since collection, with cold storage typically decelerating this process. These patterns of decay are sample dependent, and as of yet, the impacts of storage methods over time have not yet been explored in modern dental calculus samples. Understanding the taphonomic issues present upon dental calculus microbial communities would better allow us to explore the relevance of past oral microbiomes to modern human health.

Here, we assessed the impact of both time and storage techniques on microbial communities within modern dental calculus sampled over a period of six years using both shotgun and 16S ribosomal RNA amplicon metagenomic sequencing techniques. We qualitatively assessed taphonomic signatures within dental calculus stored at room temperature and –20°C freezer storage, highlighting the potential biases that may occur in the analysis of ancient oral microbiota. More broadly, this preliminary analysis aimed to examine the impact of long-term storage conditions on the reconstruction of dental calculus microbial communities.

4.3 Materials and Methods

4.3.1 Ethics Approval

All individuals recruited for this study were done so in accordance with the ethics approval obtained from the University of Adelaide Human Research Ethics Committee (H–2012-108). All samples were obtained under informed consent from healthy individuals, aged 18–50, who were not taking antibiotics at time of sampling.

4.3.2 Sample Collection and Storage

For the assessment of storage methods, supragingival dental calculus samples (n = 18) were obtained from the lingual incisors, over the course of five years, during the donor's routine dental appointment in Adelaide, Australia. The samples were removed from the tooth using a dental pick by dental professionals, and were collected into several different receptacles: transferred onto a cotton gauze and sealed in a bag (n = 13), sealed into a sterile plastic tube (n = 4), or placed directly into a dental sterilisation pouch (n = 2). After collection, the samples were randomly assigned to one of two storage conditions: indoor room temperature (∼23°C; also known as 'Room Temp' group) or a standard freezer (–20°C; also known as 'Freezer' group). Samples remained in respective storage conditions until DNA extraction. Samples and associated metadata are summarised in Table 1.

Comparative supragingival dental calculus samples (n = 18) were collected from lingual incisors of healthy volunteers, using a dental pick by a trained dental professional, at the University of Adelaide Dental School. Samples were placed into sterile 2 mL screw-cap tube and were immediately stored at –20°C until DNA extraction. All these samples were extraction within one month of collection (herein referred to as the 'Fresh' group).

4.3.3 DNA Extraction

All calculus samples were processed within a specialised clean laboratory, designed for human microbiome research, at the University of Adelaide. The laboratory is isolated from any post-PCR laboratories and has strict protocols in place to minimise human and environmental microbial contamination. Researchers working in the microbiome lab are required to wear shoe covers, two pairs of gloves, face mask, and a laboratory coat (to ensure minimum skin exposure). All surfaces are routinely cleaned with Decon 90 (Decon Laboratories Limited) or 2% bleach (NaClO) solution, and KlerAlcohol 70% v/v Isopropyl Alcohol (EcoLab Life Sciences). All consumables, disposables, tools, and instruments are wiped with 2% bleach upon en-

Sample Metadata

Sample ID	Sample Type	Storage Type	Storage Vessel	Donor ID	Gender	Year Collected
19562	Calculus	Room Temp	Cotton gauze	1	Male	2013
19563	Calculus	Room Temp	Cotton gauze	1	Male	2014
19564	Calculus	Room Temp	Plastic Bag	2	Male	2013
19566	Calculus	Room Temp	Cotton Roll	3	Male	2016
19567	Calculus	Room Temp	Cotton gauze	4	Male	2016
19568	Calculus	Room Temp	Cotton gauze	5	Female	2016
19569	Calculus	Room Temp	Cotton gauze	6	Male	2016
19570	Calculus	Room Temp	Cotton gauze	6	Male	2016
19571	Calculus	Room Temp	2mL tube	7	Female	2017
19572	Calculus	Freezer	2mL tube	1	Male	2014
19573	Calculus	Freezer	2mL tube	1	Male	2012
19574	Calculus	Freezer	Cotton gauze	8	Female	2016
19575	Calculus	Freezer	Plastic Bag	2	Male	2013
19576	Calculus	Freezer	2mL tube	3	Male	2015
19578	Calculus	Freezer	Cotton gauze	1	Male	2015
19579	Calculus	Freezer	Cotton gauze	8	Female	2017
19580	Calculus	Freezer	Cotton gauze	6	Male	2017
19581	Calculus	Freezer	Cotton gauze	5	Female	2017
18752	Calculus	Fresh	2mL tube	A	Male	2016
18754	Calculus	Fresh	2mL tube	B	Male	2016
18756	Calculus	Fresh	2mL tube	C	Male	2016
18758	Calculus	Fresh	2mL tube	D	Male	2016
18760	Calculus	Fresh	2mL tube	E	Male	2016
18764	Calculus	Fresh	2mL tube	F	Male	2016
18766	Calculus	Fresh	2mL tube	4	Male	2016
18767	Calculus	Fresh	2mL tube	G	Male	2016
18769	Calculus	Fresh	2mL tube	G	Male	2016
18771	Calculus	Fresh	2mL tube	H	Male	2016
18773	Calculus	Fresh	2mL tube	I	Female	2016
18775	Calculus	Fresh	2mL tube	J	Male	2016
18777	Calculus	Fresh	2mL tube	K	Female	2016
18781	Calculus	Fresh	2mL tube	L	Male	2016
18784	Calculus	Fresh	2mL tube	M	Male	2016
18786	Calculus	Fresh	2mL tube	8	Female	2016
18791	Calculus	Fresh	2mL tube	N	Female	2016
18793	Calculus	Fresh	2mL tube	O	Male	2016

Table 1. **Sample description.** Two individuals participating in the self-direct longitudinal calculus collection also participated in the donation of comparative fresh calculus samples, as indicated by Donor ID

try to the laboratory and are subjected to routine cleaning before, during, and after use. All sample work is carried out within still-air hoods to minimise environmental contamination; the inside of the hood, tools, and instruments are UV-irradiated for a minimum of 15 minutes before and after each use.

Once in the laboratory, all calculus samples were removed from their respective storage vessel with tweezers and placed into a sterile 2 mL tube. All workplace equipment, including the tweezers and bench-top, were treated with 5% bleach between each calculus sample. DNA was extracted from calculus using a modified in-house silica method, based on that previously developed for ancient dental calculus DNA extraction (as described in [22, 7]. In brief, dental calculus samples were decalcified and microbial cells lysed in 470 μL of 0.5 M ethylene diamine triacetic

acid (EDTA; pH 8.0) and 30 μL of 10% sodium dodecyl sulphate (SDS), treated with 20 mg/mL proteinase K, and incubated at 55°C overnight. Cell lysis products were bound to 20 μL of silica solution in the presence of 1.5 mL of buffer QG (containing guanidium thiocyanate; Qiagen). Silica-bound DNA was rinsed twice with 80% ethanol, before resuspension in 100 μL of Tris-EDTA solution. Resuspension was repeated to elute a total of 200 μL. Two sample blank extraction controls (*i.e.* extraction blank controls, or EBCs) were also processed alongside each extraction group (∼10 dental calculus samples per group).

4.3.4 Shotgun metagenomic libraries and sequencing

In preparation for shotgun sequencing, 50 μL of DNA extract was sheared using a focused-ultrasonicator (Covaris Inc.) to ∼300bp fragment lengths. 20 μL of sheared DNA extract was used to make shotgun libraries, constructed as previously described in Meyer, Sawyer, and Kircher (2011), without the enzymatic damage repair step [23, 7]. In short, DNA extracts underwent enzymatic polishing to produce blunt ended fragments, before the ligation of truncated 5-bp forward and reverse barcoded Illumina adaptors and filling of adaptor sequences. Resulting DNA fragments were purified using MinElute Reaction Clean-ups (Qiagen) after each enzymatic step, and then amplified using a polymerase chain reaction (PCR). In brief, AmpliTaq Gold reactions were done in triplicate and contained: 12.75 μL sterile H20, 2 μL of purified Library DNA, 0.25 μL of AmpliTaq Gold (Life Technologies), 2.5 μL of 10X Gold buffer, 2.5 μL MgCl2 (25 mM), 0.625 μL dNTPs (10 mM), and 1.25 μL Illumina amplification primer, and 1.25 μ L GAII Illumina indexed adaptor. Cycling conditions were as follows: 94°C for 12 minutes; 13 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 40 seconds (plus 2 seconds/cycle); and 72°C for 10 minutes. The resulting products were pooled, purified with AxyPrep magnetic beads (Axygen Scientific Inc.), and then re-amplified with GAII-indexed Illumina primers. The resulting libraries were subjected to a final purification, quantified on a TapeStation (Aligent Technologies), subsequently pooled to a final 2 nmol/L DNA concentration, and sequenced on an Illumina NextSeq, Mid Output, 150 cycle kit (Illumina) at the Australian Genome Research Facility Ltd. (AGRF) in Adelaide, Australia.

4.3.5 16S rRNA gene amplification and sequencing

All samples additionally underwent 16S ribosomal RNA (rRNA) amplification. Each sample was amplified in triplicate, alongside an additional no template control, using barcoded primers specific to the V4 region of the rRNA gene, with primers 515F (5' -GTGCCAGCMGCCGCGGTAA– 3') and 806R (5' -GGACTACHVHHHTWTCTAAT-

3') [24]. Each PCR reaction contained: 18.05 μL sterile H20, 1 μL of DNA extract, 0.25 μL of Hi-Fi taq (Life Technologies), 2.5 μL of 10X Hi-Fi reaction buffer, 1 μL MgSO4 (50 mM), 0.2 μL dNTPs (100 mM), and 1 μL each of the forward and reverse primer (10 mM). Samples were amplified under the following conditions: 95°C for 6 minutes; 37 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 90 seconds; and final step, 60°C for 10 minutes.

The resulting triplicate reactions were pooled and visualised by electrophoresis on a 2.5% agarose gel to assess the fragment sizes and quality of each sample. All resulting libraries were then quantified using a High Sensitivity dsDNA reagent kit (Qubit 2.0, Life Technologies) and pooled together at equimolar concentrations. Samples were then purified using an AMPure cleanup (Agencourt Bioscience). Final quantification and DNA sequencing was completed at AGRF, on a MiSeq, 2x150bp kit (Illumina).

4.3.6 Shotgun Bioinformatic Processing

Raw Illumina BCL files were processed through BCL2Fastq (Cassava) to convert sequences into FASTQ file format, separated by Illumina GAII index. FASTQ read files were simultaneously demultiplexed (using the unique P5/P7 barcode adaptor combinations), barcodes trimmed, and sequences collapsed with AdapterRemoval2 [25], using default parameters. All dental calculus samples underwent host read removal using KneadData [26], which aligned the sequences to the human genome (GRCh37/hg19) reference database, and removed all sequences with multiple alignments (Table S1). Taxonomic assignments were generated using MEGAN Alignment Tool (MALT; v0.3.8) [27]. MALT aligned DNA sequences against an in-house database, created using 47,696 archaeal and bacterial genome assemblies from the NCBI Assembly database [28], with BLASTn. The resulting alignment-based blast-text files were then converted to RMA files using the blast2rma script within MEGAN v6.12.8 [29], 2016), using the following lowest common ancestor (LCA) parameters: weighted-LCA, minimum percentage identity = 95%, minimum bitscore = 44, minimum E-value = 0.01, minimum support percent = 0.25.

Two shotgun calculus samples were removed from the dataset after initial analysis to minimise biases caused by unique attributes. Sample 19566 had an unusually low read count (2085 sequences) (Table S1), whereas Sample 19567 was dominated by phylum Chlamydiae (3.62% of total sequences) not previously found with great abundance within the human mouth (Figure S1). Laboratory contaminant sequences are reported in the Supplementary Materials (Table S2) but were not filtered from calculus samples.

4.3.7 16S ribosomal RNA Bioinformatic processing

Raw Illumina BCL files were converted to FASTQ file format in BCL2fastq (v.1.8.4; Illumina, San Diego, CA, USA), producing R1, R2, R3 files (forward, barcodes, and reverse). Using QIIME2 (v 2019.1) [30], raw multiplexed paired-end FASTQ files were demultiplexed by unique barcode adaptor using the EMP-paired end protocol and denoised using the Deblur algorithm QIIME2 plugin [31]). Sequences were truncated to 150bp based on the median quality score. 16S rRNA sequences were assigned to taxonomic groups using the Human Oral Microbiome Database (HOMD; v. 15.1) [32]. Contaminant taxa were assessed using the Greengenes database (v13.8) within the Supplementary Materials (Table S3), but were not filtered from calculus samples [33].

4.3.8 Statistical analyses

Differences in community diversity between storage methods were investigated at three taxonomic levels: species, genera, and phyla-level assignments. Shotgun sequences were exported from MEGAN6 at respective taxonomic-level assignments in TSV format and imported into QIIME2. Sequences from shotgun samples were then rarefied to the lowest number of assigned sequences at each taxonomic level (species-level, n = 62,579; genera-level, n = 84,982; phyla-level, n = 92,436, sequences per sample). 16S rRNA sequences were rarefied to 16,674 per sample, for every taxonomic level, the lowest number of sequences present within any sample. Samples 19566 and 19567 were removed from the 16S rRNA dataset to maintain homogeneity with the shotgun dataset, but additionally, sample 19569 was removed due to low sequence count (Table S3).

All statistical analyses were completed in QIIME2 [34], except for Kruskal-Wallis test of group significance, which was completed in QIIME1 (v.1.9.1). All beta diversity differences were measured with Bray-Curtis distance indices and significance was tested with PERMANOVA. Alpha diversity was measured using Shannon and observed species indices, with significance tested by Kruskal-Wallis nonparametric statistical test of variance. All reported p-values were false discovery rate (FDR) corrected and values < 0.05 were accepted as statistically significant.

4.4 Results

4.4.1 Typical oral microbial communities are obtained with shotgun sequencing of dental calculus

After removal of host sequences and two spurious samples (19566 and 19567), 34 dental calculus samples contained a total of 30,857,148 sequences, with a total of 19,300,197 sequences assigned to taxonomy. All dental calculus samples (n = 34) were dominated by seven phyla: Actinobacteria (39.4 ± 1.43%), Proteobacteria (27.9 ± 1.82%), Firmicutes (16.9 ± 1.34%), Bacteroidetes (11.1 ± 2.50%), Fusobacteria (2.9 ± 2.60%), Spirochaetes (1.5 ± 5.95%), and Synergistetes (0.3 ± 9.18%) (Figure 1), as expected for a typical oral microbial community [35].

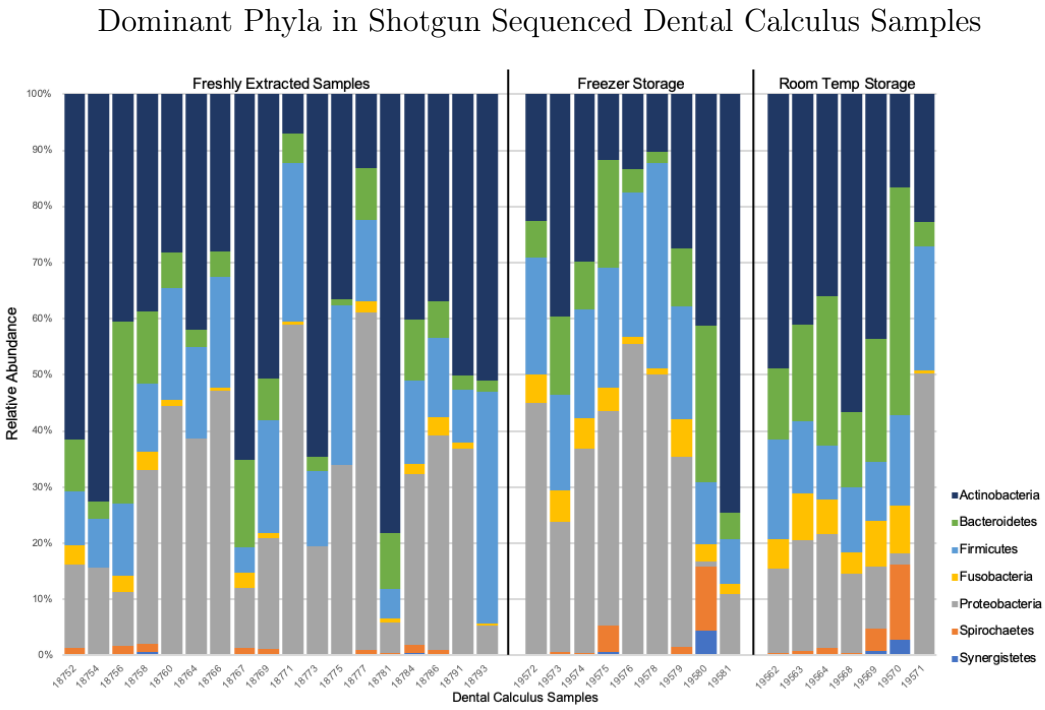

Figure 1: **The relative abundance of human dental calculus microbial phyla, sequenced with metagenomic shotgun methods.** All samples were rarefied to 92,436 sequences. Samples are grouped according to their storage method; 18 dental calculus samples underwent DNA extraction within two weeks of collection (Fresh), nine samples were stored within a standard -20°C freezer for >1 year (Freezer), and seven samples were stored at room temperature (~23°C) for >1 year (Room Temp).

We initially assessed potential biases within our dataset through the examination of known sample metadata. We found no significant differences in microbial communities between donor gender, at any taxonomic level (Male (n = 25) vs Female (n = 9); Shannon, observed species, Bray-Curtis, $p > 0.05$; Table S5a). Nor did the type of storage vessel used to collect stored calculus samples significantly impact microbial diversity or composition, at any taxonomic level (tube (n = 4), cotton-gauze (n

= 9), and plastic bag (n = 2); Table S5a; Shannon, observed species, Bray-Curtis, $p > 0.05$), indicating that such factors driving microbial diversity within this study are unlikely to be linked to sample variables

4.4.2 Storage method impacts the diversity and composition of dental calculus microbial communities

To target the microbial differences over time, only samples stored for more than two years (*i.e.* collected in 2016 or earlier) were compared to Fresh samples in all downstream analyses (Table 1). We initially assessed whether the storage method (Room Temp (n = 6), Frozen (n = 6), and Fresh (n = 18)) drove differences in microbial diversity within dental calculus samples. We detected significant differences in diversity and richness at the phylum level (Shannon, $p = 0.004$, $H = 10.87$), but found no significant differences in the presence/absence of phyla (observed species, $p = 0.09$, $H = 4.79$), suggesting that variation of phyla-level diversity is driven by abundance and richness. Moreover, differences were not observed at the species- (Shannon, $p = 0.22$, $H = 3.07$; observed species, $p = 0.18$, $H = 3.48$) or genera-levels (Shannon, $p = 0.06$, $H = 5.73$; observed species, $p = 0.26$, $H = 2.72$). Additionally, each storage method group was significant different from one another at all taxonomic levels (Bray-Curtis: species, $p = 0.02$, pseudo-$F = 1.91$; genera, $p = 0.003$, pseudo-$F = 3.14$; and phyla, $p = 0.001$, pseudo-$F = 5.58$; Figure 2). Overall, these results support potential underlying biases caused by storage method that will impact the reconstruction of microbial abundance within dental calculus.

4.4.3 Room temperature storage over time impacts microbial community reconstruction

We next examined the impact room temperature ($\sim$23°C) storage on the shotgun sequenced reconstruction of microbial communities, by comparing samples stored at room temperature for more than two years (Room Temp; n = 6) to recently collected samples (Fresh; n = 18). There was significantly greater diversity at the phylum-level within Room Temp samples relative to Fresh samples, as measured by Shannon (Table 2; $p = 0.01$, $H = 9.40$), although this was not observed at species- or genera-level assignments (Table 2; $p > 0.05$). This suggests that room temperature storage may induce greater phylum-level differences by commensurately altering the diversity at lower taxonomic levels. Next, we examined microbial compositional differences between Room Temp and Fresh samples, and noted both phyla- and genera-level differences between storage methods (Bray-Curtis; phyla, $p = 0.02$, pseudo-$F = 4.48$; genera, $p = 0.02$, pseudo-$F = 2.90$), although no significant differ-

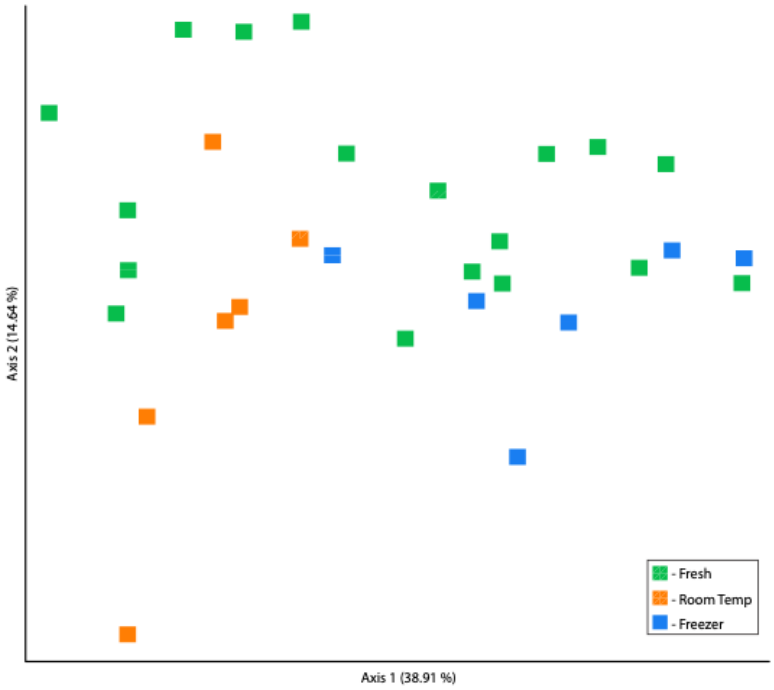

Figure 2: **Principle Coordinates Analysis (PCoA) of Bray-Curtis distances of shotgun sequenced phylum-level assignments.** Shotgun-sequenced dental calculus samples stored (>2 years) at different temperatures have distinct microbial communities. Different colours represent the different storage conditions: Freshly extracted dental calculus (Fresh, green: n = 18), samples stored for more than two years at room temperature (~23°C, Room Temp, orange: n = 6), and samples stored for more than two years in a standard freezer (–20°C, Freezer, blue: n = 6).

ences could be detected at species-level (Bray-Curtis, p = 0.11, pseudo-F = 1.55). These results suggest that long-term storage at room temperature impacts microbial communities through alterations at microbial genera.

To identify taxonomic groups potentially driving significant differences between groups, Kruskal-Wallis test of significantly different mean taxa abundances were calculated at three different taxonomic levels (phyla, genera, and species; Table 3a). Phylum Fusobacteria was significantly more abundant in Room Temp samples than in Fresh samples (p = 0.002, t = 13.02), alongside Bacteroidetes (p = 0.008, t = 9.04). Three different genera, *Fusobacterium* (p = 0.033, t = 10.84), *Leptotrichia* (p = 0.033, t = 10.01), and *Selenomonas* (p = 0.045, t = 8.67) were detected with significantly greater abundance within Room Temp samples relative to Fresh . As *Fusobacterium* and *Leptotrichia* belong to phylum Fusobacteria, this supports the previous results suggesting broader taxonomic level changes. Moreover, no specific species were identified as significantly different between groups (Table 3a; $p > 0.05$). This suggests that changes in calculus microbial composition may be largely driven by alterations to Fusobacteria during long-term room temperature storage.

Fresh (n = 18) *vs* Room Temp (n = 6)	FDR p-value	Test statistic
Species		
Shannon	0.26	2.92
Observed species	0.44	2.95
Genera		
Shannon	0.12	4.27
Observed species	0.32	1.79
Phyla		
Shannon	**0.01**	9.40
Observed species	0.10	4.51
Fresh (n = 18) *vs* Freezer (n = 6)	**FDR p-value**	**Test statistic**
Species		
Shannon	0.51	0.44
Observed species	0.26	1.07
Genera		
Shannon	0.14	2.78
Observed species	0.32	1.54
Phyla		
Shannon	0.11	3.24
Observed species	0.60	0.28
Room Temp (n = 6) *vs* Freezer (n = 6)	**FDR p-value**	**Test statistic**
Species		
Shannon	0.45	1.07
Observed species	0.44	0.61
Genera		
Shannon	0.75	0.10
Observed species	0.69	0.16
Phyla		
Shannon	0.20	1.64
Observed species	0.18	2.44

Table 2. **Pairwise PERMANOVA results for shotgun data.**

Fresh (n = 18) *vs* Room Temp (n = 5)	FDR p-value	Test statistic
Species		
Shannon	0.88	1.09
Observed species	0.53	0.87
Genera		
Shannon	0.95	0.20
Observed species	0.53	0.87
Phyla		
Shannon	0.45	1.08
Observed species	0.97	0.21
Fresh (n = 18) *vs* Freezer (n = 6)	**FDR p-value**	**Test statistic**
Species		
Shannon	0.95	0.004
Observed species	0.57	0.32
Genera		
Shannon	0.95	0.004
Observed species	0.95	0.004
Phyla		
Shannon	0.55	0.36
Observed species	0.97	0.001
Room Temp (n = 5) *vs* Freezer (n = 6)	**FDR p-value**	**Test statistic**
Species		
Shannon	0.88	0.30
Observed species	0.05	5.66
Genera		
Shannon	0.60	1.63
Observed species	0.29	2.76
Phyla		
Shannon	0.43	2.13
Observed species	0.97	0.62

Table 4. **Pairwise PERMANOVA results for 16S rRNA data.**

Table 2. and Table 4. Impact of storage conditions on dental calculus microbial communities at species-, genera-, and phyla-level assignments. PERMANOVA pairwise test for significance with FDR-corrected P-values were obtained using 999 permutations. Bold values indicate a significant result ($p > 0.05$).

4.4.4 -20°C storage over time maintains microbial diversity

Long-term freezing has shown to induce significant changes in faecal microbiota composition [36], but this has not yet been tested with oral samples. Therefore, we explored changes to the diversity and composition in dental calculus samples stored for more than two years at –20°C (Freezer; $n = 6$) compared to Fresh samples. We found no significant differences in microbial diversity within the different storage groups at any taxonomic level (Table 2; Shannon and observed species, $p > 0.05$), indicating that diversity is maintained in samples that are stored at –20°C. However, we found significant differences between the microbial composition of Freezer and Fresh samples at both the phyla- and genera-levels (Bray-Curtis, phyla, $p = 0.02$, pseudo-$F = 5.08$; genera, $p = 0.03$, pseudo-$F = 2.52$), although no significant differences were detected at the species-level (Bray-Curtis, $p = 0.06$, pseudo-$F = 2.01$). Thus, while diversity is maintained, these results suggest that storing dental calculus samples at –20°C for more than two years may influence the compositional

Kruskal-Wallis Group Significance

Table 3a. Fresh (n = 18) vs Room Temp (n = 6)

NCBI Taxonomy	test statistic	FDR corrected p-value	Average Number of Reads	
			Fresh	Room Temp
Species				
Fusobacterium nucleatum	12.00	0.055	623	3632
Capnocytophaga granulosa	10.19	0.074	38	848
Actinomyces oris	8.29	0.128	2578	336
Genera				
Fusobacterium	10.84	**0.033**	906	4278
Leptotrichia	10.01	**0.033**	204	1845
Selenomonas	8.67	**0.045**	472	2426
Phyla				
Fusobacteria	13.02	**0.002**	1249	6162
Bacteroidetes	9.40	**0.008**	7446	20372
Spirochaetes	3.68	0.129	538	3160

Table 3b. Fresh (n = 18) vs Freezer (n = 6)

NCBI Taxonomy	test statistic	FDR corrected p-value	Average Number of Reads	
			Fresh	Freezer
Species				
Actinomyces oris	10.50	0.124	2578	260
Leptotrichia sp. 212	7.86	0.229	57	750
Neisseria sicca	7.38	0.229	209	1718
Genera				
Leptotrichia	6.98	0.237	204	1393
Streptococcus	6.42	0.237	9145	17013
Capnocytophaga	4.55	0.294	2070	3581
Phyla				
Fusobacteria	7.14	**0.048**	1249	3474
Actinobacteria	6.08	**0.048**	41349	19610
Firmicutes	4.55	0.077	15110	21702

Table 3c. Room Temp (n = 6) vs Freezer (n = 6)

NCBI Taxonomy	test statistic	FDR corrected p-value	Average Number of Reads	
			Room Temp	Freezer
Species				
Ottowia sp. 894	6.80	0.361	429	6569
Fusobacterium nucleatum	6.59	0.361	3632	1135
Streptococcus sanguinis	6.56	0.361	2912	12379
Genera				
Streptococcus	8.31	0.166	5228	17013
Ottowia	6.80	0.171	432	7115
Neisseria	5.77	0.171	3852	12637
Phyla				
Proteobacteria	8.31	**0.023**	12620	38305
Firmicutes	7.41	**0.023**	12156	21702
Actinobacteria	5.03	0.058	37410	19610

Table 3. **Kruskal-Wallis taxonomic group significance analysed at species-, genera-, and phyla-level assignments with shotgun sequencing.** All sample sequences were rarefied by their taxonomic level (species n = 61,857; genera n = 85,704, and phyla n = 92,436). Bold values indicate a significant result with FDR-corrected p-value ($p > 0.05$).

reconstruction of dental calculus microbial community.

We investigated what taxa may be driving these compositional variations using Kruskal-Wallis and observed two phyla significantly different between Fresh and Freezer samples (Table 3b). Fusobacteria was significantly more abundant within Freezer samples ($p = 0.048$, $t = 7.14$), whereas Actinobacteria was significantly more abundant within Fresh samples ($p = 0.048$, $t = 6.08$). Surprisingly, there were no genera significantly associated with this change (Table 3b; $p > 0.05$). Nor were any species associated with this compositional difference (Table 3b; $p > 0.05$). These results suggest that while storage at –20°C may maintain the community composition, long-term storage will induce higher-level structural changes in dental calculus microbial community.

4.4.5 Temperature impacts microbial community over time

To further elucidate the impact of storage temperature, we compared the differences in dental calculus microbial diversity and composition between the samples stored for more than two years at difference temperatures; Room Temp compared to Freezer. Diversity within these two long-term storage methods was not significantly different at any taxonomic level (Table 2; Shannon and observed species, $p > 0.05$). As Freezer group sample diversity was not significantly different from either Fresh or Room Temp sample diversity ($p > 0.05$), but Room Temp samples were significantly more diverse than Fresh (Shannon, $p = 0.01$, $t = 9.40$), the lack of detectable difference between the two storage temperatures suggests the processes changing diversity may be occurring temporally, but –20 °C storage lessens, or slows, these mechanisms.

In contrast, the microbial community composition between Room Temp and Freezer sample groups was distinct at both phyla and genera taxonomic levels (Bray-Curtis: phyla, $p = 0.02$, pseudo-F = 10.56; genera, $p = 0.02$, pseudo-F = 5.56), although not at species-level (Bray-Curtis, $p = 0.06$, pseudo-F = 2.92). To examine this further, we explored which taxa that differed between storage groups using Kruskal-Wallis (Table 3c). We observed a significantly greater abundance of phyla Proteobacteria ($p = 0.023$, $t = 8.31$) and Firmicutes ($p = 0.023$, $t = 7.41$) within frozen dental calculus relative to those stored at room temperature, but saw no significant differences in species or genera abundances (Table 3c; $p > 0.05$). These results indicate that some sustained composition changes (*i.e.* Fusobacteria) ensue over time despite storage method, while other phyla-level variations may be more dependent upon temperature.

4.4.6 Qualitative changes over time reconstructed with shotgun sequencing

As individual donors contributed multiple dental calculus samples over the course of six years, this provided the ability to qualitatively assess a longitudinal record in both Fresh and Frozen sample datasets. Two individuals, Donor 1 and Donor 8, donated more than three times, across different storage temperatures. Donor 1 donated calculus five times over four years, from 2012 to 2015 (Table 1); two samples were stored at room temperature and three samples were stored at -20°C, providing the opportunity to determine the impact of room temperature relative to freezer storage within a single individual (Figure 3). Consistent with previous Kruskal-Wallis analyses, Fusobacteria phylum appears to increase in relative abundance with time, within both at -20°C and room temperature, whereas phyla Proteobacteria and Firmicutes appear to maintain a greater relative abundance within Freezer samples relative to Room Temp samples. This supports the hypothesis that storing dental calculus at -20°C slows—but does not prevent—taphonomic processes over time in some individuals.

Qualitative Phyla Changes over Time in Individual Donors with Shotgun Sequencing

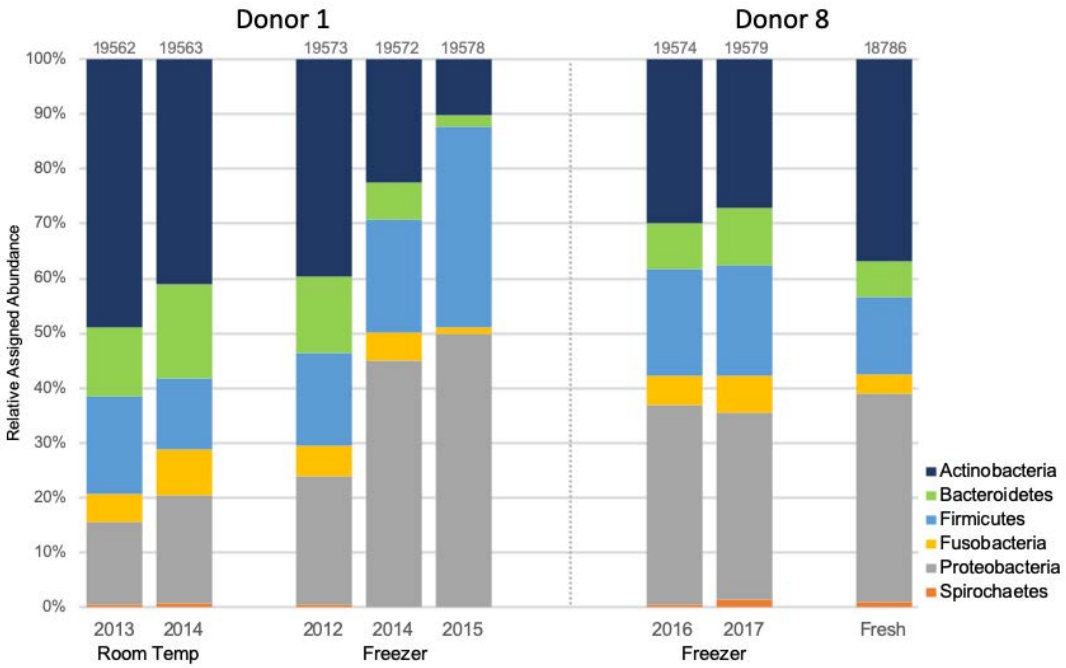

Figure 3: **The relative abundance of Donor 1 (n = 5) and Donor 8 (n = 3) dental calculus microbial phyla, with shotgun sequencing.** Samples are grouped according to their storage type and sorted through time. Donor 1 donated five individual samples of calculus across four years, and Donor 8 donated three samples across two years. This longitudinal record provides a qualitative display of compositional changes though time

Donor 8 provided three dental calculus samples over time, in which one sample was freshly extracted and two were frozen at –20°C (Figure 3). Corresponding with the significantly greater abundance of Actinobacteria phyla within Fresh dental calculus relative to Frozen (Table 3b), Donor 8 appears to have reduced abundance of Actinobacteria present within the stored freezer samples, inconsistent with Donor 1, whose abundance of Actinobacteria increasing over time within frozen samples. This would contradict the hypothetical decrease of Actinobacteria with –20°C storage, and as such, stipulates taphonomic changes are dependent upon intra-individual variation present before taphonomic processes take place.

4.4.7 16S rRNA amplicon reconstruction of dental calculus microbial communities

While 16S rRNA amplification produced pronounced biases with ancient DNA from dental calculus [7, 14], the samples within this study were stored for less than a decade, which may differ to previous assessments of amplicon based approaches [37]. Using 16S rRNA, the reconstructed oral microbial communities (n = 34) were concomitant with shotgun sequenced samples, and were dominated by the same seven phyla previously observed: Proteobacteria (37.2 ± 1.39%), Firmicutes (19.30 ± 1.18%), Actinobacteria (14.5 ± 2.59%), Bacteroidetes (13.5 ± 1.55%), Fusobacteria (12.5 ± 2.32%), Synergistetes (1.5 ± 8.39%), and Spirochaetes (1.4 ± 6.23%). However, 16S rRNA sequence assignment also presented an additional four phyla which contributing to a total of $< 1\%$ of sequences; Absconditabacteria (SR1), Chloroflexi, Gracilibacteria (GN02), and Saccharibacteria (TM7) (Figure 4).

Unlike the shotgun dataset, donor gender (Male (n = 24) vs Female (n = 9)) did significantly impact genera-level diversity as measured using observed species index (Supplementary Table 5b; $p = 0.03$, $H = 4.60$). However, gender did not significantly impact the microbial diversity at species- or phyla-levels (Table S5b; Shannon and observed species, $p > 0.05$), nor were female or male samples groups significantly different from one another at any taxonomic level as measured by Bray-Curtis (Table S5b; $p > 0.05$). It is more likely that the 16S rRNA sequencing is picking up an anomaly between the gender groups, as shotgun sequencing was unable to detect any differences between genders. The different storage vessels used to collect stored calculus samples did not significantly impact microbial diversity or composition at any taxonomic level (tube (n = 4), cotton-gauze (n = 9), and plastic bag (n = 2); Table S5b; Shannon observed species and Bray-Curtis, $p > 0.05$). Overall, these results suggest a limited impact of metadata variables influencing the storage methods differences.

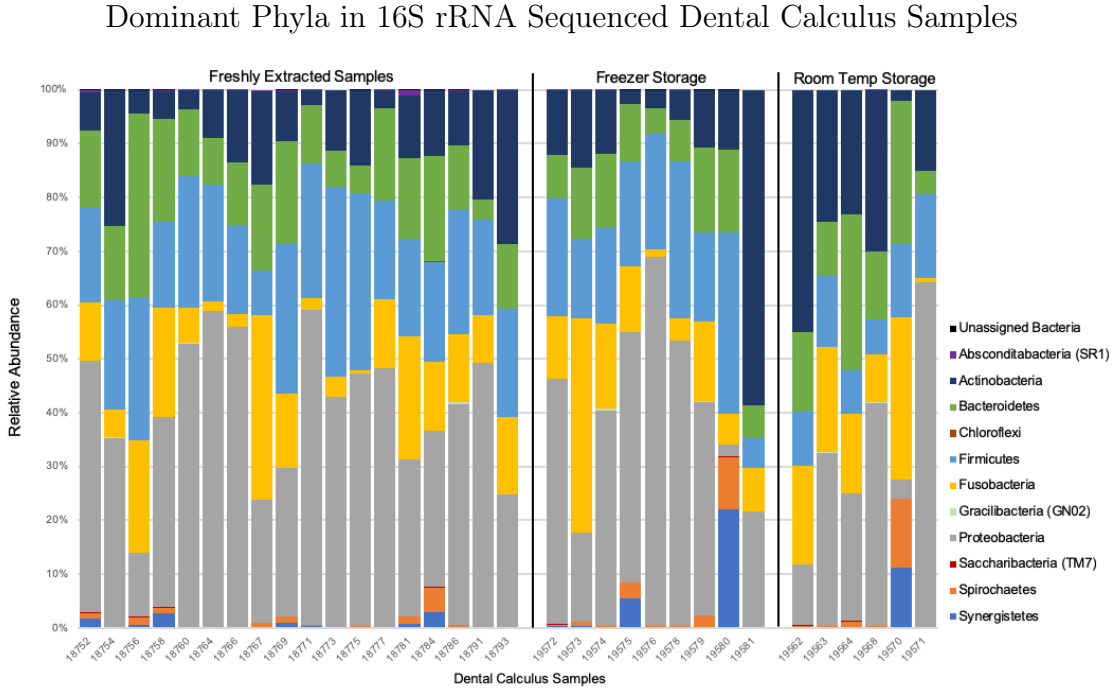

Figure 4: **The relative abundance of human dental calculus microbial phyla, sequenced with 16S ribosomal RNA amplification.** All samples were rarefied to 16,674 sequences per sample. Samples are grouped according to their storage group; 18 dental calculus samples underwent DNA extraction within two weeks of collection (Fresh), nine samples were stored within a standard -20°C freezer for >1 year (Freezer), and six samples were stored at indoor room temperature (∼23°C) for >1 year (Room Temp).

4.4.8 Storage method impacts the 16S rRNA reconstruction of dental calculus microbial communities

As we expect 16S rRNA reconstruction of degraded dental calculus material to sustain distinct alterations the community composition compared to shotgun sequencing, we re-examined the impacts of storage. Only calculus samples stored for more than two years were included in the following analyses. We first assessed any change in community variation linked to storage method, and found no significant differences in diversity driven the storage method at any of the three taxonomic levels (phyla: Shannon, $p = 0.36$, $H = 2.02$, observed species, $p = 0.83$, $H = 0.37$; genera: Shannon, $p = 0.73$, $H = 0.63$; observed species, $p = 0.44$, $H = 1.61$; species: Shannon, $p = 0.26$, $H = 1.28$; observed species, $p = 0.24$, $H = 2.87$). However, storage method groups were significantly different from one another at all taxonomic levels, as tested with Bray-Curtis (phyla, $p = 0.01$, pseudo-F $= 3.58$; genera, $p = 0.007$, pseudo-F $= 2.63$; species, $p = 0.003$, pseudo-F $= 2.19$;). These results support differences in the taphonomic effect upon the reconstruction of dental calculus microbial communities using 16S rRNA, compared to shotgun metagenomic approaches [14], and that these differences may impact the community compositional structure more than the microbial diversity.

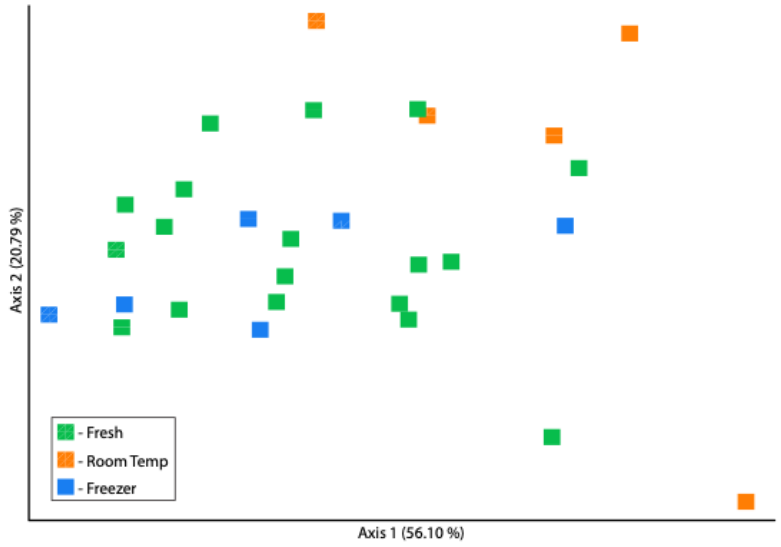

Figure 5: **Principle Coordinates Analysis (PCoA) of Bray-Curtis distances of phylum-level assignments.** 16S amplicon sequenced dental calculus samples stored (>2 years) at different temperatures have distinct microbial communities. Different colours represent the different storage conditions: Freshly extracted dental calculus (Fresh, green: n = 18), samples stored for more than two years at room temperature (~23°C, Room Temp, orange: n = 6), and samples stored for more than two years in a standard freezer (-20°C, Freezer, blue: n = 6).

4.4.9 Room temperature storage 16s rRNA reconstruction of microbial communities

To examine the impact of room temperature storage on 16S rRNA reconstruction, we compared samples stored at room temperature (Room Temp; n = 5) for two or more years to fresh extracted samples (Fresh; n = 18). No significant differences in diversity were detected at any taxonomic level between Room Temp samples and Fresh samples (Table 4; Shannon and observed species, $p > 0.05$). As phylum-level differences in diversity were detected with shotgun sequencing technique, these results suggest 16S rRNA amplification obscures, or is unable to detect, diversity differences associated with long-term, room temperature storage. Next, we found significant differences in microbial community composition between the Room Temp and Fresh samples, using Bray-Curtis, at all taxonomic levels (phyla, $p = 0.006$, pseudo-F = 5.90; genera, $p = 0.012$, pseudo-F = 3.72; species, $p = 0.006$, pseudo-F = 3.04). As species-level differences were not detected within the shotgun metagenomic dataset, these results suggest long-term storage at room temperature potentially decreases 16S rRNA sequence assignment accuracy to species-level identity, artificially inducing greater genera-level diversity differences.

Only the phylum Firmicutes was observed to significantly differ between groups,

Kruskal-Wallis Group Significance

Table 5a. Fresh (n = 18) vs Room Temp (n = 5)

HOMD Taxonomy	test statistic	FDR corrected p-value	Average Number of Reads	
			Fresh	Room Temp
Species				
Unassigned *Staphylococcus*	21.58	**0.001**	0	5
Unassigned *Acidovorax*	11.83	**0.043**	0	2
Actinomyces sp. HMT 448	11.81	**0.043**	0	265
Capnocytophaga haemolytica	11.81	**0.043**	0	33
Capnocytophaga sputigena	10.76	0.052	179	9
Genera				
Staphylococcus	16.97	**0.004**	0	5
Bergeyella	9.34	0.098	603	88
Streptococcus	8.89	0.098	2428	336
Phyla				
Firmicutes	9.80	**0.021**	3594	1735
Saccharibacteria (TM7)	4.71	0.170	6	15
Proteobacteria	4.05	0.170	6668	3753

Table 5b. Fresh (n = 18) vs Freezer (n = 6)

HOMD Taxonomy	test statistic	FDR corrected p-value	Average Number of Reads	
			Fresh	Freezer
Species				
Unassigned *Staphylococcus*	22.41	**0.001**	0	12
Unassigned *Moraxella*	17.88	**0.003**	0	2
Leptotrichia sp. HMT 217	10.80	0.098	1	118
Genera				
Staphylococcus	18.41	**0.002**	0	11
Moraxella	13.71	**0.012**	0	1
Micrococcus	9.84	**0.048**	2	0
Unassigned Betaproteobacteria	9.81	**0.048**	0	3
Pseudopropionibacterium	5.81	0.350	6	0
Phyla				
Saccharibacteria (TM7)	3.31	0.722	6	0
Bacteroidetes	2.35	0.722	2304	1621
Synergistetes	1.79	0.722	96	184

Table 5c. Room Temp (n = 5) vs Freezer (n = 6)

HOMD Taxonomy	test statistic	FDR corrected p-value	Average Number of Reads	
			Room Temp	Freezer
Species				
Ottowia sp. HMT 894	7.53	0.446	1092	12
Capnocytophaga sputigena	7.50	0.446	186	9
Unassigned *Streptococcus*	7.50	0.446	2517	316
Genera				
Saccharibacteria [G-1]	7.89	0.226	0	13
Ottowia	7.53	0.226	1082	11
Streptococcus	7.50	0.226	2536	336
Phyla				
Saccharibacteria (TM7)	8.25	**0.037**	0	15
Firmicutes	7.50	**0.037**	3507	1735
Absconditabacteria (SR1)	5.05	0.097	8	1

Table 5. Kruskal-Wallis taxonomic group significance of 16S rRNA data analysed at different taxonomic levels assigned by the Human Oral Microbiome Database (HOMD; v. 15.1) [32]. All sample sequences were rarefied to the lowest number of sequences present within a sample (n = 16,674). Bold values indicate a significant result with FDR-corrected p-value ($p > 0.05$).

observed at a lower relative abundance in Room Temp samples using Kruskal-Wallis (Table 5a; p = 0.02, t = 9.80). In contrast, a Firmicutes genus, *Staphylococcus*, was detected at a significantly greater abundance in Room Temp samples compared to Fresh (p = 0.004, t = 16.97), but its low mean relative sequence abundance would suggest minimal contribution to the microbial community differences detected. Four species were observed in Room Temp samples, but were not detected within Fresh group: Unassigned *Staphylococcus* (p = 0.001, t = 21.58), Unassigned *Acidovorax* (p = 0.043, t = 11.83), *Actinomyces* sp. HMT 448 (p = 0.043, t = 11.81), and *Capnocytophaga haemolytica* (p = 0.043, t = 11.81). Overall, unlike shotgun sequenced calculus, it appears that differences between fresh and long-term room temperature storage microbial communities are not able to be defined by particular phyla or genera, suggesting 16S rRNA reconstruction may obscure potential impact of taphonomy.

4.4.10 Long-term freezer storage maintains microbial diversity and community composition

Next, we assessed the impact of long-term –20°C storage upon dental calculus microbial communities reconstructed with 16S rRNA by comparing samples stored for more than years at –20°C (Frozen; n = 6) to those collected recently (Fresh; n = 18). As observed with the shotgun approach, microbial diversity was not impacted by long-term freezer storage at any taxonomic level (Table 4; Shannon and observed species, $p > 0.05$). However, unlike shotgun sequencing, we were also unable to detect any significant differences in the microbial community structure between Fresh and Freezer samples with Bray-Curtis (phyla, p = 0.731, pseudo-F = 0.42; genera, p = 0.36, pseudo-F = 1.07; species, p = 0.38, pseudo-F = 1.04). These results suggest 16S rRNA amplicon sequencing may mask potential taphonomic compositional changes occurring within dental calculus microbial community when stored at –20°C for more than two years.

We found no significant differences in the mean relative abundance of any phyla using Kruskal-Wallis (Table 5b; $p > 0.05$). However, despite microbial compositional similarities between samples, we detected significant differences in specific genera and species' mean relative abundance between Fresh and Frozen. Four different genera were detected at significantly different abundances between Fresh and Freezer samples, driven by the presence or absence between groups; *Staphylococcus* (p = 0.002, t = 18.41), *Moraxella* (p = 0.012, t = 13.71), and Unassigned Betaproteobacteria (p = 0.048, t = 9.81) were not detected within the Fresh samples, whereas *Micrococcus* (p = 0.048, t = 9.84) was not detected with the Freezer samples. Finally, two species, Unassigned *Staphylococcus* (p = 0.001, t = 22.41)

and Unassigned *Moraxella* (p = 0.003, t = 17.88), were detected in Frozen samples but not with Fresh samples. Significance was likely detected due to very low mean relative abundances (average of < 0.0007% of the total rarefied sequences, Table 5b) and their presence or absence between sample groups, which overall, substantiates the very limited impact of these species and genera upon overall community composition. Moreover, it supports the potential decrease of 16S rRNA sequence assignment accuracy to species-level identity, wherein both 'species' were unassigned sequence features.

4.4.11 Time in storage influenced 16S rRNA microbial community reconstruction

We investigated the specific impact of temperature by comparing long-term stored calculus samples of Room Temp (n = 5) to Freezer (n = 6), but found no significant differences in diversity at any taxonomic level between long-term storage methods (Table 4; Shannon and observed species, p > 0.05). This indicates that storage temperature alone does not influence the 16S rRNA reconstructed diversity. Yet, significant differences were detected between the different storage temperatures using Bray-Curtis, detected at all taxonomic levels (phyla, p = 0.04, pseudo-F = 4.41; genera, p = 0.01, pseudo-F = 3.68; species, p = 0.02, pseudo-F = 2.65), likely reflecting the community preservation of Freezer samples relative to Room Temp samples.

Using Kruskal-Wallis, significant differences in the mean relative abundance were detected between Room Temp and Freezer sample groups in Saccharibacteria (p = 0.037, t = 8.25) and Firmicutes phyla (p = 0.037, t = 7.50). Notably, Firmicutes was observed with significantly greater abundance within Freezer sample group relative to Room Temp samples (p = 0.04, t = 7.50), whereas, Saccharibacteria was only detected within Room Temp samples, at very low abundance within the microbial communities overall (average of < 0.0009% of the total rarefied sequences). No species or genera significantly differed in abundance between the long-term storage methods (Table 5c; p > 0.05). These results support the impact of room temperature storage upon Firmicutes abundance in the 16S rRNA reconstruction of dental calculus microbiota, as seen in the shotgun dataset.

4.4.12 Limited community changes detected over time with 16S rRNA sequencing

We assessed the qualitative phyla differences within Donor 1 and 8 samples across different storage methods with 16S rRNA sequencing (Figure 6). In Donor 1, phy-

lum Firmicutes followed the conjectured trend, with lower abundance within Room Temp samples relative to Freezer (Table 5c). Furthermore, Donor 1 showed decreasing relative abundance of Firmicutes with age within Freezer samples, which could suggest even samples stored –20°C for long periods of time may eventually procure taphonomic patterns, wherein storage at –20°C only decelerates the taphonomic process. Only one sample (Donor1; 2014 Freezer) of five had observable levels of Saccharibacteria (with very little sequence abundance attributed to this phylum; Table 5c, supporting its significance difference as symptomatic of intra-individual microbial variation.

Qualitative Phyla Changes over Time in Individual Donors with 16S rRNA Sequencing

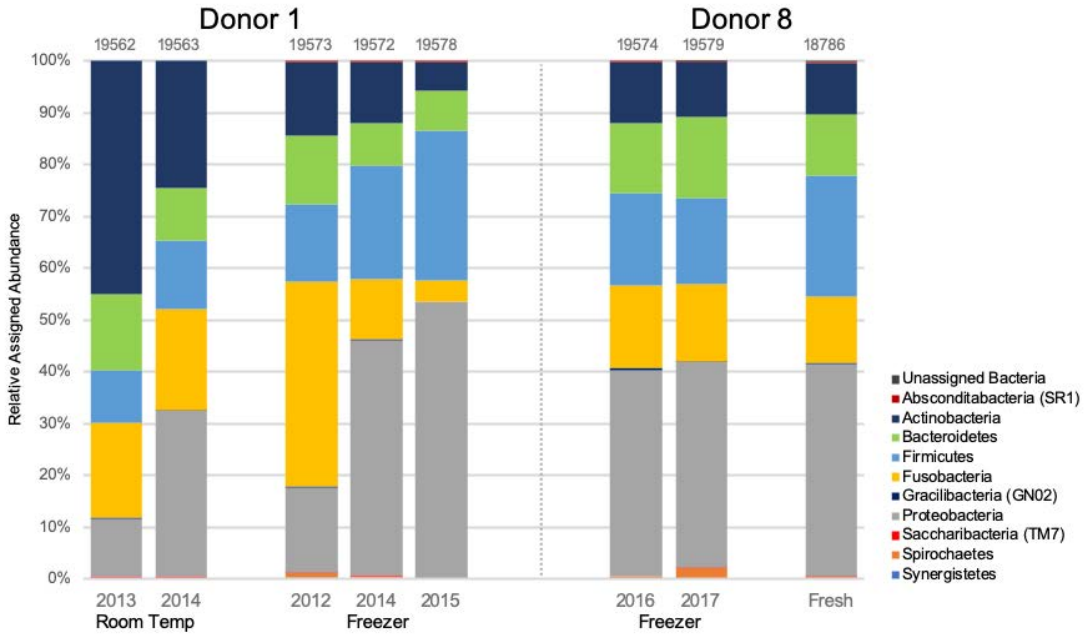

Figure 6: **The relative abundance of Donor 1 (n = 5) and Donor 8 (n = 3) dental calculus microbial phyla, with 16S rRNA amplicon sequencing.** Samples are grouped according to their storage type and sorted through time. Donor 1 donated five individual samples of calculus across four years, and Donor 8 donated three samples across two years. This longitudinal record provides a qualitative display of compositional changes though time

Saccharibacteria is present within both Freezer samples of Donor 8, but not within the Fresh sample; an alternative interpretation of this storage temperature variance is under-sampling bias of low-abundant taxa [24]. Another hypothesis could be a potential taphonomic impact of freezing producing sequence misassignment; wherein Saccharibacteria phylum was not detected with shotgun sequencing. In Donor 8, Firmicutes does not appear to be significantly impacted by –20°C, with minimal differences between Freezer samples stored a year apart (Figure 6). All three samples maintain compositional similarities, supporting the –20°C stor-

age in sustaining the calculus microbial community reconstructed with 16S rRNA sequences.

4.5 Discussion

Studies of ancient human-associated microbes are imperative to understanding how recent historical lifestyles have modified the evolutionary-relationships between human and microbes [38]. To understand the evolution, ecology, and origin of contemporary microbial communities, researchers need to be able to compare microbial communities of the past to the present compositional state; dental calculus has been shown to be promising archaeological material for this analysis [8, 6, 7]. However, the underlying contribution of taphonomic changes to the dental calculus microbial community remains largely unexplored and may significantly contribute to the conclusions made from such past to present comparisons. Within this preliminary study on the impacts of long-term storage methods on the reconstruction of dental calculus microbiota, we elucidate a number of phyla-level compositional changes with time and storage temperature, which are discussed in detail below. The analyses presented here highlight the potential underlying taphonomic biases present in ancient dental calculus research and demonstrate a need to explore taphonomic processes across ancient microbial communities more broadly.

Modern microbial research has already quantified the effect of storage conditions, over a two-week period, upon dental plaque communities [20], but this impact has not been assessed over the period of several years. Thus, our study is the first to provide insights into the taphonomic impact on the reconstruction of oral microbial communities from long-term stored samples (*i.e.* years). Of all the taphonomic differences, phylum Fusobacteria was the most significantly impacted by long-term storage, regardless of storage temperature. No literature has reported blooms of Fusobacteria previously, but current reports of time and storage temperature impacts predominantly focus on faecal material, where Fusobacteria is not a dominant phylum [16]. Furthermore, while these qualitative results of the impact of room temperature storage may have only minor implications for modern microbial research—as freezing samples immediately after collection is common-practice for modern microbiota studies [39]—these results emphasise the difficulties in comparing microbial communities from ancient and contemporary populations for paleomicrobiological research.

Our results show that there are significant changes in the diversity of the microbial communities that result from storing samples over long periods of time at room temperature ($\sim$23°C). With shotgun sequencing, we were able to detect significant alterations to phyla Fusobacteria with long-term room temperature storage.

However, as ancient DNA research relies on dental calculus collection from skeletal remains, where 'storage' is at the whim of the post-mortem environment, these alterations induced by indoor room temperature storage may not recapitulate the taphonomic changes of typical ancient dental calculus samples. Nevertheless, with the lack of species- or genera-level associations linked to microbial community differences, these results emphasise that broader microbial community level alterations will impact the comparison of ancient and contemporary microbial communities. Accordingly, this preliminary analysis suggests that researchers should look to test greater ecological shifts that differ between microbial communities, such as differences in co-occurrence relationships. For example, does the removal of significantly different phyla from a microbial community still maintain differences in the dependent ecology? Future work is needed to unravel the technicalities and patterning of the taphonomic processes within ancient dental calculus in order to annotate the impact of taphonomy within paleomicrobiological dental calculus research.

Similarly, our results indicate that dental calculus samples stored at –20°C for longer than two years will suffer compositional changes, driven by phyla Fusobacteria and Actinobacteria. Within for modern microbiome research, this impact interferes with the ability to return to long-term stored dental calculus samples to reconstruct bacterial communities using shotgun sequencing and yield the same microbial reconstruction. However, this did not appear to be problematic using 16S amplicon sequencing. Within both shotgun and 16S data, Firmicutes is seen to increase within samples stored at –20°C compared to room temperature; this has been previously noted in faecal microbial communities stored at –80°C, wherein the process of freezing increases DNA extraction of gram-positive bacterial cell walls [40, 41]. Yet, faecal material will undergo very different biomolecular taphonomic processes than stable calcified dental plaque due to the mineralisation of vast majority of plaque organisms [5]. Potentially, the microbial community differences detected between room temperature and freezer storage is driven by the developing plaque biofilm on outside of the dental calculus [42]. However, there is very limited unbiased metagenomic understanding of plaque development, with the majority of research of biofilm formation using *ex situ* modelling or DNA checkerboard hybridisation to resolve the influence of microorganisms on the outer calculus surface [43, 44]. To counter this, prospective research should look to investigate the integration of a pre-wash, prior to the DNA extraction of modern dental calculus, in how it impacts the reconstruction of both fresh and stored dental calculus communities.

Even within contemporary research, there is currently very little understood regarding the taphonomic processes *ex situ* of dental calculus microbial communities. Microscopic analysis has shown no evidence of alterations to the mineralised structure of calculus post-mortem [6]; however, this does not preclude the presence of

non-mineralised bacteria within gaps and tubular holes of the calcified matrix [42] or impacts on DNA preservation within the cellular structure. Previous work on supragingival calculus (calculus formed below the gum line, usually related to periodontal disease) found that immediately frozen oral samples maintained animate aerobic and anaerobic culturable bacteria when released from the calcified matrix [45]. However, Tan *et al.* (2004) did not investigate unculturable bacteria, which are estimated to make up more than 60% of the oral microbial community [35]. Nevertheless, this suggests that there is some duration in which bacteria captured within the calcified matrix are dormant, but the processes of extinction, predation, metabolic activity, and subsequent changes to the entire dental calculus ecological community remain unknown.

As a preliminary study, there are caveats that limit our ability to make definitive recommendations regarding taphonomic repercussions. Firstly, the results are circumscribed by the small sample size; our stored dental calculus collection was dependent upon self-directed donations over several years. Moreover, the small sample size required us to use multiple samples from the same individuals. However, while inter-individual variation could confound differences between storage method groups and freshly extracted samples (as samples originated from different donors), our results support a greater impact of storage on microbial community variation. The oral microbial community is one of the most conserved microbial ecosystems on the human body, with the smallest amount of inter- and intra-variation [46]. However, it is possible that the intra-individual variation sampled over multiple years may simulate taphonomic changes. Researchers Hall *et al.* (2017) found, within supragingival plaque, inter-individual variation was consistently stronger than the intra-individual variation, even though up to 30% of individuals experienced a significant drift in Bray-Curtis measure of microbial diversity over a period one year [47]. This makes the analysis of changes in microbial communities over time purely qualitative with regards to significant taxonomic group differences, where the extent of taphonomic change may not be able to be disassociated from the intra-individual variation over time.

4.6 Concluding remarks

Our study highlights several important considerations for studies involving both ancient dental calculus and contemporary microbiome research on modern calculus samples. Storage conditions have the potential to introduce substantial alterations to microbial community profiling based on both shotgun and 16S rRNA gene sequencing. Ideally, samples should be stored at –20°C or below immediately after collection and extracted as soon possible, limiting the time elapsed between collec-

tion and DNA extraction. Ancient or historic DNA research using dental calculus samples to reconstruction the ancient oral microbial community should also take into consideration the taphonomic impacts seen within shotgun sequenced results of room temperature samples when comparing to contemporary dental calculus samples. Despite the limitations of this study diminishing the ability to make quantitative statements regarding compositional differences, our findings suggest precautions should be taken in interpreting microbial communities' differences between calculus samples with different storage methods. These findings underpin the importance of contemporary microbiota research for bioarchaeological interpretations and to better understand the taphonomic processes in *ex-situ* dental calculus microbial communities.

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4.8 Author contributions

LSW, MHD, and JK collected dental calculus samples. ES and MHD performed laboratory work. ES and RE performed data processing and analysis. ES and LSW wrote the manuscript, all authors read and approved the final manuscript.

The authors declare that they have no competing or conflicts of interest.

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Chapter 5

Ancient DNA from dental calculus tracks microbial changes with the Industrial Revolution

Statement of Authorship

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Contribution to the Paper	Performed laboratory work and data processing. Analysed and interpreted data. Wrote the manuscript.		
Overall percentage (%)	60		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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ii. permission is granted for the candidate in include the publication in the thesis; and

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Ancient DNA from dental calculus tracks microbial changes with the Industrial Revolution

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5.1 Abstract

The sociocultural and environmental processes of Industrialisation are associated with the rapid rise of several non-communicable diseases (*e.g.* obesity, type II diabetes, heart disease, *etc.*), irrefutably altering human epidemiology [1]. Several recent studies suggest that these historic increases in 'industrial diseases' are underpinned by key changes in the human microbiome—the collection of commensal microorganisms that live within and on the human body. Contemporary populations living 'traditional' (pre-industrial) lifestyles have been shown to have significantly more microbial diversity than populations living modern industrialised lifestyles, suggesting a loss of microbial diversity with Industrialisation. However, the microbial evolutionary history of current European populations cannot be inferred from a proxy of different cultures practising unique lifestyles, bringing into question the key factors and the timing that underpin past industrial-associated changes in the microbiome and, subsequently, human health. Sequencing ancient DNA from calcified dental plaque (calculus) samples can now provide 'real-time' insights into the factors that shaped the human microbiome in the past, allowing researchers to track the impacts of past sociocultural and environmental changes through time. Here, we

examine the oral microbiomes from 128 individuals who lived before, during, and after the Industrial Revolution (IR), and describe how the microbiome was influenced by the different stages of Industrialisation (post-1800s). We identified geographic-specific changes in microbiota linked to industrial and environmental differences amongst those who lived during the IR, including unique signals in Europeans who migrated to Australia. We also identified a further historic change in human microbiome that likely began early 20th century, alongside improvements in oral hygiene and dental treatment, accounting for biases of taphonomy and disease. This study is the first to substantiate the human microbiome alterations during the IR, expanding our understanding of the ancestral European microbiome and the development of industrial diseases.

5.2 Main text

Introduction

From the beginning of the late 18th century, a revolution of human industry and manufacturing—the Industrial Revolution (IR)—transformed human behaviour and environment, leading to the development of urban centres, factory industries, built environments, advanced communication, transportation, and technology [2]. The process of Industrialisation today is associated with the increase of non-communicable diseases [3], such as obesity [4], cardiovascular disease and stroke [5], type II diabetes mellitus [6], and immunoregulatory disorders [7, 8]. It has been hypothesised that this increased prevalence of non-communicable diseases [9, 10, 11, 12] is linked to the human microbiome (*i.e.* microorganisms and respective genomic material living on and within the human body) and its evolutionary discordance with industrialised lifestyles and environments [12]. This is exemplified by studies looking at post-industrial lifestyle factors impact on the human microbiome (*e.g.* antibiotics [13]), or research into the microbiomes of people living 'traditional' lifestyles compared to those living in the US, Europe, and other conteporary industrialised societies [14, 15, 16, 17]. However, suppositions from cultural proxies are complicated by numerous factors, including the fact that many modern 'traditional' populations are genetically distinct, and likely do not recapitulate the ancestral state of industrialised European populations [18].

By utilising ancient DNA (aDNA) from ancient calcified dental plaque (calculus), we can now reconstruct 'real-time' snapshots of the human microbiome in the past [19, 20]. Initial work by Adler *et al.* (2013) reassembled the human oral microbiome extracted from dental calculus using limited subsets of ribosomal RNA, and established differences in microbial composition between pre- and post-agricultural cultures, as well as microbial alterations between Medieval and IR individuals [21]. Changes in the oral microbiome linked to Industrialisation were also noted by Weyrich *et al.* [22]. However, both studies were limited by small sample sizes and lacked comparisons between different European populations. This study aims to gain a greater understanding of the microbial changes associated with human health and disease by exploring the taxonomic changes of the human oral microbiome correlated with changing process of Industrialisation, *i.e.* alterations in physical environment (*e.g.* increased urbanisation and environmental pollutants) and human behaviour (*e.g.* antibiotics, sanitation, and hygiene).

Changes to the oral microbiome with the Industrial Revolution

We reconstructed the oral microbiome from the dental calculus of 128 individuals (68 new oral microbiomes and 60 pre-existing) who experienced the different stages of the IR, including individuals who lived prior to the IR (n = 56) in medieval England [23], Ireland [24], and Germany [25]; during the IR (∼1800–1920s; n = 37), including Germany (n = 9), Switzerland (n = 12), Australia (n = 12), and England [23] (n = 4); and those living within the recent century in France and Australia (>1900s; n = 35) (Table S1). A shotgun metagenomic sequencing approach was used to obtain an average sequencing depth 3,666,849 sequences per sample (range 23,134–18,143,046) (Table S3). All data was filtered for environmental and laboratory contaminant species, which has been shown to confound aDNA analyses of microbial communities [26, 27]. All retained samples possessed >90% non-contaminant species (Table S8) and were rarefied to 180,123 sequences per sample for downstream analyses. As prior research noted microbial community differences by tooth type within a single population [23, 28], we examined the impact of tooth type within this meta-analysis; significant differences in oral microbiome composition were linked to tooth type (Bray-Cutis PERMANOVA; p = 0.001; pseudo-F = 2.73); however, this was significantly confounded by location. Previous research [29] has observed that inter-population differences have stronger impacts upon the microbial community than intra-population tooth types; therefore, key findings were confirmed using only a single tooth type (*e.g.* only incisors; see Supplementary Materials), although all teeth were utilised for the following analyses.

Industrialisation impacted the oral microbiome composition

We first reproduced previous findings that indicated the oral microbiota of Europeans living during the IR (n = 25) were significantly different to those living before that period (n = 56) (beta-diversity; Bray-Cutis pairwise PERMANOVA; p = 0.008; pseudo-F = 3.53), confirming suspected links between change in the human microbiome and the IR [21, 22]. However, inter-population differences in geography may be compounding the IR changes observed across populations. Therefore, within our within England population, we examined only definitively pre-IR (n = 20) and IR (n = 4) individuals and indeed confirmed a significant difference oral microbiome compositions (Bray-Curtis pairwise PERMANOVA; p = 0.015, pseudo-F = 3.03), that was not confounded by cultural and environmental differences.

Unique oral microbiomes are detected in different geographic locations during the Industrial Revolution

As there is no singular model of 'Industrialisation', the processes of 'industrialising' were, and are still, experienced differently in distinct locations [30]. We examined if populations in central Europe experienced the IR differently to those in England, comparing the oral microbial diversity and composition within three separate European populations: Switzerland (n = 12), Germany (n = 9), and England (n = 4). While, no significant differences in diversity were detected between central European countries (Shannon Kruskal-Wallis; p = 0.40, H = 1.846), English and German oral microbiomes contained significantly different compositions from one another (Bray-Curtis pairwise PERMANOVA; p = 0.003, pseudo-F = 4.15). Switzerland microbiome was observed to be similar to both England (Bray-Curtis pairwise PERMANOVA; p = 0.06, pseudo-F = 2.38) and Germany (Bray-Curtis pairwise PERMANOVA; p = 0.19, pseudo-F = 1.40). The specific reasons for this remain unknown; however, differences in each countries' IR were apparent across these three countries.

The IR originated in Britain, with Industrialisation developing for a number of decades prior to the 'onset' in 1780s [31]. Switzerland and Germany remained predominately rural in early 19th century with later expansion into specialised industries; Basel (Switzerland) was textile-industry focused, especially in synthetic dye production, while Hettsedt (Germany) was primarily invested in mining and metallurgy. London (England) was infamous for its air pollution, even prior to the Industrial Revolution [32]. All of these industries would have increased production of environmental heavy metal pollutants; metallurgy was linked to increased nickel and copper pollutants, whereas increased copper, zinc, and cadmium were linked to chemical dye industries [33, 34]. Presumably, microbial similarities may be linked to shared environmental factors, such as contact with these heavy metals. Moreover, socioeconomic factors may also contribute to the microbial differences between Germany and England, as individuals were from disparate socioeconomic classes with distinct lifestyle and behavioural factors [23].

Microbial composition maintained after colonial settlement

With the increasing poverty and population size of Industrial European cities, colonial settlements became attractive economic opportunities, with increasing migration throughout the 1800s to colonies that were typically areas independent of industrial processes [35]. Here, we were able to reconstruct the oral microbiome of British settlers of the South Australian colony of Adelaide (dated 1846–1927; n = 12). Oral microbiome diversity in Australian colonists was similar to populations of

their surmised homeland, England (Shannon Kruskal-Wallis; p = 0.48; H = 0.72), as Adelaide colony was a predominately British migrant population, this suggests that migration out of Europe did not impact microbiome diversity. This is surprising, as exposure to new environments is hypothesised to lead to increased microbial diversity [36], and these results indicate that other shared lifestyle processes maintained an oral microbial community similar to the colonial homeland.

Similarly, the oral microbiome composition was similar to that observed in England (Bray-Curtis pairwise PERMANOVA; p = 0.5, pseudo-F = 0.81), perhaps reflecting their ancestral and cultural ties to their colonial homeland. In fact, the individuals examined here worshipped within an Anglican Church [37], much like those individuals of IR England [23], suggesting that they may have been committed to maintaining cultural homogeneity within settler society [38]. However, we are limited by the small sample size of the IR England population to make definitive conclusions.

Australian colonists were significantly less diverse than both German (Shannon Kruskal-Wallis; p = 0.02, H = 7.29) and Swiss IR populations (Shannon Kruskal-Wallis; p = 0.02, H = 8.00). Australian colonists also had an oral microbiome composition distinct from IR individuals of both Germany (Bray-Curtis pairwise PERMANOVA; p = 0.006, pseudo-F = 6.26) and Switzerland (Bray-Curtis pairwise PERMANOVA; p = 0.02, pseudo-F = 3.19). Several species were significantly absent in both Australian colonists and English individuals compared to German and Swiss IR individuals (n = 21): four oral species of *Selenomonas*, *Prevotella masculosa*, and *Centipedia periodontii* (Kruskal Wallis; $p < 0.05$, Table S9). Moreover, German and Swiss populations shared a greater relative abundance of 15 different genera ($p < 0.05$, Table S10) than the Australian and English populations, including *Selenomonas*, *Ottowia*, and *Streptococcus*, potentially driving the compositional differences between populations. In contrast, only three genera (*Pseudoramibacter*, *Methanobrevibacter*, and *Parvimonas*) maintained significantly greater relative abundances within the Australian colonists and English population (Figure 1).

These compositional differences appear to be linked to the dominance of (or lack of) *Methanobrevibacter* within these populations, as previously described by Farrer *et al.* [39]. Presuming *Methanobrevibacter*-dominated microbiomes persisted from England over to the colonial settlement, the environment or lifestyles of colonists appear to support this microbial composition later into the IR period. While, Farrer *et al.* had previously linked *Methanobrevibacter*-dominated microbiomes' functional potential to low-meat and high-fibre diet [39], the early Adelaide colony was renowned for their protein-heavy diets, as supported by stable isotope analysis [37].

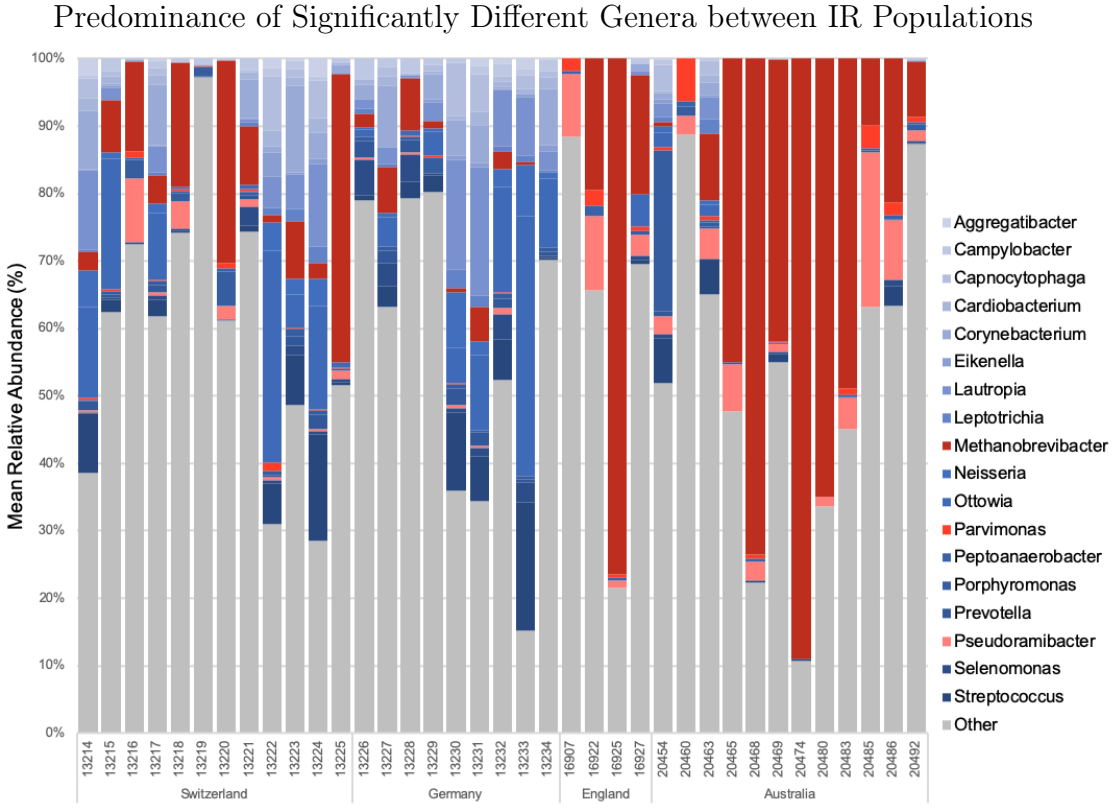

Figure 1: **Mean relative abundance of significantly different genera (Kruskal-Wallis, FDR corrected p-value < 0.05, see Table S10) between IR populations Switzerland and Germany compared to Australia and England.** All samples rarefied to 180,123 sequences. Alpha diversity differences between European populations appear to be driven by the presence or absence of genus *Methanobrevibacter*.

The modern microbiome is different from the IR microbiome

Controlling for taphonomic biases

Multiple studies have identified differences between modern and historical oral microbiomes [21, 22, 40]; however, several known biases could have driven these results which have not yet been investigated. Thus, while we initially detected significant differences in microbial composition between IR and modern healthy individuals ('healthy-modern'; Bray-Curtis PERMANOVA; p = 0.001, pseudo-F = 19.816), we sought to explore two potential key sources of bias influencing this result. First, we examined the taphonomic bias that may be influencing the composition of ancient microbiota. As long-term storage of dental calculus at room temperature has been shown to significantly alter relative abundance of Fusobacteria and Proteobacteria phyla over time (see **Chapter 4**), we controlled for taphonomy within archaeological dental calculus by removing all species within the Fusobacteria and Proteobacteria phyla. Significant differences between IR populations and healthy-modern individuals were maintained (Bray-Curtis PERMANOVA; p = 0.001, pseudo-F = 23.92), indicating this compositional difference is likely not an artefact of taphonomy.

Controlling for periodontal disease

The second key source of bias between modern and historical populations is the prevalence of oral disease within past populations and its interpretation from skeletal remains [41]. Modern microbiome research has shown differences in oral microbial composition between healthy individuals and those with periodontal disease [42, 43], although this has yet to be investigated using supragingival calculus. Here, we examined 18 modern dental calculus samples, from individuals suffering mild to advanced periodontal disease ('periodontal-modern'), for which periodontal-modern and healthy-modern populations had significant different oral microbiomes (Bray-Curtis PERMANOVA; $p = 0.04$, pseudo-$F = 2.09$).

Next, we examined all IR oral microbiomes compared to modern individuals suffering from periodontal disease and observed a significant difference between populations (Bray Curtis PERMANOVA; $p = 0.001$, pseudo-$F = 17.07$), even after correcting for taphonomy (Bray-Curtis PERMANOVA; $p = 0.001$, pseudo-$F = 20.50$). These results demonstrate that the differences between the IR and modern day cannot be explained by periodontal disease alone, suggesting that there may have been further alterations to the human microbiome following the early stages of the IR.

Differences between historic and modern populations were linked to wholesale decreases in three phyla, including Euryarchaeota, Chlorofexi, and Synergistetes taxa ($p < 1.18e^{-8}$, SI Table 11; Figure 2). Together, these results suggest that the IR has impacted and altered the human microbiome, dramatically changing the oral microbial ecology.

The inclusion of modern periodontal patients also highlights the reduction in Archaea present within the modern oral microbiome. Within our modern periodontal population, only one supragingival sample had detectable levels of *Methanobrevibacter*, whereas *Methanobrevibacter* was detected in 83% ancient and historical individuals, with 39% of individuals presenting more than >10% of absolute total sequences assigned to *Methanobrevibacter* genus (Table S3). The presence of Archaea within the supragingival calculus of living people today is limited. Several modern oral microbiome studies has identified a correlation between the presence and abundance of *Methanobrevibacter* genus within the subgingival periodontal pockets and severity of periodontal patients [44], but its overall prevalence dependent upon methodology and geographic population [45]. Moreover, this analysis has not been replicated using supragingival calculus. In contrast to the pronounced level of *Methanobrevibacter* within our IR Australian population, bioarchaeological analysis of these individuals saw very little evidence of periodontal disease within this population.

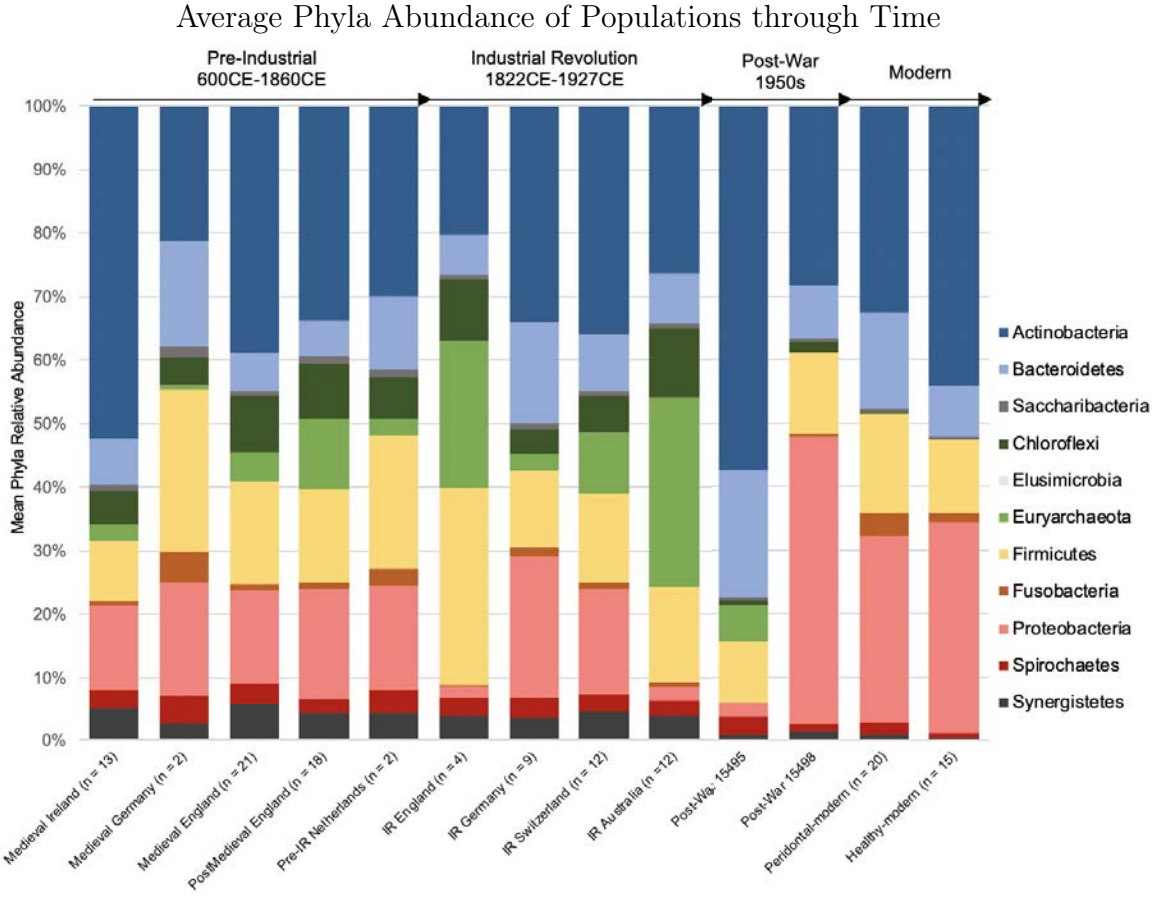

Figure 2: **Mean phyla relative abundance of the average individual oral microbiome of each geographic population, from pre-IR to modern populations**. All samples were rarefied to 180,123 sequences. Both 1950s post-war individuals included to show transition from IR to modern average composition. Phyla frequencies were generated from species assignments, and do not include unassigned reads.

Transition to modern oral microbiome began prior to the Great Acceleration

After the IR, another rapid period of change began after World War II—known as the 'Great Acceleration'—caused industrialised populations to undergo a further epoch of rapid population growth, urbanisation, and technological development [46]. As we identified differences between IR and modern populations, we sought to identify when this change began to occur. We reconstructed the oral microbiome of two French individuals who died in the early 1950s, just prior to the Great Acceleration ('post-war'). We found one individual to be more similar to the modern microbiome composition, while the other maintained a microbiome more similar to individuals of the IR (Figure 3), suggesting that changes to the modern oral microbiome was ongoing during the 1950s. Furthermore, the two post-war individuals were not significantly different from either healthy- and periodontal-modern populations (Bray Curtis PERMANOVA pairwise; healthy, $p = 0.054$, pseudo-$F = 2.50$; periodontal, p

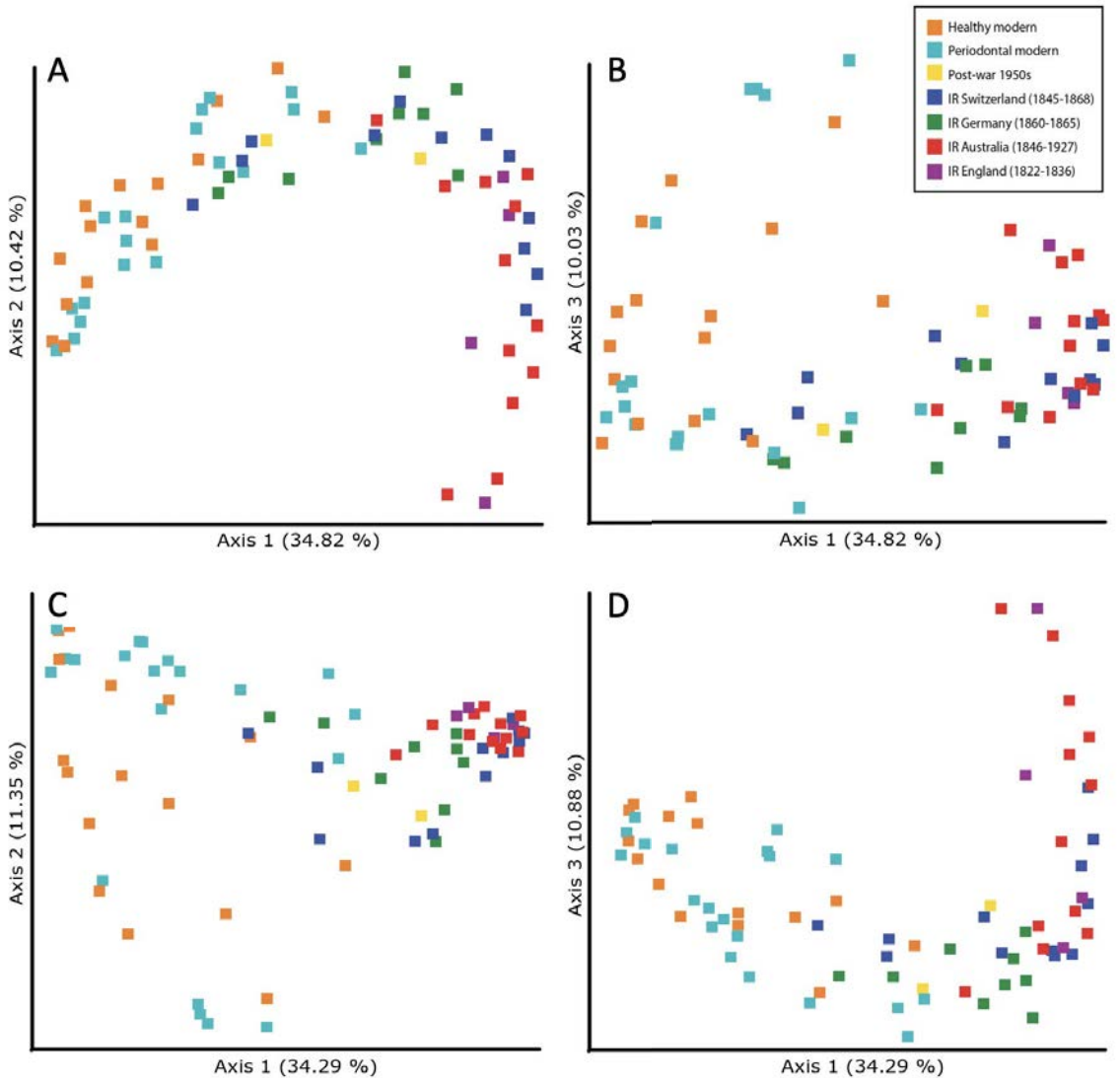

Figure 3: **Principle coordinates analysis shows microbiome transitions from Industrial Revolution (IR) populations through to modern populations, with 1950s post-war samples divided between the two groups.** Beta diversity was calculated with Bray-Curtis. (**A, B**) Plots of the first and second axis (**A**) show the progression towards the contemporary oral microbiome, and first and third axis (**B**) present the variation between modern healthy and modern periodontal disease microbiomes. All samples were rarefied 180,123. (**C, D**) After controlling for potential taphonomic biases, the axis explaining variation switch, with the first and second axis (**C**) describing the oral health of modern populations, and the first and third (**D**) support the IR to modern transition. All samples were rarefied to 143,16—due to phyla removed—but rarefaction depth was shown to not influence results (SI Table 12).

= 0.065, pseudo-F = 1.86) or IR individuals (Bray Curtis PERMANOVA pairwise; p = 0.14, pseudo-F = 1.49). However, when we accounted for taphonomy in the comparison of post-war individuals, significant differences were observed between post-war and healthy-modern individuals (Bray Curtis PERMANOVA pairwise; p = 0.008, pseudo-F = 2.81), but not between post-war and periodontal-modern individuals (Bray Curtis PERMANOVA pairwise; p = 0.07, pseudo-F = 2.17). This could suggest with the improvement in oral hygiene behaviour and treatment from post-war era induced a microbial alteration towards the healthy modern composi-

tion, but this result may alternately be an artefact of the post-war individual sample size (n = 2) and is not conclusive. Nevertheless, this suggests that the modern oral microbiome in Industrialised Europeans was established in some individuals by the 1950s.

Concluding Remarks

Our results identify key changes in the oral microbiome that are linked to human lifestyles and environments over the past 200 years. We identified unique oral microbiomes linked to areas experiencing different types of Industrialisation. Sociocultural changes introduced throughout the IR are likely to play key roles, in addition to industrial changes in the environment, such as pollutants. Evidence from ice cores has shown an increasing trend of large-scale atmospheric pollution of heavy metals such as lead, zinc, cadmium, and copper from the onset of the Industrial Revolution up until the 1960s–1970s [33, 47]. Further preliminary work using murine models supports changes within the mammalian microbiome with heavy metal exposure that induced phylum-level alterations and subsequent functional dysregulation [48, 49, 50]. As timing and amplitude differs between heavy metals pollutions differs geographically [47], this would suggest culminating exposures developing with the IR, as seen in our results. Furthermore, these geographic differences alongside individual socioeconomic status would modify personal exposures to pollutants based on accessible soil or aquatic systems, or as it were, immediate contact within workshops or factories.

Additionally, our results support a novel, additional microbial shift that occurred sometime in the past millennium, transpiring by the 1950s at least in France. This transition may have altered the microbial communities linked to periodontal disease, although further research is needed to verify these findings. Improvements in oral hygiene, oral health practices, and periodontal treatments and therapies during and after the 1950s are likely to be a significant contributor to this phenomenon [51]. Nonetheless, there are also many other sociocultural and environmental changes that flourished in the post-war period, which were introduced in the late IR era, such as, changing toothpaste ingredients to an alkaline base [52], the initial development and use of synthetic antibiotics [53], or the public adoption of synthetic organic pesticides [54], in addition to the fundamental alterations to the overall structure and dynamics of environmental ecosystems through climate change, pollution, and urbanisation [55]. In the investigation of these historical impacts, future work requires an examination of microbial adaptation with recent Industrialisation, alongside functional analyses that may reveal the mechanisms that underpin these changes at the taxonomic level. Functional potential is critical for understanding the

connections between the microbiome and chronic disease and is also more broadly needed to anticipate how microbial communities will respond to environmental and cultural changes within an increasingly Industrialised future.

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Author contributions

LSW obtained funding. FDP, JK, LSW, KD, MHD, and ES contributed to dental calculus collection. ES, AGF, and MHD performed lab work. FDP, KD, and KK assisted in anthropological and dental interpretations. ES, RE, and LSW led analysis and microbial interpretations. ES wrote the initial draft, which was edited and contributed to by all authors.

The authors declare that they have no competing or conflicts of interest.

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Discussion

Thesis Structure

The study of the human oral microbiome—whether through paleomicrobiology or contemporary dental health research—provides a new lens through which to understand and potentially contribute to improved population oral health and systemic well-being. Oral disease is the most common noncommunicable disease to affect people throughout their lifetime, causing pain, discomfort, disfigurement, and even death [1]. It is estimated that oral disease impacts over half of the world's population, with inequalities existing between different geographic and socioeconomic population groups [2]. What is especially prevalent is the increase in oral disease with increasing urbanisation, wherein the social determinants of industrialised lifestyles have detrimental repercussion impacts oral health [1]. This thesis has three main goals:

1. Provide context and perspective to microbiome science from the field of evolutionary medicine to advance contemporary public health research.

2. Investigate the interconnection between processes of industrialisation and the alteration to the human microbiome.

3. Identify promising and prospective areas for future research.

In the section below, I summarise each chapter and its greater significance.

Chapter 1

I contextualise the principles of evolutionary medicine (*i.e.* the application of modern evolutionary theory to explain human health and disease) to the investigation of the human microbiome, encompassing past environmental and sociocultural alterations which may have shaped human biological mechanisms that contribute to disease susceptibility. In this way, evolutionary medicine can be used to better understand contemporary Indigenous health by providing a contextual microbial evolutionary history.

Colonialism is known to have had many physiological and psychological impacts on Indigenous health and well-being, and I argue that contemporary Indigenous health needs to be understood within the context of the microbial evolutionary history. By defining and delimiting topically broad colonial processes and providing historical examples of potential microbial alterations, supported by recent microbiome research, I hypothesised the potential past microbial alterations that may be contributing to the health inequalities burdening Indigenous populations globally [3]. Closing the health inequality gap between Indigenous and non-Indigenous

populations requires consideration of all the likely components contributing to their health and disease.

Chapter 2

The cross-disciplinary scientific dissemination of ideas and information requires simplicity if it is to be widely accessible [4]. This chapter constitutes my effort to deliver a simple and accessible cross-disciplinary understanding of the role of evolutionary analyses of the human microbiome within of contemporary public health research (manuscript to be submitted to The Lancet journal). This is especially important within Indigenous health research, where Indigenous populations' microbial evolutionary history has been impacted by colonialism and historical subjugation, and their contemporary health status remains globally disadvantaged with serious health inequalities.

Chapter 3

Oral health research has a tendency to focus on singular pathogenic microorganisms associated with disease to make inferences about the microbial community as a whole [5]. This reductionist approach not only misrepresents the oral microbial ecology, but because scientific research tends to be concentrated upon industrialised and predominately European populations, this can lead biases in the understanding oral health and disease in difference geographic or ethnic populations [6].

This chapter represents the first study: (1) to have explored the salivary microbiome of Aboriginal Australians and Torres Strait Islanders appertaining to oral health and dental decay; and, (2) to analyse the oral microbial ecology differences following the impact of a novel longitudinal oral health treatment. Understanding how oral health treatments will impact the microbial ecology as a whole—as opposed to the study of singular 'pathogenic' microorganisms—provides greater insight into the subsequent physiological responses, potential inadvertent consequences of treatment, and prospective dental therapy targets.

Furthermore, this chapter contributes to the examination of understudied populations—such as Indigenous Aboriginal Australians and Torres Strait Islanders—which is crucial to the improvement of Indigenous health outcomes and tackling oral health inequalities.

Chapter 4

I provide the first qualitative assessment of impacts to the reconstruction of microbial communities from long-term storage upon dental calculus samples, looking at two

different standard storage conditions over a period of five years.

The growing research field of ancient human microbiomes (through the extraction and analysis of the paleomicrobiological material from dental calculus, *i.e.* calcified dental plaque) is elucidating the evolutionary history of the oral microbial. However, there is little known of the underlying biases and post-mortem nuances of dental calculus material. The processes of preservation and fossilisation (known as taphonomy) have not been analysed within dental calculus material. The interpretation and inclusion of taphonomic modifications to the reconstruction of microbial communities provided within this chapter (in which long-term room temperature storage may represent archaeological material) offer guidance for understanding and characterising the biases in comparing contemporary and ancient microbial communities.

Chapter 5

I reconstructed the historical oral microbiomes of European individuals who experienced the Industrial Revolution during their lifetime and identify the subsequent sociocultural and environmental changes caused by the processes of industrialisation that may have impacted the human oral microbiome composition. Industrialised processes changing peoples' lifestyle and modifying the environment (*i.e.* urbanisation) have been already been hypothesised to have altered the human microbiome, as evident by studies of microbial comparisons between traditional societies and cultures to industrialised ones [7, 8]. However, these modern cultural proxies cannot precisely conjecture what the past pre-industrial European microbiome composition or diversity looked like, nor reveal what historical ecological alterations assisted in the establishment of the modern oral microbiome composition diversity seen today.

By exploring the compositional changes of the human oral microbiome associated with Industrialisation, we advance our understanding of the evolutionary forces inducing ecological change. As the processes of Industrialisation have not ended with the Industrial Revolution, illuminating the preceding sociocultural and environmental changes that altered past microbiomes and physiological health may illustrate future consequences for population health research. This further improves the recognition, diagnosis, and interpretation of alterations to the oral microbial community within human health and disease.

In summary, this thesis demonstrates the broad interdisciplinary nature of understanding the human oral microbiome, the importance of advancing investigations inclusive of integral microbial ecosystem, and the contribution of evolutionary history to modern human microbiome research. In demonstrating the impacts of environmental and sociocultural-behavioural changes to the ecology of the human oral

microbiome, my thesis contributes to an array of research in the study of the human microbiome, paleomicrobiology, evolutionary medicine, public and population health, and to dentistry and general medicine, institutional academics and industry professionals alike. Equally so, understanding the human microbial ecological community within human health and disease could not be discerned without the multidisciplinary approach and interpretation. From an evolutionary perspective, uncovering past ecological changes has unequivocal relevance to the developments of future prevention and treatment of oral disease, and capacity to improve human systemic health. In this discussion chapter, I examine and explore the outcomes and interpretations of my research, presenting the ideas and prospects for future research avenues.

Influencing the oral microbiome

Within this thesis, there are two underlying primary research foci:

Firstly, by investigating the changing components of lifestyle and behaviour and their lateral impact upon human microbial ecology, we gain a better understanding of the past evolutionary history of human-microbiome interactions that have contributed to the contemporary microbial composition.

The second examines the variations of microbial ecology (both in past and present populations) for insights into mechanisms influencing the interconnection of the microbiome and human health. From the analysis of salivary microbiota composition in children associated with dental decay severity, to the historical transformation of population health in the past 200 years of industrialisation (and its direct impact upon the evolution of the oral microbiome); within this thesis and within the field of microbiome research (microbiomics), it is indisputable that human physiological health is directly tied to the human microbiome.

However, there are numerous components of human physiological health, which are often not discussed or accounted for within microbiomics research. One such factor is 'socioeconomics', which is critically pertinent to the both the investigations within this thesis and collectively within the future of human microbiome research.

Socioeconomics of the human microbiome

The combined measure of economic and social status is known as 'socioeconomic status' (SES), a complex indicator usually comprising income, education, and occupation [9]. Within this section, I will discuss SES and its respective factors applicable to the various components of this thesis, giving consideration to its position within my results, and identify prospective areas for future research.

Importantly, SES is positively associated with health; greater wealth and social position typically enjoy lower rates of morbidity or mortality within Industrialised societies [10, 11, 12]. While often discussed on an individual level, SES can also have a strong influence on health outcomes (disease, disability, and mortality) at a community-level [13]. SES explains variation in many aspects of life and lifestyles. Dietary choices, occupational activities, exposure to pollutants, psychosocial stress, and social interactions are all are reasonably well explained by an individual's SES score or other measure of resource availability [14].

This complements our current understanding of the human microbiome, in which the environment and interactions with environmental components shapes acquisition and exchanges of microbes [15, 16, 17]. Thus, SES constrains the broader social and environmental conditions able to influence the structure and composition of the microbiome, and for that reason, it becomes eminently relevant that measurements of socioeconomics are integrated into human microbiome research

SES and Indigenous health

The determinants of socioeconomics are indicative universally of health [18], especially with Indigenous population health. Indigenous populations are overrepresented within lower SES groupings, where they experience significant disadvantages across a range of indicators including education, employment, and income [**?** 19]. The consequences of Colonialism (see **Chapter 1**) and the incorporation of Indigenous peoples into the construction of the nation state (or complete lack of incorporation in some cases) was shaped by varying degrees of violence, dislocation, and cultural oppression, that structures the marginalisation, denigration, or the suppression of Indigenous communities today [20].

Marginalisation and its associated stressors and anxieties can alter the body on a fundamental biological and biomolecular level, impacting immune response, growth and metabolic processes [21, 22]. Contemporary Indigenous health is impacted by a range of culturally-specific historical trauma, such as a loss of language, environmental deprivation or separation from land, or more widely, a spiritual disconnect, that has modified neuroendocrine and psychological functional ramifications [21, 19]. These impacts can be intergenerational, passed along by epigenetic alterations, or through the human microbiome [23, 24, 25]. Thus, the integration of SES within microbiome research and associated health status of Indigenous populations is confounded by the ensuing impacts of historical trauma.

Within **Chapter 1**, I suggest SES is not a confounding factor within the discussion of the microbial contribution to Indigenous health, but rather compounding factor to Indigenous health disparities. The SES of Indigenous populations is rem-

nant of the historical colonisation wherein the colonial structures have maintained material and symbolic (*i.e.* political) privileges, but is also emblematic of an ongoing system of oppression [26]. If intergenerational impacts from historical alterations of colonialism contribute to the discordance of the human microbiome with physiological health, we have to assume that the continuity of oppression (*i.e.* generalised lower SES than their non-Indigenous counterparts) within colonial societies defines contemporary Indigenous health.

In other words, there is no certainty in completely disentangling SES factors in the study of the Indigenous human microbiome. However, this should not preclude its integration within analyses; the only way to understand how health and the microbiome are shaped by differential psychosocial, physical, and chemical environments linked to ethnicity and SES is through the inclusion of individuals from diverse ancestral, cultural, and social backgrounds [27].

SES in microbiome research

Thus far, research on SES and the human microbiome is relatively limited. Miller *et al.* (2016) found lower neighbourhood SES was positively associated with lower colonic microbiota diversity and unevenness [28]. This was supported by Bowyer *et al.* (2019), in finding reduced microbial diversity in lower SES individuals even after adjusting for individual health status and diet [29]. The incorporation of SES within the analysis of human microbiome associated with health and disease becomes vitally important in the understanding of biological mechanisms underlying the relationships between disease risk and environmental factors.

Even though oral health research has long acknowledged the impact of SES upon disease prevalence [30], only two studies have included SES in their analysis of the oral microbiome. Belstrm *et al.* (2014) noted approximately 20 percent of the variation of bacterial profiles within saliva could be attributed to SES, despite no detectable impact of body mass index, alcohol consumption, or diet [31]. In a larger analysis of community SES, Renson *et al.* (2018) also detected a number of microbial taxa associated with sociodemographic variables, consistent with health inequalities [32]. Notably, within this meta-analysis, the researchers were able to detect disease-associated microbial differences between ethnic populations, suggesting the oral disease state was driven by different microbial ecologies [32]. However, one of the difficulties with large-scale cross-sectional studies is their limited resolution in narrowing the role of oral microbiota in health inequalities. Therefore, prospective studies which allow for controlling population demographics along with repeated measurement are preferable for identification of etiological factors.

In the investigation of microbiota associated with oral health and disease, **Chap-**

ter 3 concentrates on a subset of Aboriginal Australians and Torres Strait Islanders, sharing environmental locality, and lifestyle and socioeconomic factors [33]. Aboriginal Australians and Torres Strait Islanders—who live across a wide range of locations, belong to many distinctive descendent groups, with diverse sociocultural and environmental interactions. As a collective population, they exhibit greater rates of oral disease (including that of dental decay and periodontal disease) than their non-Indigenous counterparts [34]. Disentangling the socioeconomic influences from the role of culture and environment within Australian populations becomes important for government programs and public health initiatives targeted towards the education, preventative action, and treatment of oral disease within Aboriginal Australians and Torres Strait Islanders.

Within the Aboriginal Australians and Torres Strait Islanders of the Northern Peninsula Area (NPA), Queensland (**Chapter 3**), we observed very little impact a novel caries preventative intervention treatment on the abundance of *Streptococcus mutans* bacteria within the salivary microbial community. *S. mutans* is the predominant target in dental decay intervention treatments, with the design and development of therapies concentrating on the biophysical properties of *S. mutans* that augment dental decay [35, 36]. In fact, many developed dental treatments focus on isolated oral 'pathogenic' species in the aetiology of decay, without accounting for the oral ecological ensemble [37].

Thus, within the study of Aboriginal Australian and Torres Strait Islander children of the NPA community, we saw the preventative intervention treatment improving oral health and decreasing the prevalence and severity of dental decay, but not impacting *S. mutans* abundance. This raises questions about the microbial ecological of the community involved with dental decay progression and the extraneous cultural and socioeconomic impacts that shape this microbial community. The complex interactions of the oral microbiome, influenced by social and physical environmental factors, alongside biological processes, may be unique to this particular population. Only by extricating the socioeconomic factors relevant to the cultural- or regional-specific influence upon oral microbial ecology, are we better equipped to tackle the population-wide Aboriginal and Torres Strait Islander disparities in oral health.

SES in past populations

The SES role within health inequalities is not a product of contemporary society; health inequalities were far more pronounced in the past than they are today [38]. While the Industrial Revolution brought about nationwide economic benefits and diffusion of wealth among the general public, it also resulted in inconceivable hard-

ships for the lower socioeconomic classes dependent upon industrial occupations for survival [38, 39]. The historical working class of the 19th century were not just spatially differentiated in where they lived and worked, but the adversity and hardships of working-class labour, inadequate diets, unsanitary conditions, rampant endemic diseases and occupational trauma, physically demarcated lower SES individuals [38, 40]. These SES inequalities would be cultural and environmentally differentiated, creating and shaping vast SES differences across populations and ethnicities.

I suspect the geographic differences detected among the oral microbiomes of individuals from the period of Industrial Revolution were driven by SES differences where the cultural processes of industrialisation varied. However, in the analysis of historical microbiomes, SES of an individual is specified at best by mortuary records, but more often inferred by bioarchaeological interpretations of skeletal remains [41]. To the best of our knowledge, the vast majority of individuals analysed from the Industrial Revolution period were of lower socioeconomic classes (see **Chapter 5 Supplementary Materials**). Through this supervised consideration, our analysis of historical low SES Industrial populations and the microbial differences between them, contribute to a greater comprehension of cultural and environmental drivers shaping the human microbiome (discussed in further detail in following sub-sections) as defined by their SES. This also cultivates questions to the intergenerational consequences of the ancestral SES upon the contemporary microbial composition of descents, wherein social status becomes enmeshed within the biological components of microbial ecology. As the same processes that contributed to the onset of the Industrial Revolution still persisted through time, alterations to cultural socioeconomics and continued escalation of accessible wealth to the ordinary individual continued to improve and alter human physiological health and disease in conjunction with the human microbiome [42, 43].

This societal improvement led to the development of numerous public health initiatives, altering epidemiological patterns and driving the transition towards the present-day microbiome [42, 44]. Our analysis of two individuals living through the early 19th century up until post-World War II 1950s, suggests a secondary alteration transitioning the early Industrial Revolution microbial composition to the contemporary periodontal disease-associated microbiome, but potentially dissimilar to the modern healthy microbiome (see **Chapter 5**). Rather, the contemporary 'healthy' human microbiome is not analogous to the ancient or historical 'healthy' microbiome. However, this analysis may be distorted by our sample of predominantly low-SES populations. Until we can better account for the influence of SES, both in past and present populations, we may not be able to fully capitalise on the ways in which the microbiome can inform our understanding of the causes and consequences

of disease risks. Ideally, SES needs to be controlled within microbiome research, in the search for etiological factors and disease associations. Where SES cannot be supervised within microbiome analysis, it needs to be integrated and accounted for. However, with the difficulties in being able to tease apart the nuances of socioeconomics, by narrowing down on the factors that are influenced by socioeconomics—as such as diet or exposure to environmental pollutants—we may better exemplify the contribution of SES to human microbiome and health.

Diet impacts the human oral microbiome

Diet, nutrition, and subsistence has been shown to impact the oral microbial ecosystem and the oral environment, with direct associations to oral disease. Some areas of nutritional analysis have well-known impacts on the oral microbial ecology, such as the increased consumption of dietary carbohydrates, wherein the breakdown of sugars encourages plaque development through microbial ecological changes that lead to the onset or worsening of dental decay [45, 46]. Other dietary components are not yet fully understood, such as the associations between periodontal health and nutritional consumption, with a strong association between obesity and periodontal disease [47], and an inverse relationship between high protein intake and periodontitis [48]. Even still, the modification of dietary patterns has documented consequences for oral and systemic health; from major changes in subsistence, such as the shift from foraging to agriculture [49, 50, 51], or cultural alterations, such the impact of colonisation upon Indigenous populations [52, 53]. There is still much to unravel regarding to cultural dietary and the nutritional factors that influence oral microbial ecology.

The evolution of human subsistence patterns correlates with the major alterations in the evolutionary history of the human oral microbiome. The introduction of a predominantly carbohydrate-based diet, approximately ten thousand years ago (*i.e.* the Agricultural Revolution), was shown to have altered the oral microbial composition with an increased number of periodontal-associated and decay-associated microorganisms [49]. Notably, the transition to agriculture appears to have developed an ecosystem inclusive of a newly dominant Fusobacteria phylum [49]. Dental research has observed Fusobacteria as the key component of greater dental plaque construction, acting as a 'coaggregation bridge' in the biofilm formation with non-specific, multi-species binding [54]. Increased plaque formations are associated with increased dental decay, wherein the microbial biofilm maintains a micro-environment with acidic conditions inducing enamel breakdown [46]. The archaeological record observes increased rates of dental decay with the onset of agriculture, but moreover, notes the presence of carious lesions were common even within hunter-gatherer subsistence societies reliant upon high-carbohydrate plants (especially with sticky

texture, *e.g.* dates or figs) [53].

Rates of dental decay climbed alongside the Industrial Revolution. With technological advances in milling and food processing and the establishment of the New World sugar industry, individuals increasingly gained access to refined carbohydrates and sugar [55, 45, 56]. Sugar consumption from the 17$^{\text{th}}$ century went from nearly zero to, on average, 10 pounds (4.54 kg) per person annually, and by the 19$^{\text{th}}$ century this increased to about 20 pounds (9.07 kg) [45]. Prior to the mid-19$^{\text{th}}$ century, sugar was a luxury commodity afforded only by the wealthy high-socioeconomic classes, but with the revolution of processing led to the economical production of sugar, available to all SES classes [57].

One of the key questions in **Chapter 5**, in the inquiry of the apparent dichotomy of oral ecological communities (*i.e. Methanobrevibacter*-dominant and the auxiliary composition), is how the role of sugar might be participating in what appears to be a progressive loss of *Methanobrevibacter* dominance. Research supports the fermentation of dietary sugars (especially sucrose, fructose, and glucose) into acidic by-products, lowering the salivary pH below 5.0 to induce demineralisation (dental decay) [46]. But for optimal growth for *Methanobrevibacter* species, these organisms prefers a more alkaline pH 6.9–7.4 environment [58]. With increasing volume and presumably frequency of sugar intake, the microbial community is frequently disturbed by pH fluctuations. Consequently, the ecological community alters with the proliferation of acid-tolerating microorganisms, which would detrimentally impact ecology dominated by *Methanobravibacter* [46].

Sugar consumption

The historical increased intake of dietary sugars throughout the 19$^{\text{th}}$ and 20$^{\text{th}}$ centuries would likely have driven numerous cultural factors, for which we unfortunately lack both historical and anthropological information to support a sugar-driven hypothesis. For example, we observed the dominance of *Methanobrevibacter* oral ecology within Australian colonialists. We know these individuals were of low-SES, buried within the 'pauper' section of the Anglican Church cemetery between 1846–1927 [59], but we have limited information of the foodstuffs available within their lifetime. At the formation of early Australian colonies in New South Wales, sugar was privately traded with regularity, with a per capita consumption of sixty pounds (27 kg) by 1800s [60]. Sugar was initially linked to status; given as a reward to convicts for good behaviours, and sugar rations as part of wage payments [60]. Sugar's high-powered status appears to be maintained within New South Wales colonies until the late-1880s when the sugar cane economy crashed after the introduction of low-cost beet sugar [60]. Conceivably, access to sugar within the South Australian colony of Adelaide likened to that of New South Wales, and our South Australian

low-SES colonialists may not have had access to a consistent or frequent supply of sugar. Unfortunately, while I was unable to establish historical evidence regarding the South Australian diets of the mid-19th to the early 20th centuries, future analyses may be able to derive dietary information from the functional analysis of oral microbiomes (see below section '**Functional analysis of microbiomes**' for further discussion).

With now ubiquitous access to sugar, Industrial societies today present a distinct reversal of SES and sugar consumption, with lower SES is linked to greater sugar and refined cereal grains consumption [61]. In Australia, low SES was correlated with both the increased consumption of sugar-sweetened beverages and greater rates of dental decay, both of which were higher in rural or remotes regions [62]. This was notably an influence on the rates of dental decay of Aboriginal Australian and Torres Strait Islander children of the NPA region (**Chapter 3**), with a high proportion of children consuming soft drinks, adding sugar to hot drinks and cereals, or consuming syrup, jam, and sweet spreads on a daily basis [63]. While detailed dietary data was collected, there was only a small subset of children who did not consume sugar on a frequent basis, impacting our ability to statistically test the dietary influence within the **Chapter 3** analysis. Moreover, measurements of salivary pH were not taken in the 2017 sampling year, which would be representative of sugar intake and ecological pressures on the microbial community. Despite the omitted pH and dietary assessment within **Chapter 3**, the overall significance of the improved oral health (*i.e.* lower rates of dental decay and severity in children receiving Intervention treatment) suggests that preventative action within communities suffering from severe rates of dental decay and unequal oral health access benefit from immediate action of novel treatments and post hoc microbial investigations.

Exposure to environmental pollutants

Environmental pollutants are chemicals or substances that end up in the environment as a result of human activity; pollutants can be naturally occurring matter or energies, but are considered contaminants at excess levels in which the environment cannot process or neutralize harmful by-products [64]. Human activity has produced environment pollutants since the Bronze Age (∼3,600 BCE), with a notable steady increase of anthropogenic lead pollution parallel to the growing sophistication of metallurgy [65].

However, the onset of Industrial Revolution was a turning point in output of heavy metal pollutants, notable bismuth, copper, zinc, nickel and cadmium [66, 67, 68]. Further technological developments saw the production of persistent organic pollutants and synthetic long-lasting compounds in the late Industrial era [69]. Since the Great Acceleration, the exponential increase in environmental pol-

lution has already shown detrimental structural and functional damage to Earth's ecosystems and climate [70]. Concerns regarding the impact of environmental pollution on human health—through uptake of water, air, food, and medication—are increasing with mounting evidence of pollutant exposure leading to the development of numerous disorders, including obesity, metabolic syndrome, and type II diabetes [71, 72].

Pollutants impacting the human microbiome

Currently, there is no research on the impact of environmental pollutants on the human oral microbiome. However, there have been studies looking at the impact of pollutants causing alterations to the gut microbiome of murine models [73, 74]. Heavy metal exposure has been shown to induce alterations in the composition and functionality of the gut microbiome, prompting physiological disorders, such as disrupted metabolic functionality and nutrient absorption [72]. Exposure to arsenic significantly decreased bacterial phylum Firmicutes, and increased phylum Bacteroidetes, parallel with detected alterations in metabolite production [75]. Correspondingly, Dheer *et al.* (2015) supported phyla-level microbial compositional changes, noting associated alterations of the microbial functional capacity and increased microbial gene expression of nitrogen reductase, which was linked to the increased nitrate and nitrite levels in the ecological environment [76]. Thus, there is probable cause for environmental pollutants impacting the oral microbiome directly (*i.e.* pollutants interact with microorganisms in the oral cavity, *e.g.* breathing air pollution) or indirectly through the initial alterations to the gut microbial community [77].

With the increasing rates of heavy metal pollutants, populations of the Industrial Revolution would have had very little prior exposure to such toxicity, supplying a new ecological pressure upon the human microbiome. From **Chapter 5**, the microbial ecological differences across geographic regions may have been driven by varying national advances and specialisations of industry, producing distinctive pollutant profiles. Another hypothesis regarding the lost ecological dominance of *Methanobrevibacter* could be the increasing heavy metal pollutants present in the environment. While speculative, there could be direct impact of heavy metal ions upon *Methanobrevibacter*, as copper, nickel, and zinc metals had an observed impact upon methanogenesis [78]. But this was seen to be strain dependent, and to my knowledge, no literature has investigated the impacts of heavy metals on oral *Methanobrevibacter* strains. Another hypothetical impact of heavy metals upon the oral ecosystem by indirectly influencing the oral environment. Research has noted the correlation between increased blood lead levels (from the ingestion or inhalation of lead-containing substances, or transferred from mother to foetus) and increased

prevalence of dental decay [79, 80]. Causation has not been elucidated, but it is hypothesised that lead ions compete for calcium binding sites in salivary gland cells, causing hypofunction and diminishing salivary flow rates, precipitating alterations in the microbial community [81].

While the conjectured heavy-metal contact with ecological loss of *Methanobrevibacter*-dominance is only hypothetical, there are known impacts of pollutants upon the environmental microbiome; *e.g.* the impact of mercury on soil microbial community structures [82], or the soil microbial functional adaptation to increased heavy metal exposure [83]. While there is very limited analysis on environmental pollutants impacting the human microbiome, there are hypotheses that suggest a direct correlation between environmental microbial diversity and human microbial diversity [84, 85], Ideally, investigating the *Methanobrevibacter* pollutant hypothesis would require a far greater temporal and spatial geographic sampling, where further archaeological or geohistorical information could illuminate past cultural practices and behaviours of the individuals studied. However, even with the additional sampling of ancient individuals, the reality is that very little is known about the various mechanisms underlying bidirectional interactions between environmental pollutants and human-associated microbiota [86].

In a world with ever-increasing urbanisation and industrialisation, future research to comprehend the microbial alterations consequent of pollutant exposure is critical for human health and the interconnection of microbial ecosystems, functionality, and biochemical interactions. Prospective work should look towards human rural and urban microbiome population differences in microbial functionality (not solely composition [87]), especially in regards to environmental chemical contaminants. Moreover, investigations should look to large-scale analysis of present environmental contaminants in soil, water, or air, using geo-databases, such as World Health Organization Air Pollution database [88] and the combination of publicly available microbiome datasets, such as the Human Microbiome Project [89] or the American Gut Project [90]. While a future of genetically modifying microbial biocatalytic functions—as to reduce toxic or cariogenic pollutants to non-toxic harmless derivatives—is a long way away, even just basic preliminary analyses are needed before we can begin conceptualising theoretical engineering of microorganisms as potential health treatments or solutions for pollutant biodegradation [91, 92].

Working with Ancient Dental Calculus

Metagenomic analyses of paleomicrobiological material, like any new field of research, has a number of elemental issues afflicting the analysis and interpretation of ancient oral microbiomes. Within this thesis, I observed and critically evaluated

three main issues: (1) the dependence and reliance upon constructed databases, (2) the limited understanding of taphonomic biases upon dental calculus microbiomes, and (3) the ascertainment bias of sample collection. Below, I will discuss each issue in greater detail.

Database bias

There is a vast amount of microbial diversity that remains uncharacterised on Earth, which hinders the ability to accurately reconstruct ancient microbial communities when reliant upon genome databases curated from modern microbiology research [93, 94]. This is exemplified by how fast microorganisms can evolve, with rapid generation times and large population size, allowing for rapid genetic adaptions [95]. Therefore, sequencing historical microorganisms and matching them to modern genomes can lead to misidentification and misinterpretation, and with ancient DNA (aDNA) analyses of past microbiomes only stipulating what microorganism remained, not what taxa were lost. Unfortunately, within **Chapter 5**, this bias will have impacted my ability to analyse the cultural and temporal differences of alterations to ancient oral microbiomes and to those ecological changes at the onset of Industrialisation. This can be observed by the number of sequences unassigned, for which an average of 41% sample reads could not be assigned taxonomy (**Chapter 5 Supplementary Materials**, Table S8).

Database bias not only impacts our ability to accurately reconstruct the oral microbial community but affects our ability to reconstruct the microbial species function. Microbial species which are retained through time are likely to procure genetic adaptations to environmental changes, but we are unable to detect them within the contemporary species genomes (*e.g.* *Methanobrevibacter oralis* which has been observed in the oral cavity since Neanderthals [51]). Such that, future paleomicrobiology research efforts need to concentrate on bioinformatic approaches (*e.g.* binning-assembly or *de novo* assembly) for reconstruction of ancient draft genomes [96]. The benefit for these analyses is two-fold: firstly, we gain greater insight into the bacterial evolution and gene content over time. Secondly, this moves towards a further characterisation of the human oral microbiome and advances our comprehension of the microbial role in human oral health and disease.

Taphonomic bias

Taphonomy is the processes of fossilisation and decay that biases preservation of microbial taxa [97]. Within ancient paleomicrobiological research, this bias will alter the accurate reconstruction of the pre-mortem microbial community. Paleomicrobiological research of fossilised faecal material (coprolites) have shown phyla-level

microbial differences are resilience to taphonomic processes [98], however, there have been limited investigations into how taphonomy might impact the ancient dental calculus microbiome. Research supports the viability of culturable bacteria within the exterior of the calculus matrix [99, 100], which would support the survival of taxa within the lacunae and channels of the otherwise calcified mass. Warinner *et al.* (2014) observed little post-mortem alteration to dental calculus using various microscopy and spectroscopy analyses; confirmation of the little to no post-mortem alterations impacting the mineralised matrix [101]. But this does not exclude the potential influence, and subsequent modifications of taphonomy upon non-calcified microorganisms, that will affect the genetic reconstruction of the oral microbiome.

My study was one of the first to analyse the impact of storage condition and time upon the reconstruction of dental microbiome communities (**Chapter 4**). While experimental investigations of taphonomy in living organisms and modern environments may not recapitulate the exact taphonomic alterations to the reconstruction of archaeological dental calculus microbiomes, they can advise ancient dental calculus research to the taxonomic differences driven by evolutionary changes versus those induced by taphonomy. However, a long-term study is needed to explore these taphonomic effects in the context of the soil environment. Body farms (such as the Australian Facility for Taphonomic Experimental Research [102]) could be essential in the archaeological analysis of dental calculus, but would also have applicability in forensic research. With the sampling accessibility of the oral cavity, oral bacteria could act as indicators to time since death [103]. Furthermore, studies looking at these taphonomic processes could be used to model such changes for the development of bioinformatic techniques to detect and account for alterations of the microbiome historically.

Sampling bias

Sampling bias, or ascertainment bias, is a systematic distortion in the measurement of a true phenomenon due to the way a sample is collected [104]. This is a well-documented bias in bioarchaeology, known as the osteological paradox, wherein the interpretations of past epidemiological trends cannot discern the underlying vulnerabilities of a skeletal populations [105]. For instance, the health of adolescences living in the early Adelaide colony of Australia cannot be determined from osteological material, because only deceased adolescences are being studied. Likewise, ancient oral paleomicrobiology suffers from a similar bias, in that sampled dental calculus samples are only representative of the microbial plaque developed over an indeterminate amount of time. This build up will temporally vary between individuals, impacted by intra-individual physiological and biological factors, impacting

the microbial community accessible and the microbiome that is reconstructed.

Furthermore, we are only able to sample calculus from individuals who have formed sufficient calculus build-up, usually excluding children who generally have less calculus formation [106]. Calculus formation and incidence can be shaped by systemic factors; aspects of health and disease can influence salivary pH or salivary flow rates, impacting the calcification of dental plaque [106]. Archaeological dental calculus collection further depends on the oral health of an individual for remaining teeth (which can be influenced both by pre-mortem and post-mortem loss).

Research in **Chapter 5** was impacted by these ascertainment biases; access to ancient oral microbiomes is dependent upon access to bioarchaeological material. Our skeletal material was generally biased toward lower socioeconomic status, potentially influencing our temporal cross-comparisons. As previously mentioned, this could be circumvented by improving modern-day sampling from a range of SES, geographic locations, as well as age groups, so that we may better anticipate the biases and influence of such factors on the oral microbiome. Furthermore, **Chapter 5** noted the impact of different sampling schemes across skeletal populations (*e.g.* collecting all samples from molar teeth) impacted the ability to perfectly test for potential oral geographical influences of tooth type in the oral microbial communities, which have been detected within single population analyses [107] (see **Chapter 5 Supplementary materials**). Ideally, future paleomicrobiological research will be able to build upon the available sample size that work towards improving methodologies to account for, or statistically model, the impact of ascertainment.

Future directions for ancient paleomicrobiological research

There is still much to learn in dealing with the confounding factors that can mislead paleomicrobiological findings and research. The unfortunate publication bias within scientific research as a whole, which pushes researchers to publish mostly positive results without highlighting the negative outcomes [108], suppresses methodological or investigative flaws and impeding sufficient comprehension. Sometimes this had led to been erroneous claims [109, 110, 111], whether by honest mistake or misconduct within research practices, these studies illustrate the difficulties of innovative explorations of unchartered territory. However, we gain to learn from even the most controversial claims. Only by tackling these issues head on, critically evaluating and highlighting the pitfalls of such biases, can we determine the necessary actions needed to rectify them. The evaluation of paleomicrobiology biases and the inconsistencies in our knowledge contributes to the growing foundation of paleomicrobiomics, to ensure the reliability of future research.

Greater contribution to understanding oral health

Although oral commensal microorganisms are culpable within oral disease, they also characterise and cultivate oral health. Oral microbiota are responsible for colonisation resistance, inhibiting the establishment of invading pathogens [112]) or by behaving antagonistically [113]. Moreover, there are implications for oral microbiota contributing towards greater systemic health through nitrate metabolism, essential for cardiovascular health [114, 115].

Thus, the transformation of oral microbial ecology towards oral disease is dependent upon the complex interactions between host susceptibility and environmental factors, and by advancing our knowledge of such interplay endorses the enrichment of global oral health research. Here, I will broaden the discussion of five main factors that I determined and contributed within this thesis that support the improvement of global oral health: (1) improving the foundational understanding of oral microbiota involved in health and disease, (2) inclusion of diverse ethnic populations, (3) advise on whole-community investigations within dental research, (4) recommend future functional analyses, and (5) evaluate the evolutionary history of the human oral microbiome.

Foundational understanding of human oral microbiota

This thesis builds on the foundational knowledge of the human oral microbiome, both past and present populations, that underpin future advances and novel dental treatments to improve oral health. Both **Chapter 3** and **Chapter 5** describe the basics of oral microbial composition of previously unexplored communities and populations, which may necessitate understanding for oral health. Undeterred by the technological and scientific developments in dental health treatments, overall improvements in oral health tend to be the resultant of general progress in living standards and livelihood conditions, rather than clinical interventions of dentistry [116]. Moving away from predominately preventative and band-aid solutions, research into the oral microbiome looks to resolve how we can better treat oral health and disease at the causative-level.

One such innovative method is looking at designing an oral 'microbiota transplant'. Founded on principles of bacteriotherapy, a microbial transplant (*e.g.* faecal transplantation) from a healthy donor with an endogenous microbiome community is prescribed to an afflicted patient (*e.g. Clostridium difficile*-associated disease) in order to re-establish a health-associated microbial ecology [117]. If the oral microbial ecology of an individual suffering from an oral disease is persistently unable to restore a healthy composition or its functional potential, a whole-community trans-

plant may regenerate the oral health state. For less detrimental circumstances, the development of an oral prebiotic or probiotic could be used for the maintenance or restoration of ecological balance or enhance the existing beneficial microbial community [118].

The movement towards a personalised dental treatment would have extensive effects for all of healthcare, not just dentistry. Oral microbiota and health are tightly linked to systemic health through inflammatory mediators, the immune system, or even bacteraemia [118]. Notably, there have been observed changes in the oral microbial community associated with alterations to the gut microbiome [119, 77]. Thus, the oral ecology is critical in understanding the interconnection of the human microbiome as a whole and the physiological relationship to health. Medicine could focus on the management and treatment of the oral microbial community to prescribe good systemic health.

Ascertainment bias in modern microbiome research

Prior discussions of ascertainment bias in regard to aDNA and sample collection is also relevant to modern microbiome research. Ascertainment bias is prevalent in modern microbiome research, especially for Indigenous populations, where study populations are mainly of European descent [120]. Determining the role of microbial diversity within human health and disease will be hindered by inadequate investigation into human diversity [6]. Such explorations are vital in the understanding of rare variants, as microbiome variation is inclusive of individual biological processes, localised environmental factors, and sociocultural lifestyle behaviour [6]. The under-representation of low socioeconomic and minority groups misrepresents our perception of the human microbiome, and such investigations into these populations is paramount for improving the health inequalities that plague them (see **Chapter 2**).

However, researchers need to recognise the ethical issues underlying this missing inclusivity: many of these minority populations, especially those who identify as Indigenous, are historically disadvantaged [120]. There are established procedures in biomedical research for protecting vulnerable groups (see [121]), nevertheless, microbiome research should be built upon an 'ethics of care' framework that emphasises a mutually beneficial relationship [122]. This could be done by designing a microbiome study that additionally addresses specific community health concerns; much like the novel preventative intervention treatment for dental decay among children of the NPA region (**Chapter 3**). Studying the salivary microbiota of these Aboriginal Australian and Torres Strait Islander children was a by-product of oral health research that aimed to directly address the eminent rates of dental decay within

the community [123]. Ensuring microbiome research is working alongside Indigenous communities to incorporate relevance and perceived benefit to the community in question, alongside the culturally appropriate management of research practices, methodology, and results is fundamental to these analyses [120, 121].

Whole community analysis of oral health and disease

Many investigations into the oral microbial community within dental health research still utilise low resolution techniques, such as PCR-DGGE or DNA-microbe arrays, omitting the ability to identify all present taxa within the sample or fully characterise the microbial ecology [124]. These studies often concentrate on the formerly identified taxa associated with oral disease, neglecting unknown or unclassified microorganisms. By definition, these taxa have an unidentified contribution to the ecological community; individual microorganisms within these ecologies could have disproportionate influence upon microbiome functionality or impacts upon physiological health, and precluding them from analysis is counterproductive for oral health research.

Despite some of the drawbacks of 16S ribosomal RNA (rRNA; as covered in **Chapter 3**), 16S rRNA sequencing is a straightforward and economical solution for microbial ecology analysis that can produce species-level resolution. Within **Chapter 3**, we were able to identify several microorganisms associated with severe dental decay (*Lactobacillus salivarus*, *Lactobacillus reuteri*, *Lactobacillus gasseri*, *S. mutans*, *Prevotella multisaccharivorax*, and *Mitsuokella* HMT species 131), for which most had not been linked to dental decay before. Intriguingly, some of these taxa have been previously associated with periodontal disease; as Aboriginal Australians suffer significantly greater rates of periodontal disease relative to non-Indigenous Australians, these microorganisms may be indicative of their prospective susceptibility [125]. But without whole community analysis, these connections and intersections between oral health and microbial ecologies remain hidden and ignored.

Future work should look towards improving whole community resolution. Particularly, shotgun metagenomic sequencing techniques, which can provide superior oral microbiome analyses with accurate strain-level classifications, the possibility to extract whole genes, and functional analysis of the metagenome [126]. As methodologies continue to advance with decreasing costs, whole community analysis should be applied to widely surveyed oral disease mechanisms, especially in populations suffering from health inequalities.

Functional analysis of microbiomes

While changes in microbial composition can affect the functionality of the microbiome, the inverse is also true; the loss of unnecessary functional properties can induce changes in the microbiome composition [127]. Therefore, it is imperative that knowledge of both microbiome composition and function be established. Understanding the functional features of the microbiota has a greater capability to provide ecological comprehension, and thus, illuminating the functional potential of an oral ecosystem may be more meaningfully associated with physiological health status. However, research is limited in the availability of bioinformatic programs able to address microbial functions.

Currently, methods only used function-based gene screening, which can determine what genes are present in the entire sample and how those functionalities or protein pathways differ between samples and environments [128]. Unfortunately, we are still unable to link the sequenced functional information with specific microorganisms, limiting the annotation a singular microbiota's role within the ecosystem as a whole. Alternatively, by employing *de novo* assembly methodologies to create draft genomes from metagenomic sequencing, gene annotation can illuminate the potential functions of specific microorganisms [129]. Nonetheless, this method will not advise on genes that are actually expressed (*i.e.* used for functional output) within the microbial ecosystem. This could be done with transcriptomics in parallel to microbiome reconstruction; analysing the RNA sequences (gene transcripts) can explain the proportional gene expression within a microbial community and the community function at time of sampling [128].

Better still, metaproteomic techniques, measuring the proteins expressed within the environment, can provide more precise functional information, as the presence of gene transcripts does not necessarily indicate protein expression [130]. Equally so, metabolomics—the study of the intermediates or end products of cellular metabolisms (known as metabolites) within an environment—can accurate quantify the biological interactions within the ecosystem of both the host and their microbiota [131]. The integration of these more informative methodologies increase our understanding of the activities and dynamics of microbial communities, that provide insights into microbial functionality.

Incorporating evolutionary history

The progression of industrialisation globally, and the subsequent shift in industrial disease patterns, is occurring at a faster rate in developing countries—for which the developmental status usually referring to its industrial status—than it did within Europe more than half a century ago [132]. In **Chapter 5**, I explored how Indus-

trialisation impacted oral microbiota for the first time. This information provides new insights into how detrimental these industrial changes were to the human microbiome, coordinating with the increasing burden of industrial disease; a major public health threat that demands immediate and effective action. Understanding the processes that altered the human microbiome in the past are critical for understanding the alterations occurring in places anew, establishing such environmental and cultural influences upon the human microbiome promotes the development of preventive measures and controls to counter the epidemic of chronic disease [133]. While I emphasise this at a broad public health scale; the same patterns are characteristic of global oral disease (especially untreated dental decay and periodontitis) [134].

Evolutionary history also plays a significant role in shaping the human microbiome composition and function; as stated earlier, Indigenous health is directly tied to their unique evolutionary histories. Current assessments of geographic and ethnic differences in the microbiome—based on diet, subsistence, and dental disease—suggest that while generalisations may not be applied universally, knowing what patterns to look for guides population-specific analyses and allows for more precise inferences regarding the roles of evolutionary forces on the expression and composition of both the oral and human microbiome.

Conclusion

While each chapter maintains its own significance and contribution to the field of oral microbiomes, together this work identifies and describes the impact of sociocultural and environmental changes, consequent of industrialisation, on the human oral microbial composition. From this work three themes have emerged:

Evolutionary medicine informs our understanding of the human microbiome: I look at microbial changes in both contemporary and historic populations to investigate the nuances of oral health and disease, and the greater changes to the oral microbial ecology through time. Moreover, improving our methodological ability to make such population comparisons.

Integrating cross-disciplinary understanding for public health research: By contributing to the fields of both contemporary and ancient microbiome research, I attempt to address and fill the gaps of our knowledge that will bring about greater comprehension in the future of human microbiome research to inform population and public health.

Importance of oral microbiome research: The data presented in this thesis provides a greater understanding of the human oral microbial community, both through evolutionary time and within understudied contemporary populations. The

contribution of my thesis to facilitates insights into our understanding of oral microbial ecology and its relationship to human oral and systemic health.

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Supplementary Materials I
Chapter 4

Sample Info		Knead Data Output							Sequence Assignment		
Sample ID	Sample Group	Unaligned Sequences	Unaligned Percentage (%)	Sequences aligned = 1 (n)	Sequences aligned = 1 (%)	Sequences aligned > 1 (n)	Sequences aligned >1 (%)	Overall alignment rate	Raw Sequencing Reads	Taxonomy Assigned Reads	Percentage Assigned
18752	Calculus_Fresh	3644282	56.47	2152806	33.36	655894	10.16	43.53%	1500000	808939	54%
18754	Calculus_Fresh	16473077	81.94	2843880	14.15	785708	3.91	18.06%	1500000	926210	62%
18756	Calculus_Fresh	1162304	38.04	1384861	45.33	508198	16.63	61.96%	1162304	679319	58%
18758	Calculus_Fresh	2567777	65.08	1030205	26.11	347363	8.8	34.92%	1500000	719067	48%
18760	Calculus_Fresh	12829921	88.18	1301635	8.95	418851	2.88	11.82%	1500000	867804	58%
18764	Calculus_Fresh	1611610	25.85	3676170	58.97	946334	15.18	74.15%	1500000	983014	66%
18766	Calculus_Fresh	1103739	89.68	95590	7.77	31448	2.56	10.32%	1103739	709395	64%
18767	Calculus_Fresh	4466633	47.95	3819006	41	1028685	11.04	52.05%	1500000	871577	58%
18769	Calculus_Fresh	2997397	71.82	866727	20.77	309116	7.41	28.18%	1500000	915270	61%
18771	Calculus_Fresh	4616490	94.29	216797	4.43	62902	1.28	5.71%	1500000	915444	61%
18773	Calculus_Fresh	3757006	62.89	1686816	38.24	530140	8.87	37.11%	1500000	838267	56%
18775	Calculus_Fresh	719877	99.03	5375	0.74	1693	0.23	0.97%	719877	594914	83%
18777	Calculus_Fresh	5922132	93.34	311946	4.92	110273	1.74	6.66%	1500000	935450	62%
18781	Calculus_Fresh	1497628	78.8	296878	15.62	106118	5.58	21.20%	1497628	961680	64%
18784	Calculus_Fresh	6257755	96.09	192221	2.95	62094	0.95	3.91%	1500000	955946	64%
18786	Calculus_Fresh	11077891	90.47	920931	7.52	245893	2.01	9.53%	1500000	955089	64%
18791	Calculus_Fresh	7865569	94.53	356146	4.28	98756	1.19	5.47%	1500000	939165	63%
18793	Calculus_Fresh	475986	81.82	78983	13.58	26791	4.61	18.18%	475986	389063	82%
18794	ExtractionBlankControl	13217	98.27	179	1.33	53	0.39	1.73%	13217	11721	89%
18795	ExtractionBlankControl	31023	99.02	236	0.75	70	0.22	0.98%	31023	19404	63%
18796	ExtractionBlankControl	37849	100	1	0	0	0	0.00%	37849	34487	91%
18798	ExtractionBlankControl	21687	59.52	12110	33.24	2638	7.24	40.48%	21687	18109	84%
18800	ExtractionBlankControl	939	100	0	0	0	0	0.00%	939	890	95%
19562	Calculus_RoomTemp	344732	49.43	281708	40.4	70936	10.17	50.57%	344732	218187	63%
19563	Calculus_RoomTemp	291542	65.65	123748	27.86	28820	6.49	34.35%	291542	188712	65%
19564	Calculus_RoomTemp	180573	33.82	286646	53.69	66688	12.49	66.18%	180573	94577	52%
19566	Calculus_RoomTemp	2085	96.04	69	3.18	17	0.78	3.96%	2085	1319	63%
19567	Calculus_RoomTemp	1198758	66.17	504855	27.87	107964	5.96	33.83%	1198758	529378	44%
19568	Calculus_RoomTemp	849496	66.26	357338	27.87	75318	5.87	33.74%	849496	515677	61%
19569	Calculus_RoomTemp	207206	86.35	27008	11.25	5755	2.4	13.65%	207206	108681	52%
19570	Calculus_RoomTemp	303381	61.2	155263	31.32	37054	7.48	38.80%	303381	110594	36%
19571	Calculus_RoomTemp	357869	93.11	21997	5.72	4468	1.16	6.89%	357869	195356	55%
19572	Calculus_Freezer	661823	88.92	67213	9.03	15273	2.05	11.08%	661823	400816	61%
19573	Calculus_Freezer	307595	79.99	60311	15.68	16651	4.33	20.01%	307595	184976	60%
19574	Calculus_Freezer	487815	96.68	13623	2.7	3154	0.63	3.32%	487815	286547	59%
19575	Calculus_Freezer	406545	92.19	27901	6.33	6533	1.48	7.81%	406545	205384	51%
19576	Calculus_Freezer	354716	84.09	53176	12.61	13943	3.31	15.91%	354716	257543	73%
19578	Calculus_Freezer	475130	98.53	5588	1.16	1486	0.31	1.47%	475130	343482	72%
19579	Calculus_Freezer	441933	91.05	35436	7.3	7982	1.64	8.95%	441933	272648	62%
19580	Calculus_Freezer	298763	80.44	60031	16.16	12639	3.4	19.56%	298763	121291	41%
19581	Calculus_Freezer	428493	94.48	20939	4.62	4115	0.91	5.52%	428493	299416	70%
19808	ExtractionBlankControl	312	75.18	83	20	20	4.82	24.82%	312	147	47%
19810	ExtractionBlankControl	823	90.34	71	7.79	17	1.87	9.66%	823	76	9%

Supplementary Table 1. Kneaddata output and Sequence Assignment. Shotgun sequences underwent host read removal, presenting the percentage of sequences aligning to the human genome (GRCh37/hg19) reference database. Sequences were then aligned against an in-house database created using 47,696 archaeal and bacterial genome assemblies from the NCBI Assembly database (Eisenhofer and Weyrich, 2018).

Identified species of the shotgun sequenced Control Samples

Assigned Species	18794	18795	18796	18798	18800	19808	19810
Sulfurihydrogenibium azorense	0	0	0	0	0	2	0
Chryseobacterium bovis	0	0	0	0	0	1	0
Cloacibacterium normanense	0	0	0	0	0	2	0
Flavobacterium sp. Leaf359	0	0	0	0	0	1	0
Bosea sp. Root483D1	0	0	0	0	0	1	0
Bosea sp. UNC402CLCol	0	0	0	0	0	1	0
Janthinobacterium sp. KBS0711	0	0	0	0	0	1	0
Escherichia coli	0	0	0	0	0	1	0
Xenorhabdus bovienii	0	0	443	0	0	0	0
Acinetobacter beijerinckii	0	0	0	0	0	2	0
Acinetobacter ursingii	0	0	0	0	0	1	0
Moraxella osloensis	0	0	0	0	0	12	3
Pseudomonas aeruginosa	0	0	0	0	0	1	0
Pseudomonas putida	0	0	0	0	0	1	0
Pseudomonas sp. GM24	0	0	33708	0	0	0	0
Pseudomonas sp. HMSC08G10	0	0	0	0	0	0	2
Pseudomonas stutzeri	0	0	0	0	0	0	2
Stenotrophomonas acidaminiphila	0	0	0	0	0	1	0
Xanthomonas campestris	0	0	0	0	0	3	0
Tepidiphilus margaritifer	0	0	0	0	0	7	0
Tepidiphilus thermophilus	0	0	0	0	0	53	0
Corynebacterium imitans	0	151	0	0	0	0	0
Corynebacterium kroppenstedtii	0	0	0	0	0	1	0
Corynebacterium urealyticum	0	141	0	0	0	0	0
Rothia aeria	0	0	0	0	0	1	0
Bacillus thermoamylovorans	0	0	0	0	0	1	0
Gemella haemolysans	0	0	0	0	0	2	0
Enterococcus faecalis	11678	0	0	0	883	0	0
Enterococcus faecium	0	17244	119	17839	0	0	0
Streptococcus sanguinis	0	0	0	0	0	2	0
Streptococcus vestibularis	0	0	0	0	0	0	2
Blautia obeum	0	0	0	0	0	1	0
Thermoanaerobacterium thermosaccharolyticum	0	0	0	0	0	1	0

Supplementary Table 2. Blank control samples species composition. List of assigned species identified within shotgun sequenced extraction blank controls. Most samples appear to be dominated by only one or two species.

Sample Info		QIIME2 Proccessing			
Sample ID	Sample Group	Demultiplexed Sequencing Reads	Delur Denoised Reads	Assigned Reads	Percentage Assigned (Demultiplexed/Assigned)
18752	Calculus_Fresh	265679	161639	160892	61%
18754	Calculus_Fresh	123775	74099	73373	59%
18756	Calculus_Fresh	418069	237248	235245	56%
18758	Calculus_Fresh	109572	53447	52474	48%
18760	Calculus_Fresh	139260	73004	71822	52%
18764	Calculus_Fresh	136190	82939	80863	59%
18766	Calculus_Fresh	126497	76576	76062	60%
18767	Calculus_Fresh	109076	68905	67574	62%
18769	Calculus_Fresh	163333	89258	87237	53%
18771	Calculus_Fresh	122548	58170	57377	47%
18773	Calculus_Fresh	129734	79199	76910	59%
18775	Calculus_Fresh	132814	85047	84375	64%
18777	Calculus_Fresh	122748	79195	76474	62%
18781	Calculus_Fresh	141157	86698	86293	61%
18784	Calculus_Fresh	182021	91708	91044	50%
18786	Calculus_Fresh	156394	88735	88193	56%
18791	Calculus_Fresh	126514	64063	63727	50%
18793	Calculus_Fresh	145058	87793	87339	60%
18794	Extraction Blank Control	1711	629	625	37%
18795	Extraction Blank Control	3291	556	547	17%
18796	Extraction Blank Control	1438	150	136	9%
18798	Extraction Blank Control	20055	16707	16707	83%
18800	Extraction Blank Control	3008	220	217	7%
19562	Calculus_RoomTemp	73429	41814	41472	56%
19563	Calculus_RoomTemp	27528	16838	16674	61%
19564	Calculus_RoomTemp	38250	21033	20538	54%
19567	Calculus_RoomTemp	62987	37834	37523	60%
19568	Calculus_RoomTemp	57960	33250	32901	57%
19569	Calculus_RoomTemp	8	0	0	0%
19570	Calculus_RoomTemp	67797	42054	41791	62%
19571	Calculus_RoomTemp	68611	33895	33568	49%
19572	Calculus_Freezer	107451	58812	58504	54%
19573	Calculus_Freezer	83258	48240	47767	57%
19574	Calculus_Freezer	82804	43703	43054	52%
19575	Calculus_Freezer	71151	41385	40956	58%
19576	Calculus_Freezer	67915	42313	41667	61%
19578	Calculus_Freezer	58276	27875	27664	47%
19579	Calculus_Freezer	92357	49231	48700	53%
19580	Calculus_Freezer	59808	35966	35705	60%
19581	Calculus_Freezer	74167	43403	43083	58%
19808	Extraction Blank Control	12	8	4	33%
19810	Extraction Blank Control	20924	14954	14944	71%

Supplementary Table 3. Percentage of assigned 16S rRNA sequences. Sequencing reads from 16S rRNA amplification underwent demultiplexing by unique barcode adaptors using the EMP-paired end protocol and denoised using the Deblur algorithm QIIME2 plugin. Sequences were truncated to 150 bp (based on the median quality score) before being assigned taxonomy using the Human Oral Microbiome Database (HOMD; v. 15.1) (Chen et al., 2010).

Identified species of the 16S rRNA sequenced Control Samples

OUT Assignment	18794	18795	18796	18798	18800	19808	19810
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium;s__	0	0	0	620	0	0	0
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Corynebacteriaceae;g__Corynebacterium;s__durum	0	2	0	0	0	0	0
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Dietziaceae;g__Dietzia;s__	0	2	0	0	0	0	0
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Micrococcaceae;g__Rothia;s__aeria	0	2	2	0	0	0	0
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Propionibacteriaceae;g__Propionibacterium;s__acnes	0	26	0	0	0	0	0
k__Bacteria;p__Actinobacteria;c__Coriobacteriia;o__Coriobacteriales;f__Coriobacteriaceae;g__;s__	0	2	0	0	0	0	0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Porphyromonas;s__	0	0	2	0	0	0	0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Porphyromonadaceae;g__Porphyromonas;s__endodontalis	0	0	3	0	0	0	0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella;s__	0	0	17	0	0	0	0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella;s__intermedia	0	0	2	0	0	0	0
k__Bacteria;p__Bacteroidetes;c__Bacteroidia;o__Bacteroidales;f__Prevotellaceae;g__Prevotella;s__melaninogenica	0	0	6	0	0	0	0
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Hymenobacter;s__	0	0	31	0	0	0	0
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Capnocytophaga;s__	0	0	0	0	0	2	0
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Capnocytophaga;s__ochracea	0	0	3	0	0	0	0
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];g__;s__	0	25	0	0	0	0	0
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__[Weeksellaceae];g__Cloacibacterium;s__	0	0	0	0	0	0	49
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Paenibacillaceae;g__Paenibacillus;s__	0	0	0	14586	0	0	0
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Staphylococcaceae;g__Staphylococcus;__	0	246	0	0	0	0	7
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;__	0	2	0	0	0	0	0
k__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales;f__Streptococcaceae;g__Streptococcus;s__	0	0	4	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Peptostreptococcaceae;g__;s__	0	0	2	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Ruminococcaceae;g__Clostridium;s__hungatei	0	0	37	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Schwartzia;s__	0	0	2	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Selenomonas;s__	0	0	5	0	0	0	0
k__Bacteria;p__Firmicutes;c__Clostridia;o__Clostridiales;f__Veillonellaceae;g__Veillonella;s__dispar	0	0	0	115	0	0	0
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Fusobacteriaceae;g__Fusobacterium;s__	0	2	5	3	0	0	0
k__Bacteria;p__Fusobacteria;c__Fusobacteriia;o__Fusobacteriales;f__Leptotrichiaceae;g__Leptotrichia;s__	0	0	3	0	0	0	0
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__;g__;s__	0	0	0	0	27	0	0
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Bradyrhizobiaceae;__;__	9	0	0	0	188	0	0
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Brucellaceae;g__Ochrobactrum;s__	0	0	0	0	0	0	70
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;g__;s__	0	0	0	0	0	0	52
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae;__;__	0	46	0	162	0	0	10334
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__Novosphingobium;s__	0	0	0	0	0	0	249
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;__;__	65	133	0	180	0	0	0
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Comamonas;s__	0	0	0	0	0	0	159
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Comamonadaceae;g__Tepidimonas;s__	0	0	0	0	0	0	147
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Cupriavidus;s__	43	0	0	0	0	0	0
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Oxalobacteraceae;g__Ralstonia;s__	243	10	0	577	0	0	0
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;__;__	0	2	0	0	0	0	0
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__Eikenella;s__	0	0	0	0	0	2	0
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Neisseriales;f__Neisseriaceae;g__Neisseria;s__	0	3	0	0	0	0	0
k__Bacteria;p__Proteobacteria;c__Epsilonproteobacteria;o__Campylobacterales;f__Campylobacteraceae;g__Campylobacter;s__	0	4	0	0	0	0	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__Aggregatibacter;s__	0	3	0	0	2	0	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__Aggregatibacter;s__segnis	0	0	4	0	0	0	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pasteurellales;f__Pasteurellaceae;g__Haemophilus;s__parainfluenzae	0	3	2	3	0	0	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;__;__	0	0	0	0	0	0	39
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__;s__	265	0	0	0	0	0	3802
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas;s__	0	0	0	0	0	0	36
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadales;f__Pseudomonadaceae;g__Pseudomonas;s__stutzeri	0	34	0	0	0	0	0
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema;s__	0	0	0	461	0	0	0
k__Bacteria;p__Spirochaetes;c__Spirochaetes;o__Spirochaetales;f__Spirochaetaceae;g__Treponema;s__socranskii	0	0	2	0	0	0	0
k__Bacteria;p__Synergistetes;c__Synergistia;o__Synergistales;f__Dethiosulfovibrionaceae;g__TG5;s__	0	0	2	0	0	0	0
k__Bacteria;p__Tenericutes;c__Mollicutes;o__RF39;f__;g__;s__	0	0	2	0	0	0	0

Supplementary Table 4. Blank control samples species composition. List of assigned species identified within 16s rRNA amplified extraction blank controls. Taxonomy was assigned using Greengenes database (v13.8) (McDonald et al., 2012). A number of assigned species have been previously identified as part of the typical human oral microbial community (Dewhirst et al., 2010).

Alpha and beta diversity tests for significance of metadata variables

Shotgun Data	t-statistic	FDR corrected p-value
Species-Level		
Storage Vessel		
Shannon	0.7676	0.68
Observed species	0.4511	0.80
Bray-Curtis	1.2895	0.18
Gender		
Shannon	0.5215	0.47
Observed species	0.8101	0.37
Bray-Curtis	1.1303	0.31
Genus-Level		
Storage Vessel		
Shannon	0.7219	0.40
Observed species	0.3159	0.85
Bray-Curtis	1.5966	0.15
Gender		
Shannon	0.3661	0.55
Observed species	1.8807	0.17
Bray-Curtis	0.6971	0.62
Phylum-Level		
Storage Vessel		
Shannon	3.4147	0.18
Observed species	4.7551	0.09
Bray-Curtis	1.7433	0.18
Gender		
Shannon	0.7714	0.38
Observed species	0.6122	0.43
Bray-Curtis	0.7827	0.44

A. Shotgun data

16S rRNA Data	t-statistic	FDR corrected p-value
Species-Level		
Storage Vessel		
Shannon	1.0625	0.59
Observed species	3.2082	0.20
Bray-Curtis	1.0401	0.37
Gender		
Shannon	1.8889	0.17
Observed species	2.9524	0.09
Bray-Curtis	0.9601	0.48
Genus-Level		
Storage Vessel		
Shannon	0.4625	0.79
Observed species	5.0437	0.08
Bray-Curtis	1.0723	0.35
Gender		
Shannon	1.3742	0.24
Observed species	4.6014	**0.03**
Bray-Curtis	0.9142	0.50
Phylum-Level		
Storage Vessel		
Shannon	2.5556	0.28
Observed species	0.0483	0.98
Bray-Curtis	0.9251	0.49
Gender		
Shannon	2.1176	0.15
Observed species	0.4938	0.48
Bray-Curtis	1.8446	0.15

B. 16S rRNA data

Supplementary Table 5. Alpha and beta diversity tests for significance of categorical variables 'Storage Vessel' and 'Gender' using PERMANOVA. At each taxonomic level PERMANOVA significance was calculated for alpha (Shannon and Observed species) and beta (Bray-Curtis) diversity metrics, to identify the potential influence of metadata variables on the microbial communities of dental calculus. Bold values indicate a significant result ($p<0.05$); p-values are FDR corrected.

Supplementary Materials II
Chapter 5

I Archaeological Context and Site Information

A total number of 130 dental calculus samples were analysed within this study, including: 56 ancient European samples, 37 historical Industrial Revolution (IR) samples, two post-war samples, 15 healthy modern samples, and 20 samples from modern periodontal patients. Of this, 60 dental calculus samples (56 ancient and 4 IR samples) were downloaded from published sources (Table S1). The Australian Centre for Ancient DNA (ACAD) and museum collection sample numbers, sample information, estimated date and age, and classifications based on geography and culture are provided in Table S1. The archaeological and anthropological context of individuals from historic and 1950s populations are described within the following sections.

Historical Australian samples

Dental calculus samples were obtained from individuals of the early Adelaide colony, buried at The Anglican Church of St Mary's (herein referred to as St Mary's), Adelaide, South Australia. St Mary's Church was established in 1846; its unmarked burial 'free grounds' were used from 1846 to 1927. Excavation began in 1999, undertaken by the Archaeology Department at the Flinders University under the direction of Dr William H. Adams. The 70 burials were osteologically analysed in a PhD thesis [1]. In 2004, the 70 individuals were moved from storage at the University of Adelaide to a specially designed subterranean storage facility [2]. In 2017, the crypt was reopened, and 36 individual skulls were moved to the University of Adelaide for further analysis, including the sampling of dental calculus. Only 15 skulls had dental calculus present (likely due to the age of the individuals; 18 of the 36 individuals were estimated to be younger than 13 years); in some cases, multiple dental calculus samples (from different teeth) from the same individual were collected.

Historical documentation and skeletal evidence indicate the majority of individuals buried in the free grounds were farmers or labourers (*i.e.* working class individuals) with good/adequate nutrition, but poor social conditions, especially in regards to hygiene [1]. There was a high incidence of child mortality in the free grounds at St Mary's prior to 1875; likely a result of infectious diseases for which people of the time had little understanding or no effective treatment for. Disease was the clear cause of death for most adult individuals in the free grounds, as historical records indicate 41.8–73.1% of individuals died from infectious causes, which is corroborated by minimal evidence of skeletal peri-mortem trauma.

Historical records and skeletal analyses of geographic origin denote the majority of the St Mary's adults to have immigrated to Australia from England. Dietary

habits remained distinctly English, and consumption of meat became especially important for Australian Europeans. Stable isotope analysis (backed by historical accounts) indicated the average adult diet of St Mary's individual would have consisted of approximately 32% seafood, 60% terrestrial meat (*e.g.* cattle, sheep) and 8% terrestrial vegetation (*e.g.* wheat, barley). All individuals had dental decay, and there was no evidence of fillings or other restorative procedures observed within this population. Periodontal disease played a less significant role in oral disease within this population, with ante-mortem tooth loss linked to the high incidence of carious lesions [1].

Historical German samples

Dental calculus samples from IR German samples were attained from Hettstedt, of the Mansfeld distinct in Saxony-Anhalt, Germany. Details of archaeological context and sample descriptions have been previously described in Weyrich *et al.* (2017) [3]. In brief, Hettstedt burials were osteologically analysed in an unpublished Master thesis by Klapdohr *et al.* in 2013 (see Weyrich *et al.* [3] for details). This population was characterised socioeconomically by mining and metallurgical work, supported by dominantly carbohydrate-based diet. Tooth loss and dental decay was prevalent, with approximately 20% of adults exhibiting a severe degree of plaque formation [3].

Historical Swiss samples

Swiss dental calculus samples were obtained from the former Basel Hospital Cemetery (St. Johanns Park), Bürgerspital hospital of Basel, Switzerland. Bürgerspital hospital was the first modern hospitals for the lower classes of Basel, in use from 1845 to 1868. Of the 1061 individuals excavated, 220 individuals were curated by the Natural History Museum, Basel, as part of the Spitalfriedhof St Johann Known Age Collection, representing those individuals who could be identified by both grave and hospital registers. Switzerland (notable the city of Basel) was a predominantly textile industry, with the introduction of chemical factories in mid 19^{th} century [4]. The production of synthetic dyes along the River Rhine was known for dumping chemical waste straight into the aquatic systems [5].

1950s French samples

French dental calculus samples were collected from individuals who donated their bodies to science, now part of the Georges Olivier collection at the Musee du l'Homme of Paris, France. Calculus was collected from 13 different individuals, all middle-age adults, living in Paris at the time of their death.

Table S1. **Sample Information**

Study ID Number	Sample Type	Museum ID	Museum	Geographic Location	Specific Location	Archaeological Site	Estimated Date (CE)	Period	Anthropological Reference	Calculus Sample Reference
13214	Dental Calculus	795	Natural History of Basel Museum, Switzerland	Switzerland	Basel	Basel Hospital Cemetery	1845-1868	PostIndustrial	Unpublished; Natural History of Basel Museum, Switzerland	This study
13215	Dental Calculus	776	Natural History of Basel Museum, Switzerland	Switzerland	Basel	Basel Hospital Cemetery	1845-1868	PostIndustrial	Unpublished; Natural History of Basel Museum, Switzerland	This study
13216	Dental Calculus	837	Natural History of Basel Museum, Switzerland	Switzerland	Basel	Basel Hospital Cemetery	1845-1868	PostIndustrial	Unpublished; Natural History of Basel Museum, Switzerland	This study
13217	Dental Calculus	734	Natural History of Basel Museum, Switzerland	Switzerland	Basel	Basel Hospital Cemetery	1845-1868	PostIndustrial	Unpublished; Natural History of Basel Museum, Switzerland	This study
13218	Dental Calculus	764	Natural History of Basel Museum, Switzerland	Switzerland	Basel	Basel Hospital Cemetery	1845-1868	PostIndustrial	Unpublished; Natural History of Basel Museum, Switzerland	This study
13219	Dental Calculus	801	Natural History of Basel Museum, Switzerland	Switzerland	Basel	Basel Hospital Cemetery	1845-1868	PostIndustrial	Unpublished; Natural History of Basel Museum, Switzerland	This study
13220	Dental Calculus	733	Natural History of Basel Museum, Switzerland	Switzerland	Basel	Basel Hospital Cemetery	1845-1868	PostIndustrial	Unpublished; Natural History of Basel Museum, Switzerland	This study
13221	Dental Calculus	842	Natural History of Basel Museum, Switzerland	Switzerland	Basel	Basel Hospital Cemetery	1845-1868	PostIndustrial	Unpublished; Natural History of Basel Museum, Switzerland	This study
13222	Dental Calculus	781	Natural History of Basel Museum, Switzerland	Switzerland	Basel	Basel Hospital Cemetery	1845-1868	PostIndustrial	Unpublished; Natural History of Basel Museum, Switzerland	This study
13223	Dental Calculus	777	Natural History of Basel Museum, Switzerland	Switzerland	Basel	Basel Hospital Cemetery	1845-1868	PostIndustrial	Unpublished; Natural History of Basel Museum, Switzerland	This study
13224	Dental Calculus	834	Natural History of Basel Museum, Switzerland	Switzerland	Basel	Basel Hospital Cemetery	1845-1868	PostIndustrial	Unpublished; Natural History of Basel Museum, Switzerland	This study
13225	Dental Calculus	822	Natural History of Basel Museum, Switzerland	Switzerland	Basel	Basel Hospital Cemetery	1845-1868	PostIndustrial	Unpublished; Natural History of Basel Museum, Switzerland	This study
13226	Dental Calculus	19	Johannes Gutenberg University, Mainz, Germany	Germany	Hettstedt	Hettstedt Gymnasium	1860-1865	PostIndustrial	Unpublished; Klapdohr 2013 (Thesis)	This study
13227	Dental Calculus	23	Johannes Gutenberg University, Mainz, Germany	Germany	Hettstedt	Hettstedt Gymnasium	1860-1865	PostIndustrial	Unpublished; Klapdohr 2013 (Thesis)	This study
13228	Dental Calculus	34	Johannes Gutenberg University, Mainz, Germany	Germany	Hettstedt	Hettstedt Gymnasium	1860-1865	PostIndustrial	Unpublished; Klapdohr 2013 (Thesis)	This study
13229	Dental Calculus	40	Johannes Gutenberg University, Mainz, Germany	Germany	Hettstedt	Hettstedt Gymnasium	1860-1865	PostIndustrial	Unpublished; Klapdohr 2013 (Thesis)	This study
13230	Dental Calculus	43	Johannes Gutenberg University, Mainz, Germany	Germany	Hettstedt	Hettstedt Gymnasium	1860-1865	PostIndustrial	Unpublished; Klapdohr 2013 (Thesis)	This study
13231	Dental Calculus	50	Johannes Gutenberg University, Mainz, Germany	Germany	Hettstedt	Hettstedt Gymnasium	1860-1865	PostIndustrial	Unpublished; Klapdohr 2013 (Thesis)	This study
13232	Dental Calculus	85	Johannes Gutenberg University, Mainz, Germany	Germany	Hettstedt	Hettstedt Gymnasium	1860-1865	PostIndustrial	Unpublished; Klapdohr 2013 (Thesis)	This study
13233	Dental Calculus	88	Johannes Gutenberg University, Mainz, Germany	Germany	Hettstedt	Hettstedt Gymnasium	1860-1865	PostIndustrial	Unpublished; Klapdohr 2013 (Thesis)	This study
13234	Dental Calculus	93	Johannes Gutenberg University, Mainz, Germany	Germany	Hettstedt	Hettstedt Gymnasium	1860-1865	PostIndustrial	Unpublished; Klapdohr 2013 (Thesis)	This study
20454	Dental Calculus	B83	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20455	Dental Calculus	B83	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20456	Dental Calculus	B83	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20457	Dental Calculus	B83	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20459	Dental Calculus	B59	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20460	Dental Calculus	B53c	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20462	Dental Calculus	B66b	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20463	Dental Calculus	B66b	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20465	Dental Calculus	B9	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20466	Dental Calculus	B9	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20467	Dental Calculus	B23	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20468	Dental Calculus	B5	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20469	Dental Calculus	B61	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20472	Dental Calculus	B72	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20473	Dental Calculus	B6	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20474	Dental Calculus	B6	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20475	Dental Calculus	B6	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20476	Dental Calculus	B6	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20477	Dental Calculus	B6	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20480	Dental Calculus	B68	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20482	Dental Calculus	B78	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20483	Dental Calculus	B73	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20485	Dental Calculus	B73	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study

Table S1. **Sample Information**

Study ID Number	Sample Type	Museum ID	Museum	Geographic Location	Specific Location	Archaeological Site	Estimated Date (CE)	Period	Anthropological Reference	Calculus Sample Reference
20486	Dental Calculus	B57	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20487	Dental Calculus	B57	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20490	Dental Calculus	B57	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20492	Dental Calculus	B79	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
20493	Dental Calculus	B79	Flinders University, Adelaide, Australia	Australia	Adelaide	St Marys Anglican Church	1846-1927	PostIndustrial	Anson 2004 (Thesis)	This study
13227B	Dental Calculus	23	Johannes Gutenberg University, Mainz, Germany	Germany	Hettstedt	Hettstedt Gymnasium	1860-1865	PostIndustrial	Klapdohr 2013 (Thesis)	Weyrich et al. 2017
13229B	Dental Calculus	40	Johannes Gutenberg University, Mainz, Germany	Germany	Hettstedt	Hettstedt Gymnasium	1860-1865	PostIndustrial	Klapdohr 2013 (Thesis)	This study
13230B	Dental Calculus	43	Johannes Gutenberg University, Mainz, Germany	Germany	Hettstedt	Hettstedt Gymnasium	1860-1865	PostIndustrial	Klapdohr 2013 (Thesis)	Weyrich et al. 2017
13232B	Dental Calculus	85	Johannes Gutenberg University, Mainz, Germany	Germany	Hettstedt	Hettstedt Gymnasium	1860-1865	PostIndustrial	Klapdohr 2013 (Thesis)	Weyrich et al. 2017
B61	Dental Calculus	B61	University of Zürich's Institute of Anatomy,	Germany	Dalheim	St. Petri church	950–1200	Medieval	Read. 2010. *in* Medieval Lough Cé: history, archaeology, and landscape	Mann et al. 2018
G12	Dental Calculus	G12	University of Zürich's Institute of Anatomy,	Germany	Dalheim	St. Petri church	950–1200	Medieval	Read. 2010. *in* Medieval Lough Cé: history, archaeology, and landscape	Mann et al. 2018
KT05	Dental Calculus	Burial 102	Institute of Technology Sligo, Republic of Ireland	Ireland	Kilteasheen	Bishop's Seat	661-1275	Medieval	Read. 2010. *in* Medieval Lough Cé: history, archaeology, and landscape	Mann et al. 2018
KT08	Dental Calculus	Burial 27	Institute of Technology Sligo, Republic of Ireland	Ireland	Kilteasheen	Bishop's Seat	661-1275	Medieval	Read. 2010. *in* Medieval Lough Cé: history, archaeology, and landscape	Mann et al. 2018
KT09	Dental Calculus	Burial 122	Institute of Technology Sligo, Republic of Ireland	Ireland	Kilteasheen	Bishop's Seat	661-1275	Medieval	Read. 2010. *in* Medieval Lough Cé: history, archaeology, and landscape	Mann et al. 2018
KT13	Dental Calculus	Burial 27	Institute of Technology Sligo, Republic of Ireland	Ireland	Kilteasheen	Bishop's Seat	1162-1275	Medieval	Read. 2010. *in* Medieval Lough Cé: history, archaeology, and landscape	Mann et al. 2018
KT14	Dental Calculus	Burial 63	Institute of Technology Sligo, Republic of Ireland	Ireland	Kilteasheen	Bishop's Seat	661-1275	Medieval	Read. 2010. *in* Medieval Lough Cé: history, archaeology, and landscape	Mann et al. 2018
KT24	Dental Calculus	Burial 86-91	Institute of Technology Sligo, Republic of Ireland	Ireland	Kilteasheen	Bishop's Seat	661-1275	Medieval	Read. 2010. *in* Medieval Lough Cé: history, archaeology, and landscape	Mann et al. 2018
KT25	Dental Calculus	Burial 32	Institute of Technology Sligo, Republic of Ireland	Ireland	Kilteasheen	Bishop's Seat	661-1275	Medieval	Read. 2010. *in* Medieval Lough Cé: history, archaeology, and landscape	Mann et al. 2018
KT26	Dental Calculus	Burial 124	Institute of Technology Sligo, Republic of Ireland	Ireland	Kilteasheen	Bishop's Seat	661-1275	Medieval	Read. 2010. *in* Medieval Lough Cé: history, archaeology, and landscape	Mann et al. 2018
KT28	Dental Calculus	Burial 87	Institute of Technology Sligo, Republic of Ireland	Ireland	Kilteasheen	Bishop's Seat	661-1275	Medieval	Read. 2010. *in* Medieval Lough Cé: history, archaeology, and landscape	Mann et al. 2018
KT29	Dental Calculus	Burial 62	Institute of Technology Sligo, Republic of Ireland	Ireland	Kilteasheen	Bishop's Seat	661-1275	Medieval	Read. 2010. *in* Medieval Lough Cé: history, archaeology, and landscape	Mann et al. 2018
KT31	Dental Calculus	Burial 44	Institute of Technology Sligo, Republic of Ireland	Ireland	Kilteasheen	Bishop's Seat	661-1275	Medieval	Read. 2010. *in* Medieval Lough Cé: history, archaeology, and landscape	Mann et al. 2018
KT32	Dental Calculus	Burial 8	Institute of Technology Sligo, Republic of Ireland	Ireland	Kilteasheen	Bishop's Seat	661-1275	Medieval	Read. 2010. *in* Medieval Lough Cé: history, archaeology, and landscape	Mann et al. 2018
KT36	Dental Calculus	Burial 71	Institute of Technology Sligo, Republic of Ireland	Ireland	Kilteasheen	Bishop's Seat	661-1275	Medieval	Read. 2010. *in* Medieval Lough Cé: history, archaeology, and landscape	Mann et al. 2018
S108	Dental Calculus	S108	University of Leiden, Netherlands	Netherlands	Middenbeemster	Middenbeemster cemetery	1829–1866	PreIndustrial	Waters-Rist & Hoogland. 2013. International Journal of Paleopathology	Ziesemer et al. 2015
S454	Dental Calculus	S454	University of Leiden, Netherlands	Netherlands	Middenbeemster	Middenbeemster cemetery	1856	PreIndustrial	Waters-Rist & Hoogland. 2013. International Journal of Paleopathology	Ziesemer et al. 2016
16892	Dental Calculus	155	Museum of London, England	England	London	Crossbones	1598-1853	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16893	Dental Calculus	119	Museum of London, England	England	London	Crossbones	1598-1853	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16894	Dental Calculus	114	Museum of London, England	England	London	Crossbones	1598-1853	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16896	Dental Calculus	101	Museum of London, England	England	London	Crossbones	1598-1853	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16897	Dental Calculus	28	Museum of London, England	England	London	Crossbones	1598-1853	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16898	Dental Calculus	2	Museum of London, England	England	London	Crossbones	1598-1853	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16899	Dental Calculus	32	Museum of London, England	England	London	Crossbones	1598-1853	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16900	Dental Calculus	6	Museum of London, England	England	London	Crossbones	1598-1853	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16901	Dental Calculus	99	Museum of London, England	England	London	Crossbones	1598-1853	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16903	Dental Calculus	356	Museum of London, England	England	London	St Benet Sherehog Church	1673	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16905	Dental Calculus	24	Museum of London, England	England	London	St Benet Sherehog Church	1670-1853	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16906	Dental Calculus	712	Museum of London, England	England	London	St Benet Sherehog Church	1670-1853	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16907	Dental Calculus	601	Museum of London, England	England	London	St Benet Sherehog Church	1825	PostIndustrial	Farrer et al. 2018	Farrer et al. 2018
16911	Dental Calculus	12	Museum of London, England	England	London	St Benet Sherehog Church	1670-1853	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16913	Dental Calculus	726	Museum of London, England	England	London	St Benet Sherehog Church	1280-1666	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16914	Dental Calculus	1601	Museum of London, England	England	London	St Benet Sherehog Church	1250-1500	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16915	Dental Calculus	1566	Museum of London, England	England	London	St Benet Sherehog Church	1250-1500	PostMedieval	Farrer et al. 2018	Farrer et al. 2018

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Study ID Number	Sample Type	Museum ID	Museum	Geographic Location	Specific Location	Archaeological Site	Estimated Date (CE)	Period	Anthropological Reference	Calculus Sample Reference
16916	Dental Calculus	67	Museum of London, England	England	London	St Benet Sherehog Church	1280-1666	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16917	Dental Calculus	1511	Museum of London, England	England	London	St Benet Sherehog Church	1280-1666	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16918	Dental Calculus	1570	Museum of London, England	England	London	St Benet Sherehog Church	1280-1666	PostMedieval	Farrer et al. 2018	Farrer et al. 2018
16919	Dental Calculus	1018	Museum of London, England	England	London	Chelsea Old Church	1700-1850	PreIndustrial	Farrer et al. 2018	Farrer et al. 2018
16920	Dental Calculus	198	Museum of London, England	England	London	Chelsea Old Church	1732	PreIndustrial	Farrer et al. 2018	Farrer et al. 2018
16921	Dental Calculus	505	Museum of London, England	England	London	Chelsea Old Church	1700-1850	PreIndustrial	Farrer et al. 2018	Farrer et al. 2018
16922	Dental Calculus	654	Museum of London, England	England	London	Chelsea Old Church	1827	PostIndustrial	Farrer et al. 2018	Farrer et al. 2018
16923	Dental Calculus	353	Museum of London, England	England	London	Chelsea Old Church	1700-1850	PreIndustrial	Farrer et al. 2018	Farrer et al. 2018
16925	Dental Calculus	622	Museum of London, England	England	London	Chelsea Old Church	1836	PostIndustrial	Farrer et al. 2018	Farrer et al. 2018
16926	Dental Calculus	1023	Museum of London, England	England	London	Chelsea Old Church	1700-1850	PreIndustrial	Farrer et al. 2018	Farrer et al. 2018
16927	Dental Calculus	722	Museum of London, England	England	London	Chelsea Old Church	1822	PostIndustrial	Farrer et al. 2018	Farrer et al. 2018
16930	Dental Calculus	13872	Museum of London, England	England	London	St Mery Graces	1400-1538	Medieval	Farrer et al. 2018	Farrer et al. 2018
16931	Dental Calculus	6351	Museum of London, England	England	London	St Mery Graces	1350-1400	Medieval	Farrer et al. 2018	Farrer et al. 2018
16933	Dental Calculus	12403	Museum of London, England	England	London	St Mery Graces	1400-1538	Medieval	Farrer et al. 2018	Farrer et al. 2018
16937	Dental Calculus	13898	Museum of London, England	England	London	St Mery Graces	1400-1538	Medieval	Farrer et al. 2018	Farrer et al. 2018
16938	Dental Calculus	12339	Museum of London, England	England	London	St Mery Graces	1400-1538	Medieval	Farrer et al. 2018	Farrer et al. 2018
16939	Dental Calculus	12356	Museum of London, England	England	London	St Mery Graces	1400-1538	Medieval	Farrer et al. 2018	Farrer et al. 2018
16940	Dental Calculus	7268	Museum of London, England	England	London	St Mery Graces	1350-1400	Medieval	Farrer et al. 2018	Farrer et al. 2018
16941	Dental Calculus	13622	Museum of London, England	England	London	St Mery Graces	1400-1538	Medieval	Farrer et al. 2018	Farrer et al. 2018
16942	Dental Calculus	12497	Museum of London, England	England	London	St Mery Graces	1400-1538	Medieval	Farrer et al. 2018	Farrer et al. 2018
16944	Dental Calculus	7358	Museum of London, England	England	London	St Mery Graces	1350-1400	Medieval	Farrer et al. 2018	Farrer et al. 2018
16948	Dental Calculus	7202	Museum of London, England	England	London	St Mery Graces	1350-1400	Medieval	Farrer et al. 2018	Farrer et al. 2018
16949	Dental Calculus	12400	Museum of London, England	England	London	St Mery Graces	1400-1538	Medieval	Farrer et al. 2018	Farrer et al. 2018
16950	Dental Calculus	6210	Museum of London, England	England	London	St Mery Graces	1350-1400	Medieval	Farrer et al. 2018	Farrer et al. 2018
8812	Dental Calculus	J2360	Jewbury Cemetery, England	England	York	Jewbury Cemetery	1250	Medieval	Adler et al. 2013	Weyrich et al. 2017
8824	Dental Calculus	J2454	Jewbury Cemetery, England	England	York	Jewbury Cemetery	1250	Medieval	Adler et al. 2013	Weyrich et al. 2017
15501	Dental Calculus	35020	The Musée de l'Homme, Paris, France	France	Paris	The Musée de l'Homme	1950	Post-War	NA	This study
15498	Dental Calculus	35023	The Musée de l'Homme, Paris, France	France	Paris	The Musée de l'Homme	1950	Post-War	NA	This study
15495	Dental Calculus	35004	The Musée de l'Homme, Paris, France	France	Paris	The Musée de l'Homme	1950	Post-War	NA	This study
15500	Dental Calculus	35026	The Musée de l'Homme, Paris, France	France	Paris	The Musée de l'Homme	1950	Post-War	NA	This study
15494	Dental Calculus	34993	The Musée de l'Homme, Paris, France	France	Paris	The Musée de l'Homme	1950	Post-War	NA	This study
18752	Dental Calculus	H1	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18754	Dental Calculus	H2	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18756	Dental Calculus	H3	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18758	Dental Calculus	H4	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18760	Dental Calculus	H5	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18764	Dental Calculus	H7	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18766	Dental Calculus	H8	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18767	Dental Calculus	H8	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18769	Dental Calculus	H9	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18771	Dental Calculus	H10	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18773	Dental Calculus	H11	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18775	Dental Calculus	H12	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18777	Dental Calculus	H13	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study

Table S1. **Sample Information**

Study ID Number	Sample Type	Museum ID	Museum	Geographic Location	Specific Location	Archaeological Site	Estimated Date (CE)	Period	Anthropological Reference	Calculus Sample Reference
18781	Dental Calculus	H15	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18784	Dental Calculus	H16	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18786	Dental Calculus	H17	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18791	Dental Calculus	H20	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18793	Dental Calculus	H21	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19767	Dental Calculus	P1	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19768	Dental Calculus	P1	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19769	Dental Calculus	P2	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19770	Dental Calculus	P2	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19771	Dental Calculus	P3	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19772	Dental Calculus	P4	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19773	Dental Calculus	P5	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19774	Dental Calculus	P6	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19775	Dental Calculus	P7	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19776	Dental Calculus	P8	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19777	Dental Calculus	P9	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19778	Dental Calculus	P10	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19779	Dental Calculus	P10	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19780	Dental Calculus	P11	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19781	Dental Calculus	P11	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19782	Dental Calculus	P12	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19783	Dental Calculus	P12	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19784	Dental Calculus	P13	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19785	Dental Calculus	P13	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19786	Dental Calculus	P13	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19787	Dental Calculus	P14	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19788	Dental Calculus	P14	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19789	Dental Calculus	P14	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19790	Dental Calculus	P14	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19791	Dental Calculus	P15	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19792	Dental Calculus	P16	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19793	Dental Calculus	P17	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19794	Dental Calculus	P17	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19795	Dental Calculus	P18	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19796	Dental Calculus	P18	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19797	Dental Calculus	P19	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19798	Dental Calculus	P20	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19799	Dental Calculus	P21	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19800	Dental Calculus	P22	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19801	Dental Calculus	P23	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
19802	Dental Calculus	P23	University of Adelaide Dental School, Australia	Australia	Adelaide	NA	Modern	Modern	NA	This study
18433	Environment Control	NA	NA	Hungary	Bács-Kiskun	Fajsz Garadomb	Modern	Environment	NA	Abdul-Aziz 2019 (Thesis)
20050	Environment Control	NA	NA	Australia	Australia	Tasmania	Modern	Environment	NA	Haberle et al. 2019
20051	Environment Control	NA	NA	Australia	Australia	Tasmania	Modern	Environment	NA	Haberle et al. 2019

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Study ID Number	Sample Type	Museum ID	Museum	Geographic Location	Specific Location	Archaeological Site	Estimated Date (CE)	Period	Anthropological Reference	Calculus Sample Reference
20052	Environment Control	NA	NA	Australia	Australia	Tasmania	Modern	Environment	NA	Haberle et al. 2019
20053	Environment Control	NA	NA	Australia	Australia	Tasmania	Modern	Environment	NA	Haberle et al. 2019
20055	Environment Control	NA	NA	Australia	Australia	Tasmania	Modern	Environment	NA	Haberle et al. 2019
20056	Environment Control	NA	NA	Australia	Australia	Tasmania	Modern	Environment	NA	Haberle et al. 2019
20057	Environment Control	NA	NA	Australia	Australia	Flinders Island	Modern	Environment	NA	Haberle et al. 2019
20058	Environment Control	NA	NA	Australia	Australia	Flinders Island	Modern	Environment	NA	Haberle et al. 2019
20059	Environment Control	NA	NA	Australia	Australia	Flinders Island	Modern	Environment	NA	Haberle et al. 2019
20060	Environment Control	NA	NA	Australia	Australia	Flinders Island	Modern	Environment	NA	Haberle et al. 2019
20061	Environment Control	NA	NA	Australia	Australia	Flinders Island	Modern	Environment	NA	Haberle et al. 2019
20062	Environment Control	NA	NA	Australia	Australia	Flinders Island	Modern	Environment	NA	Haberle et al. 2019
20063	Environment Control	NA	NA	Australia	Australia	Flinders Island	Modern	Environment	NA	Haberle et al. 2019
20064	Environment Control	NA	NA	Australia	Australia	Flinders Island	Modern	Environment	NA	Haberle et al. 2019
20065	Environment Control	NA	NA	Australia	Australia	Flinders Island	Modern	Environment	NA	Haberle et al. 2019
20066	Environment Control	NA	NA	Australia	Australia	Flinders Island	Modern	Environment	NA	Haberle et al. 2019
20067	Environment Control	NA	NA	Australia	Australia	Flinders Island	Modern	Environment	NA	Haberle et al. 2019
20068	Environment Control	NA	NA	Australia	Australia	Flinders Island	Modern	Environment	NA	Haberle et al. 2019
Air	Environment Control	NA	NA	Environment	USA	U	Modern	Environment	NA	Weyrich et al. 2017
Braccish Water	Environment Control	NA	NA	Environment	USA	U	Modern	Environment	NA	Weyrich et al. 2017
ForestSoil	Environment Control	NA	NA	Environment	USA	U	Modern	Environment	NA	Weyrich et al. 2017
Fresh GroundWater	Environment Control	NA	NA	Environment	USA	U	Modern	Environment	NA	Weyrich et al. 2017
Grassland Soil	Environment Control	NA	NA	Environment	USA	U	Modern	Environment	NA	Weyrich et al. 2017
19181	Laboratory Control	NA	ACAD Ancient DNA Laboratory	Australia	Adelaide	NA	NA	Modern	NA	This study
13224EBC	Laboratory Control	NA	ACAD Ancient DNA Laboratory	Australia	Adelaide	NA	NA	Modern	NA	This study
20495	Laboratory Control	NA	ACAD Ancient DNA Laboratory	Australia	Adelaide	NA	NA	Modern	NA	This study
20520	Laboratory Control	NA	ACAD Ancient DNA Laboratory	Australia	Adelaide	NA	NA	Modern	NA	This study
18796	Laboratory Control	NA	Modern Microbiome Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	This study
18800	Laboratory Control	NA	Modern Microbiome Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	This study
18794	Laboratory Control	NA	Modern Microbiome Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	This study
18795	Laboratory Control	NA	Modern Microbiome Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	This study
18798	Laboratory Control	NA	Modern Microbiome Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	This study
16972	Laboratory Control	NA	ACAD Ancient DNA Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	Farrer et al. 2018
15522	Laboratory Control	NA	ACAD Ancient DNA Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	Farrer et al. 2018
17004	Laboratory Control	NA	ACAD Ancient DNA Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	Farrer et al. 2018
17034	Laboratory Control	NA	ACAD Ancient DNA Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	Farrer et al. 2018
17229	Laboratory Control	NA	ACAD Ancient DNA Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	Farrer et al. 2018
17232	Laboratory Control	NA	ACAD Ancient DNA Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	Farrer et al. 2018
17234	Laboratory Control	NA	ACAD Ancient DNA Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	Farrer et al. 2018
17252	Laboratory Control	NA	ACAD Ancient DNA Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	Farrer et al. 2018
17254	Laboratory Control	NA	ACAD Ancient DNA Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	Farrer et al. 2018
17580	Laboratory Control	NA	ACAD Ancient DNA Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	Farrer et al. 2018
17673	Laboratory Control	NA	ACAD Ancient DNA Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	Farrer et al. 2018
19804	Laboratory Control	NA	Modern Microbiome Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	This study
19834	Laboratory Control	NA	Modern Microbiome Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	This study
19837	Laboratory Control	NA	Modern Microbiome Laboratory, Adelaide,	Australia	Adelaide	NA	NA	Modern	NA	This study

II Methods and Materials

103 dental calculus samples processed for this study, including four post-1800s populations from Australia (n = 28), Switzerland (n = 12), Germany (n = 10), and France (n = 5), 54 modern Australian dental calculus samples from healthy donors (n = 18) and periodontitis patients (n = 36). All samples and their respective extraction controls were maintained through to contamination filtering, before removing duplicates (*i.e.* multiple calculus samples from the same individual), contaminated samples, or samples not meeting metadata criteria (*i.e.* modern subgingival calculus samples).

Historical dental calculus collection and DNA extraction

Sampling of the St Mary's skulls were completed at the University of Adelaide, using sterile procedures previously described in Weyrich *et al.* (2015) [6]. In brief, a sterile dental pick was used to detach the calculus from tooth surface, collecting the calculus fragments into a labelled non-breakable container for transport (*e.g.* a sterile plastic 2 mL screw cap tube or plastic bag). The metadata regarding the sampled individual (*e.g.* specifics of the oral location of calculus), the sampling environment, and the collector was recorded in detail at time of sampling. German and Swiss dental calculus were sampled using the described techniques at their respective locations housing the skeletal remains.

Dental calculus samples were transported to the quarantine facility for ancient DNA at University of Adelaide, Australia. Conditions are typical of an ancient DNA laboratory, as specified in Weyrich *et al.* (2017) [3]. Prior to entry into the ancient DNA laboratory, sample bags were bleached and UV irradiation for 15 minutes to minimise introduced exogenous microbial contamination. Samples were stored at 4°C until DNA extraction.

Prior to DNA extraction, a large fragment of the sampled dental calculus deposit was isolated and decontaminated through exposure to high-intensity UV radiation for 15 minutes on each side, to reduce environmental contaminant DNA present on the outside of the dental calculus fragment. Following UV treatment, the fragment was immersed in approximately 2 mL of bleach (5% (w/v) sodium hypochlorite) in a sterile petri dish for 3 minutes, then submerged in ethanol (80%) for 1 minute to remove any residual chemicals (*i.e.* bleach) [7]. The fragment was then transferred to a 2 mL screw-cap tube and crushed into a non-uniform powder ready for DNA extraction.

Each sample was extracted using an in-house silica-based extraction method, previously described in Brotherton *et al.* [8], but with modified buffer volumes to

account for smaller sample size, as described in Weyrich *et al.* [3]. Two sample blanks controls (also know as extraction blank controls; EBCs) were extracted alongside each batch (first and last sample, EBC1 and EBC2 respectively), with no more than 14 calculus samples extracted together to reduce potential cross-contamination. EBCs were treated as samples, undergoing the identical experimental procedures as the dental calculus samples from extraction, to through library preparation, and sequencing.

Modern dental calculus sample collection and extraction

Fresh supragingival dental calculus (n = 18) was collected from orally healthy volunteers (aged 18–50) at the University of Adelaide School of Dentistry clinic, obtained under informed consent, as previously described in **Chapter 5**.

Periodontal dental calculus samples (n = 36) were collected in Adelaide, from patients under examination at a private dental practice. Samples were collected by dental professional, using a dental pick following standard calculus removal procedures, and placed in sterile 2 mL screw-cap tubes for transport.

All modern dental calculus samples were transported to a specialised clean laboratory facility, designed for human microbiome research at the University of Adelaide, and upon arrival, stored at −20°C until DNA extraction. The modern microbiome laboratory is isolated from any post-PCR laboratories and has strict protocols in place to minimise entry of human and bacterial contamination. Researchers in the modern microbiome lab are required to wear shoe covers, two pairs of gloves, face mask, and laboratory coat (ensuring minimum skin exposure while working). All surfaces are routinely cleaned with Decon 90 (Decon Laboratories Limited) or 2% bleach (NaClO) solution, with KlerAlcohol 70% v/v Isopropyl Alcohol (EcoLab Life Sciences). All consumables, disposables, tools and instruments are externally bleached on entering the lab and then subjected to routine cleaning before, during and after use. All sample work is carried out within the PCR hoods to minimise environmental contamination; the inside of the PCR hood, tools and instruments are UV-radiated for a minimum of 15 minutes before and after use.

DNA was extracted using a modified in-house silica method, based on that previously developed for ancient dental calculus DNA extraction (as described in [3, 8] and optimised for modern dental calculus (as discussed in **Chapter 4**). In brief, dental calculus samples were decalcified and microbial cells lysed in 470 μL of 0.5 ethylene diamine triacetic acid (EDTA; pH 8.0), and 30 μL of 10 % sodium dodecyl sulphate (SDS), and treated with 20 mg/mL proteinase K, then incubated at 55°C for overnight. Cell lysis products were bound to 20 μL of silica solution in the presence of 1.5 mL of QG buffer (Qiagen) containing guanidium thiocyanate.

Silica-bound DNA was then rinsed with 80 % ethanol twice, before re-suspending in 100 μL of Tris-EDTA solution. Re-suspension is repeated to elute 200 μL total of DNA. Two EBCs were processed alongside each extraction group (no more than 14 calculus samples per batch), and treated as samples, from extraction to sequencing.

Shotgun library preparation and sequencing

Modern dental calculus DNA extracts underwent fragmentation prior to library preparation; 50 μL of extract was sheared using focused-ultrasonicator (Covaris Inc.) to ∼300 bp fragment lengths. Both historical and modern dental calculus metagenomic shotgun libraries were constructed within their respective laboratories, using the same protocol described previously in Kirche, Martin, and Sawyer (2011), but without the enzymatic damage repair step [9, 3]. In short, 20 μL of DNA extract was used in enzymatic polishing to produce blunt ended fragments, before the ligation of truncated 7-bp forward and reverse barcoded Illumina adaptors, finishing by filling in the gaps between the adaptor sequences and the DNA sequence. MinElute clean-ups (Qiagen) were completed after both enzymatic polishing and barcode ligation steps. Historical dental calculus libraries were amplified in triplicate by PCR for 13 cycles with Illumina amplification primers [10]. Each PCR reaction contained: 13.25 μL sterile H20, 5 μL of Library DNA, 0.25 μL of Hi-Fi taq (Life Technologies), 2.5 μL of 10X Hi-Fi buffer, 1.25 μL MgSO4 (50 mM), 0.25 μL dNTPs (100 mM), and 1.25 μL each of the forward and reverse primers. Cycling conditions were as follows: 94°C for 12 minutes; 13 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 40 seconds (plus 2 seconds/cycle); and 72°C for 10 minutes. PCR products were pooled and cleaned with AxyPrep magnetic beads (Axygen Scientific Inc.). Modern libraries were amplified, and ancient libraries re-amplified, with GAII Indexed Illumina primers [10], using the above cycling conditions and a modified PCR reaction: 12.75 μ L sterile H20, 2 μL of purified Library DNA, 0.25 μL of AmpliTaq Gold (Life Technologies), 2.5 μL of 10X Gold buffer, 2.5 μL MgCl2 (25 mM), 0.625 μL dNTPs (10 mM), and 1.25 μL Illumina amplification primer, and 1.25 μL GAII Illumina indexed adaptor. All libraries were purified again prior to quantification using TapeStation (Aligent), then pooled for a final 4 nmol/L DNA concentration, before sequencing on Illumina NextSeq, Mid Output 150 cycles, or HiSeq X Ten (Illumina).

Downloaded dental calculus metagenomic data

60 shotgun-sequenced dental calculus samples from previously published datasets were downloaded to include within this meta-analysis; sample information, geographic and cultural information, and respective publication are listed in Table S1.

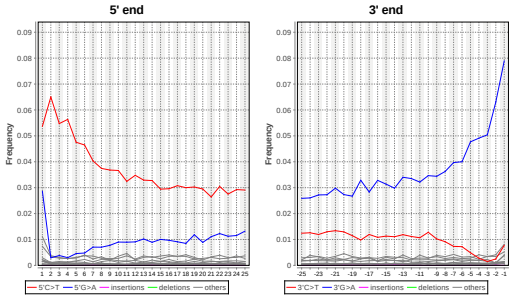
This includes 41 dental calculus samples from Medieval to Industrial England [11]; 13 samples from Medieval Ireland [12]; two samples from Medieval England [3]; two samples from Medieval Germany [13], and two samples from pre-Industrial Netherlands [14]. Three dental calculus samples from Weyrich *et al.* were included in the contamination analysis [3], but were removed from further downstream analysis, as the calculus samples were sampled from the same individuals re-extracted in this study (ACAD samples 13232, 13227, and 13230). One of the datasets [11] included 11 extraction blank controls which were included in decontam analysis (**Section III**).

As no archaeological material included a soil sample from their respective site, a number of environmental and soil samples were downloaded to act as a proxy for environmental contaminants; further sample details, geographic information, and respective publications are listed in Table S1. This included one soil sample from Hungary [15], six soil core samples from North Tasmania and 12 soil cores from Flinders Island, Tasmania, Australia [16], and five various environmental samples [3].

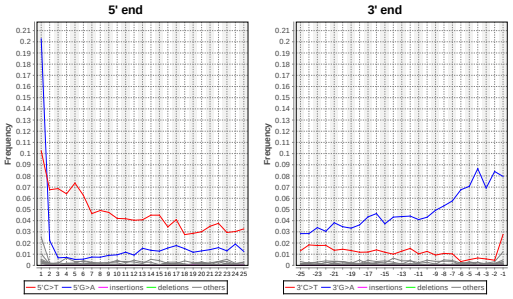
Bioinformatic Analysis

Raw fastQ files were trimmed, demultiplexed, and collapsed using AdapterRemoval v2 [17] based on the unique forward and reverse barcodes. All modern samples underwent host read removal using KneadData [18], which aligns sequencing reads to the human genome (GRCh37/hg19) reference database and removes all sequences with one or more alignments (Table S2), before being subsampled to 1.5 million reads. Taxonomic composition was generated from collapsed reads sequenced data using MEGAN Alignment Tool (MALT) v 0.3.8 [19]. MALT aligns DNA reads from samples against an in-house database created using 47,696 archaeal and bacterial genome assemblies from the NCBI Assembly database [20]. The resulting alignment based blast-text files were then converted in RMA files using the blast2rma script included with the program MEGAN v 6.12.8 [21] with the following Last Common Ancestor (LCA) parameters: Weighted-LCA=80% , minimum bitscore=42, minimum E-value=0.01, minimum support percent=0.1. Historical samples were assess for ancient DNA authenticity by estimation of cytosine deamination using DamageProfiler [22] on *Anaerolineaceae* oral taxon 439 (Figure S1).

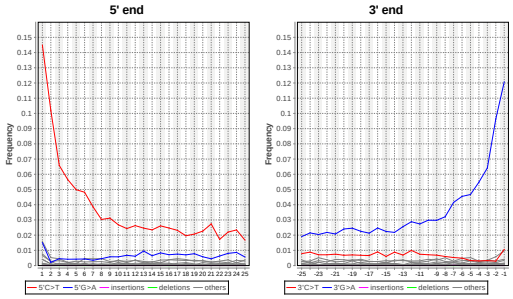
20455_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 56,399 (100.0% of all input reads) | Specie: null



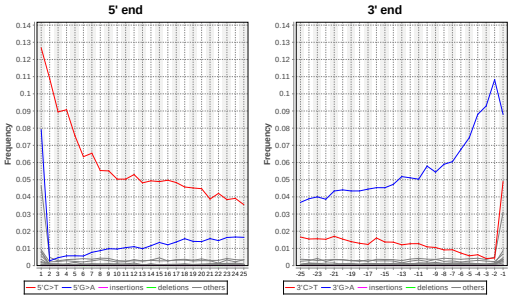
20454_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 7,223 (100.0% of all input reads) | Specie: null



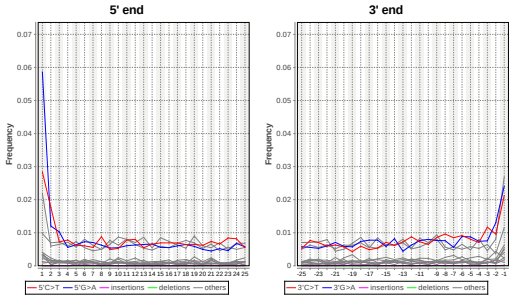
20457_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 17,008 (100.0% of all input reads) | Specie: null



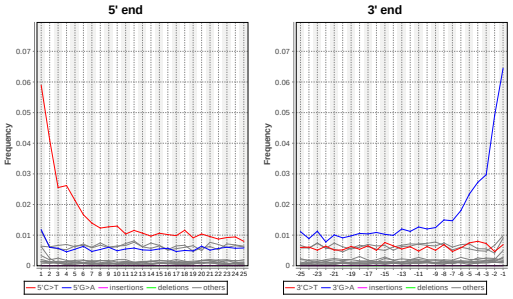
20456_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 46,699 (100.0% of all input reads) | Specie: null



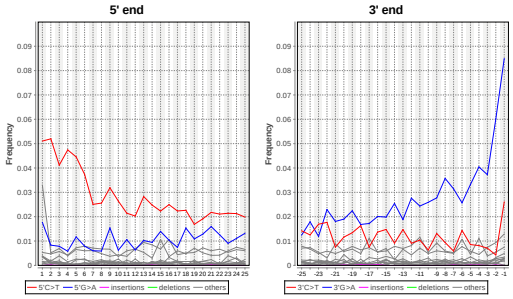
20463_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 23,380 (100.0% of all input reads) | Specie: null



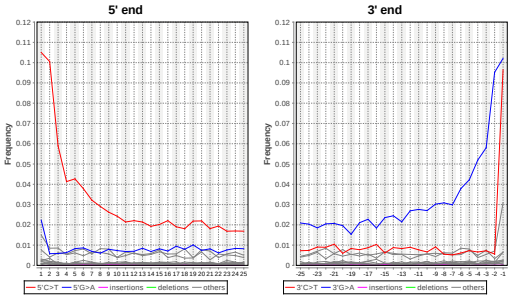
20460_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 53,038 (100.0% of all input reads) | Specie: null



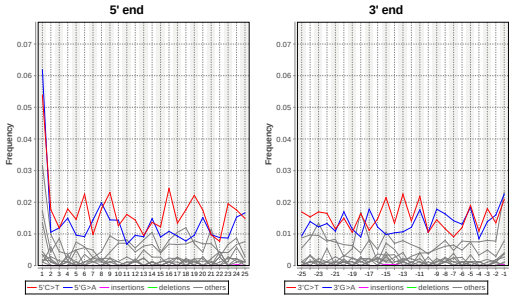
20465_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 7,089 (100.0% of all input reads) | Specie: null



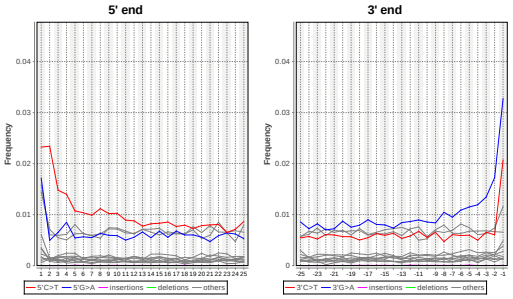
20462_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 14,031 (100.0% of all input reads) | Specie: null



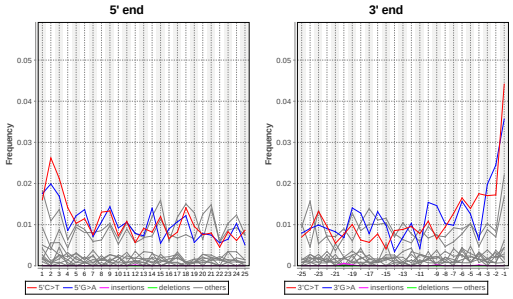
20467_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 3,267 (100.0% of all input reads) | Specie: null



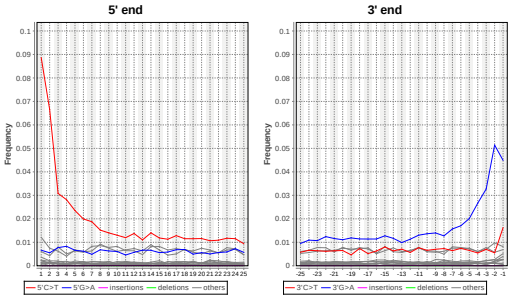
20466_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 68,191 (100.0% of all input reads) | Specie: null



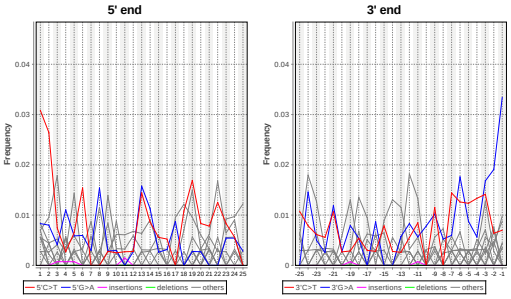
20469_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 5,155 (100.0% of all input reads) | Specie: null



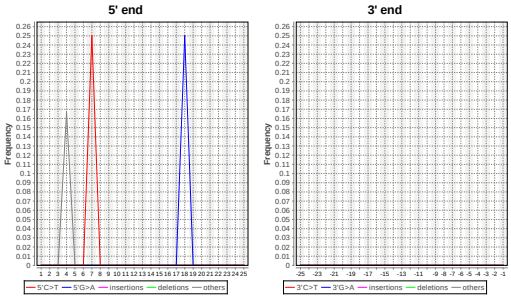
20468_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 34,457 (100.0% of all input reads) | Specie: null



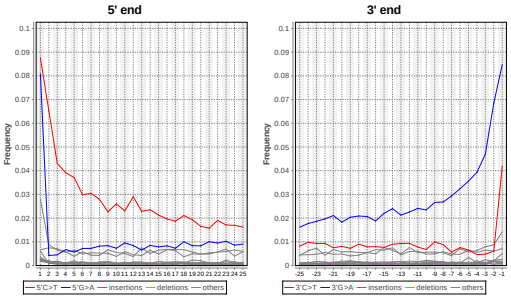
20473_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 1,365 (100.0% of all input reads) | Specie: null



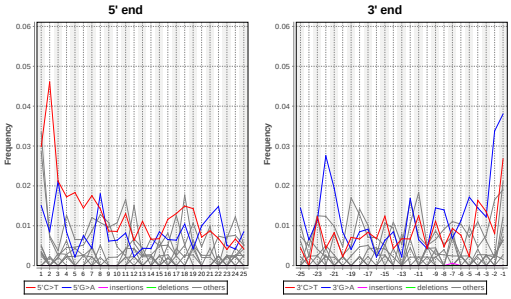
20472_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 8 (100.0% of all input reads) | Specie: null



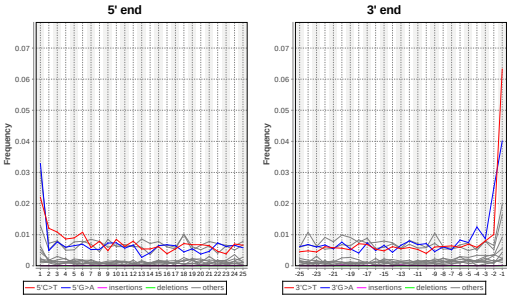
20475_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 23,718 (100.0% of all input reads) | Specie: null



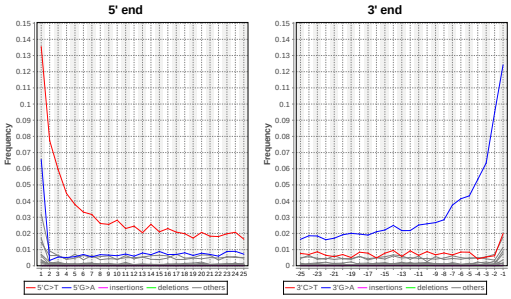
20474_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 1,736 (100.0% of all input reads) | Specie: null



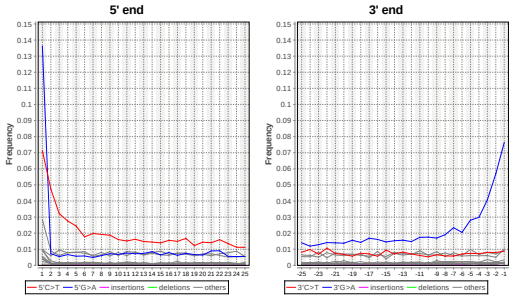
20480_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 17,394 (100.0% of all input reads) | Specie: null



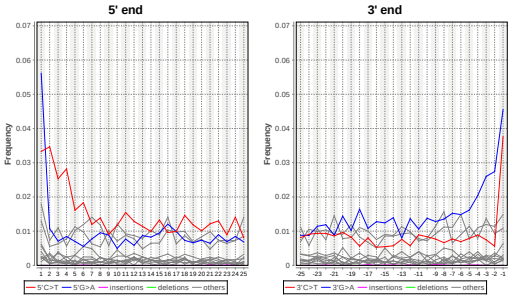
20476_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 22,077 (100.0% of all input reads) | Specie: null



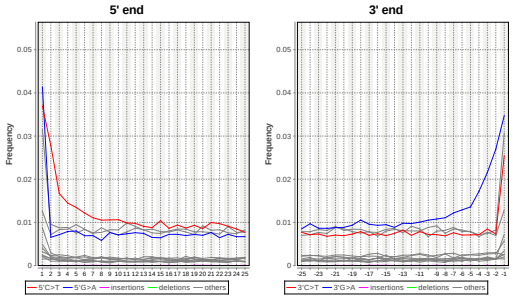
20483_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 21,949 (100.0% of all input reads) | Specie: null



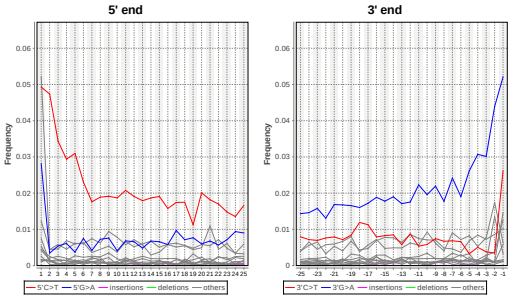
20482_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 9,173 (100.0% of all input reads) | Specie: null



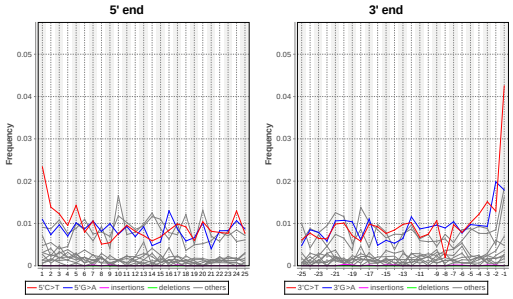
20486_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 125,484 (100.0% of all input reads) | Specie: null



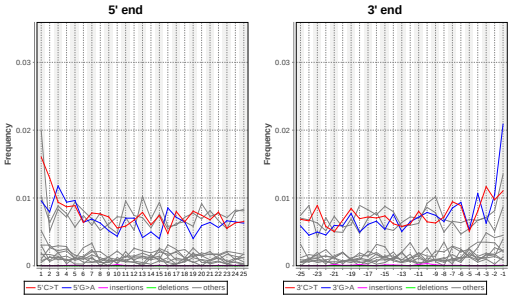
20485_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 11,686 (100.0% of all input reads) | Specie: null



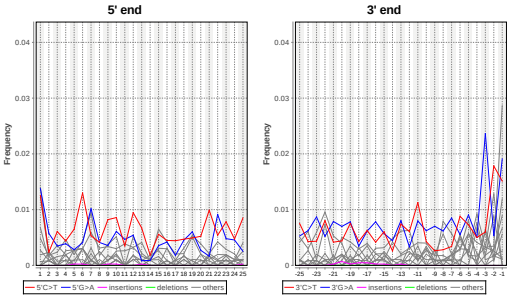
20490_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 6,119 (100.0% of all input reads) | Specie: null



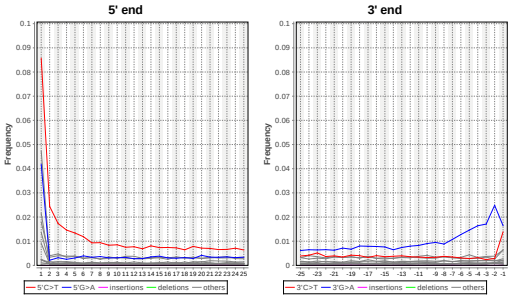
20487_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 15,370 (100.0% of all input reads) | Specie: null



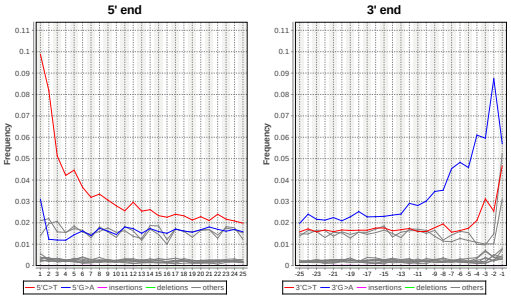
20493_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 4,288 (100.0% of all input reads) | Specie: null



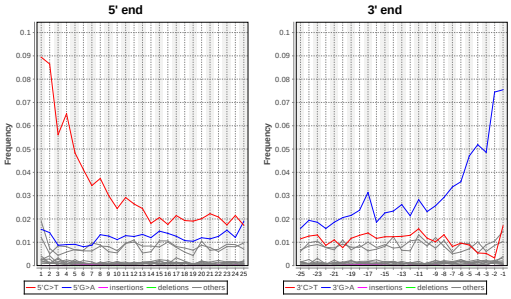
20492_StMarys_AustralianColonialists-A439-MQ1-sorted
Number of used reads: 104,077 (100.0% of all input reads) | Specie: null



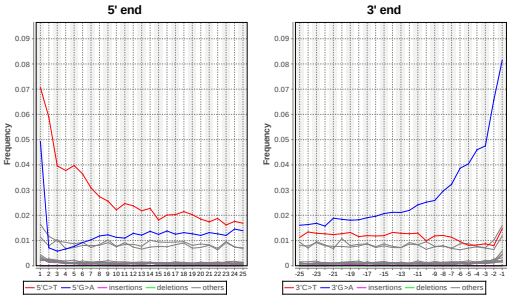
13215_Switzerland_IndustrialEra-A439-MQ1-sorted
Number of used reads: 47,879 (100.0% of all input reads) | Specie: null



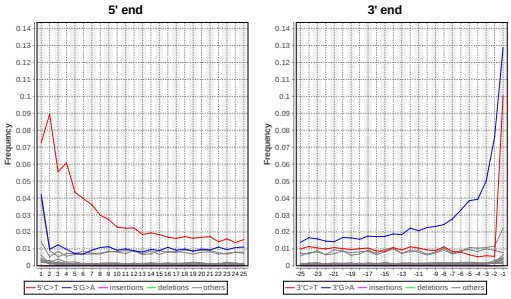
13214_Switzerland_IndustrialEra-A439-MQ1-sorted
Number of used reads: 12,288 (100.0% of all input reads) | Specie: null



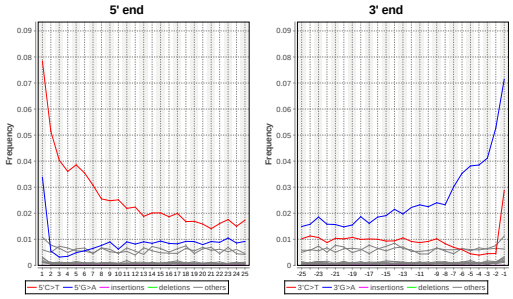
13217_Switzerland_IndustrialEra-A439-MQ1-sorted
Number of used reads: 68,167 (100.0% of all input reads) | Specie: null



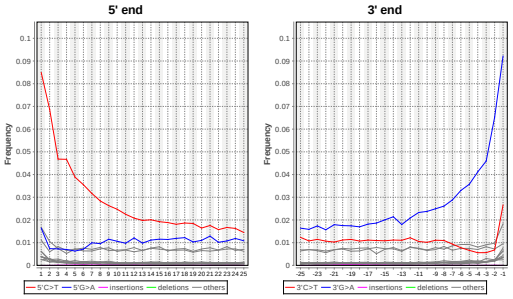
13216_Switzerland_IndustrialEra-A439-MQ1-sorted
Number of used reads: 63,868 (100.0% of all input reads) | Specie: null



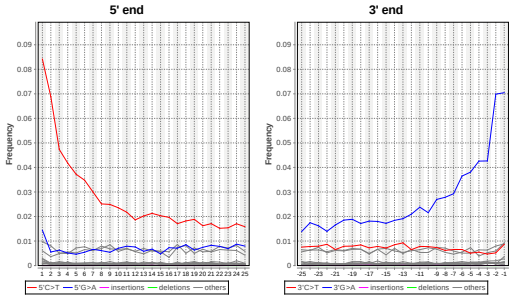
13219_Switzerland_IndustrialEra-A439-MQ1-sorted
Number of used reads: 41,318 (100.0% of all input reads) | Specie: null



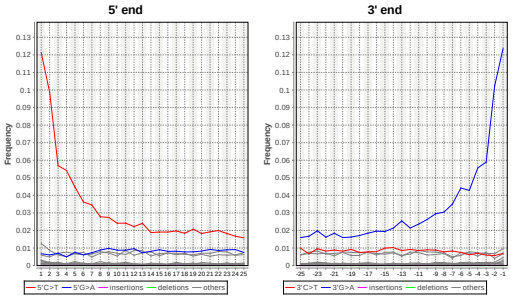
13218_Switzerland_IndustrialEra-A439-MQ1-sorted
Number of used reads: 101,623 (100.0% of all input reads) | Specie: null



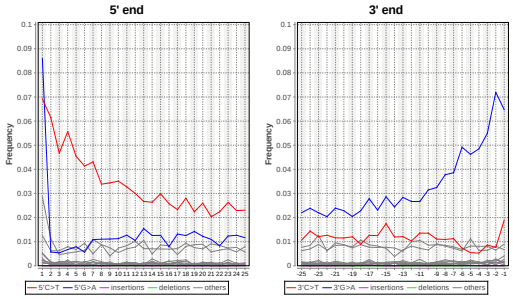
13221_Switzerland_IndustrialEra-A439-MQ1-sorted
Number of used reads: 27,523 (100.0% of all input reads) | Specie: null



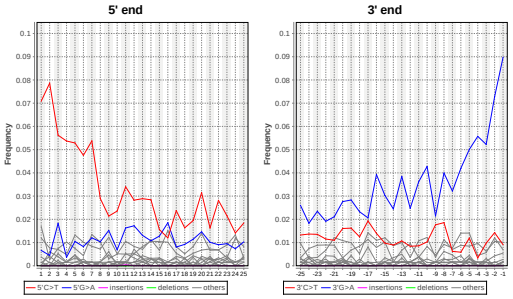
13220_Switzerland_IndustrialEra-A439-MQ1-sorted
Number of used reads: 33,539 (100.0% of all input reads) | Specie: null



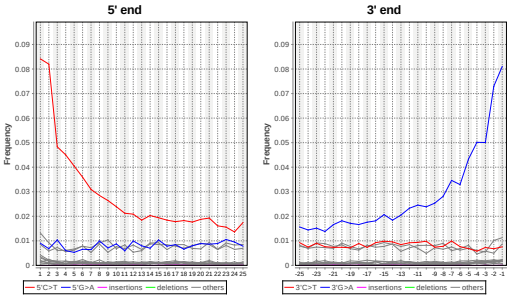
13223_Switzerland_IndustrialEra-A439-MQ1-sorted
Number of used reads: 14,346 (100.0% of all input reads) | Specie: null



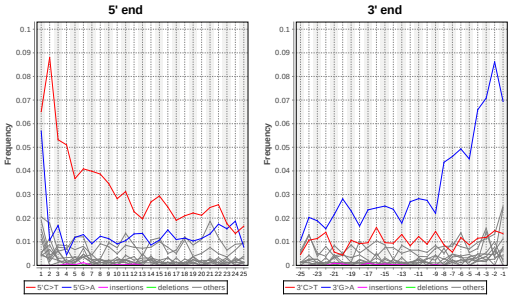
13222_Switzerland_IndustrialEra-A439-MQ1-sorted
Number of used reads: 2,582 (100.0% of all input reads) | Specie: null



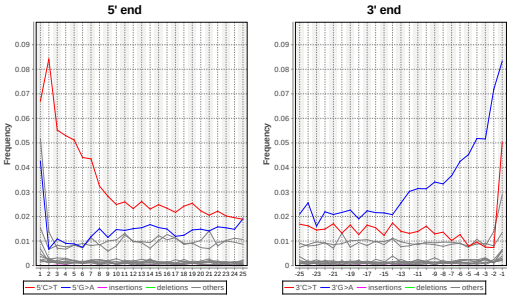
13225_Switzerland_IndustrialEra-A439-MQ1-sorted
Number of used reads: 25,352 (100.0% of all input reads) | Specie: null



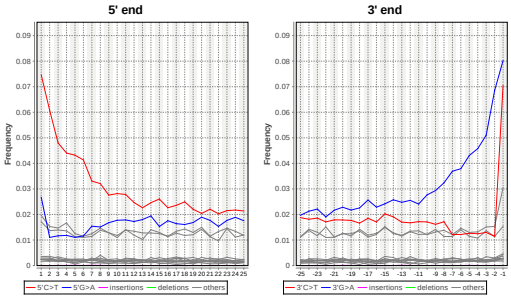
13224_IndRev_Switzerland-A439-MQ1-sorted
Number of used reads: 3,421 (100.0% of all input reads) | Specie: null



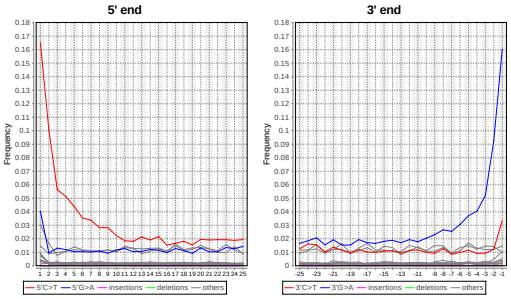
13227_Germany_IndustrialEra-A439-MQ1-sorted
Number of used reads: 19,668 (100.0% of all input reads) | Specie: null



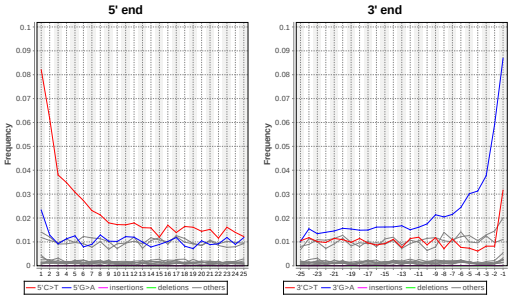
13226_Germany_IndustrialEra-A439-MQ1-sorted
Number of used reads: 45,224 (100.0% of all input reads) | Specie: null



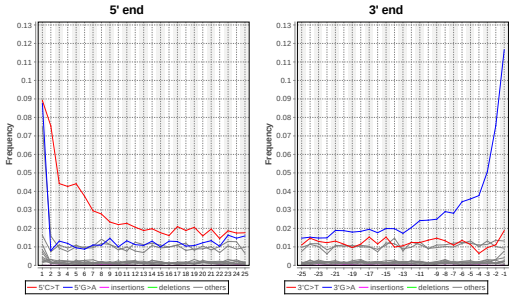
13229_Germany_IndustrialEra-A439-MQ1-sorted
Number of used reads: 28,007 (100.0% of all input reads) | Specie: null



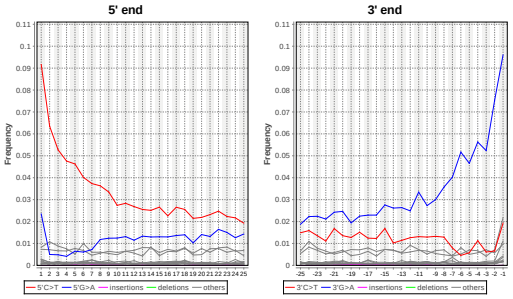
13228_Germany_IndustrialEra-A439-MQ1-sorted
Number of used reads: 19,888 (100.0% of all input reads) | Specie: null



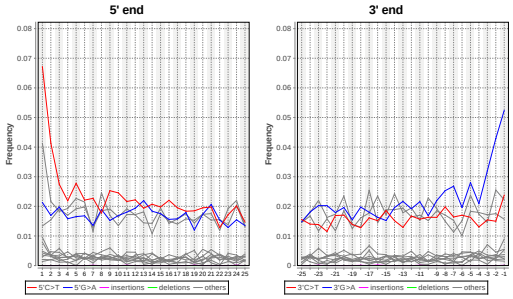
13231_Germany_IndustrialEra-A439-MQ1-sorted
Number of used reads: 19,892 (100.0% of all input reads) | Specie: null



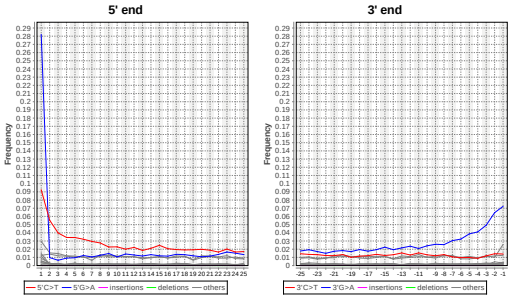
13230_Germany_IndustrialEra-A439-MQ1-sorted
Number of used reads: 18,255 (100.0% of all input reads) | Specie: null



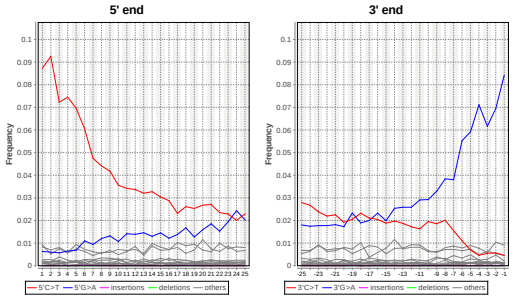
13233_Germany_IndustrialEra-A439-MQ1-sorted
Number of used reads: 10,125 (100.0% of all input reads) | Specie: null



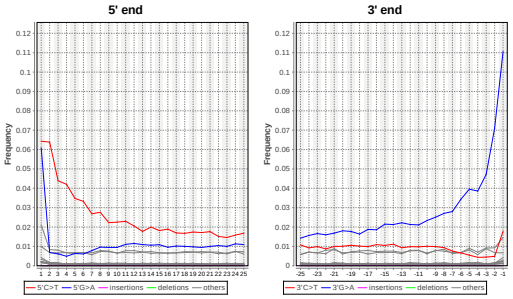
13232_Germany_IndustrialEra-A439-MQ1-sorted
Number of used reads: 26,519 (100.0% of all input reads) | Specie: null



50s1_15494.collapsed_rmdup-MQ1-sorted
Number of used reads: 18,475 (100.0% of all input reads) | Specie: null



13234_Germany_IndustrialEra-A439-MQ1-sorted
Number of used reads: 84,155 (100.0% of all input reads) | Specie: null



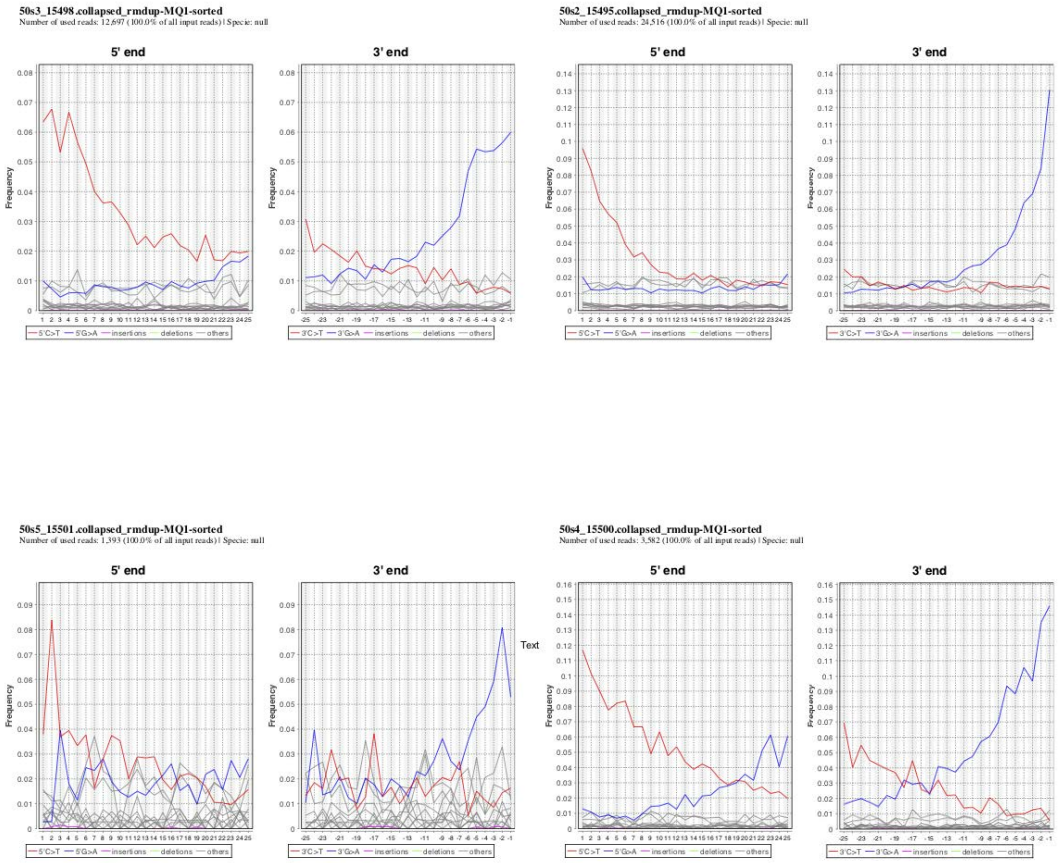


Figure S1. Damage plots of all historical samples extracted (n = 52). Damage estimation of cytosine deamination using DamageProfiler of *Anaerolineaceae* oral taxon 439

All dental calculus samples (n = 172) were filtered of environmental and laboratory contaminant taxa, described in detail within Section III. In brief, initial identification of any dental calculus sample identified to be more similar to EBCs or environmental samples using Bray-Curtis beta diversity within a principle coordinates analysis (PCoA) or hierarchical clustering (UPGMA tree) was removed from further analysis. Next, using the decontam R package, contaminant species were calculated by prevalence within dental calculus samples and control samples (*i.e.* environmental samples or laboratory blank controls) [23]. Finally, using QIIME2 [24], sequences assigning to any of the decontam identified species were removed from dental calculus samples. Any calculus sample found to have more than 10% of their sequencing reads removed by filtering were also removed from downstream analysis, as the biological signal may have potentially been distorted by contamination (Table S2).

After visualisation, species-level assignments were exported from MEGAN into QIIME2 [25]. All statistical analyses were completed in QIIME2, except for Kruskal Wallis test of group significance, which was completed in QIIME1 (v.1.9.1). All sequences were rarefied to the lowest number of reads within a historical sample

Table S2. KneadData Statistics. Sequencing reads aligned to the human genome (GRCh37/hg19) reference database and removes all sequences with one or more alignments.

Sample ID	Sequences did not align human genome	(%) Unaligned Sequences	Sequences aligned = 1	Sequences aligned = 1 (%)	Sequences aligned > 1	Sequences aligned >1 (%)	Overall alignment rate
18752_HealthyModern_1_Calculus	3644282	56.47	2152806	33.36	655894	10.16	43.53%
18754_HealthyModern_2_Calculus	16473077	81.94	2843880	14.15	785708	3.91	18.06%
18756_HealthyModern_3_Calculus	1162304	38.04	1384861	45.33	508198	16.63	61.96%
18758_HealthyModern_4_Calculus	2567777	65.08	1030205	26.11	347363	8.8	34.92%
18760_HealthyModern_5_Calculus	12829921	88.18	1301635	8.95	418851	2.88	11.82%
18764_HealthyModern_7_Calculus	1611610	25.85	3676170	58.97	946334	15.18	74.15%
18766_HealthyModern_8a_Calculus	1103739	89.68	95590	7.77	31448	2.56	10.32%
18767_HealthyModern_8b_Calculus	4466633	47.95	3819006	41	1028685	11.04	52.05%
18769_HealthyModern_9_Calculus	2997397	71.82	866727	20.77	309116	7.41	28.18%
18771_HealthyModern_10_Calculus	4616490	94.29	216797	4.43	62902	1.28	5.71%
18773_HealthyModern_11_Calculus	3757006	62.89	1686816	38.24	530140	8.87	37.11%
18775_HealthyModern_12_Calculus	719877	99.03	5375	0.74	1693	0.23	0.97%
18777_HealthyModern_13_Calculus	5922132	93.34	311946	4.92	110273	1.74	6.66%
18781_HealthyModern_15_Calculus	1497628	78.8	296878	15.62	106118	5.58	21.20%
18784_HealthyModern_16_Calculus	6257755	96.09	192221	2.95	62094	0.95	3.91%
18786_HealthyModern_17_Calculus	11077891	90.47	920931	7.52	245893	2.01	9.53%
18791_HealthyModern_20_Calculus	7865569	94.53	356146	4.28	98756	1.19	5.47%
18793_HealthyModern_21_Calculus	475986	81.82	78983	13.58	26791	4.61	18.18%
19767_ModernPerio_1	522533	98.05	7640	1.43	2775	0.52	1.95%
19768_ModernPerio_2	693950	96.96	17644	2.47	4142	0.58	3.04%
19769_ModernPerio_3	533598	66.8	212938	26.66	52287	6.55	33.20%
19770_ModernPerio_4	504145	87.59	58813	10.22	12600	2.19	12.41%
19771_ModernPerio_5	479474	92.52	31026	5.99	7735	1.49	7.48%
19772_ModernPerio_6	573102	99.5	2226	0.39	681	0.12	0.50%
19773_ModernPerio_7	625631	95.13	26590	4.04	5435	0.83	4.87%
19774_ModernPerio_8	605075	92.89	35113	5.39	11201	1.72	7.11%
19775_ModernPerio_9	581843	96.16	17592	2.91	5631	0.93	3.84%
19776_ModernPerio_10	494696	95.72	17566	3.4	4547	0.88	4.28%
19777_ModernPerio_11	824570	90.21	65587	7.18	23939	2.62	9.79%
19778_ModernPerio_12	1251970	93.59	69427	5.19	16342	1.22	6.41%
19779_ModernPerio_13	1217403	98.61	12747	1.03	4476	0.36	1.39%
19780_ModernPerio_14	994875	98.18	14702	1.45	3728	0.37	1.82%
19781_ModernPerio_15	550995	85.57	76958	11.95	15922	2.47	14.43%
19782_ModernPerio_16	648308	97.76	10874	1.64	3967	0.6	2.24%
19783_ModernPerio_17	827074	99.23	4527	0.54	1899	0.23	0.77%
19784_ModernPerio_18	19940	94.79	895	4.25	201	0.96	5.21%
19785_ModernPerio_19	934968	86.98	115386	10.73	24580	2.29	13.02%
19786_ModernPerio_20	544425	99.29	3055	0.56	834	0.15	0.71%
19787_ModernPerio_21	562260	99.78	965	0.17	298	0.05	0.22%
19788_ModernPerio_22	459807	92.87	28478	5.75	6807	1.37	7.13%
19789_ModernPerio_23	693123	96.02	23569	3.27	5139	0.71	3.98%
19790_ModernPerio_24	871478	99.42	4005	0.46	1104	0.13	0.58%
19791_ModernPerio_25	521033	88.99	47581	8.13	16865	2.88	11.01%
19792_ModernPerio_26	933977	89.34	82969	7.94	28501	2.73	10.66%
19793_ModernPerio_27	459335	92.66	28279	5.7	8102	1.63	7.34%
19794_ModernPerio_28	468098	86.91	56703	10.53	13815	2.56	13.09%
19795_ModernPerio_29	533622	96.53	14442	2.61	4749	0.86	3.47%
19796_ModernPerio_30	563683	94.77	23670	3.98	7467	1.26	5.23%
19797_ModernPerio_31	408738	74.97	99976	18.34	36467	6.69	25.03%
19798_ModernPerio_32	86552	92.12	5882	6.26	1521	1.62	7.88%
19799_ModernPerio_33	537553	96.61	14168	2.55	4678	0.84	3.39%
19800_ModernPerio_34	311903	53.99	169224	29.29	96599	16.72	46.01%
19801_ModernPerio_35	551503	98.28	7139	1.27	2514	0.45	1.72%
19802_ModernPerio_36	345396	56.9	207903	34.25	53688	8.84	43.10%
19804_EBC1_B1ModernPerio	113	97.41	3	2.59	0	0	2.59%
19834_EBC1_B2ModernPerio	7947	98.39	88	1.09	42	0.52	1.61%
19837_EBC1_B3ModernPerio	63	100	0	0	0	0	0.00%
18794_HealthyModern_EBC1	13217	98.27	179	1.33	53	0.39	1.73%
18795_HealthyModern_EBC1	31023	99.02	236	0.75	70	0.22	0.98%
18796_HealthyModern_EBC1	37849	100	1	0	0	0	0.00%
18798_HealthyModern_EBC1	21687	59.52	12110	33.24	2638	7.24	40.48%
18800_HealthyModern_EBC1	939	100	0	0	0	0	0.00%

(180,183 sequences). Alpha diversity was measured using Shannon and observed species, and pairwise comparisons calculated with Kruskal-Wallis. Beta diversity was measured with Bray-Curtis distance index. Pairwise comparisons of beta diversity between groups was measured with PERMANOVA [26]. All p-values were false discovery rate corrected (reported p-value), and significant $p < 0.05$.

III Contaminant filtering with decontam

Visualisation of highly contaminated dental calculus samples

Using Bray-Curtis dissimilarity matrix, all historical and ancient calculus samples (n = 113), which included a number of duplicate individuals not used in downstream analysis (Table S1), alongside laboratory controls (n = 15), and environmental controls (n = 24), are visualised in MEGAN6 using principle coordinates analysis (PCoA) plot in order to identify samples that are poorly preserved and/or highly contaminated.

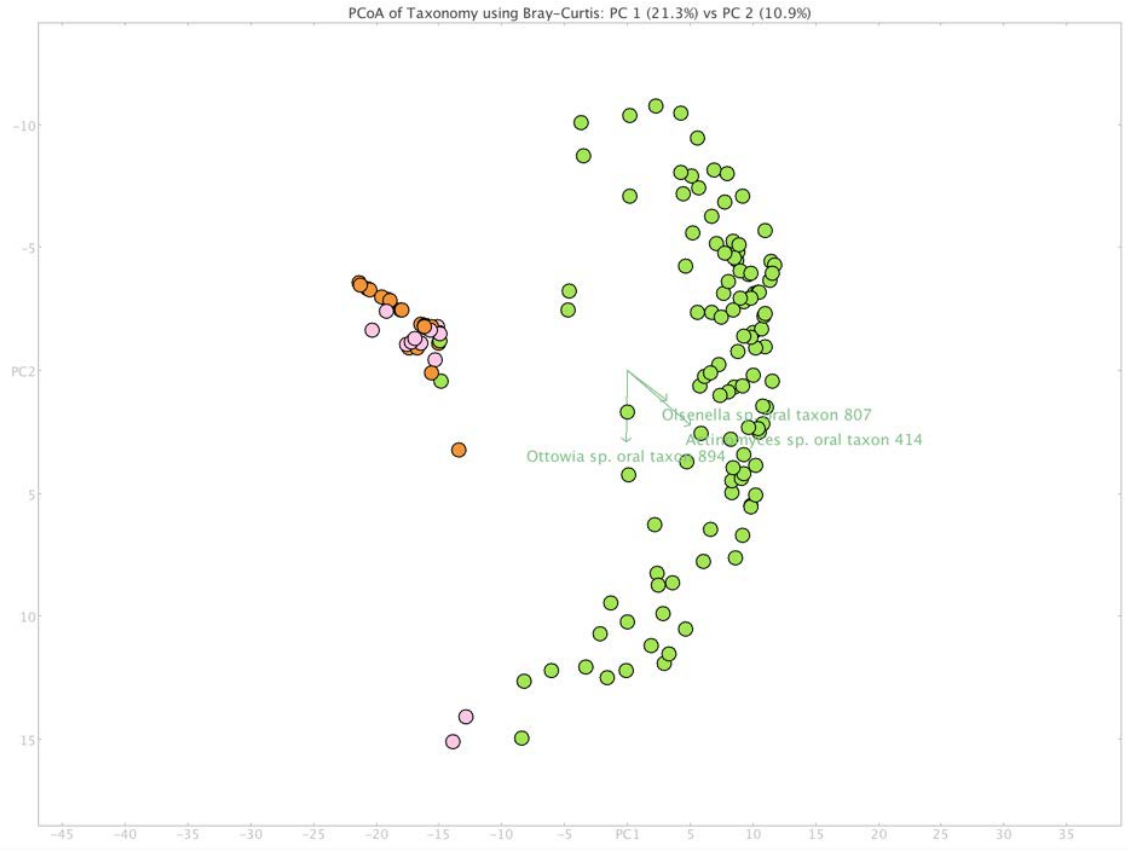


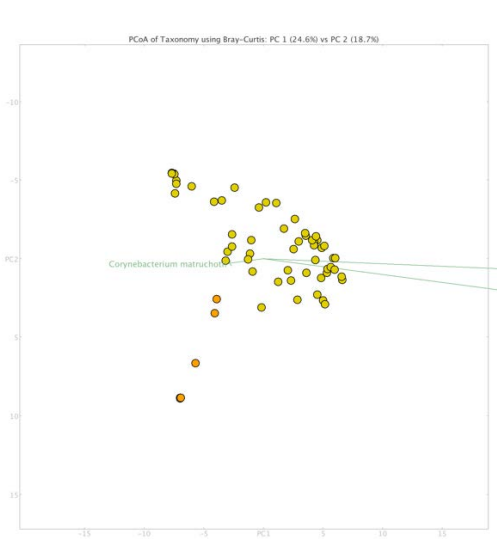
Figure S2. PCoA of Taxonomy using Bray-Curtis. All historical and ancient calculus samples (Green; n = 113), laboratory controls (Pink; n = 15), and environmental controls (Orange; n = 24) are plotted in three-dimensional space relative to their dissimilarity to one another. [Screenshot from MEGAN6 2019-02-18]

Two calculus samples, 13232 and 20459 (located within the cluster of control samples, Figure S2), were removed from further analysis due to their similarities with the laboratory and environmental control samples.

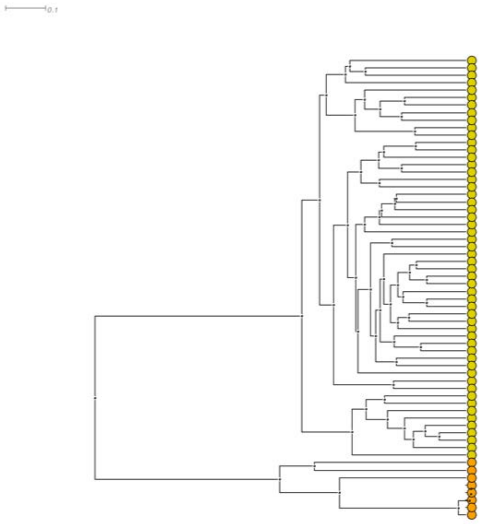
While the similarities between laboratory control samples 20520 and 17673 (bottom most laboratory control samples located at 15% on PC2 axis, Figure S1) to the dental calculus samples suggest potential cross-contamination, it is not possible to distinguish this from a PCoA plot alone. 20520 and 17673 were samples were re-

tained for decontam analysis. Furthermore, dental calculus samples 20472 and 20490 (located half-way between the clustering of control samples and the dental calculus samples, Figure S1) were thought to be either highly contaminated or contain a very weak biological signal and were retained for Decontam analysis.

No modern calculus samples were removed from further analysis as dental calculus samples clustered separately from laboratory controls (Figure S3).



3a. Unable to discriminate potentially contaminated calculus samples using PCoA plot of species taxonomy



3b. Using hierarchical clustering (UPMGA tree) confirmed the laboratory controls clustered separately from the dental calculus samples.

Figure S3. Visualisation of modern dental calculus samples (Yellow; n = 54) and laboratory controls (Orange; n = 8) using Bray-Curtis dissimilarity matrix. [Screenshots from MEGAN6 2019-02-18].

Identifying contaminant species with decontam

Decontam implements a statistical classification to identify of contaminant taxa based on prevalence within a defined set of 'negative controls' relative to the 'biological sample'. Biom tables of species-level taxonomy is exported from MEGAN6, then imported into R. Multiple decontam tests were run to separate samples by DNA extraction laboratory and respective laboratory controls and maximise the ability to detect contamination prevalence.

Historical and ancient calculus vs laboratory controls

Decontam was first analysed using only calculus samples extracted at the University of Adelaide's specialised ancient DNA laboratory (n = 89), with respective laboratory controls (n = 15). Using a stringent threshold of 0.7, a total of 159 taxa were

identified as contaminants, in such that the taxa were more prevalence in laboratory control samples than within dental calculus samples (Table S4). Of these 159 species, eight taxa were not assigned to species-level, thus the only remaining 151 species were used for downstream filtering. A total of 307 assigned taxa were unable to be classified due to their prevalence in only one sample.

Historical and ancient calculus vs environmental controls

Only dental calculus samples collected from archaeological skeletal remains were included within the comparison with environmental controls. Decontam was ran for all ancient and historic calculus samples (extracted and downloaded; n = 111) vs environmental control samples (n = 24) at species level. A total of 440 assigned taxa were unable to be classified due to their presence in only one sample. Using a stringent threshold of 0.6, a total of 179 taxa were identified as 'contaminants', in such that their prevalence greater in soil samples than within dental calculus samples (Table S5). Of this, 15 taxa were not assigned to species-level, thus the remaining 164 species were used for downstream filtering.

Modern dental calculus vs laboratory controls

To identify contaminants specific to University of Adelaide's specialised modern microbiome laboratory, only modern dental calculus samples (n = 54) were run alongside their respective laboratory controls (n = 8). Using a stringent threshold of 0.6, a total of 11 taxa were identified as 'contaminant' taxa, in such that sequence prevalence was greater in laboratory control samples than within dental calculus samples (Table S6). However, one taxon, *Actinobaculum* sp. oral taxon 183, identified as a 'contaminant' despite its prior identification within the dental plaque [27]. Furthermore, only 11 contaminants appears to be an unusually low number relative to previous research on contaminant profiles [28], or even the prior two decontam analyses of ancient and historic calculus. Potentially, the calculation of prevalence between 54 dental calculus with only eight laboratory controls limits the ability to identify cross-contamination. Thus, in order to get a clearer signal of contaminants within the modern calculus (despite the difference between laboratories) decontam analysis was rerun using all extracted ancient, historic, and modern dental calculus samples and all respective laboratory controls to increase statistical power.

Historical, ancient, and modern calculus vs laboratory controls

All ancient/historical (n = 89) and modern (n = 54) samples with their respective extraction blank controls (n = 23) were analysed together (N =166) in decontam. Using a threshold of 0.6, 96 contaminant taxa were identified, with only four taxa

not identified to the species level. A total of 340 taxa were unable to be classified due to their presence in only one sample (Table S7).

In comparison between the three different decontam tests (Historical and ancient calculus, Modern calculus, and Historical, ancient, and modern calculus; groupings referred hereinafter to as 'Ancient', 'Modern' and 'All-calculus' respectively), all taxa from the Modern contaminant list, except *Actinobaculum* sp. oral taxon 183, were identified as a contaminants within Ancient and/or All-calculus decontam lists. An additional nine taxa were identified as contaminants within the All-calculus decontam list that had not been previously identified within either Modern or Ancient decontam results. Yet, 71 different taxa had been identified within the Ancient decontam results that were not identified by All-calculus. Therefore, in order to filter dental calculus samples, all contaminants identified in all decontam tests were filtered from all dental calculus samples.

Remove contaminant sequences

As 1950s France (n = 5) dental calculus samples did not have available extraction blank controls, nor were at any point buried and in contact with the environment, these samples were not included within any decontam analysis.

All contaminants identified through the combination of both environmental and laboratory control decontam results totalled 418 taxa, in which 102 species were duplicated across the multiple decontam tests. Accordingly, a total of 286 species were used to filter contaminants from all dental calculus samples.

Removing contamination with QIIME2

Filtering historical and ancient dental calculus

After exporting all historical and ancient calculus samples (n = 116) from MEGAN6, all sequences assigned taxonomy at species-level were imported into QIIME2 (v. 2019.1), with a total of 428 species assignments, and a total sequence count of 176,437,951. Taxa classified as 'contaminants' from decontam analysis were filtered from the calculus samples, leaving a total of 299 species assignments with 174,720,382 sequences.

Any sample with more than 10% of total sequences filtered were removed from downstream analyses. Four historical calculus samples and three 1950s samples were subsequently removed. Furthermore, all duplicate samples (*i.e.* dental calculus samples from different teeth of the same individual) were removed from the filtered dataset. A total of 95 dental calculus samples, containing 221 species, with a total of 166,448,366 sequences, were maintained for downstream analysis.

Filtering modern dental calculus

Modern dental calculus samples (n = 54) had a total of 228 species assignments and a total sequence count of 28,530,844. Filtering all species classified by decontam as contaminants left 206 species and a total sequence count of 27,742,736. Consistent with the stringent cut off used with historical and ancient samples, two modern dental calculus samples with more than 10% of total sequences filtered were removed from downstream analyses. Finally, after the removal of all duplicate samples, a total of 35 modern dental calculus samples containing 185 species assignments, with a total of 21,408,656 sequences, maintained for downstream analysis.

Table S3. **Total sequences assigned to species taxonomy** Before filtering contaminants. Supplied electronically: **5_Supplementary_TableS3.csv**

Table S4. **Decontam output:** for historical and ancient calculus vs laboratory controls. Supplied electronically: **5_Supplementary_TableS4.csv**

Table S5. **Decontam output:** for historical and ancient calculus vs environmental controls. Supplied electronically: **5_Supplementary_TableS5.csv**

Table S6. **Decontam output:** for modern dental calculus vs laboratory controls. Supplied electronically: **5_Supplementary_TableS6.csv**

Table S7. **Decontam output:** for historical, ancient, and modern calculus vs laboratory controls. Supplied electronically: **5_Supplementary_TableS7.csv**

Table S8. **Dental calculus samples sequence information (n = 170).** Including raw sequencing, percentage assigned taxonomy, and contaminant sequences removed. Supplied electronically: **5_Supplementary_TableS8.csv**

IV Oral geography biases microbial communities

Oral geography has been previously noted in literature to impact the microbial plaque communities forming the tooth surface [29], linked to the biochemical and biophysical properties of the oral cavity [30]. Within dental calculus research, this may confound correlations with external variables, and up until recently [11], had not been tested with ancient dental calculus. The following analysis looked to test the statistical significance of tooth type within each dataset, and where significance was found, we controlled for tooth type by subsequently processing the tooth type-specific samples independently. However, due to the differing sampling schemes used between geographic groups and published datasets, it becomes difficult to disentangle potential cultural or geographic signals from interpopulation oral geography.

Significance of tooth type in Ancient and Historic datasets

In the analysis of biases present within the dataset, oral geography, or tooth type (molar, premolar, canine, or incisor) from which the calculus was sampled, from was found statistically significant within the ancient and historic dataset with both alpha (Shannon Kruskal-Wallis, $p = 0.0008$, $H = 21.02$; observed species Kruskal-Wallis, $p = 0.00002$, $H = 29.42$) and beta diversity (Bray-Curtis PERMANOVA, $p = 0.001$, pseudo-$F = 2.74$).

Alpha diversity detected differences between incisors ($n = 19$) and molars ($n = 38$) (Shannon Kruskal-Wallis pairwise, $p = 0.04$, $H = 5.30$; observed species Kruskal-Wallis pairwise, $p = 0.01$, $H = 10.14$). Beta diversity as measured by Bray-Curtis dissimilarity metric noted differences between canines ($n = 7$) and molars ($n = 38$) (pairwise PERMANOVA, $p = 0.04$, pseudo-$F = 2.59$), incisors ($n = 19$) and molars ($n = 38$) (pairwise PERMANOVA, $p = 0.01$, pseudo-$F = 4.79$), incisors ($n = 19$) and premolars ($n = 9$) (pairwise PERMANOVA, $p = 0.04$, pseudo-$F = 2.95$), and between molars ($n = 38$) and premolars ($n = 9$) (pairwise PERMANOVA, $p = 0.05$, pseudo-$F = 2.38$).

Significant differences between pre-IR and IR populations with single tooth type

To test difference between teeth type, twenty samples were removed from the following analyses due to missing metadata, including all IR Switzerland ($n = 12$), pre-IR Netherlands ($n = 2$), Medieval Germany ($n = 2$), and IR Germany ($n = 2$).

Furthermore, Australian IR samples were included (n = 12) as an IR population to increase statistical power.

All samples collected from a molar tooth (n = 38) were tested for microbial differences between pre-IR (n = 33) and IR individuals (n = 5); which included Medieval Ireland (n = 13), Medieval to pre-IR England (n = 20), and IR Australia (n = 3), IR Germany (n = 1), and IR England (n = 1). As seen in tests including all teeth, there were no significant differences detected in alpha diversity (Shannon Kruskal-Wallis, p = 0.68, H = 0.17; observed species Kruskal-Wallis, p = 0.44, H = 0.61). Furthermore, beta diversity supported significant differences between pre-IR (n = 33) and post-IR (n = 5) oral microbial communities using Bray-Curtis (PERMANOVA pairwise, p = 0.023, pseudo-F = 2.38).

These tests were repeated using all samples collected from incisor teeth (n = 19), which includes populations IR Germany (n = 5), IR England (n = 1), IR Australia (n = 3), and Medieval to pre-IR England (n = 10) samples. Again, no significant alpha diversity differences were detected between pre-IR (n = 10) and IR (n = 9) populations (Shannon Kruskal-Wallis, p = 0.87, H = 0.03; observed species Kruskal-Wallis, p = 0.49, H = 0.48). Significant differences were detected between pre-IR (n = 10) and post-IR (n = 9) oral microbial communities using Bray-Curtis (PERMANOVA pairwise, p = 0.033, pseudo-F = 2.31).

In testing the pre-IR and IR differences within England only, there was no significant differences detected between tooth type with either alpha or beta diversity (Shannon Kruskal-Wallis, p = 0.11, H = 6.13; observed species Kruskal-Wallis, p = 0.15, H = 5.37; Bray-Curtis PERMANOVA, p = 0.08, pseudo-F = 1.61).

Tooth type had no impact on IR geographic differences

Due to the missing metadata of the Switzerland IR population, we could not test IR geographic differences without the inclusion of the Australian IR populations (n = 12). We found no support for differences driven by impact of tooth type between three populations, Australia (n = 12), England (n = 4), and Germany (n = 7), in alpha diversity (Shannon Kruskal-Wallis, p = 0.08, H = 5.13; observed species Kruskal-Wallis, p = 0.68, H = 1.52). Furthermore, we did not detect any significant impact of tooth type driving microbial differences with beta diversity (Bray-Curtis PERMANOVA, p = 0.122, pseudo-F = 1.45).

Significance of tooth type in historic and modern dataset

Significant differences were detected between different tooth types within the modern and historic dataset using alpha (Shannon Kruskal-Wallis, p = 0.03, H = 12.28; Observed species Kruskal-Wallis, p = 0.02, H = 13.26) and beta diversity (Bray-

Curtis PERMANOVA, p = 0.01, pseudo-F = 3.02).

Despite the significance of tooth type groups with alpha diversity, no pairwise comparisons between different tooth types showed any significant difference (Shannon Kruskal-Wallis, p > 0.18, H range = 0.02–3.63; observed species Kruskal-Wallis, p > 0.29, H range = 0.05–1.98). Beta diversity pairwise comparisons showed significant differences between incisors (n = 35) and molars (n = 9) (Bray-Curtis pairwise PERMANOVA, p = 0.015, pseudo-F = 4.74), and between incisors (n = 35) and premolars (n = 8) (Bray-Curtis pairwise PERMANOVA, p = 0.015, pseudo-F = 4.94).

Significant differences between modern and IR populations with single tooth type

As the majority of healthy-modern samples were collected from the incisor teeth (n = 14), we could only test differences between healthy-modern and IR populations with an incisor dataset. All IR Switzerland (n = 12) were excluded, leaving Australia (n = 3), Germany (n = 5), and England (n = 1). We confirm there were no significant alpha differences detected between healthy-modern individuals (n = 14) and IR individuals (n = 9) (Shannon Kruskal-Wallis, p = 0.71, H = 0.14; observed species Kruskal-Wallis, p = 0.16, H = 1.93). Furthermore, we were able to detect significant beta diversity differences with Bray-Curtis (PERMANOVA, p = 0.002, pseudo-F = 9.61).

Likewise, using only incisors from modern periodontal patients (periodontal-modern; n = 12), we were able to reproduce the same results found with all-teeth found between IR individuals (n = 9; Australia (n = 3), Germany (n = 5), and England (n = 1)). Firstly, we did not detect any significant differences in alpha diversity (Shannon Kruskal-Wallis, p = 0.71, H = 0.14; Observed species Kruskal-Wallis, p = 0.52, H = 0.41). But, differences between periodontal-modern and IR populations were still supported by Bray-Curtis (PERMANOVA, p = 0.001, pseudo-F = 11.90).

Despite the smaller dataset, molar teeth also replicated previous results between periodontal-modern (n = 4) and IR populations (total n = 5; Australia (n = 3), Germany (n = 1), and England (n = 1)). No significant alpha diversity differences could be detected between groups (Shannon Kruskal-Wallis, p = 0.14, H = 2.16; observed species Kruskal-Wallis, p = 1, H = 0). Yet, the microbial community differences between periodontal-modern and IR individuals were supported by Bray-Curtis (PERMANOVA, p = 0.025, pseudo-F = 3.90).

Differences maintained after correcting for taphonomy

After correcting for taphonomy (**Section VI**), beta diversity differences between modern populations and IR individuals using a single tooth dataset were retested. Healthy-modern individuals (n = 14) and IR individuals (n = 9) sampled from incisors, retained significance beta diversity differences with Bray-Curtis (PERMANOVA, p = 0.001, pseudo-F = 10.59) after correcting for taphonomy.

Correspondingly, periodontal-modern (n = 12) and IR individuals (n = 9) sampled from incisors maintained significant beta diversity differences with Bray-Curtis (PERMANOVA, p = 0.001, pseudo-F = 13.89) after correcting for taphonomy. Furthermore, the molar dataset from periodontal-modern (n = 4) and IR populations (n = 5) upheld beta diversity differences (Bray-Curtis PERMANOVA, p = 0.025, pseudo-F = 4.09).

Post-war samples suggest transition with single tooth type

As both 1950s post-war individuals were both sampled from molar teeth, we were unable to test for differences using any health-modern samples. We tested significant differences between post-war (n = 2), periodontal-modern (n = 4) and IR populations (total n = 5; Australia (n = 3), Germany (n = 1), and England (n = 1)), and again, found no significant alpha diversity differences between groups (Shannon Kruskal-Wallis, p = 0.10, H = 4.66; observed species Kruskal-Wallis, p = 0.64, H = 0.89). While beta diversity supported significant differences between populations (Bray-Curtis PERMANOVA, p = 0.01, pseudo-F = 2.53), we found no significant differences between post-war individuals (n = 2) and periodontal-modern (n = 4) (Bray-Curtis pairwise PERMANOVA, p = 0.19, pseudo-F = 1.76), nor between post-war (n = 2) and IR individuals (n = 5) (Bray-Curtis pairwise PERMANOVA, p = 0.19, pseudo-F = 1.51). These results are maintained after correcting for taphonomy, with beta diversity supporting significant differences between populations (Bray-Curtis PERMANOVA, p = 0.015, pseudo-F = 2.51). Yet, no significant differences were detected between post-war individuals (n = 2) and periodontal-modern (n = 4) (Bray-Curtis pairwise PERMANOVA, p = 0.26, pseudo-F = 1.75), nor between post-war (n = 2) and IR individuals (n = 5) (Bray-Curtis pairwise PERMANOVA, p = 0.38, pseudo-F = 1.15).

Species Group Significance

	Statistics			Mean Relative Sequences			
Species	**Test-Statistic**	**P**	**FDR_P**	**Switzerland**	**Germany**	**Australia**	**England**
Campylobacter gracilis	22.13121	0.00006	0.01109	1133.17	1884.44	145.08	240.50
Cardiobacterium valvarum	17.70176	0.00051	0.02956	1101.75	1033.33	22.17	0.00
Selenomonas sp. oral taxon 138	17.38255	0.00059	0.02956	0.00	386.89	0.00	0.00
Pseudoramibacter alactolyticus	16.49318	0.00090	0.02956	2375.75	442.78	7388.25	7868.50
[Eubacterium] sulci	16.48130	0.00090	0.02956	370.17	29.56	472.17	2385.00
Ottowia sp. oral taxon 894	16.28923	0.00099	0.02956	13403.33	16506.78	495.75	1673.75
Bacteroidetes oral taxon 274	15.98258	0.00114	0.02956	2871.83	6061.67	1514.83	898.50
Lautropia mirabilis	15.57654	0.00138	0.03133	5594.00	11373.00	644.58	239.75
Selenomonas sp. oral taxon 892	14.95511	0.00186	0.03624	209.58	374.00	0.00	0.00
Prevotella sp. oral taxon 472	14.79321	0.00200	0.03624	557.42	1016.11	87.08	0.00
Anaerovorax odorimutans	14.31787	0.00250	0.03780	398.00	0.00	460.75	3010.50
Selenomonas sp. oral taxon 920	14.31530	0.00251	0.03780	29.50	318.56	0.00	0.00
Selenomonas sp. oral taxon 126	13.93728	0.00299	0.04165	45.00	405.00	0.00	0.00
Prevotella maculosa	13.52039	0.00364	0.04701	0.00	272.56	0.00	0.00
Selenomonas noxia	13.30338	0.00402	0.04781	287.25	630.33	45.75	0.00
[Eubacterium] infirmum	13.19865	0.00423	0.04781	614.08	230.11	767.58	2804.00
Corynebacterium matruchotii	12.91228	0.00483	0.04817	5495.58	5907.22	327.83	365.50
Centipeda periodontii	12.85155	0.00497	0.04817	36.08	204.44	0.00	0.00
Peptostreptococcaceae bacterium oral taxon 113	12.72747	0.00526	0.04817	3564.50	785.78	3095.25	7274.75
Capnocytophaga sp. ChDC OS43	12.70384	0.00532	0.04817	995.50	1257.00	186.17	0.00
Catonella morbi	12.44252	0.00601	0.05133	649.50	1193.56	276.17	1539.50
Peptoniphilus sp. oral taxon 386	12.21475	0.00668	0.05133	454.25	32.67	451.58	2531.50
[Eubacterium] brachy	12.19943	0.00673	0.05133	1058.17	408.00	1394.67	6458.25
Leptotrichia buccalis	12.17517	0.00681	0.05133	391.92	710.67	91.17	0.00
Prevotella saccharolytica	12.08328	0.00710	0.05143	218.50	530.22	25.33	0.00

Table S9. Kruskal-Wallis Group Significance calculated at species-level Differences in the mean relative abundance of sequences between between IR populations

Genera Group Significance

	Statistics			Mean Relative Sequences	
Genera	**Test-Statistic**	**P**	**FDR_P**	**Swiss&Germany**	**Australia&England**
Campylobacter	22.88632	0.00000	0.00012	1779.76	185.13
Cardiobacterium	14.62265	0.00013	0.00251	1553.90	122.06
Ottowia	14.98335	0.00011	0.00251	14733.38	790.25
Pseudoramibacter	14.24547	0.00016	0.00251	1547.33	7508.31
Selenomonas	14.01048	0.00018	0.00251	2212.71	224.13
Prevotella	13.25631	0.00027	0.00312	1598.95	99.00
Corynebacterium	12.73143	0.00036	0.00351	5901.43	337.25
Lautropia	12.49995	0.00041	0.00351	8070.71	543.38
Neisseria	11.04205	0.00089	0.00683	3275.67	160.06
Capnocytophaga	9.85883	0.00169	0.01136	3197.57	558.31
Eikenella	9.73136	0.00181	0.01136	705.10	79.94
Streptococcus	9.39523	0.00218	0.01251	7686.57	1597.31
Leptotrichia	9.19784	0.00242	0.01286	1226.52	278.06
Peptoanaerobacter	7.61333	0.00579	0.02856	94.90	428.75
Porphyromonas	7.43517	0.00640	0.02942	1543.24	2731.75
Methanobrevibacter	7.12216	0.00761	0.03283	12033.57	50823.38
Aggregatibacter	6.69319	0.00968	0.03928	822.43	15.69
Parvimonas	6.15172	0.01313	0.05033	464.57	1639.63

Table S10. Kruskal-Wallis Group Significance calculated at genera-level. Differences in the mean relative abundance of sequences between between IR populations

Phyla Group Significance

	Statistics			Mean relative sequences	
Phyla	**Test-Statistic**	**p-value**	**FDR p-value**	**Modern sample**	**IR samples**
Chloroflexi	57.67420	0.00000	0.00000	327.19	13354.78
Euryarchaeota	52.48263	0.00000	0.00000	0.00	28835.51
Synergistetes	38.28626	0.00000	0.00000	1072.59	7094.54
Proteobacteria	20.66869	0.00001	0.00002	54312.38	21141.73
Spirochaetes	17.91130	0.00002	0.00005	2464.76	5289.27
Fusobacteria	13.17193	0.00028	0.00052	4567.08	1514.68
Candidatus Saccharibacteria	9.74873	0.00179	0.00282	814.84	1426.32
Elusimicrobia	2.02740	0.15448	0.21242	0.00	45.65
Bacteroidetes	0.77630	0.37827	0.45581	22922.14	17938.03
Actinobacteria	0.66621	0.41438	0.45581	68651.08	55309.38
Firmicutes	0.33452	0.56301	0.56301	24990.95	28153.27

Table S11. Kruskal-Wallis Group Significance calculated at phyla-level. Differences in the mean relative abundance of sequences between between IR populations

V Correcting for taphonomic bias

The processes of decay and preservation upon archaeological materials, such as dental calculus, is known as taphonomy. It has been suggested that taphonomy could potential influence the microbial community within the post-mortem environment, biasing microbial community reconstruction [12, 14]. In fact, analyses of long-term room temperature storage of dental calculus revealed significance differences between relative abundance of phyla Fusobacteria, Proteobacteria, and Bacteroidetes (see **Chapter 4** for details). This means significant differences detected between historical and modern dental calculus samples may be influenced by taphonomy. Here, we tried to account for taphonomy within our analysis of historical and modern microbiomes.

Testing for taphonomy

As the relative abundance between three main phyla—Fusobacteria, Proteobacteria, and Bacteroidetes—were potentially influenced by taphonomic processes (**Chapter 4**), we initially looked to see what significant differences at the phyla level could be detected between our modern population (n = 38) and our historic IR population (n = 37). With Kruskal-Wallis group significance, we found significant differences in the mean relative abundance of Fusobacteria and Proteobacteria phyla between groups (Table S11; Kruskal-Wallis; Fusobacteria, $p = 0.0005$, $t = 13.17$; Proteobacteria, $p = 0.00002$, $t = 20.67$). Furthermore, the difference in Proteobacteria relative abundance replicated taphonomic patterns of with a lower mean relative abundance within the historic IR population compared to modern populations. However, Fusobacteria also had a lower mean relative abundance in IR compared to modern populations, opposite to what was expected with taphonomic processes. Conceivably, as previous ancient dental calculus research [7] has noted an increase in Fusobacteria through time associated with the consumption of carbohydrate sugars, the mean relative abundance of Fusobacteria phyla may be confounded by temporal patterns of microbial community alterations. Therefore, we proceeded with the analysis under the assumption that both Fusobacteria and Proteobacteria may be influenced by taphonomy.

We reran all analyses comparing modern and historic populations by removing all species within Fusobacteria and Proteobacteria phyla. However, due to the removal of assigned sequences, rarefaction depth was lowered to 143,674 sequences per sample, the lowest sequencing depth of any sample within the historic dataset. Ensuring this lowered rarefaction depth did not significantly alter the reported results, we report the historical and modern comparisons below (Table S12)

at 180,183 rarefactions, 143,674 rarefactions, and taphonomy-corrected 143,674 rarefactions. Post-war sample comparisons were most impacted by rarefaction depths or taphonomy-correction, indicating the insufficient statistical power. Tests comparing the larger datasets showed negligible differences between rarefaction depth, supporting the taphonomy-correction depth to be sufficient to support the overall differences between modern and historic populations.

Healthy-modern (n = 15) vs IR (n = 37)

Bray-Curtis	q value	pseudo-F
180,183 rarefaction	**0.001**	19.8160
143,674 rarefaction	**0.001**	19.7711
taphonomy-corrected 143,674 rarefaction	**0.001**	22.6270

Periodontal-modern (n = 20) vs IR (n = 37)

Bray-Curtis	**q value**	**pseudo-F**
180,183 rarefaction	**0.001**	17.0713
143,674 rarefaction	**0.001**	17.0745
taphonomy-corrected 143,674 rarefaction	**0.001**	20.5033

Post-war (n = 2) vs IR (n = 37)

Bray-Curtis	**q value**	**pseudo-F**
180,183 rarefaction	0.144	1.4919
143,674 rarefaction	0.140	1.4978
taphonomy-corrected 143,674 rarefaction	0.118	1.5630

Periodontal-modern (n = 20) vs Post-war (n = 2)

Bray-Curtis	**q value**	**pseudo-F**
180,183 rarefaction	0.0648	1.8559
143,674 rarefaction	0.0804	1.8636
taphonomy-corrected 143,674 rarefaction	0.0744	2.1776

Healthy-modern (n = 15) vs Post-war (n = 2)

Bray-Curtis	**q value**	**pseudo-F**
180,183 rarefaction	0.054	2.5109
143,674 rarefaction	**0.047**	2.5143
taphonomy-corrected 143,674 rarefaction	**0.008**	2.8119

Table S12. Bray-Curtis pairwise PERMANOVA at different rarefaction depths. q-value denotes FDR corrected p-value; significant q-values are bold ($q < 0.05$). Post-war sample comparisons were impacted by differing rarefaction depths due to the sample size ($n = 2$).

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