# Insights from a multidisciplinary approach to Indigenous microbiome research

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# Abstract

In recent decades, advances in high-throughput DNA sequencing and other techniques have ushered in a marked expansion of research and public interest in microbial communities. Within this trend, studies of the human microbiome – the microorganisms living in and on the human body together with their genes and environment – have shifted our understanding of health and disease, and even of what it means to be human. Research has revealed the fundamental importance of the microbiome in supporting human health and homeostasis through nutrient acquisition, immune training, and protection against infectious disease. Insight has also been gained into links between the microbiome and many chronic diseases and health conditions, as well as the myriad mutable lifestyle and environmental factors that can influence the microbiome. Targeting therapies at these microbial communities therefore appears to hold tremendous medical potential.

Indigenous peoples have arguably been underserved by human microbiome research thus far – despite Indigenous people in many countries being disproportionately affected by chronic health conditions that microbiome-based therapies hold promise for treating. What evidence has been collected to date suggests that Indigenous groups may harbour microbiota distinct from those of non-Indigenous counterparts, with the causes and health implications of this distinction being poorly understood. Further research to improve understanding of the roles that microbiomes play in Indigenous health will be important for ensuring that benefits from future microbiome-based therapies and diagnostics accrue to Indigenous people rather than reinforcing existing health inequities. Additional benefits of microbiome research for Indigenous communities could include new microbiome knowledge relevant to community priorities, educational and capacity-building opportunities, and intellectual property generation and commercial benefit.

However, many ethical concerns can also arise from microbiome research, particularly in Indigenous contexts. For example, stigmatisation, cultural harm, and exclusion from sovereignty or control over data, samples and intellectual property relating to the microbiome are among the risks that Indigenous people must weigh when considering participation in microbiome research projects. These risks extend outside the sphere of human-associated

microbiota into environmental metagenomic research, and are compounded by a general lack of cultural competency training and attention to Indigenous perspectives in the microbiome field as a whole.

Multidisciplinary approaches that engage both microbiome science and the ethical, legal and social implications of such research represent a promising way forward. To this end, this thesis presents a multidisciplinary investigation of Indigenous microbiome research, defined as microbiome or metagenomic research that involves Indigenous people as research participants or stakeholders. Chapters include both scientific studies of oral microbiota and oral health in Aboriginal Australians and Torres Strait Islanders, and discussion and analysis of (bio)ethical and social implications of microbiome research for Indigenous peoples. Further integration of microbiome science, bioethics and Indigenous leadership and perspectives holds promise for realising the benefits and minimising the harms of future research.

# Thesis declaration

I 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. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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# Thesis introduction

Nearly every surface on the planet is covered by microorganisms that fulfil functions essential to human and planetary health, from oxygen production and nitrogen fixation to nutrient acquisition and immune development. We are constantly interacting with microorganisms, yet it is easy for this fact to escape our conscious awareness. Nevertheless, recent years have seen an explosion of interest in the huge diversity of the microbial world. Technical advances in culture-independent approaches to microbiology have enabled investigation of microorganisms that are difficult or impossible to culture in laboratory settings and facilitated the study of microbial communities in addition to individual species. In the burgeoning field of metagenomics, DNA sequence analysis allows the reconstruction of taxonomic composition, evolutionary relationships, and functional potential from complex microbial communities without the need for culturing. Employing similar approaches to metatranscriptomics (studying microbial gene expression through RNA transcripts) and metabolomics (studying metabolites produced by microorganisms or present in their environment) can provide even greater insight into microbial species and communities. Through the technical improvement and decreasing costs of culture-independent approaches, the study of many microbial ecosystems has become far more tractable.

One such system is the human microbiome, a collective term for the microorganisms that live in and on the human body (microbiota), together with their genetic material, environment and theatre of activity (Berg et al. 2020). Distinct microbial communities are found at different sites in the body, such as the gastrointestinal tract, oral cavity, and skin (The Human Microbiome Project Consortium 2012). Our microbial companions participate in food digestion, vitamin synthesis, protection against infectious disease, and immune system training and modulation (Guarner and Malagelada 2003; Abt and Pamer 2014; Leger et al. 2017; Hooper, Littman, and Macpherson 2012; Gensollen et al. 2016). Growing evidence indicates that human microbiomes also modulate or contribute to many non-communicable diseases and health conditions, including inflammatory bowel diseases, oral and colorectal cancers, diabetes, periodontal disease, dental caries, eczema, psoriasis, and mental health conditions (Chuong et al. 2018; Wang and Ganly 2014; Fong, Li, and Yu 2020; Vallianou, Stratigou, and Tsagarakis 2018; Abusleme et al. 2013; Burne et al. 2012; Weyrich et al. 2015; Clapp et al. 2017). Human-

associated microbial communities can respond to a wide range of influences over an individual's lifetime, such as diet, medication, and other cultural, lifestyle and environmental factors (Bokulich et al. 2016; Blaser 2016; Muegge et al. 2011; Zimmer et al. 2012; David et al. 2014; Korpela et al. 2016; 2018). A degree of host genetic influence or vertical transmission of microbiota has also been proposed for some body sites (Blekhman et al. 2015; Corby et al. 2007; Demmitt et al. 2017; Gomez et al. 2017; Li et al. 2007; Shaw et al. 2017; Stahringer et al. 2012; Winkelhoff and Boutaga 2005). Researchers are currently exploring several avenues to modulate or improve human microbiota to support health, including probiotics (health-promoting microorganisms), prebiotics (substances that promote the growth of health-associated microbial species), postbiotics (health-promoting microbial metabolites usually produced by the microbiome), and microbiota transplants (Mimee, Citorik, and Lu 2016; Fong, Li, and Yu 2020; Wong and Levy 2019; Sokol et al. 2020; Nascimento 2017). Other recent studies have investigated the microbial interactions between humans and our physical environment, hypothesising that exposure to highly diverse environmental microbes may contribute to human wellbeing (Mills et al. 2017; Selway et al. 2020). Together with the comparative ease of study thanks to recent technical advances, this combination of importance for human health and apparent amenability to manipulation has made microbiomes a highly attractive prospect for research.

When I took microbiology as one of my undergraduate majors, however, the curriculum largely focused on individual pathogens and model organisms. The history of microbiology, as it is classically told, begins with Antonie van Leeuwenhoek, the 17$^{th}$-century Dutch textile merchant who made the first recorded observations of bacteria and protozoa under microscopes he had built himself (Opal 2009). Leeuwenhoek used his microscopes to study materials as diverse as rain and lake water, insects, and bodily fluids; he was apparently driven largely by curiosity about the natural world:

> "My work, which I've done for a long time, was not pursued in order to gain the praise I now enjoy, but chiefly from a craving after knowledge, which I notice resides in me more than most other men." Leeuwenhoek, Letter of 12 June 1716, cited in (Lane 2015).

The better part of the next three centuries saw most prominent microbiologists on a journey to unravel the origins, transmission, and prevention of infectious diseases. This is what I was taught at university – how the two giants of 19th-century microbiology, Louis Pasteur and Robert Koch, along with their colleagues and students, validated the germ theory of disease and pioneered pure culture and other laboratory techniques that allowed them to isolate and study microbial pathogens (Opal 2009). We learned about Koch's famous postulates for verifying a single infectious organism as the cause of a given disease. We were trained in a style of microbiology that was detailed, precise, and tended towards the reductive. I dutifully memorised different cell wall structures, secretion systems, infection pathways, virulence factors and classes of antibiotics; in laboratory practicals, I learned how to streak an agar plate and how to design a plasmid gene expression vector. These topics are undeniably important, but in retrospect only exposed me to a small section of the microbial world.

Against this background, a guest lecture on the evolution and functions of the human microbiome in the final year of my undergraduate degree was revelatory. Instead of looking species by species and disease by disease, suddenly I saw communities, ecology, and interconnection. And why had no one ever told me before about the massive numbers of microbial cells and species in my body, whose functions I had never appreciated – who might hold the key to solving numerous diseases and alleviating suffering? The lecture set me on a research path leading to my present thesis, via an Honours project focusing on the human oral microbiome and Indigenous oral health in Australia (Handsley-Davis 2016). That experience helped shape my cultural awareness and my conviction that ethical and social questions are as important as scientific ones, setting the foundation for my PhD project.

The overall aim of my PhD is to take a multidisciplinary approach towards Indigenous microbiome research, which I define broadly as microbiome and metagenomic research that involves Indigenous people as research participants or stakeholders. Indigeneity is a complex concept that encompasses diverse peoples and contains an element of self-identification and contextual specificity. Rather than attempting to conclusively define "Indigenous", the United Nations Permanent Forum on Indigenous Issues has identified a series of elements or characteristics that are often true, but not essential, of peoples who define themselves as Indigenous (United Nations Secretariat of the Permanent Forum on Indigenous Issues 2009).

These elements include: self-identification as Indigenous; historical continuity with pre-colonial and/or pre-settler societies; strong links to territories and surrounding natural resources; distinct social, economic or political systems and language, culture, and beliefs; and resolve to maintain and reproduce ancestral environments and systems as distinctive peoples and communities (United Nations Permanent Forum on Indigenous Issues 2009). As noted in the first item, the right of peoples to define their own membership and identity is key. Individuals or groups may prefer terminology that emphasises diversity over pan-Indigeneity (e.g. First Nations), or that refer to a specific nation or polity (e.g. Arrernte, Cherokee), over the broader term 'Indigenous', or use all of the above depending on context (L. Pearson 2021; Charron 2019; Peters and Mika 2017).

There are many different First Nations of the continent now known as Australia and nearby islands. The term 'Indigenous Australians' encompasses Aboriginal Australians, the first peoples of mainland Australia and Tasmania, and Torres Strait Islanders, the first peoples of the Torres Strait Islands or Zenadth Kes between north-eastern Australia and Papua New Guinea, with each of these broad groupings containing many cultures and nations (Australian Institute of Aboriginal and Torres Strait Islander Studies (AIATSIS) 2021). Like many other Indigenous peoples worldwide, Aboriginal Australians and Torres Strait Islanders have experienced a history of invasion, genocide and forced assimilation since European colonisation, and currently face ongoing health disparities related to colonisation, trauma and discrimination (Gracey and King 2009; King, Smith, and Gracey 2009; Valeggia and Snodgrass 2015; Anderson et al. 2016; Mitrou et al. 2014).

Improving Indigenous health has been a concern of Australian governments only since the 1970s, after the historic 1967 referendum paved the way for the rise of the Indigenous community-controlled health sector (National Aboriginal Community Controlled Health Organisation (NACCHO) 2021; O. Pearson et al. 2020). Indigenous health research efforts have focused largely on infectious and chronic disease, although there is increasing interest in the health benefits of engaging with emerging sciences including genetics, epigenetics and the microbiome (Kowal 2012; QIMR Berghofer 2019; Warin, Kowal, and Meloni 2019; Rogers et al. 2019). Indigenous microbiome research has the potential to bring considerable benefits both in Australia and elsewhere. Many chronic non-communicable diseases linked to the human

microbiome disproportionately impact Indigenous peoples.[1] Understanding how commensal microbes protect against or mediate disease, and how the microbiota can be altered and manipulated, supports the development of new microbiota-based therapies and health interventions. Meanwhile, current evidence indicates that lifestyle and heritage[2] factors can influence the human microbiome, and by implication both disease risk and the effectiveness of treatments that target the microbiome. Therefore, a failure to include Indigenous peoples in human microbiome research is likely to further entrench disparities in health outcomes through unequal access to new knowledge and effective treatments based on the microbiome (Rogers et al. 2019; Nath et al. 2021). Microbiome research also presents opportunities for non-medical benefits to Indigenous peoples. Examples could include microbiome research collaborations on topics of interest to Indigenous communities, whether focused on human health or environmental microbiomes; education and capacity-building for Indigenous communities and individuals; and sharing of material and non-material benefits arising from research, such as new knowledge or intellectual property and material benefits from eventual research commercialisation.

While recognising these benefits, stakeholders in this field equally need to be proactive in confronting the ethical questions and concerns that arise from microbiome research, especially in Indigenous contexts. We must contend with the history of unethical research on Indigenous bodies and data in closely related fields, which is already being echoed in some contemporary human microbiome research.[3] Certain risks relating to group identification, reification of racial categories or stereotypes, cultural harm, or stigmatisation when participating in research are more salient for minority or marginalised groups, such as Aboriginal and Torres Strait Islander peoples in the Australian context, than for majority groups. Biopiracy (enrichment of non-Indigenous actors using resources rightfully belonging to Indigenous peoples) and other exploitation of Indigenous peoples and resources is also a concern. Doing Indigenous microbiome research properly requires respect for Indigenous views and perspectives, as well as honest engagement with these ethical issues. Particularly when new research techniques are

[1] These links are reviewed in Chapter I of this thesis.
[2] The word 'heritage' is here intended to accommodate both biological and cultural elements that may be passed on over generations. This idea will be explored further in later chapters and in the thesis Discussion.
[3] This point is expanded upon in Chapter IV of this thesis.

pioneered or increase in popularity, stakeholders need to consider whether new ethical questions have emerged and whether practices need to be updated to address these questions. Furthermore, work must be approached in a culturally aware manner, not by assuming that ethical norms and standards agreed upon by colonial and Western institutions can be transferred wholesale to groups with different history, culture, and experiences.

In my thesis, I aim to present new knowledge and communicate the promise of Indigenous microbiome research while maintaining a clear-eyed view of the ethical risks and issues involved. Thesis chapters span scientific investigations of oral microbiota and oral health in Aboriginal Australians and Torres Strait Islanders as well as commentaries and bioethical discussions that aim to raise awareness and suggest constructive approaches to ethical and social implications of Indigenous microbiome research. A brief summary of each thesis chapter is presented below.

*Chapter I: The role of the oral microbiota in chronic non-communicable disease and its relevance to the Indigenous health gap in Australia*

This piece, published in *BMC Oral Health*, reviews literature related to the underlying themes and motivation of my overall research project (Handsley-Davis, Jamieson, et al. 2020). The review draws together several background threads, including the Indigenous health gap in Australia, links between the oral microbiota and chronic disease, knowledge about oral microbiota of Indigenous peoples generally, and the relevance of oral microbiota research to Indigenous health. This review outlines the gaps in knowledge and presents a case for oral microbiota research as an emerging field with many potential medical and non-medical benefits for Indigenous peoples if implemented appropriately.

*Chapter II: Heritage-specific oral microbiota in Indigenous Australian dental calculus*

This study, submitted to *Evolutionary Medicine and Public Health*, adds to a growing body of evidence of unique microbiota in Indigenous peoples. We compare oral (dental calculus) microbiota in Indigenous Australian and non-Indigenous adults for the first time, leading to identification of significant microbiota differences between Indigenous and non-Indigenous

participants that were robust to stratification by periodontal disease status. Interestingly, significant differences in oral microbiota diversity and composition were also identified between Indigenous Australians living in two different regions of Australia with considerably different environments and traditional cultural practices. We hypothesise that the microbiota differences observed may be linked to heritage, shaped through connections to Country (i.e., traditional homelands) and apparently persistent through colonisation and industrialisation. Expanding understanding and awareness of these microbiota differences and unique signatures in Indigenous Australians is important for ensuring equitable access to effective future microbiome-based research and therapies.

*Chapter III: Biocultural drivers of salivary microbiota in Australian Aboriginal and Torres Strait Islander children*

Informed by the findings of Chapter II, this study published in *Frontiers in Oral Health* shifts to a more specific context, exploring detailed factors associated with oral microbiota variation in a remote Indigenous Australian community experiencing high levels of dental caries (Handsley-Davis et al. 2021). In this chapter, biological, behavioural and socioeconomic factors were linked to salivary microbiota diversity and composition. This information forms a baseline for longitudinal studies of oral disease and oral health interventions and for understanding which taxa or mechanisms mediate associations between the oral microbiota and dental caries. This work could also be expanded upon to directly influence the development of new microbiome-based therapies.

*Chapter IV: Ethics of microbiome ownership for Indigenous peoples*

In this chapter, in preparation for submission to *Nature Reviews Microbiology*, we move from scientific investigations of oral microbiota in Aboriginal Australians and Torres Strait Islanders to consideration of some key ethical issues in global Indigenous microbiome research. Microbiome ownership is a key issue that influences the distribution of benefits and harms from microbiome research. This issue deserves unique attention in Indigenous contexts for several reasons. First, Indigenous peoples harbour unique microbiota signatures and experience a high level of interest from researchers as a result. Second, concepts and systems governing

ownership and intellectual property are culturally shaped, and hence frameworks developed in a Western context are not necessarily suitable or acceptable for Indigenous contexts. Third, Indigenous scholars and communities worldwide have articulated the importance of Indigenous data sovereignty, which has implications for the ownership and governance of microbiome data and samples. Finally, Indigenous peoples have historical and ongoing experience of mistreatment and unethical research, which must be considered when framing ownership claims. This chapter analyses ethical issues under the theme of microbiome ownership at different stages of Indigenous microbiome research, beginning with the framing of research questions, contemporary trends in the field, data management and stewardship in current and future studies, and progressing to anticipated research translation and commercialisation. We review the current legal and ethical landscape of Indigenous ownership of knowledge and resources, intellectual property, and data sovereignty, and evaluate how the microbiome fits into this landscape. The chapter concludes with some future directions and recommendations for ethical research practices and benefit-sharing in Indigenous microbiome research.

*Chapter V: Researchers using environmental DNA must engage ethically with Indigenous communities*

My final thesis chapter, published in *Nature Ecology & Evolution*, expands the conversation beyond the human body into the burgeoning field of environmental DNA (eDNA) research (Handsley-Davis, Kowal, et al. 2020). eDNA analysis is a powerful tool that can be used to answer questions of mutual interest to researchers and Indigenous communities. However, as always, emerging scientific techniques raise new ethical questions. In this commentary, we use a hypothetical project analysing eDNA from soil around Aboriginal Australian birthing trees as a case study to illustrate ethical issues arising from eDNA research on Indigenous lands. We highlight potential risks and benefits of such research and argue for the prioritisation of Indigenous engagement and perspectives. While standards of ethical engagement with Indigenous stakeholders are increasingly recognised in human health research, our commentary argues that these are by no means limited to this field.

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# Chapter I: The role of the oral microbiota in chronic non-communicable disease and its relevance to the Indigenous health gap in Australia

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REVIEW Open Access

# The role of the oral microbiota in chronic non-communicable disease and its relevance to the Indigenous health gap in Australia

Matilda Handsley-Davis[1*], Lisa Jamieson[2], Kostas Kapellas[2], Joanne Hedges[2] and Laura S. Weyrich[1,3*]

**Abstract**

**Background:** Aboriginal Australians and Torres Strait Islanders (hereafter respectfully referred to as Indigenous Australians) experience disproportionately poor health and low life expectancy compared to non-Indigenous Australians. Poor oral health is a critical, but understudied, contributor to this health gap. A considerable body of evidence links poor oral health to increased risks of other chronic non-communicable conditions, such as diabetes, cardiovascular disease, chronic kidney disease, and poor emotional wellbeing.

**Main:** The oral microbiota is indisputably associated with several oral diseases that disproportionately affect Indigenous Australians. Furthermore, a growing literature suggests direct and indirect links between the oral microbiota and systemic chronic non-communicable diseases that underpin much of the Indigenous health gap in Australia. Recent research indicates that oral microbial communities are shaped by a combination of cultural and lifestyle factors and are inherited from caregivers to children. Systematic differences in oral microbiota diversity and composition have been identified between Indigenous and non-Indigenous individuals in Australia and elsewhere, suggesting that microbiota-related diseases may be distinct in Indigenous Australians.

**Conclusion:** Oral microbiota research involving Indigenous Australians is a promising new area that could benefit Indigenous communities in numerous ways. These potential benefits include: (1) ensuring equity and access for Indigenous Australians in microbiota-related therapies; (2) opportunities for knowledge-sharing and collaborative research between scientists and Indigenous communities; and (3) using knowledge about the oral microbiota and chronic disease to help close the gaps in Indigenous oral and systemic health.

**Keywords:** Indigenous health, Australia, Microbiota, Microbiome, Oral health, Chronic disease

## Background

Aboriginal Australians and Torres Strait Islanders (hereafter respectfully referred to as Indigenous Australians) experience disproportionately poor health and lower life expectancy compared to non-Indigenous Australians. This has been described by the Australian Government as the Indigenous health gap [1]. Gaps in life expectancy and disease burden are largely driven by chronic non-communicable diseases (NCDs). Although many factors contributing to this health gap are well-characterised, there is scant description of the oral microbiota in Indigenous Australians. This is despite growing evidence linking oral microbiota to many oral and systemic chronic NCDs. To contribute to this knowledge gap, we review: (1) current evidence of the extent and causes of the Indigenous health gap in Australia; (2) the oral microbiota, its role in oral and systemic disease, and its influencing factors; and (3) the oral microbiota of Indigenous peoples in Australia and elsewhere. We propose that the oral

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microbiota is an important, yet under-studied, concept in Indigenous health research and outline both medical and non-medical potential benefits to Indigenous peoples that could result from microbiota research.

#### The Indigenous health gap in Australia

Indigenous Australians comprise two distinct First Nations groups in contemporary Australia. Aboriginal Australians are the first peoples of mainland Australia and Tasmania, while Torres Strait Islanders are the first peoples of islands located in the Torres Strait between Australia and Papua New Guinea [2]. The health disparities experienced by Indigenous Australians in comparison to the non-Indigenous population are well-documented. In 2018, the age-standardised death rate for Indigenous Australians was 1.7 times that of non-Indigenous people, while Indigenous Australians had a life expectancy approximately 8 years less than that of their non-Indigenous counterparts [3]. A large proportion of this death and disease burden can be attributed to NCDs such as cancers, diabetes, cardiovascular disease, ischaemic heart disease, chronic respiratory diseases, and poor mental health [4, 5]. Furthermore, several chronic NCDs exhibit both a higher incidence and higher death rate in Indigenous Australians compared to the non-Indigenous population. For example, Indigenous Australians have an incidence of diabetes 3.5 times higher, and a death rate from diabetes approximately 5 times higher, than non-Indigenous Australians. The incidence and death rate for Indigenous Australians from chronic kidney disease are 3.5 times and 2.6 times higher, respectively, than those of non-Indigenous Australians [4]. Addressing the burden of chronic NCDs is therefore a priority in closing the Indigenous health gap in Australia.

Although less widely documented, poor oral health is another chronic NCD prevalent among Indigenous Australians. Unfortunately, representative nationwide data on the oral health status of Indigenous Australians is limited. The 2017–18 National Survey of Adult Oral Health (NSAOH) reported that only 1.7% of participants who underwent dental examinations identified as Indigenous, compared to 2.4% of the Australian population in the 2016 census [6]. Due to this small sample size and lack of representativeness, it is difficult to draw firm conclusions about the oral health of Indigenous Australians from this survey, highlighting the need for more effective approaches to data collection on Indigenous Australian oral health.

Nevertheless, available evidence supports the existence of a substantial oral health gap within the overall Indigenous health gap, with Indigenous Australians experiencing a high incidence and severity of oral diseases including dental caries (tooth decay), periodontal (gum) disease, and head and neck cancers [4, 7–12]. An earlier NSAOH conducted in 2004–2006 reported an Indigenous participation rate similar to that identified in the 2001 census [13]. The 2004–2006 NSAOH found that Indigenous Australians had "disproportionately elevated rates of tooth loss, untreated decay and tooth wear" and experienced worse oral health overall than non-Indigenous counterparts [7]. Specifically, 57% of Indigenous participants in the 2004–2006 NSAOH experienced untreated dental caries, more than twice the proportion of non-Indigenous participants. The 2004–2006 NSAOH additionally found that Indigenous Australians experienced a high prevalence of moderate or severe periodontal disease compared to non-Indigenous counterparts [7]. A smaller convenience study of young adults in an Aboriginal birth cohort in the Northern Territory found a prevalence of untreated tooth decay 3.1 times higher, and of moderate or severe periodontal diseases 10.8 times higher, compared to age-matched NSAOH participants [8]. Research with otherwise healthy Aboriginal Australian adults in the Northern Territory found that 88% had moderate or severe periodontitis, equivalent to 3.5 times the estimated national average [9]. This suggests that the prevalence and severity of periodontal disease among Indigenous Australians may be much higher than what has been captured in nationwide surveys that do not specifically seek out Indigenous participants.

Indigenous Australians also experience a high burden of oral cavity and oropharyngeal cancers [10]. A recent review found Indigenous Australians are diagnosed with head and neck cancers at twice the rate of non-Indigenous Australians [4]. As of 2011, oral cavity and oropharyngeal cancers were responsible for 7% of the total cancer burden for Indigenous Australians living in the state of Queensland, sharing third place with colorectal cancers [11]. Data from the state of South Australia indicate that head and neck cancers represent approximately 8% of cancer diagnoses in Aboriginal South Australians, compared to only approximately 2% of cancer diagnoses in non-Indigenous South Australians [12]. Furthermore, Aboriginal South Australians were more likely to be diagnosed with cancer at a later stage and had a significantly lower 5-year survival rate from cancer compared to non-Indigenous counterparts [12]. Reflecting these state-level findings, cancer has recently overtaken cardiovascular diseases as the leading cause of Indigenous deaths nationwide [3]. Therefore, despite gaps in the data, an overall picture emerges of considerably poorer oral health in Indigenous Australians in comparison to the overall Australian population.

Oral health is a particularly important, yet understudied, contributor to the Indigenous health gap due to the numerous links between oral and systemic health.

Periodontal disease has been identified as a risk factor for many chronic NCDs, including diabetes [14–17], chronic kidney disease [18], cardiovascular disease [19, 20], rheumatoid arthritis [21–24], and head and neck cancers [25, 26]. The mouth can also act as a reservoir for bacteria that can cause dangerous infections elsewhere in the body, such as infective endocarditis [27, 28]. Furthermore, poor oral health frequently has negative impacts on mental wellbeing and quality of life. For example, in a recent survey of urban Indigenous Australian adults seeking primary healthcare, approximately 40% of women and 25% of men reported recently experiencing oral pain or discomfort [29]. Similar proportions reported recently experiencing discomfort when eating because of oral health problems, while approximately 24% of women and 14% of men reported recent sleep disruption due to pain from oral health problems [29]. Poor oral health has been associated with anxiety, depression, and suicidal thoughts in young Aboriginal Australian adults [30]. Therefore, improving oral health could lead to a lowered risk of chronic disease and infection as well as improved social and emotional wellbeing and overall quality of life for Indigenous Australians.

These observed disparities in both oral and overall health result from a complex mixture of factors. It is well established that people living in rural and remote areas of Australia often have poorer health outcomes than their urban counterparts. This is due, in part, to these areas having less health infrastructure and a smaller health workforce, particularly of specialist medical professionals [31]. A larger proportion of the Indigenous Australian population lives in outer regional or remote areas (39% of the Indigenous population vs 9.5% of the non-Indigenous population), which may contribute to health disparities between Indigenous and non-Indigenous Australians [32]. However, while many Indigenous Australians experience socioeconomic disadvantage or health challenges related to geographical remoteness, these factors do not fully explain the health gap. While Indigenous Australians are more likely than non-Indigenous Australians to live in rural and remote areas, the majority (60.1%) reside in metropolitan locations or inner regional areas [32]. A study of public dental patients in Australia found that Indigenous Australians experienced noteworthy disparities in oral health even among an economically disadvantaged cohort [33]. Important physical risk factors for poor oral and overall health, such as overweight and obesity, tobacco smoking, and high levels of free sugar consumption, are comparatively prevalent among Indigenous Australians [4, 34]. Socioeconomic and cultural factors, including education and employment, the economic and social costs of obtaining healthcare, and racism and lack of cultural competence in the healthcare system, can prevent Indigenous Australians from seeking and receiving timely and appropriate care [7, 35–37]. Mistrust of the Australian government, unemployment, poor housing, high alcohol and sugar consumption, poor communication of health information, and lack of cultural and linguistic sensitivity in the Australian healthcare system have all been identified by Indigenous individuals and healthcare workers as factors contributing to the health burdens of Indigenous Australians [38–40].

Despite a range of initiatives attempting to address identified contributing factors, progress in closing the health gap to date has been limited [3, 36, 41, 42]. The Council of Australian Governments (COAG) first committed at the end of 2007 to an intention to close the gap in Indigenous life expectancy by 2030 [43]. Specific targets towards this goal, including closing the gap in life expectancy by 2031 and halving gaps in child mortality, literacy and numeracy skills, and employment by 2018, were set by COAG in 2008 [43]. In 2013, several new initiatives were announced by the Australian Government, including the National Aboriginal and Torres Strait Islander Health Plan 2013–2023, a renewed national partnership agreement on closing the gap in Indigenous health outcomes, and an implementation plan for the National Aboriginal and Torres Strait Islander health plan [43]. However, an independent 10-year review of COAG's Closing the Gap (CTG) strategy highlighted discontinuity, funding cuts, frequent changes in political leadership, and other factors that contributed to failures to meet CTG targets, concluding that "[b]y 2014–15, the CTG Strategy as a coherent, national response to Indigenous disadvantage was effectively over" [44]. The most recent Australian Government report on CTG noted that, while some improvements had been achieved in addressing socioeconomic determinants of health, such as educational attainment, the gaps in Indigenous adult and child and mortality rates had not narrowed and the target to close the gap in life expectancy was not on track [3]. In recognition of this lack of progress, Australian state and federal governments have recently announced a 'reset' of CTG targets with increased emphasis on Indigenous community control of efforts to close the gap, albeit without additional funding [45]. Inclusion of novel health-related factors, such as the human microbiota, in the Indigenous health management armamentarium could help to further strengthen efforts to close the health gap.

#### The human oral microbiota in oral and systemic disease

The human oral microbiota refers to the collection of micro-organisms living in the human oral cavity. Within this collection, there are several distinct communities of microbes that establish themselves in different habitats.

The composition of these communities differs among different sites, such as the teeth, gingiva (gums), tongue, saliva, and the buccal mucosa (inside of the cheek) [46–49]. Dental plaque, a microbial biofilm that grows on the surface of teeth, has a particularly distinct composition [47, 50]. Teeth are the only surface in the body that do not regularly shed cells, allowing a complex biofilm to flourish on the tooth surface [51]. Gram-positive primary colonisers such as *Streptococcus* and *Actinomyces* species first bind directly to the acquired pellicle, a thin layer of salivary glycoproteins on the tooth surface [52]. As the plaque community grows, it can incorporate 'bridging' organisms, such as *Fusobacterium*, that bind to both primary and late colonisers, and Gram-negative late colonisers such as *Porphyromonas* species [52, 53]. Mature dental plaque is a highly structured community in which bacteria work together to optimise their access to appropriate resources [54]. Dental plaque, in the absence of regular oral hygiene, calcifies into a hardened calcium phosphate matrix known as dental calculus or tartar [55]. In this process, microbial DNA can be preserved in dental calculus for thousands of years, allowing for the study of microbial communities through time [56]. Despite these useful qualities of dental plaque and calculus communities, lower invasiveness and ease of collection has favoured the use of saliva as a proxy for studying the oral microbiota as a whole in modern-day populations. Although saliva may not represent a true microbial 'community', it can provide broad insight into the microbes present throughout the oral cavity [47, 50].

Oral microbial communities have been implicated in the most common oral diseases: dental caries, periodontal disease, and oral cancers [57]. Dental caries affects approximately 90% of the global population and can be highly resistant to interventions [57–59]. This disease is primarily caused by oral bacteria fermenting sugars to acid, which in turn dissolves the enamel microstructures in the outer layer of the tooth to enable bacterial entry deeper into the tooth structure [60]. *Streptococcus mutans* and related oral bacteria ('mutans streptococci') have traditionally been considered the microorganisms responsible for caries [61–63]. However, this paradigm has been challenged by culture-independent research demonstrating that mutans streptococci can be detected in the oral microbiota of caries-free individuals and identifying alternative bacterial species associated with caries [46, 64]. Hence, the oral health field has witnessed an increasing focus on the overall ecology of the oral microbiota in relation to caries, in particular the balance between acid-producing, acid-tolerant and alkali-producing species and metabolic processes [65–68].

Periodontal disease refers to a group of conditions characterised by inflammation of the gingiva (gums), which can range from relatively mild and reversible gingivitis to severe periodontitis involving tissue destruction and loss of bone and teeth [69]. This inflammation is thought to constitute an immune reaction by the human host against resident oral bacteria. Although periodontitis is often characterised as a "polymicrobial infection" [70], a mixture of microbial, host, and environmental factors are likely required to produce the disease [71, 72]. Identification of specific pathogenic species in periodontal disease [73, 74], such as the 'red complex' bacteria, has been complicated by culture-free microbiota studies, which have both expanded the list of putative periodontal pathogens [75–77] and identified changes in oral microbiota community structure, ecological diversity, and the relative abundance of specific microbial taxa in periodontal disease compared to periodontal health [78–81]. These findings have formed the basis for new theories regarding periodontal disease, including the concepts of the 'pathobiont'—a species that is normally harmless but has the potential to cause disease in a changed environment—and the 'keystone pathogen'—a species that can cause disease, even when present in low abundance, through its interactions with other microbes and with the host immune system [82, 83].

Recent research also supports a role for the oral microbiota in oral cancers. Several studies have found links between periodontal disease and increased risk of oral cancer [25, 26, 84]. These links may be mediated by the production of carcinogenic compounds by the oral microbiota, direct carcinogenic effects of specific microbes (particularly viruses), or the chronic inflammation resulting from periodontal disease [25, 85]. Although the role of the oral microbiota in oral cancer is a relatively new field of study, differences in the relative abundance of specific bacterial taxa and the presence of unique taxa in oral cancer compared to clinically healthy sites have been reported [86, 87], suggesting that the topic warrants further attention. For all three of these diseases, a deeper understanding of the role of the oral microbiota is needed to improve our ability to treat and prevent these conditions, especially in groups, such as Indigenous Australians, who disproportionately experience poor oral health.

In addition to its roles in widespread oral diseases, the oral microbiota has direct and indirect impacts on many systemic diseases. On the one hand, there is considerable evidence to support a role for specific oral species in the initiation or progression of certain non-oral conditions. A classic example is infective endocarditis, a life-threatening illness frequently triggered by oral *Streptococcus* species [28, 88]. Although these species typically live harmlessly in the oral cavity, they can cause severe disease when they escape into the bloodstream (termed

bacteraemia) and colonise injured heart endothelial tissue [88]. Another common oral species, *Fusobacterium nucleatum*, is frequently isolated from colorectal tumours and is thought to promote colorectal cancer through several mechanisms, including promotion of a pro-inflammatory host response (reviewed in [89]) and tumour microenvironment [90], acceleration of cancer cell proliferation through activation of Wnt/beta-catenin signalling [91], interference with the host immune response [92, 93] and induction of chemotherapy resistance [94]. It has recently been shown that *F. nucleatum* strains from patient colorectal tumours are also found in saliva from the same individual, and most likely reach tumours by escape from the mouth into the bloodstream [95, 96]. A third example is the emerging evidence of a possible relationship between *Porphyromonas gingivalis*, an oral species associated with periodontal disease, and Alzheimer's disease (reviewed in [97]). *P. gingivalis* can escape from the mouth into the blood and colonise other tissues, while *P. gingivalis* products have been identified in brain autopsy specimens of humans and mice with Alzheimer's disease. Therefore, while considerable research is needed to identify specific oral bacterial species and fully elucidate mechanisms linking them to various systemic conditions, current evidence suggests that oral bacteria may play an important role in several diseases.

More generally, the association of periodontal disease with many chronic systemic conditions suggests that the oral microbiota as a whole may exert a substantial influence on overall health [98–100]. Although the mechanisms that underlie these links are not fully understood, some hypotheses exist. Firstly, the observed relationship between periodontal and systemic diseases could be based on shared risk factors, such as smoking [20, 101]. Secondly, periodontal inflammation and bleeding could facilitate bacteraemia caused by oral bacteria which, in turn, contributes to the initiation and maintenance of systemic disease, as in the examples discussed in the previous paragraph [100, 102]. Thirdly, the long-term inflammation stimulated by periodontal disease in the mouth could affect systemic immune function, contributing to disease [16, 19, 100, 102–104]. For instance, recent evidence suggests that oral microbiota in periodontal disease may inhibit normal macrophage polarisation and function [105]. Finally, a combination of these mechanisms could also act in tandem to promote disease. A summary of representative recent publications on proposed links between the oral microbiota and non-oral or systemic NCDs is given in Table 1.

#### The oral microbiota of Indigenous peoples

The many host-related and environmental factors that shape the composition and activity of the oral microbiota represent an area of active research. Microbial community composition at different oral sites is influenced by local ecological factors such as nutrient and oxygen availability, pH, and salivary flow rate [106, 107]. The oral microbiota appears to be at least partly vertically transmitted from parent to child, although the relative importance of inherited host genetic factors versus parent-to-child transfer of microbes through physical contact and shared environment is debated [108–115]. A small number of studies have reported correlations between specific dietary nutrients and oral microbiota composition and diversity in modern humans, while major shifts in the oral microbiota composition of ancient Europeans have been attributed to dietary changes following the introduction of agriculture [116–118]. Current evidence suggests that the oral microbiota is resilient to the effects of short-term antibiotic use, but clinical reports indicate that long-term, heavy antibiotic use can damage endogenous oral bacterial communities, in addition to having systemic impacts on the gut and inflammation [60, 119]. Therefore, current evidence indicates that the oral microbiota is influenced by a combination of vertical transmission, host genetics, and lifestyle-related factors.

This evidence raises the pertinent question of whether the oral microbiota differs among human populations with different histories and cultures. Most oral microbiota research is conducted among people of predominantly European descent living industrialised lifestyles [120, 121]. However, reconstructions of ancient oral microbiota have identified shifts in microbiota composition that may be linked to major changes in lifestyle, such as meat consumption and transition to agriculture [116, 122]. In three studies of the oral microbiota of modern-day Indigenous populations living hunter-gatherer lifestyles in Venezuela, the Philippines, and Uganda, saliva samples from Indigenous individuals exhibited a microbial composition distinct from that of industrialised or farming populations [123–125]. A fourth study found that Cheyenne and Arapaho Native Americans residing in towns in western Oklahoma had much higher variability in oral microbiota composition than did non-Native individuals living nearby [120]. All four of these studies established differences in within-individual diversity (alpha diversity) and inter-individual variability (beta diversity) of the oral microbiota when comparing Indigenous to non-Indigenous individuals [120, 123–125]. Preliminary work in Australia shows that the oral microbiota of Indigenous Australians living industrialised lifestyles differ significantly from those of non-Indigenous Australians [126]. Although current knowledge is not extensive and suffers from some limitations, it seems credible that Indigenous individuals may harbour oral microbiota that

**Table 1 Recent publications on direct and indirect links between oral microbiota and systemic NCDs**

| Publication | Non-oral disease(s) linked to oral microbiota | Proposed mechanism(s) |
|---|---|---|
| *Infective endocarditis* | | |
| Kawamata et al. [27] [original research] | Infective endocarditis | Bacteraemia |
| Cahill and Prendergast [88] [review] | Infective endocarditis | Bacteraemia, colonisation of thrombus following endothelial injury |
| *Colorectal cancer* | | |
| Flemer et al. [141] [original research] | Colorectal cancer | Colonisation of colorectal tumour, shared risk factors, possibly Western diet |
| Komiya et al. [95] [original research] | Colorectal cancer | Colonisation of colorectal tumour from origins in the mouth |
| Abed et al. [96] [original research] | Colorectal cancer | Bacteraemia, colonisation of colorectal tumour via bloodstream |
| *Central nervous system diseases* | | |
| Ryder [97] [review] | Alzheimer's disease | Bacteraemia, infiltration of central nervous system, gingipain activity |
| Ballini et al. [105] [original research] | Central nervous system diseases | Perturbed host tissue homeostasis and immune response, chronic host inflammatory response, carriage of pathogens between body sites by macrophages |
| *Cardiovascular disease* | | |
| Lockhart et al. [101] [review] | Cardiovascular disease | Shared risk factors, systemic host inflammatory response |
| Schenkein and Loos [104] [review] | Cardiovascular disease | Systemic host inflammatory response, cross-reactive antibody production, promotion of dyslipidaemia, shared genetic susceptibility factors |
| Bansal et al. [102] [review] | Cardiovascular disease | Host inflammatory response, direct induction of inflammatory mediators by bacteria or bacterial products |
| Carrizales-Sepùlveda et al. [19] [review] | Cardiovascular disease | Systemic host inflammatory response, oxidative stress, cross-reactive autoantibody production, shared risk factors |
| *Diabetes mellitus* | | |
| Lalla and Papapanou [16] [review] | Diabetes | Systemic host inflammatory response, increased proinflammatory cytokine production |
| *Rheumatoid arthritis* | | |
| Horz and Conrads [142] [review] | Rheumatoid arthritis | Host immune response |
| Bingham and Moni [22] [review] | Rheumatoid arthritis | *Porphyromonas gingivalis* enzyme activity, host inflammatory response |
| *Mutiple systemic diseases* | | |
| Kim and Amar [103] [review] | Cardiovascular disease, diabetes, adverse pregnancy outcomes, osteoporosis | Direct invasion of host tissues, systemic host inflammatory response, changes in host proinflammatory cytokine and hormone levels, shared risk factors |
| Borgnakke [98] [review] | Cardiovascular disease, diabetes, metabolic syndrome, non-alcoholic fatty liver disease, rheumatoid arthritis, chronic kidney disease | Bacteraemia, microbial dysbiosis, host inflammatory response, indirect stimulation of immune response by bacterial products |
| Guimarães et al. [99] [book chapter] | Diabetes, cardiovascular disease | Bacteraemia, host inflammatory response |
| Joshipura and Andriankaja 2016 [143] [book chapter] | Cardiometabolic conditions | Common risk factors, host inflammatory response |

Representative articles and book chapters published since 2005 are listed, along with the non-oral disease(s) and proposed mechanistic links with the oral microbiota discussed in each publication

differ in important ways from those of non-Indigenous individuals, even in a shared context of industrialisation.

A number of factors could plausibly influence the Indigenous Australian oral microbiota in unique ways. For example, smoking is more common among Indigenous Australians than in the overall Australian population, and has previously been shown to influence the composition of the oral microbiota [79, 127]. Diet may also influence the oral microbiota and is likely to vary among Indigenous Australians compared to non-Indigenous

Australians. For instance, health survey data has reported that many Indigenous Australians obtain a comparatively large proportion (on average, 41%, compared to 35% for the overall Australian population) of their dietary energy from 'discretionary' foods such as confectionery, snack foods and alcohol [4, 128]. Reported consumption of free sugars is relatively high among Indigenous Australians [4], which may impact the oral microbiota by, for example, favouring the growth of sugar-metabolising bacteria. Traditional foods such as native game meats, certain insects, and native plants are rarely eaten by non-Indigenous Australians and may impart a unique effect on the oral microbiota, whether through the introduction of different environmental microbes or through other nutrients or compounds that could affect resident microbial communities.

Finally, the evolutionary history of the microbiota in Australia may explain some differences between the oral microbiota of Indigenous and non-Indigenous Australians. Archaeological and genetic evidence suggests that Aboriginal Australians had lived on Country (i.e. in specific homelands), in relative isolation from the rest of the world, for at least 50,000 years prior to European arrival [129, 130]. Under this scenario, we might expect that the microbiota of Indigenous Australians became uniquely adapted to the Australian environment and to aspects of human lifestyles, such as diet and medicines. Alternatively, even without specific adaptation, a process of random microbiota 'drift' as people settled in specific homelands may have led to variation in microbiota across the continent. As current evidence suggests that the oral microbiota is partly heritable, such historic variation in the microbiota could explain differences between the oral microbiota of Indigenous and non-Indigenous Australians, even when living relatively similar industrialised lifestyles today. Given the links between the oral microbiota and oral and systemic disease, such differences could have important implications for Indigenous health in terms of both disease risk and response to treatments. Indeed, Skelly et al. [121] have previously argued that the traumatic experience of colonisation and its possible impacts on the human microbiome are likely to have health consequences for Indigenous people today.

### Harnessing oral microbiota research to benefit Indigenous communities

There are numerous potential benefits from investigating the oral microbiota in Indigenous Australians. As reviewed above, a significant body of evidence points towards a central role for the oral microbiota in shaping oral and overall health. The ability to understand and shape the microbiome to support human health holds considerable promise for addressing the burden of microbiome-mediated diseases, perhaps especially for groups whose access to medical care is compromised by structural issues such as geographic remoteness. Novel microbiome-based therapies, such as oral microbiota transplants [131, 132] and probiotic mouthwash or lozenges [133–138], are of increasing interest in the field of oral health promotion. However, factors including poor historical and continuing research practices when engaging with Indigenous communities have led to a dearth of studies examining how the human microbiome mediates disease in Indigenous populations [139]. Therefore, there is currently little understanding of how existing treatments may influence the microbiota of Indigenous populations and few attempts to verify that microbiome-based therapies will be effective for these groups. Explicit inclusion of Indigenous Australians in oral microbiota research could help strengthen efforts to close the Indigenous health gap and to ensure that future health benefits stemming from microbiota research in general are equitably shared. To realise this goal, it will be crucial to continue to strive for ethical and culturally appropriate conduct of research, including commitment to the involvement and leadership of Indigenous researchers and communities in study design, data management and knowledge translation [106, 115].

Beyond these potential longer-term benefits, there are numerous opportunities for microbiota research to be a positive for Indigenous communities in the short term. Importantly, it may be some time before robust and sufficiently powered microbiota research is carried out, analysed, and translated into new therapies or healthcare approaches with tangible benefits for Indigenous communities [121]. Valid concerns have previously been raised about the dangers of over-promising therapeutic benefits of microbiota research to research participants, especially for marginalised groups such as Indigenous peoples [140]. However, research could be designed in such a way as to provide other, non-medical benefits for Indigenous Australians in the shorter term. Oral microbiota research could present opportunities for collaborative research partnerships between scientists and Indigenous communities, for two-way outreach and knowledge-sharing, and for scientific training and capacity-building among Indigenous Australian communities through programs such as the Summer Internship for Indigenous Peoples in Genomics (SING) Australia.

## Conclusion

Chronic systemic NCDs such as cardiovascular disease, diabetes, chronic kidney disease, and cancer disproportionately impact Indigenous Australians and have known associations with poor oral health. The human oral microbiota is linked to the pathogenesis of oral diseases that

severely affect Indigenous Australians. Current evidence suggests that oral microbiota composition and diversity differ between Indigenous and non-Indigenous people in Australia and elsewhere. Therefore, the oral microbiota and oral health are likely to be directly linked to oral and systemic health outcomes in Indigenous peoples. Appropriate inclusion of Indigenous Australians in oral microbiota research and further investigation of these links between the oral microbiota and oral and systemic disease is an important emerging strategy to help close the Indigenous health gap in Australia.

**Abbreviations**
CTG: Closing the gap; NCD: Non-communicable disease; NSAOH: National Survey of Adult Oral Health.

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**Authors' contributions**
MHD undertook the initial review of literature and wrote the manuscript. LJ, KK, JH and LSW provided substantive feedback on language and content of the manuscript. All authors read and approved the final manuscript.

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**Competing interests**
MHD and LSW are members of the Organising Committee and faculty for the Summer Internship for Indigenous Peoples in Genomics (SING) Australia initiative mentioned in this manuscript. SING Australia is a capacity-building program for Aboriginal and Torres Strait Islander people interested in genomics and related fields. MHD is employed on a casual basis as a research and administrative assistant for SING Australia. The authors declare that they have no other competing interests.

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## Publisher's Note

# Chapter II: Heritage-specific oral microbiota in Indigenous Australian dental calculus

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

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**Heritage-specific oral microbiota in Indigenous Australian dental calculus**

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**Abstract**

Background and objectives: Aboriginal Australians and Torres Strait Islanders (hereafter respectfully referred to as Indigenous Australians) experience a high burden of chronic non-communicable diseases (NCDs). Increased NCD risk is linked to oral diseases mediated by the oral microbiota, a microbial community influenced by both vertical transmission and lifestyle factors. As an initial step towards understanding the oral microbiota as a factor in Indigenous health, we present the first investigation of oral microbiota in Indigenous Australian adults.

Methodology: Dental calculus samples from Indigenous Australians with periodontal disease (PD) (n=13) and non-Indigenous individuals both with (n=18) and without PD (n=20) were characterized using 16S rRNA gene amplicon sequencing. Alpha and beta diversity, differentially abundant microbial taxa, and taxa unique to different participant groups were analysed using QIIME2.

Results: Samples from Indigenous Australians were more phylogenetically diverse (Kruskal-Wallis $H=19.86$, $p=8.3 \times 10^{-6}$), differed significantly in composition from non-Indigenous samples (PERMANOVA pseudo-$F=10.42$, $p=0.001$), and contained a relatively high proportion of unique taxa not previously reported in the human oral microbiota (e.g. *Endomicrobia*). These patterns were robust to stratification by PD status. Oral microbiota diversity and composition also differed between Indigenous individuals living in different geographic regions.

Conclusions and implications: Indigenous Australians may harbour unique oral microbiota shaped by their long relationships with Country (ancestral homelands). Our findings have implications for understanding the origins of oral and systemic NCDs and for inclusion of Indigenous peoples in microbiota research, highlighting the microbiota as a novel field of enquiry to improve Indigenous health.

**Keywords (3-10)**

microbiota, microbiome, Indigenous Australian, Aboriginal Australian, oral health, evolutionary medicine

**Lay summary**

The community of microorganisms in the mouth (oral microbiota) has recently been linked to several chronic diseases that disproportionately impact Indigenous Australians. In this study, oral microbiota differ significantly between Indigenous Australians and non-Indigenous

counterparts, suggesting the microbiota could be a novel factor with potential to improve Indigenous health outcomes.

## Background and objectives

Around the world, Indigenous peoples experience a disproportionate burden of chronic non-communicable diseases (NCDs), such as diabetes and cardiovascular disease [1–6]. This pattern is prominent in Australia, where Aboriginal Australians and Torres Strait Islanders (hereafter respectfully referred to as Indigenous Australians) experience markedly higher rates of chronic NCDs and lower life expectancy compared to the overall population [4,7]. Indigenous Australians also disproportionately experience poor oral health [8–11], reinforcing the overall health gap; for example, periodontal disease (PD) increases the risk and severity of diabetes, chronic kidney disease, and cardiovascular disease [12–14].

Complex factors create and sustain these gaps in oral and overall health. A relatively large proportion of Indigenous Australians (39%) live in outer regional and remote areas of Australia, compared to only 9.5% of non-Indigenous Australians [15]. Residents of these areas often experience poor health outcomes, influenced by limited healthcare infrastructure, higher costs of healthcare delivery, and a lack of specialist medical practitioners [16]. However, many other factors affect the health of both rural and urban Indigenous Australians, including lack of access to healthcare, mistrust of the Australian government or of healthcare providers, lack of cultural safety in the healthcare system, socioeconomic status, and communication barriers between Indigenous patients and healthcare providers [6,11,17–21]. Despite increasing knowledge of these causes of the health gap in Australia, government initiatives to address these issues and close the gap have resulted in limited success [6,7,22,23].

Alongside these social, cultural, and economic factors, additional mechanisms may also play a role in Indigenous health disparities. Diverse communities of microorganisms (microbiota) live within the human body and play key roles in health and disease, yet they have not been investigated in the context of the Indigenous health gap. In the mouth, oral microbiota are linked with oral diseases, including PD, dental caries, and oral cancers [24]. Oral microbiota likely further contribute to the link between PD and increased risk of chronic systemic NCDs

[24]. Hence, exploring oral microbiota may reveal new pathways to improve both oral and systemic health outcomes for Indigenous Australians.

However, developing microbiota-based health interventions first requires a detailed understanding of the factors that affect oral microbiota. Oral microbiota are influenced by numerous factors, such as vertical transmission, diet, medical treatments, and environmental and behavioural context [25]. However, these factors have only recently been investigated in Indigenous Australians, and only in the context of childhood caries [26,27]. Further, the impact of the broader evolutionary history of microbiota has not yet been explored in Indigenous Australians. The upheaval of European invasion and rapid industrialization in the past two centuries, following a long period of at least 45,000 years of co-evolution between humans and microbes in Australia, may have perturbed Indigenous Australian microbiota, resulting in an evolutionary mismatch between host and microbiota that contributes to today's Indigenous health gap [28]. Only a handful of studies to date have investigated the oral microbiota of Indigenous peoples around the world [29–32], while fewer still have explicitly examined the impact of recent and profound lifestyle shifts on the microbiota of Indigenous individuals [28].

We propose that the oral microbiota may be an important, yet critically under-studied, contributor to Indigenous health disparities [24,28]. We conducted a pilot study to compare oral microbiota preserved in dental calculus (calcified dental plaque) – a long-term record of oral microorganisms in the mouth – from Indigenous and non-Indigenous Australians with periodontal disease, as well as from periodontally healthy non-Indigenous individuals. We examine and discuss the possibility for unique microbiota patterns linked to ethnicity or heritage. We use 'ethnicity' to express participants' self-identification as Indigenous Australian or non-Indigenous, as this term encompasses both shared ancestry and culture, and 'heritage' to convey the idea of both genetic (human and microbial) and cultural information passed on across generations, without privileging one or the other in our discussion. Further, we explored distinct microorganisms that may be associated with PD in Indigenous Australians, laying the basis for future work identifying microbial mechanisms that may underpin this disease and the broader Indigenous health gap.

## Methodology

**Ethical approval and consent to participate**

Indigenous Australian participants participated in a study on the association between periodontal treatment and cardiovascular health in Indigenous Australians [33]. This study was approved by the Human Research Ethics Committee of the Northern Territory Department of Health and Menzies School of Health Research (09/95), the Central Australian Human Research Ethics Committee (2009.11.05), Northern Territory Correctional Services Research Committee (no number), University of Adelaide Human Research Ethics Committee (179.2009), and the Aboriginal Health Council of South Australia (04-09-311). Collection of samples from non-Indigenous participants was approved by the University of Adelaide Human Research Ethics Committee (H-2012-108). All study participants gave informed consent before participating, and research was conducted in accordance with the World Medical Association Declaration of Helsinki (version VII, 2008).

**Sample collection**

Supragingival dental calculus was collected using sterile hand scalers and frozen to await DNA extraction. Samples were collected from Indigenous Australian participants (n=13) living in either Central Australia (n=7) or the Northern Territory's Top End region (n=6) (Table 1). Samples from periodontally healthy non-Indigenous participants (n=20) were collected at the University of Adelaide Dental Simulation Clinic, and samples from non-Indigenous participants with periodontal disease (n=18) at a private dental clinic in metropolitan South Australia (Table 1).

**DNA extraction, amplification and sequencing**

Genomic DNA was extracted from dental calculus in clean laboratory facilities at the Australian Centre for Ancient DNA using an in-house in-solution silica-binding method [34]. Extraction blank controls (EBCs) were processed alongside samples for each extraction, with an average of two EBCs for every five samples. Barcoded amplicon libraries targeting the V4 region of the prokaryotic 16S ribosomal RNA (rRNA) encoding gene region were constructed as previously published [35], with no-template amplification controls (NTCs) processed alongside the biological samples. Double-stranded DNA was quantified for each sample using Qubit (ThermoFisher Scientific). PCR products were pooled at equal relative concentrations, cleaned

using Ampure magnetic beads (New England Biolabs), and quantified using TapeStation (Agilent) and qPCR, then combined into a single DNA sequencing library. Paired-end 150 bp sequencing was performed on an Illumina MiSeq at the Australian Genome Research Facility (AGRF).

**Bioinformatic and statistical analysis**

Raw BCL data files were converted to FASTQ using bcl2fastq Conversion Software (Illumina), and forward and reverse reads joined with fastq-join (Bioconda). All subsequent data processing and analysis was undertaken using QIIME2 (2020.2 release) [36]. Merged reads were imported into QIIME2, then demultiplexed and quality filtered. Strain-level amplicon sequence variants (ASVs or 'features') were obtained using the QIIME2 Deblur plugin. After filtering to remove very low-abundance features (minimum frequency of 10), representative sequences were placed in a 16S rRNA phylogeny (Greengenes 13.8) using the QIIME2 SEPP plugin, and features were taxonomically classified using a pre-fitted Naïve Bayesian classifier trained on the Greengenes 13.8 database.

Alpha diversity (Faith's phylogenetic diversity [37]) and beta diversity (unweighted UniFrac distance [38]) values were calculated for all dental calculus samples and controls, subsampling at 400 sequences per sample in order to retain a reasonable number of negative controls (n=10). Differences between samples and controls were statistically evaluated using Kruskal-Wallis (alpha diversity) and PERMANOVA (beta diversity) tests. Negative controls were subsequently removed from the feature table used for downstream analysis.

Alpha and beta diversity metrics for dental calculus samples only were calculated, visualized, and tested for statistical significance as described above, with the subsampling depth increased to 10,000 sequences per sample. We verified using a lower subsampling depth (1800 sequences per sample) that two Indigenous Australian samples that contained fewer than 10,000 sequences (A11 and A12) clustered with the other Indigenous Australian samples in Principal Coordinates Analysis (PCoA), consistent with the broad conclusions of this manuscript (Figure S1). For statistical tests, an FDR-corrected p-value was used for comparisons across more than two groups [39]. After removing features present in less than 10% of samples, features that differed significantly in abundance across sample groups were identified using the QIIME2

ANCOM plugin with default parameters [40]. We identified and characterized features found uniquely in a) Indigenous Australian samples, b) non-Indigenous samples, c) samples from the Top End region, and d) samples from Central Australia as described in the Supplementary Methods. A detailed record of QIIME2 commands and parameters is provided as supplementary material (File S1).

The feature table, sample metadata, taxonomic information, Faith PD values, and unweighted UniFrac PCoA results were imported from QIIME2 into RStudio using qiime2R [41,42]. Figures were constructed using qiime2R, ggplot2 [43] and RColorBrewer [44,45].

## Results

### Overview of study participants and sample composition

Our overall study cohort comprised 52 adult individuals living in Australia (Table 1, overleaf). The Indigenous Australian group comprised 13 individuals who lived in either Central Australia (n=7) or the Top End region of the Northern Territory (n=6) and were assessed by an oral health professional as experiencing periodontal disease. The non-Indigenous group comprised 39 individuals who lived in South Australia and did not identify as Indigenous Australian. Of these, 20 individuals were assessed by a dentist as periodontally healthy; the remaining 19 non-Indigenous participants were assessed as experiencing periodontal disease. Overall, dental calculus samples were dominated by typical human oral taxa, such as *Proteobacteria* (accounting for 35.4% of sequences across all dental calculus samples), *Firmicutes* (23.9%), *Bacteroidetes* (16.1%), *Fusobacteria* (10.9%), and *Actinobacteria* (9.7%), with 11 remaining phyla contributing approximately 4% of total sequences (Figure 1, page 50). We confirmed that samples differed significantly from negative controls (Table S1) in both diversity and composition (Figure S2 and Supplementary results).

**Table 1. Demographic characteristics of study participants.** *Summary of key demographic characteristics of study participants used in oral microbiota analyses: specific sampling location (the Northern Territory's Top End region, Central Australia, or metropolitan South Australia), self-identified ethnicity (Indigenous Australian or non-Indigenous), and diagnosis of periodontal disease (PD) as assessed by an oral health professional (yes (Y) or no (N)).*

| ID | Location | Ethnicity | PD status | ID | Location | Ethnicity | PD status |
|---|---|---|---|---|---|---|---|
| A1 | Top End | Indigenous Australian | Y | 14C | South Australia | Non-Indigenous | N |
| A2 | Top End | Indigenous Australian | Y | 15C | South Australia | Non-Indigenous | N |
| A3 | Top End | Indigenous Australian | Y | 16C | South Australia | Non-Indigenous | N |
| A4 | Top End | Indigenous Australian | Y | 17C | South Australia | Non-Indigenous | N |
| A5 | Top End | Indigenous Australian | Y | 19C | South Australia | Non-Indigenous | N |
| A6 | Top End | Indigenous Australian | Y | 20C | South Australia | Non-Indigenous | N |
| A7 | Central Australia | Indigenous Australian | Y | 21C | South Australia | Non-Indigenous | N |
| A8 | Central Australia | Indigenous Australian | Y | 19767 | South Australia | Non-Indigenous | Y |
| A9 | Central Australia | Indigenous Australian | Y | 19770 | South Australia | Non-Indigenous | Y |
| A10 | Central Australia | Indigenous Australian | Y | 19771 | South Australia | Non-Indigenous | Y |
| A11 | Central Australia | Indigenous Australian | Y | 19772 | South Australia | Non-Indigenous | Y |
| A12 | Central Australia | Indigenous Australian | Y | 19773 | South Australia | Non-Indigenous | Y |
| A13 | Central Australia | Indigenous Australian | Y | 19774 | South Australia | Non-Indigenous | Y |
| 1C | South Australia | Non-Indigenous | N | 19775 | South Australia | Non-Indigenous | Y |
| 2C | South Australia | Non-Indigenous | N | 19777 | South Australia | Non-Indigenous | Y |
| 3C | South Australia | Non-Indigenous | N | 19778 | South Australia | Non-Indigenous | Y |
| 4C | South Australia | Non-Indigenous | N | 19780 | South Australia | Non-Indigenous | Y |
| 5C | South Australia | Non-Indigenous | N | 19782 | South Australia | Non-Indigenous | Y |
| 6C | South Australia | Non-Indigenous | N | 19785 | South Australia | Non-Indigenous | Y |
| 7C | South Australia | Non-Indigenous | N | 19786 | South Australia | Non-Indigenous | Y |
| 8Ca | South Australia | Non-Indigenous | N | 19790 | South Australia | Non-Indigenous | Y |
| 9C | South Australia | Non-Indigenous | N | 19792 | South Australia | Non-Indigenous | Y |
| 10C | South Australia | Non-Indigenous | N | 19793 | South Australia | Non-Indigenous | Y |
| 11C | South Australia | Non-Indigenous | N | 19796 | South Australia | Non-Indigenous | Y |
| 12C | South Australia | Non-Indigenous | N | 19799 | South Australia | Non-Indigenous | Y |
| 13C | South Australia | Non-Indigenous | N | 19801 | South Australia | Non-Indigenous | Y |

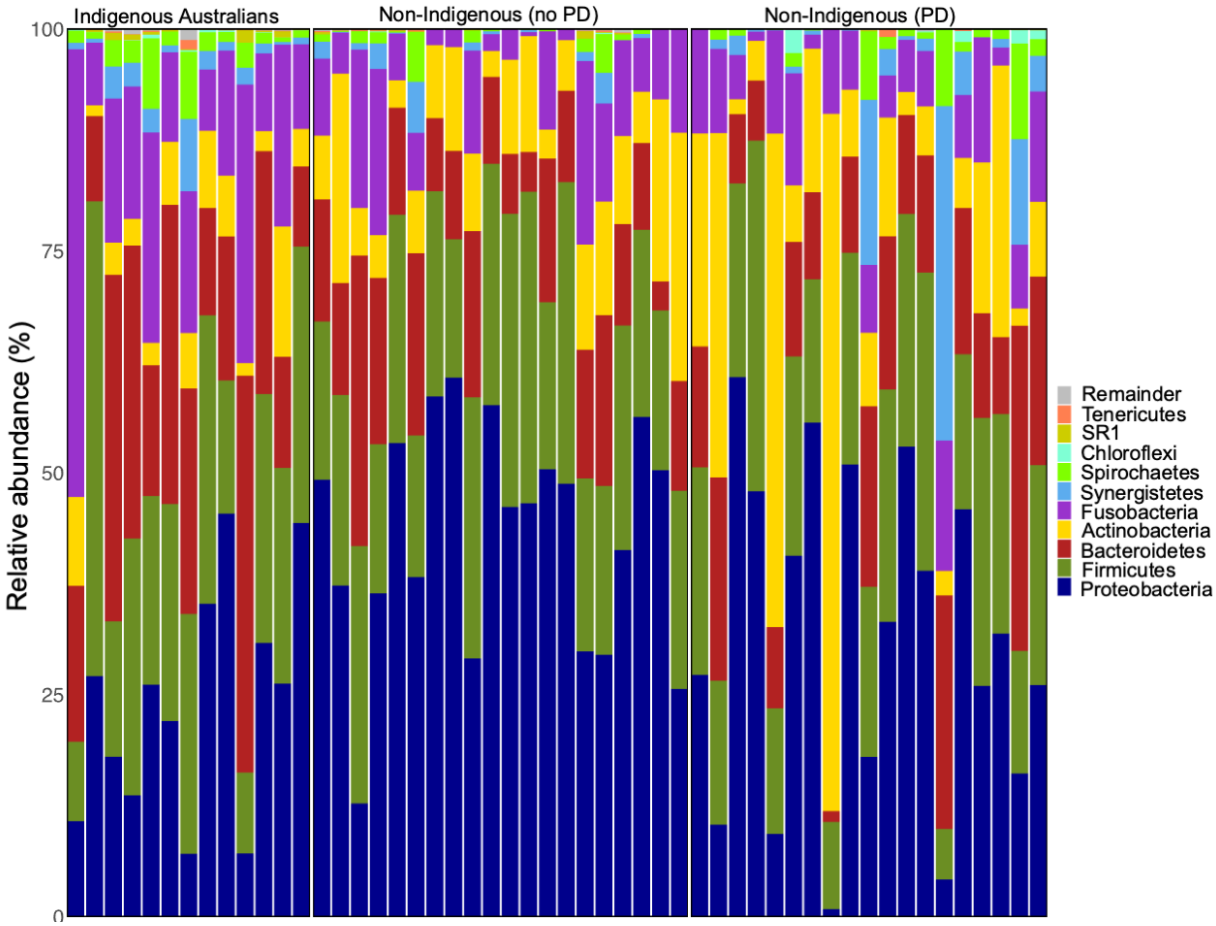


**Figure 1. Dental calculus samples are dominated by typical oral taxa.** *Relative abundance of microbial phyla in all dental calculus samples. Each bar represents a single sample. Samples were dominated by Proteobacteria, Firmicutes, Bacteroidetes, Fusobacteria and Actinobacteria.*

**The diversity and composition of dental calculus microbiota of Indigenous Australian participants differs significantly from that of non-Indigenous participants**

We initially compared the diversity and composition of all dental calculus samples based on self-reported ethnicity (Indigenous Australian or non-Indigenous). Samples from Indigenous Australians had significantly higher phylogenetic diversity (Kruskal-Wallis H=19.86, $p=8.3 \times 10^{-6}$) and differed significantly in composition from the non-Indigenous samples (PERMANOVA pseudo-F=10.42, p=0.001) (Figure 2, overleaf).

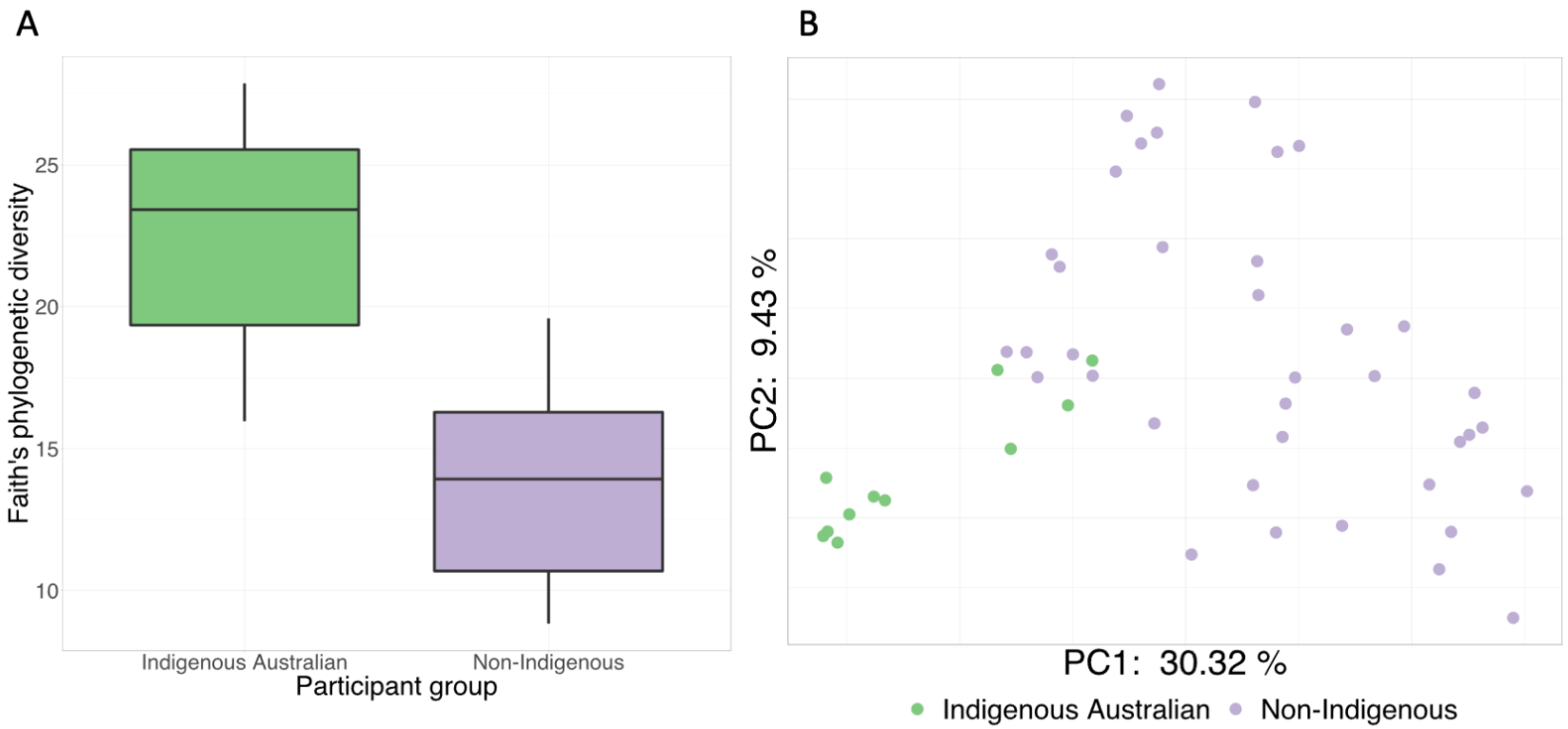


**Figure 2. Oral microbiota diversity and composition differs significantly between Indigenous Australian and non-Indigenous individuals.** *(A) Faith's phylogenetic diversity subsampled to 10,000 sequences per sample. Samples from Indigenous Australians have significantly higher diversity than samples from non-Indigenous individuals (Kruskal-Wallis H=19.86, p=8.3 x $10^{-6}$). (B) Principal coordinates analysis (PCoA) of unweighted UniFrac distances, subsampled to 10,000 sequences per sample. Samples from Indigenous Australians cluster towards one end of PC1 and differ significantly in composition from samples from non-Indigenous individuals (PERMANOVA pseudo-F=10.42, p=0.001).*

To better understand the taxa underlying these differences in diversity and composition, we used ANCOM to identify microbial features (amplicon sequence variants) that differed significantly in abundance between the Indigenous and non-Indigenous groups. A single feature in the genus *Porphyromonas* was significantly more abundant in Indigenous Australians than in non-Indigenous Australians (W=379). BLAST searches revealed that the 16S rRNA sequence associated with this feature was a 100% match to multiple *Porphyromonas gingivalis* and unspecified *Porphyromonas* sequences present in the National Centre for Biotechnology Information (NCBI) and HOMD databases.

**Indigenous Australians harbor more unique oral microbes than non-Indigenous individuals**

We next examined the absolute presence or absence of features unique to either non-Indigenous individuals or Indigenous Australians in this dataset. We identified 125 microbial features that were uniquely found in non-Indigenous individuals and present in at least two samples (Table S2); of these, the top 5 most abundant features were classified as *Streptococcus*, *Rothia aeria*, *Peptostreptococcaceae*, *Streptococcus anginosus*, and *Fusobacterium* taxa. Of the 125 total features, 105 (84%) were known human oral taxa. Of the remaining 20 features, 14 (11% of total) have been identified in studies investigating laboratory contamination and were potentially contaminants [46,47], leaving 6 putatively oral but previously unknown unique non-Indigenous features (5% of total). These comprised features in the genera *Blvii28* (n=4), *Dietzia*, and *Jeotgalicoccus*. As the non-Indigenous group contained individuals both with and without PD, we also examined the 70 features unique to non-Indigenous individuals with PD only (Table S3). The top 5 most abundant features in this group were classified as *Fusobacteria* (n=2), *Blvii28*, *Neisseria oralis*, and *Leptotrichia*. Among the 70 total features, 62 (89%) were known human oral taxa and 5 (7%) were likely contaminants. The 3 remaining features (4% of total) were all classified as members of the genus *Blvii28*. Overall, the features uniquely found in non-Indigenous individuals were dominated by previously described human oral taxa.

In Indigenous Australians, we identified 171 unique microbial features present in at least two samples (Table S4). The top 5 most abundant features unique to Indigenous Australians were classified as *Mogibacteriaceae*, *Porphyromonas*, *[Tissierellaceae]*, *Desulfomicrobiaceae*, and *Methanobrevibacter*. Altogether, 141 (82%) features were known human oral taxa, while 10 features (6%) were likely contaminants. A remaining 20 features (12%) were putatively oral but

previously unknown – more than double the proportion of features in this category uniquely identified in the non-Indigenous group. Of these 20 features, the top 5 most abundant were classified as *Syntrophomonas*, *BS11*, *ML615J-28* (n=2), and *Endomicrobia*. Taken together, these results suggest that Indigenous and non-Indigenous individuals may harbour unique strains of oral microbes. The samples from Indigenous Australians contained a relatively high proportion of unique microorganisms not found in current human oral microbiome literature or reference databases.

**Indigenous Australians and non-Indigenous individuals with periodontal disease harbor microbiota differences**

To control for possible biases in our results caused by PD status, we sought to explore the impacts of PD on the oral microbiota differences we observed between Indigenous and non-Indigenous participants. We first divided the samples into three groups according to self-identified ethnicity and PD status: Indigenous Australians with periodontal disease PD (IPD), non-Indigenous individuals with PD (NPD), and non-Indigenous individuals without PD (NH) (Figure 3, overleaf).

Consistent with previous results, samples from Indigenous Australians had significantly higher phylogenetic alpha diversity than samples from non-Indigenous Australians (pairwise Kruskal-Wallis tests: IPD vs NH $H=15.38$, $p=1.3 \times 10^{-4}$; IPD vs NPD $H=16.73$, $p=1.3 \times 10^{-4}$; NPD vs NH $H=0.41$, $p=0.52$) (Figure 3A). Clear and statistically significant clustering according to these categories was observed in PCoA based on unweighted UniFrac distances (pairwise PERMANOVA tests: IPD vs NH pseudo-$F=10.14$, $p=0.0015$; IPD vs NPD pseudo-$F=8.96$, $p=0.0015$; NPD vs NH pseudo-$F=2.45$, $p=0.008$) (Figure 3B). ANCOM testing highlighted the same *Porphyromonas* feature previously identified as significantly more abundant in Indigenous than non-Indigenous samples. This feature was significantly more abundant in IPD than in NPD and was absent from the NH group ($W=353$).

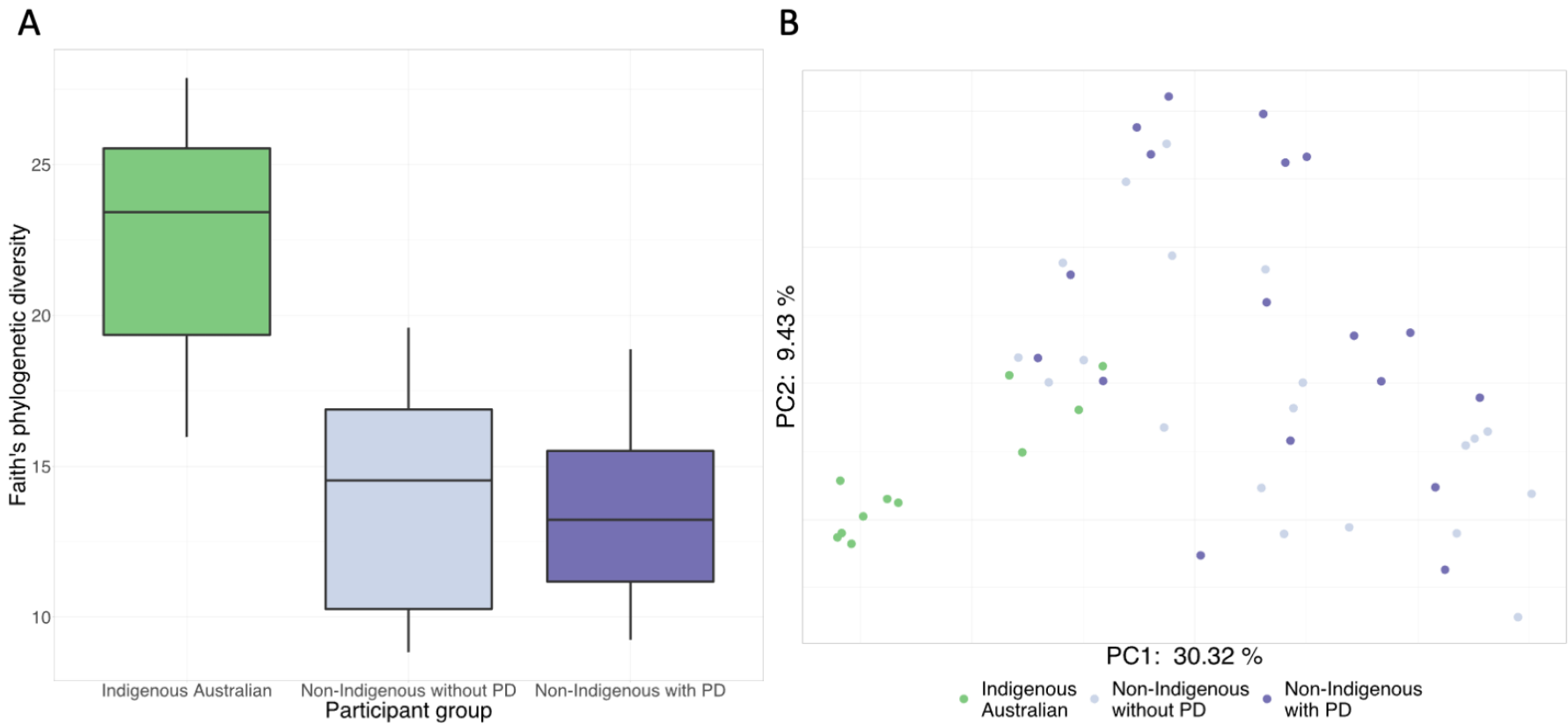


**Figure 3. Differences between Indigenous and non-Indigenous oral microbiota are robust to PD status.** *(A) Faith's phylogenetic diversity subsampled to 10,000 sequences per sample. Samples from Indigenous Australians have significantly higher diversity than samples from both non-Indigenous individuals without PD (Kruskal-Wallis H=15.38, p=1.3 x $10^{-4}$) and non-Indigenous individuals with PD (H=16.73, p=1.3 x $10^{-4}$), while diversity of samples from non-Indigenous individuals did not differ significantly according to PD status (H=0.41, p=0.52). (B) Principal coordinates analysis (PCoA) of unweighted UniFrac distances, subsampled to 10,000 sequences per sample. Samples from Indigenous Australians cluster towards one end of PC1 and differ significantly in composition from samples from both non-Indigenous individuals without PD (pairwise PERMANOVA pseudo-F=10.14, p=0.0015) and non-Indigenous individuals with PD (pseudo-F=8.96, p=0.0015). A less pronounced, but still significant, difference in composition was observed between non-Indigenous individuals with and without PD (pseudo-F=2.45, p=0.008).*

We next examined the oral microbiota in Indigenous and non-Indigenous individuals with PD, excluding periodontally healthy non-Indigenous individuals. The IPD group had significantly higher alpha diversity than the NPD group (Kruskal-Wallis H=17.1, p=3.5 x $10^{-5}$) (Figure S3A); the two groups again differed significantly in microbiota composition (PERMANOVA pseudo-F=9.26, p=0.001) (Figure S3B). Using ANCOM, we identified two features that were significantly more abundant in the IPD group than in the NPD group: the same *Porphyromonas* feature previously identified (W=335) and a *Clostridiales* feature (W=299). Overall, these results suggest that PD status alone did not drive the microbiota differences observed between Indigenous and non-Indigenous participants. However, this interpretation would be clarified by further research investigating oral microbiota in periodontally healthy Indigenous Australians.

**Oral microbiota diversity and composition may vary across Australian regions**

As microbiota differences between Indigenous and non-Indigenous participants were not associated with PD status, we explored other factors that may contribute to unique signatures in the oral microbiota of Indigenous Australians. As heritage is deeply linked to connection to Country (ancestral homelands) for Indigenous Australians, we first investigated geographic differences between our sample collection sites. Significant differences linked to sampling region were identified across the whole dataset (i.e. differences between the Northern Territory's Top End region (TE), Central Australia (CA), or metropolitan South Australia (SA)). All locations significantly differed in alpha diversity, with CA having the highest diversity and SA (i.e. non-Indigenous individuals) the lowest (pairwise Kruskal-Wallis tests: CA vs TE H=4.8, p=0.028; CA vs SA H=12.95, p=0.001; TE vs SA H=9.69, p=0.003) (Figure S4A). Next, we tested the effect of specific sampling region on microbiota composition. While the largest differences were between SA and the other two locations (i.e. between non-Indigenous and Indigenous Australian individuals) (pairwise PERMANOVA tests: CA vs SA pseudo-F=8.49, p=0.0015; TE vs SA pseudo-F=4.7, p=0.0015), a significant difference in composition was also observed between CA and TE (PERMANOVA pseudo-F=2.59, p=0.016) (Figure S4B). Together, these findings suggest that oral microbiota diversity and composition among Australians may be linked to geographic location. However, due to the small number of samples available from TE (n=6) and CA (n=5), these results are preliminary and need further verification.

Lastly, we examined unique microbial features that were specific to each sampling location. The same *Porphyromonas* feature previously identified as significantly more abundant in Indigenous Australians significantly varied in abundance according to location (ANCOM W=351); specifically, this feature was in very low abundance in SA, intermediate abundance in CA, and highest abundance in TE. We also characterized features that were uniquely present in samples collected from Indigenous Australians at a given location. We identified only 3 features unique to the Top End that were found in at least two samples; these were respectively classified as members of the order *Bacteroidales* and the genera *Eikenella* and *TG5* (Table S5), which are all known human oral taxa. In contrast, we identified 57 features that were uniquely found in Central Australia and present in at least two samples (Table S6). The top 5 most abundant of these unique features were classified as *Porphyromonas*, *Desulfomicrobiaceae*, *Leptotrichia*, *Desulfovibrio* and *Peptoniphilus*, with the *Porphyromonas* and *Desulfomicrobiaceae* features being the same two identified in the top 5 most abundant unique features in Indigenous Australians overall. Altogether, 49 (86%) of the unique Central Australian features were classified as known human oral taxa and none were classified as likely contaminants, leaving 8 features (14%) classified as putatively oral but previously unknown taxa. This last group included features classified as *Syntrophomonas*, *BS11*, *Endomicrobia*, *OPB56*, and a member of the family *p-2534-18B5*. Several of these were also among the most abundant previously unknown unique features in Indigenous Australians above, implying that many of these features may be localized to the Central Desert. These findings suggest that regional variation may play a role in shaping the oral microbiota among Indigenous Australians.

**Conclusions and implications**

This study characterizes the oral microbiota in a cohort of Indigenous Australian and non-Indigenous adults, addressing a critical first step in identifying previously undescribed mechanisms that may contribute to disparities in Indigenous oral and overall health. Both the diversity and composition of oral microbiota of Indigenous Australians differed significantly from that of non-Indigenous participants, regardless of PD status. Unique microbial features not previously described in the human oral microbiota were also found in Indigenous Australians, and these features differed between Indigenous Australians living in different locations. Together, these results lay the groundwork to better understand how the microbiota influences health and disease.

In our study, oral microbiota of Indigenous Australians had significantly higher alpha diversity and a distinct composition compared to non-Indigenous individuals (Figures 2-3), as well as a relatively high proportion of unique taxa not previously observed in the human oral microbiota, including *Syntrophomonas*, *BS11*, *ML615J-28* and *Endomicrobia* (Tables S4-S6). Previous studies of oral microbiota in Indigenous peoples have reported differences in alpha diversity [30–32] and distinct microbiota composition [30,31,48] compared to non-Indigenous individuals. However, with the exception of work by Ozga and colleagues [32], these studies typically compare Indigenous and non-Indigenous groups with markedly different lifestyles and subsistence strategies (e.g. industrialized vs hunter-gatherer), making it difficult to differentiate the impacts of *lifestyle* (e.g. different diets), environment (e.g. exposure to certain places), and *inheritance* (e.g. vertical transmission of microbiota adapted to specific lifestyles and environments) independently or concurrently. In contrast, both Indigenous and non-Indigenous participants in our study have industrialized lifestyles. Our findings therefore support the idea that the life and experiences of an individual's ancestors, and not only current individual lifestyle, may help to shape the oral microbiota [28].

We hypothesize that these differences may be explained by a mechanism of microbiota inheritance across generations, influenced by the evolutionary history of the oral microbiota in different locations. We found significant differences in diversity and composition between Indigenous Australian oral microbiota from different sampling locations (the Northern Territory's Top End and Central Australia) (Figure S4). Geographic variation in oral microbiota was previously reported among hunter-gatherers and traditional farmers living in the Philippines [30]. Indigenous Australians have close connections to Country: in many cases, the ancestors of living Indigenous Australians lived in a particular location for at least 45,000 years, even if recent colonial disruptions mean that not all Indigenous Australians live on Country today [49]. During this time, Indigenous Australians' microbiota could have adapted to specific environments or cultural practices such as diets or traditional medicines.

For example, an *Endomicrobia* feature was uniquely detected in samples from Indigenous Australians in Central Australia (Table S6). *Endomicrobia* species are typically intracellular symbionts that live in the guts of termites and other wood-eating insects [50,51] and allow the

host to digest cellulose. Termites are a traditional food for some Aboriginal Australians in the Central Desert region [52–54], and termite mounds are also used in traditional medicine throughout the Northern Territory [55], suggesting possible mechanisms for the introduction of termite-associated species into the oral microbiota. Therefore, we propose that heritage may play a role in the maintenance of *Endomicrobia* and other unique oral species in the oral microbiota of Indigenous Australians living industrialized lifestyles. This concept of heritage may encompass factors including transgenerational microbiota inheritance, the experiences of ancestors, and ongoing connection to Country and cultural practices, which will require further research to fully understand. Using dental calculus, which preserves microbiota over longer time spans than dental plaque or saliva, may have also provided unique insights into the heritage of oral microbes. We acknowledge that this is only a pilot study with a small number of samples and lacks detailed familial information. Nevertheless, this study opens the door for future research investigating the evolutionary history of unique oral microbes, their connection to heritage, and their roles in health and disease.

While this study was not designed to directly investigate microbial mechanisms that underpin periodontal disease (PD), we did observe some possible links between our microbiota data and PD. A microbial feature classified as *Porphyromonas* was significantly higher in abundance in the microbiota of Indigenous Australians. *P. gingivalis* is thought to play an important role in tissue destruction during PD progression [56]. This species has been shown to impair innate immunity in mice with knock-on effects for the oral microbiota [57], leading to the hypothesis that *P. gingivalis* is a 'keystone pathogen' in PD [58]. Several features uniquely identified in samples from Indigenous Australians (e.g. *Mogibacteriaceae*, *Porphyromonas*, *Tissierellaceae*, *Desulfomicrobiaceae* and *Methanobrevibacter* (Table S4)) may also be associated with PD [59–64]. This finding may point to the importance of specific strains of periodontal pathogens or unique manifestations of PD in Indigenous Australians, which may be related to a mismatch between the evolution of the oral microbiota in Australia and the lifestyle and environmental exposures experienced by Indigenous Australians today. If so, a deeper understanding of these differences could be important to improve early diagnosis and identify successful treatment options for PD in Indigenous Australians. While outside the scope of this study, the health implications of high diversity and unique composition of oral microbiota in Indigenous Australians are worthy of further investigation.

Building on the results presented here, future work could characterize oral microbiota in more Indigenous Australian communities to better understand how oral microbial communities develop and function in relation to heritage, environment, and oral and systemic health. Additionally, shotgun metagenomic sequencing could be used to obtain strain-level and functional information, assemble microbial genomes, and examine the evolutionary history of the oral microbiota in Australia using ancient dental calculus samples. As recently suggested by Benezra, transdisciplinary research projects that combine scientific and social science approaches to better understand the social, cultural and environmental factors that shape microbiota are another important avenue for future work [65]. As microbiota research continues to gain clinical relevance, understanding how these microbes function and interact with the host will be crucial to inform the most effective treatment and prevention strategies for NCDs that disproportionately impact Indigenous peoples.

**Funding and conflicts of interest**

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**Data availability**

The microbial 16S V4 region amplicon datasets generated using samples from non-Indigenous participants during the current study are available in the Qiita repository (https://qiita.ucsd.edu/) under the Study ID 13416. Study data relating to Indigenous Australian participants are not freely available because of ethical and data protection constraints. These de-identified data are stored at the University of Adelaide and cannot be sent outside the institution. Proposals to access data for further analyses should initially be addressed to Lisa Jamieson (lisa.jamieson@adelaide.edu.au) and will be reviewed by the Indigenous Reference Group and research team.

Supplementary information for this manuscript is available for review by thesis examiners at the following link: https://figshare.com/s/d7a985ede00975844b0a

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# Chapter III: Biocultural drivers of salivary microbiota in Australian Aboriginal and Torres Strait Islander children

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# Statement of Authorship

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# Biocultural Drivers of Salivary Microbiota in Australian Aboriginal and Torres Strait Islander Children

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Australian Aboriginal and Torres Strait Islander children experience unacceptably high rates of dental caries compared to their non-Indigenous Australian counterparts. Dental caries significantly impacts the quality of life of children and their families, particularly in remote communities. While many socioeconomic and lifestyle factors impact caries risk, the central role of the oral microbiota in mediating dental caries has not been extensively investigated in these communities. Here, we examine factors that shape diversity and composition of the salivary microbiota in Aboriginal and Torres Strait Islander children and adolescents living in the remote Northern Peninsula Area (NPA) of Far North Queensland. We employed 16S ribosomal RNA amplicon sequencing to profile bacteria present in saliva collected from 205 individuals aged 4–17 years from the NPA. Higher average microbial diversity was generally linked to increased age and salivary pH, less frequent toothbrushing, and proxies for lower socioeconomic status (SES). Differences in microbial composition were significantly related to age, salivary pH, SES proxies, and active dental caries. Notably, a feature classified as *Streptococcus sobrinus* increased in abundance in children who reported less frequent tooth brushing. A specific *Veillonella* feature was associated with caries presence, while features classified as *Actinobacillus/Haemophilus* and *Leptotrichia* were associated with absence of caries; a *Lactobacillus gasseri* feature increased in abundance in severe caries. Finally, we statistically assessed the interplay between dental caries and caries risk factors in shaping the oral microbiota. These data provide a detailed understanding of biological, behavioral, and socioeconomic factors that shape the oral microbiota and may underpin caries development in this group. This information can be used in the future to improve tailored caries prevention and management options for Australian Aboriginal and Torres Strait Islander children and communities.

**Keywords: bacteria, community dentistry, dental caries, ecology, microbiology**

## INTRODUCTION

Dental caries is a highly prevalent oral disease that severely impacts children and families' quality of life [1, 2]. Australian Aboriginal and Torres Strait Islander children have higher rates of dental caries, and of untreated caries, than non-Indigenous Australian children [3]. Additionally, Aboriginal and Torres Strait Islander children in remote communities experience worse oral health than their urban counterparts [4, 5]. For example, a 2006 survey of child caries experience in a remote Aboriginal and Torres Strait Islander community in the Northern Peninsula Area (NPA) of Far North Queensland found that caries experience in NPA children was over four times the national average [6]. Caries incidence in this community remained unacceptably high as of 2015 [7]. Risk factors for caries include lifestyle (e.g., oral hygiene, diet, fluoride exposure) and underlying host susceptibility (e.g., immune factors, prevalence of caries-promoting oral bacteria) [8, 9]. Many of these factors are prevalent for Aboriginal and Torres Strait Islander children [10].

Recent research describes how caries is mediated by the oral microbiota – the community of microorganisms inhabiting the human mouth [11]. Although the factors shaping Aboriginal and Torres Strait Islander child oral microbiota have not yet been explored, changes in microbiota composition associated with caries initiation and progression have previously been reported in other groups [12–16]. Many host-related and environmental factors, including vertical transmission, diet, and antibiotic use have been reported to influence the diversity and composition of an individual's oral microbiota [17–23]. In turn, these factors may contribute to broader-scale microbiota differences among members of human groups with different heritage and lifestyles [24, 25]. For example, studies from Venezuela, the Philippines and Uganda have reported differences in saliva microbiota diversity and composition between Indigenous groups with hunter-gatherer lifestyles and counterparts living agricultural or industrialized lifestyles [26–28]. Furthermore, our previous work identified systematic differences between the oral microbiota of Indigenous Australian and non-Indigenous adults, including differences linked to oral health [29]. These findings raise the possibility that Aboriginal and Torres Strait Islander children may have unique oral microbiota signals linked to caries development.

Here, we investigate the oral microbiota in children aged 4–17 from the NPA using stimulated saliva and extensive metadata from dental examinations and participant questionnaires. Our objective was to examine whether any metadata factors were linked to salivary microbiota diversity and composition and thereby to better understand how the microbiota, lifestyle and socioeconomic factors, and oral disease interact in this population. We explored factors influencing the salivary microbiota and identified factors linked to dental caries in the absence of regular professional dental care. Our study provides important baseline data to better understand how the oral microbiota and its relationship to caries is shaped in a remote Aboriginal and Torres Strait Islander community and how the oral microbiota may be harnessed to improve Indigenous oral health. Such data will be crucial to ensure that future microbiota-aware therapies for dental caries and other oral diseases are also relevant to these communities.

## MATERIALS AND METHODS

### Ethical Approval

In planning the study, extensive consultations were held with Elders and community members. Data reported in this manuscript are from a single baseline survey of oral and general health, conducted in 2015, prior to the implementation of preventive measures against dental caries. Feedback from the overall study was provided to the community in 2018 and 2019. 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, and with support of the Principal and teaching staff. We have worked closely with Community Health staff, and regularly consulted with the Mayor and Community over the years.

### Participant Recruitment and Data Collection

Participating children aged 4–17 were recruited and sampled at a single timepoint as the baseline for a larger clinical trial examining the impact of an annual caries preventive intervention in the NPA (ANZCTR no. ACTRN12615000693527, registered 3 July 2015) [4]. Saliva samples were collected by chewing on paraffin wax for 5 min while expectorating into a sterile cup. Total saliva volume produced was recorded and 2 mL of saliva was transferred to an OMNIgene OM-501 collection tube (DNA Genotek) and stored according to manufacturer's instructions. Samples were collected throughout school hours; due to complex field conditions, information on last food intake was not collected. Dental examinations were performed by trained and calibrated examiners as previously described [30]. For microbiota analyses, caries severity categories were assigned based on International Caries Detection and Assessment System (ICDAS) II codes [31] as follows: 0 = sound, 1–2 = incipient, 3–4 = moderate, 5–6 = severe; caries status was assigned based on ICDAS II codes as follows: 0 = caries-free, 1–6 = caries-active; categories were assigned to saliva samples based on the highest ICDAS II code recorded for that child. As compressed air was not available during examinations, teeth were dried with gauze prior to ICDAS assessment. Questionnaires detailing oral health behaviors, diet, emotional well-being, and oral health impact on quality of life were completed by participants or caregivers (**Supplementary Table 3**). Specifically, the validated CHU-9D [32] and OHIP-14 [33] systems were used to collect data on general child quality of life and oral health-related quality of life.

## DNA Extraction and Sequencing

A total of 255 saliva samples were used for DNA extraction and sequencing, from participants whose parents had consented to oral microbiota analysis. Genomic DNA was extracted from saliva samples in a clean facility at the University of Adelaide using the Roche High Pure PCR Template Preparation Kit (Roche Life Sciences). Two extraction blank controls (EBCs, i.e., empty tubes) were included for every 22 saliva samples. The V4 region of the bacterial 16S rRNA gene was amplified using uniquely barcoded reverse primers for each sample, as previously described [34]. No-template controls (NTCs) were processed alongside each amplification. Amplified, barcoded DNA was quantified using Qubit (ThermoFisher Scientific), pooled at equal relative concentrations, cleaned using Ampure magnetic beads (New England Biolabs), and quantified using TapeStation (Agilent). Paired-end 150 bp sequencing was performed using an Illumina MiSeq.

## Sequence Data Processing

Data were processed using QIIME2 (v. 2018.8) [35]. Briefly, sequences were demultiplexed, denoised using the *q2-deblur* plugin [36], and assigned taxonomy using a classifier trained on the SILVA 132 database, selected as the most suitable taxonomic database as it contains sequences from a wide range of sample types. Key taxonomic results were also compared against the Human Oral Microbiome Database (HOMD) v15.1 (**Supplementary Tables 4, 6**). Five samples were removed from further analysis due to insufficient data or withdrawal of consent. Amplicon sequence variants (referred to herein as microbial "features") observed <3 times were removed from the dataset. Detection and removal of putative contaminant features at the 0.5 threshold (i.e., features that were more prevalent in negative controls than in samples were considered contaminants) was performed using *qiime2R* [37] and *decontam* [38].

## Microbiota Analysis

Data were analyzed using QIIME2 (v. 2019.7). Only samples with at least 30,000 sequences per sample were retained for microbiota analysis, leading to the removal of 42 saliva samples due to insufficient sequence depth; three samples from individuals who had contributed two saliva samples each were also removed, leaving a total of 205 samples. Samples with unknown or unrecorded values for a given metadata factor were removed prior to analysis, and categorical metadata factors needed at least 10 samples in each group for significance testing. Alpha diversity (within sample diversity; Faith's phylogenetic diversity [39]) and beta diversity (between sample diversity; unweighted UniFrac [40]) were calculated and statistically examined at the feature (amplicon sequence variant) level using the *q2-diversity* plugin, randomly subsampling data to 30,000 sequences per sample. These metrics were chosen because they incorporate phylogenetic as well as non-phylogenetic information about the diversity of the samples. Statistical significance was determined non-parametrically using Spearman correlation (for continuous variables) or Kruskal-Wallis (for categorical variables) [41] tests for alpha diversity and adonis tests [42, 43] for beta diversity. Metadata categories that returned a significant or near-significant result in adonis tests were further investigated using PERMANOVA [42] and permdisp tests [44]. Features with <10 observations and/or present in <5 samples were removed prior to ANCOM testing using *q2-composition ancom* to identify features that differed significantly in abundance across categorical sample groups [45]. Figures were constructed in RStudio [46] using the *qiime2R* [37] and *ggplot2* packages [47], or downloaded from QIIME2 View and edited for clarity using Inkscape 2.0.

# RESULTS

## Sociodemographic Characteristics of Participants

A total of 205 saliva samples with corresponding metadata were used for microbiota analysis (**Table 1**). Although specific ethnicity data was not collected alongside individual saliva samples, within the NPA community 49.5% identified as Aboriginal Australian and Torres Strait Islander, 46.4% identified as Torres Strait Islander only, 1% identified as Aboriginal Australian only, and 3.1% identified as neither. A full list of metadata factors tested in microbiota analysis is given in **Supplementary Table 1**; further details on values recorded for continuous metadata variables are given in **Supplementary Table 2**.

## Background DNA Had a Limited Effect on Salivary Microbiota

Because environmental and laboratory-based contamination can significantly impact microbiota studies [48, 49], we used negative controls (EBCs and NTCs) to track contamination in our study. We verified significant differences in diversity ($H = 62.67$, $p = 2.44 \times 10^{-15}$) and composition ($R^2 = 0.21$, $p = 0.001$) (**Supplementary Figure 1A**) between saliva and negative controls. We used *decontam* to statistically identify and remove 39 contaminant microbial features [38] (**Supplementary Figure 1B**; **Supplementary Table 3**).

## The Salivary Microbiota of NPA Children Is Dominated by Typical Human Oral Taxa

Following removal of putative contaminant features, we summarized the taxonomic composition of the 205 saliva samples that formed the core of our microbiota analysis (**Figure 1**). The samples were dominated by the phyla *Proteobacteria* (30%), *Bacteroidetes* (26%), *Firmicutes* (25%), *Actinobacteria* (12%), and *Fusobacteria* (6%), with sequences assigned to *Epsilonbacteraeoata*, *Spirochaetes*, *Patescibacteria*, *Tenericutes*, *Synergistetes*, *Cyanobacteria*, *Chloroflexi*, and unassigned *Bacteria* making up the remaining 1%. At the genus level, the salivary microbiota was dominated by *Prevotella* (18%), *Neisseria* (14%), *Haemophilus* (12%), *Streptococcus* (9%), *Rothia* (8%), *Veillonella* (6%), *Fusobacterium* (4%), *Alloprevotella* (3%), *Porphyromonas* (3%), *Gemella* (2%), *Granulicatella* (2%), *Leptotrichia* (2%), *Actinomyces* (2%), and *Aggregatibacter* (2%), with various genera accounting for 1% or less of total sequences

**TABLE 1 |** Sociodemographic characteristics of study participants.

| Category | Mean | Standard deviation | Range |
|---|---|---|---|
| **Continuous variables** | | | |
| Age | 8.53 | 3.53 | 4–17 |
| Saliva pH | 7.05 | 0.49 | 5.4–7.8 |
| Saliva flow rate | 5.77 | 3.00 | 0.5–9.5 |
| Total carious surfaces | 9.68 | 9.8 | 0–62 |

| Category | Values | *n* | % |
|---|---|---|---|
| **Categorical variables** | | | |
| **Sex** | | | |
| | Female | 115 | 56.1% |
| | Male | 90 | 44.0% |
| **Caries status** | | | |
| | Caries-free | 17 | 8.3% |
| | Caries-active | 184 | 90% |
| | Unknown or not recorded | 4 | 2.0% |
| **Caries severity** | | | |
| | Caries-free (ICDAS 0) | 17 | 8.3% |
| | Incipient caries (ICDAS 1–2) | 37 | 18.0% |
| | Moderate caries (ICDAS 3–4) | 47 | 22.9% |
| | Severe caries (ICDAS 5–6) | 100 | 48.8% |
| | Unknown or not recorded | 4 | 2.0% |
| **Household size** | | | |
| | 1–5 | 86 | 42.0% |
| | 6–10 | 96 | 46.8% |
| | More than 10 | 10 | 4.9% |
| | Unknown or not recorded | 13 | 6.3% |
| **Household employment status** | | | |
| | No people work | 18 | 8.8% |
| | At least one person works | 172 | 83.9% |
| | Unknown or not recorded | 15 | 7.3% |
| **Soft drink consumption** | | | |
| | Yes | 156 | 76.1% |
| | No | 41 | 20.0% |
| | Unknown or not recorded | 8 | 3.9% |
| **Daily toothbrushing** | | | |
| | Less than once | 16 | 7.8% |
| | Once | 34 | 16.6% |
| | Twice | 130 | 63.4% |
| | More than twice | 17 | 8.3% |
| | Unknown or not recorded | 8 | 3.9% |
| Total | | 205 | 100% |

*A summary of important sociodemographic characteristics of individuals who donated the 205 saliva samples used for microbiota analysis is presented in this table. For continuous variables, the mean, standard deviation and range values are reported, rounded to two decimal places. A detailed record of data for continuous variables is presented in* ***Supplementary Table 2****. For continuous categories, the number of samples and percentage of the total in each possible value are reported; percentage values are rounded to one decimal place and may not add to exactly 100%.*

making up the remaining 13%. Taxonomic assignment of sequences using HOMD in place of SILVA yielded nearly identical classifications (**Supplementary Table 4**).

## Age Significantly Impacts Salivary Microbiota in NPA Children

We tested all available metadata factors (**Supplementary Table 1**) for associations with changes in microbial diversity (alpha diversity) (**Table 2**; **Figure 2**), composition (beta diversity) (**Table 2**) and abundance of microbial features (**Table 3**). Participant age (Spearman $\rho = 0.4$, $p = 0.0$; $R^2 = 0.048$, $p = 0.001$) and examination date ($H = 26.7$, $p = 8 \times 10^{-4}$; $R^2 = 0.101$, $p = 0.001$) were significantly related to both diversity and composition (**Table 2**). A feature classified as uncultured *Actinomyces* [identified as *Actinomyces* using HOMD (**Supplementary Table 6**)] was significantly associated with examination date (**Table 3**); this feature was observed on 16 of the 26 different examination dates and varied in abundance. Additionally, salivary pH and flow rate were significantly associated with microbiota diversity (Spearman $\rho = 0.18$, $p = 0.011$ and $\rho = 0.28$, $p = 1 \times 10^{-4}$, respectively) and composition ($R^2 = 0.013$, $p = 0.007$ and $R^2 = 0.028$, $p = 0.001$) (**Table 2**). However, multi-factor adonis tests indicated that both examination date ($R^2 = 0.063$, $p = 0.025$) and salivary flow rate ($R^2 = 0.013$, $p = 0.005$) were confounded with age although examination date retained some independent explanatory power ($R^2 = 0.075$, $p = 0.001$). Salivary pH remained independently significant in the multi-factor test ($R^2 = 0.011$, $p = 0.014$) (**Table 2**). We accounted for age in subsequent beta diversity (i.e., composition) analyses using multi-factor adonis tests (**Table 2**), and report only age-adjusted results (where applicable) in the remainder of the text.

## Behavioral Factors Impact Salivary Microbiota Diversity and Composition

We investigated associations between the salivary microbiota and behavioral factors known to impact caries risk. The number of times per day a child reported brushing their teeth was significantly associated with alpha diversity ($H = 8.68$, $p = 0.034$) (**Table 2**). Children who reported brushing their teeth less than once per day harbored higher diversity and exhibited less inter-individual variation in alpha diversity than children who reported more frequent tooth brushing (**Figure 2**). A feature classified as *Streptococcus sobrinus* was significantly more abundant in the saliva of children who reported brushing their teeth less than once per day (**Table 3**). However, tooth brushing was not associated with significant change in overall composition. Of dietary variables collected, only self-reported soft (i.e., carbonated) drink consumption approached a significant association with microbiota composition ($R^2 = 0.007$, $p = 0.067$), an effect that may have been confounded by other factors such as age and caries severity (**Table 2**).

## Socioeconomic Factors Are Linked to Salivary Microbiota

We used self-reported questionnaire data on household size and employment status in the child's household as proxies for socioeconomic status (SES). Employment status was significantly related to alpha diversity ($H = 4.79$, $p = 0.029$), with children who reported no people in their household working having higher

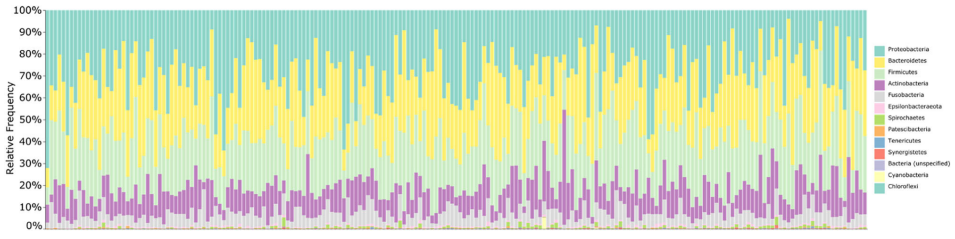


**FIGURE 1 |** Taxonomic summary of saliva samples. Bar chart summarizing the taxonomic composition of each saliva sample at the phylum level. Bars are ordered by participant age; taxonomy was assigned to 16S V4 amplicon sequences using the SILVA 132 database. Overall, samples are dominated by the phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Fusobacteria*.

average diversity and lower inter-individual variability than those who reported at least one person working (**Table 2**; **Figure 2**). Household size was also generally associated with higher average diversity (H = 6.58, $p = 0.037$) (**Table 2**; **Figure 2**); however, no pairwise significant differences in diversity were observed between groups. Both employment status ($R^2 = 0.011$, $p = 0.009$; permdisp F = 8.68, $p = 0.008$) and household size ($R^2 = 0.015$, $p = 0.054$) were significantly related to microbiota composition (**Table 2**). Specifically, significant differences in composition were found between children who reported living with 1–5 people and those who reported living with 6–10 (pairwise PERMANOVA FDR-corrected $p = 0.039$) or more than 10 people (pairwise PERMANOVA FDR-corrected $p = 0.041$). However, ANCOM testing did not identify any microbial features that changed significantly in abundance across these groups.

## Dental Caries Is Associated With Salivary Microbiota Composition

Finally, we explored associations between caries and the salivary microbiota. Total number of carious surfaces was significantly associated with alpha diversity (Spearman $\rho = 0.2$, $p = 0.005$) (**Table 2**). Variations in composition were associated with caries status (i.e., caries-free or caries-active) ($R^2 = 0.008$, $p = 0.052$), caries severity ($R^2 = 0.02$, $p = 0.034$), and number of carious surfaces ($R^2 = 0.014$, $p = 0.002$) (**Table 2**). Pairwise tests comparing the caries-free or incipient caries groups with the moderate or severe caries groups approached significance (pairwise PERMANOVA FDR-corrected $p = 0.072$). We used ANCOM differential abundance testing to identify microbial features driving these compositional changes (**Table 3**). The *Actinobacillus porcinus* feature previously associated with examination date was significantly associated with caries status and was more abundant in the caries-free group (W = 81) (**Table 3**). Members of the genera *Leptotrichia* (W = 26) and *Veillonella* (W = 19) were significantly more abundant in the caries-free and caries-active groups, respectively (**Table 3**). A microbial feature identified as *Lactobacillus gasseri* was significantly associated with caries severity (W = 208), specifically with the severe caries group (**Table 3**).

We further sought to understand whether caries severity interacted with other factors significantly or near-significantly related to salivary microbiota composition using multi-factor adonis tests (**Table 2**). Caries severity consistently explained 2.1–3.2% of variation in the dataset and was significantly associated with composition ($p < 0.05$). Self-reported soft drink consumption co-varied with caries severity ($R^2 = 0.008$, $p = 0.074$) (**Table 2**). However, saliva pH ($R^2 = 0.015$, $p = 0.004$), saliva flow rate ($R^2 = 0.025$, $p = 0.001$), household size ($R^2 = 0.016$, $p = 0.043$) household employment status ($R^2 = 0.012$, $p = 0.004$), and total carious surfaces ($R^2 = 0.023$, $p = 0.001$) were significantly related to composition, independent of caries severity (**Table 2**).

## DISCUSSION

This is the first study to examine the biological, behavioral, and socioeconomic factors driving overall salivary microbiota diversity and composition in Aboriginal and Torres Strait Islander children and adolescents. Although Aboriginal and Torres Strait Islander children, especially those living in remote communities, are at high risk of caries [3–5, 10], little is known about the role of the oral microbiota and how it may mediate disease in this group [50]. While several recent studies have investigated oral microbiota in Indigenous individuals around the world, many have focused primarily on characterizing differences between human groups living different lifestyles [26–28]. Only a handful of studies have examined links between oral microbiota and oral health in Indigenous populations [13, 50]. In our study, we found that salivary microbiota diversity in Aboriginal and Torres Strait Islander children and adolescents from the NPA is linked to age, salivary characteristics, number of carious surfaces, toothbrushing behaviors, and SES (**Table 2**; **Figure 2**). The composition of salivary microbiota is related to age, salivary characteristics, self-reported soft drink consumption, SES, and dental caries (**Table 2**). We identified microbial features that significantly varied in abundance according to examination date, toothbrushing behavior, caries status, and caries severity (**Table 3**). We acknowledge that the number of caries-active individuals in this study clearly

**TABLE 2 |** Metadata factors linked to significant differences in alpha and beta diversity across sample groups.

| **Alpha diversity (Faith's phylogenetic diversity)** | | | | | | |
|---|---|---|---|---|---|---|
| **Category** | **Spearman correlation test results** | | | | | |
| | **ρ (rho)** | ***p*** | | | | |
| Age | 0.39 | 0.0 | | | | |
| Saliva pH | 0.18 | 0.011 | | | | |
| Saliva flow rate | 0.28 | $1 \times 10^{-4}$ | | | | |
| Total carious surfaces | 0.2 | 0.005 | | | | |
| | **Kruskal-Wallis test results** | | | | | |
| | **H** | ***p*** | | | | |
| Daily toothbrushing | 8.68 | 0.034 | | | | |
| Examination date | 26.7 | $8 \times 10^{-}4$ | | | | |
| Household employment status | 4.79 | 0.029 | | | | |
| Household size | 6.58 | 0.037 | | | | |
| **Category** | **Adonis test results, not accounting for age** | | **Adonis test results, after accounting for age** | | **Adonis test results, after accounting for caries severity** | |
| | $R^2$ | ***p*** | $R^2$ | ***p*** | $R^2$ | ***p*** |
| **Beta diversity (unweighted UniFrac distance)** | | | | | | |
| Age | 0.046 | 0.001 | NA | NA | 0.043 | 0.001 |
| Examination date | 0.101 | 0.001 | 0.075 | 0.001 | 0.093 | 0.001 |
| Saliva pH | 0.013 | 0.007 | 0.011 | 0.014 | 0.015 | 0.004 |
| Saliva flow rate | 0.028 | 0.001 | 0.006 | 0.23 | 0.025 | 0.001 |
| Soft drink consumption | 0.008 | 0.05 | 0.007 | 0.067 | 0.008 | 0.074 |
| Household size | 0.019 | 0.011 | 0.015 | 0.054 | 0.016 | 0.043 |
| Household employment status | 0.012 | 0.013 | 0.011 | 0.009 | 0.012 | 0.004 |
| Caries status | 0.009 | 0.039 | 0.008 | 0.052 | NA | NA |
| Caries severity | 0.025 | 0.007 | 0.02 | 0.034 | NA | NA |
| Total carious surfaces | 0.025 | 0.001 | 0.014 | 0.002 | 0.023 | 0.001 |

*To test for significant differences in alpha diversity, a Spearman correlation test was used for numerical metadata categories; Kruskal-Wallis tests were used for categorical metadata categories. To test for significant differences in beta diversity, adonis tests were used for both numerical and categorical metadata categories. The results of each significant test for both alpha and beta diversity are displayed; categories that did not return a significant result for a given test are excluded from the relevant section of the table.*

outweighs the caries-free group; however, this was a population-based study, not focused on the recruitment of caries-active children. Accordingly, our results should be interpreted to identify these factors within a population where caries is highly prevalent. While saliva does not represent a singular, structured oral microbial community, it is easy and non-invasive to collect and provides a broad overview of the microbes present in the oral cavity [51]. Our findings contribute to a novel understanding of the mechanistic associations between biological, behavioral and socioeconomic factors, the oral microbiota, and dental caries in this population of children, with implications for the microbiota-aware treatment and prevention of oral diseases.

Behavioral factors, such as toothbrushing frequency and soft drink consumption, were linked to changes in microbiota diversity and composition in our dataset. The ability of microbes to grow at a given site depends on environmental factors, such as pH and oxygen or nutrient availability, which may be altered by host behavior. Less frequent brushing was linked to higher microbial alpha diversity in our study (**Figure 2**). Regular tooth brushing interrupts the accumulation of microbial species on the surfaces of the oral cavity and thereby lowers the overall diversity of microbes found in saliva. Further, a feature classified as *Streptococcus sobrinus* was significantly more abundant in the salivary microbiota of children who reported brushing their teeth less than once per day (**Table 3**). *S. sobrinus* has long been associated with dental caries in the literature and is thought to aggravate caries when found in association with other cariogenic species such as *Streptococcus mutans* [52]. Of interest, a recent study of supragingival plaque microbiota in non-Indigenous Australian children also reported that *Streptococcus* abundance decreased according to tooth brushing frequency [53]. However, *S. mutans* itself was not significantly associated with dental caries or other metadata factors in our microbiota analysis. Soft drink consumption, which was widespread within our study population (**Table 1**), decreases environmental pH in the mouth through the introduction of free sugars that microbes ferment to acid [11]. The established impact of environmental pH on oral microbial communities is further supported by our result that salivary pH was significantly associated with salivary microbiota composition and diversity (**Table 2**). Overall, our findings suggest that daily behavioral activities linked to caries development can impact child salivary microbiota in this population. Understanding which microbes respond to specific factors such as tooth brushing could be translated into personalized medical approaches in the future, providing tailored recommendations relevant to the individual's microbiota composition.

We also found that socioeconomic factors, such as household size and employment status of household members, were associated with salivary microbiota diversity and composition in NPA children (**Table 2**). It is well-established that low SES increases caries risk in children [3, 8, 10]. However, whether microbial mechanisms mediate this risk is less well-understood. Johansson et al. demonstrated differences in dental plaque microbiota richness and composition between a low-SES, high-caries population in Romania and a high-SES, low-caries population in Sweden [8]. This comparison is confounded by large cultural, geographic, and historical differences, so it is of interest that we, for the first time, demonstrate that a similar pattern in salivary microbiota in a comparatively homogeneous population. Here, children who reported no one in their household working had less variability in microbial composition compared to the group who reported at least one person working. However, given that household size co-varied with caries severity in our dataset and that significant differences in dispersion linked to household employment status were identified by permdisp testing, these findings require further investigation to determine whether these factors are mechanistically linked to caries.

In our study population, multiple measures of caries were significantly associated with salivary microbiota composition

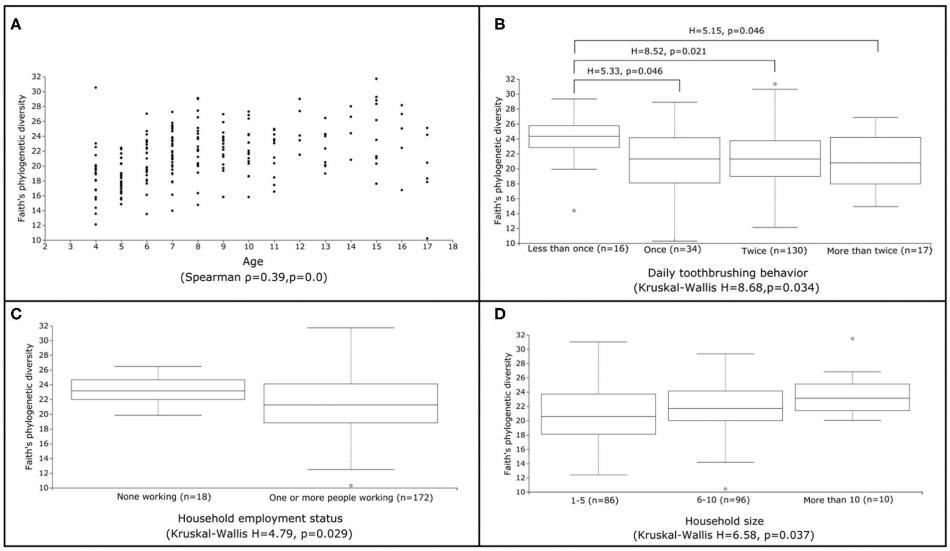


**FIGURE 2 |** Factors linked to significant differences in alpha diversity. Scatter **(A)** and box **(B-D)** plots illustrating the range of Faith's phylogenetic diversity values for selected metadata categories identified as significantly linked to alpha diversity (**A**: age, **B**: daily toothbrushing behavior, **C**: household employment status, **D**: household size). Results of overall Spearman correlation (continuous variables) and Kruskal-Wallis (categorical variables) tests are also displayed, along with any statistically significant (FDR $p < 0.05$) pairwise Kruskal-Wallis test comparisons between groups. All other pairwise Kruskal-Wallis test comparisons were not statistically significant.

(**Table 2**), supporting previous findings that salivary microbiota correlates with caries status [14]. In particular, pairwise comparisons of the caries-free or incipient caries groups to the moderate or severe caries groups approached significance, suggesting that progression of caries to more advanced stages may involve a distinct shift in microbiota composition. Studies of dental plaque have identified shifts in microbial community composition related to caries progression [13, 15, 54]; such patterns may be less obvious, but still present, when sampling saliva rather than tooth surfaces [55, 56]. A feature classified as *Lactobacillus gasseri* was significantly associated with caries severity in our dataset and was most abundant in the severe caries group (**Table 3**). *L. gasseri* has previously been identified in the human mouth [57]; as a lactic acid bacterium, it likely participates in sugar fermentation and hence, caries promotion. A *Veillonella* feature was significantly more abundant in caries-active samples, while features classified as *Leptotrichia* and *Actinobacillus porcinus* were more abundant in the caries-free group (**Table 3**). Numerous studies have reported that *Veillonella* or *Veillonellaceae* species are associated with dental caries [12–14, 16, 54]. *Veillonella* species use lactic acid as their primary energy source and therefore are closely associated with caries-promoting species that produce lactate [12, 58]. Because of this association, *Veillonella* levels in plaque and saliva have been suggested as a biomarker for future caries risk even at apparently healthy sites [16, 54, 58]. Of interest, a *Veillonella* species was previously identified as significantly more abundant in the dental plaque microbiota of Canadian First Nations children with early childhood caries compared to caries-free counterparts [13]. The association of *Leptotrichia* species with oral health and disease is less clear; some species in this genus may be disease-associated and others health-associated [8, 54]. The importance of the *Actinobacillus porcinus* feature in oral health is also difficult to interpret, as this species is not typically found in the human oral microbiota. However, the 16S amplicon sequence associated with this feature was classified as *Haemophilus* using the Human Oral Microbiome Database (**Supplementary Table 6**). Other recent publications have reported that *Haemophilus* is found in higher relative abundance in the saliva of caries-free children and adults compared to those with caries [56, 59], although this association is not universal [13]. Better characterization of the oral health relevance of *Leptotrichia* and *Haemophilus* strains present in the NPA child population could be useful in understanding microbial oral health and informing new therapeutic strategies.

While this study is the first of its kind, there are several limitations. While our study used saliva samples to profile the oral microbiota, samples of plaque biofilm from specific tooth sites might reveal closer associations between the microbiota and

**TABLE 3 |** Microbial features differing significantly in abundance across groups identified by ANCOM.

| Category | Feature Taxonomy | ANCOM W-value | Prevalence (no. of samples detected in) | Abundance (no. of sequences across all samples) | Group association |
|---|---|---|---|---|---|
| Daily toothbrushing | *Streptococcus sobrinus* | 279 | 51 | 433 | Brush teeth less than once per day |
| Caries status | *Actinobacillus porcinus* | 81 | 5 | 8,649 | Caries-free |
| | *Leptotrichia* | 26 | 11 | 121 | Caries-free |
| | *Veillonella* uncultured organism | 19 | 195 | 24,662 | Caries-active |
| Caries severity | *Lactobacillus gasseri* | 208 | 44 | 1,660 | Severe caries |
| Examination date | *Actinomyces* uncultured bacterium | 622 | 59 | 3,978 | Unclear |

*Specific microbial features significantly associated with daily toothbrushing behavior, caries status, caries severity, and examination date are listed below. The metadata category, brief feature taxonomy as assigned using the SILVA 132 database, the reported W-value from ANCOM testing, overall prevalence (i.e., number of samples the feature was detected in), overall abundance (i.e., total number of sequences associated with the feature), and group association (the sample group in which the feature was most abundant) are displayed for each significant feature. Samples with unknown or unrecorded values for a given category were removed prior to ANCOM testing. Individual IDs and full taxonomy strings for significant features are given in* ***Supplementary Table 5****; comparison of taxonomic classification of the significant features using the SILVA and HOMD databases is given in* ***Supplementary Table 6****.*

disease [51, 60]. However, plaque collection was not practicable under the field conditions of our study. Saliva samples, especially after a period of chewing wax, which would dislodge much adherent biofilm [61], give an overview of oral microbiota [51, 55, 60, 62] and can be related to overall caries experience and activity of the individual child. In addition, some metadata information was collected using self-administered questionnaires, data on last meal before sample collection was not collected, and our dataset lacked sampling controls to detect contamination at the sampling site. In relation to this last point, investigation of sampling controls by our group in later years of the clinical trial demonstrated minimal overlap between saliva and sampling controls, indicating negligible contamination of saliva during sampling [50]. We also saw that some significant factors co-varied, making it difficult to distinguish the specific mechanisms underpinning each factor, i.e., whether these factors work together or independently to influence microbiota composition, or are both correlated with some other, unmeasured factor. For example, examination date was significant in explaining variation in both alpha and beta diversity (**Table 2**) and was associated with a significant change in abundance of a specific *Actinomyces* feature (**Table 3**). However, further investigation suggested that examination date was partially confounded with age, meaning that these associations may not be directly linked to examination date but an artifact of collecting samples from children of different ages at different schools on different days. In another example, we found that soft drink consumption co-varied with caries severity (**Table 2**), making it difficult to discern whether either of these factors acts independently. Further research into each of these factors is needed to better understand their contributions to oral microbiota diversity and composition.

For the first time, we describe the salivary microbiota of Aboriginal and Torres Strait Islander children living in a remote location with limited access to dental care. We identify relationships between the salivary microbiota, dental caries, and known caries risk factors such as behavioral activities and SES. Given the importance of the oral microbiota for oral health, refining our understanding of oral microbial communities and how they mediate oral health and disease could be key to informing better treatment and prevention strategies, particularly in populations at high risk of oral disease. This understanding may be especially important for the oral health of Australian Aboriginal and Torres Strait Islander peoples, as early evidence suggests a distinctive relationship between these peoples and their associated oral microbes [29]. Datasets such as ours form a baseline for longitudinal studies of caries prevention and will be key in ensuring that new microbiome-based or microbiome-aware therapies are also applicable to Indigenous communities and do not damage or disrupt Indigenous microbiota. Future research toward this goal could include the investigation of different sample types, such as dental plaque, that allow for a more structured view of oral microbial communities; inclusion of more Aboriginal and Torres Strait Islander communities across Australia; employment of more precise sequencing techniques, such as shotgun metagenomics, to obtain species-level identification of oral microbes; and collection of more detailed metadata to support a finer-scale understanding of the relationship between oral microbiota and, for example, diet.

## DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because of ethical and privacy constraints and respect for principles of Indigenous data sovereignty. Data may be made available for further analyses on a case-by-case basis subject to HREC review and community approval. Requests to access the datasets should be directed to Newell W. Johnson, n.johnson@griffith.edu.au.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Griffith University Human Research Ethics Committee, Far North Queensland (FNQ) Human Research Ethics Committee, Department of Education and Training

(Queensland Government), and Torres and Cape Hospital and Health Service. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

## AUTHOR CONTRIBUTIONS

MH-D performed data analysis and interpretation and wrote the manuscript. ES performed lab work and data processing and critically revised the manuscript. NJ contributed to study conception and design, data acquisition and interpretation, and critically revised the manuscript. KK and RL contributed to data acquisition and critically revised the manuscript. JK contributed to study conception and design, ethical approvals, data acquisition, and critically revised the manuscript. LW contributed to study conception and design, contributed to data interpretation, and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/froh.2021.641328/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

# Chapter IV: Ethics of microbiome ownership for Indigenous peoples

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*Manuscript in preparation for submission*

# Statement of Authorship

| | |
|---|---|
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| | | | |
|---|---|---|---|
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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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**Ethics of microbiome ownership for Indigenous peoples**

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**Background**

The human microbiome is defined as a collection or community of microorganisms (microbiota) that reside in or on the human body together with their genetic material, environment, and theatre of activity (Berg et al. 2020). This microbiome performs important physiological functions with profound effects on human health (Fan and Pedersen 2021; Willis and Gabaldón 2020; Pascal et al. 2018). The greatest microbial biomass in the human body is found in the gut, which has been the focus of most human microbiome research to date. Consequently, gut microbial communities have been linked to many disease states, including inflammatory bowel diseases, diabetes, other autoimmune conditions, allergies, and mental health conditions (Chuong et al. 2018; Vallianou, Stratigou, and Tsagarakis 2018; Luca and Shoenfeld 2019; Pascal et al. 2018; Eisenstein 2020; Clapp et al. 2017). Oral and skin microbial communities have also been linked to diseases such as periodontal disease, dental caries, eczema, and psoriasis (Abusleme et al. 2013; Ai et al. 2017; Burne et al. 2012; Weyrich et al. 2015). Therefore, understanding the microbiome and its interactions with human health represents an exciting

avenue for research and the eventual development of microbiome-based therapies, such as pre- and probiotics, microbiota transplant, or therapeutic microbiota modulation. Interest in this field is reflected in a high level of research and commercial investment: as of 2019, more than $1.7 billion USD had been spent on human microbiome research and more than $3 billion invested in gut microbiome-related biotechnology ventures (Proctor et al. 2019; Li et al. 2020).

A key step towards translational microbiota research is understanding the complex factors that shape human-associated microbial communities. Some gut microbial taxa have speciated alongside humans and other primate hosts, consistent with refinement in mutually beneficial evolutionary trajectories likely centred on host physiology and ecological niches (Moeller et al. 2016; Nishida and Ochman 2019; Moran, Ochman, and Hammer 2019). At the scale of an individual lifetime, microbiota acquisition begins at birth (Dominguez-Bello et al. 2010), and microbial communities across the body are shaped by a variety of influences, including diet, medication, other lifestyle and environmental factors, and possibly host genetics (Bokulich et al. 2016; Blaser 2016; Muegge et al. 2011; Zimmer et al. 2012; David et al. 2014; Korpela et al. 2016; 2018; Blekhman et al. 2015; Corby et al. 2007; Demmitt et al. 2017; Gomez et al. 2017; Shaw et al. 2017; Stahringer et al. 2012). Awareness of these multiple inputs operating on the human microbiome has prompted research interest in microbiota variations in humans across different lifestyles, ancestries, and environments, in order to better understand the factors that shape the microbiome in health and disease and their underlying mechanistic relationships. As part of this research effort, a growing number of studies have specifically targeted the microbiomes of Indigenous peoples around the world. Researchers expect that such studies will contribute to general understanding of human microbiota variation and factors that influence the microbiome, as well as supporting the eventual development of medical benefits.

However, the process of unlocking understanding of the microbiome's role in human development, health, and disease raises important ethical questions and concerns. Despite the popular framing of the gut microbiome as a "new" or "forgotten" human "organ" (O'Hara and Shanahan 2006; Baquero and Nombela 2012; Clarke et al. 2014; Marchesi et al. 2016; Margo 2019), host and microbial materials are often treated as disconnected during research, perhaps contributing to underappreciation of ethical issues. Questions about microbiome ownership have important implications for deciding who receives any future economic, social and health

benefits arising from human microbiome research (Hawkins and O'Doherty 2011; Schwab et al. 2013; Slashinski et al. 2012). For microbiome research to have the best possible chance of meeting its potential to improve human health and wellbeing, we must consider and adequately address the ethics of how microbiome research is performed and to whose benefit or detriment. Here, we approach the question of microbiome ownership with a specific focus on the situation for Indigenous peoples, whom we see as having been largely excluded from conversations on microbiome research despite being a focal point of such work. The structure of our paper loosely follows the expected trajectory of research on Indigenous peoples' microbiomes and explores major ethical issues relating to the theme of ownership at each stage. We begin with the framing of research questions and interpretation of current results, proceed to data handling and governance, and then to research translation, patenting, and commercialisation. To our knowledge, this paper represents the first focused discussion of microbiome ethics and ownership in an Indigenous context.

## Ethical and social issues in framing and interpreting research on Indigenous peoples' microbiomes

A considerable number of studies have aimed to characterise and understand the microbiota of Indigenous peoples. For the gut microbiota, this has typically meant comparing non-Indigenous individuals living industrialised lifestyles in the United States (US) or Europe to Indigenous individuals living hunter-gatherer or small-scale agriculturalist lifestyles in the Global South (Filippo et al. 2010; Yatsunenko et al. 2012; Schnorr et al. 2014; Obregon-Tito et al. 2015; Martínez et al. 2015; Clemente et al. 2015; Girard et al. 2017). Collectively, these studies have reported higher gut microbiota diversity and unique microbial community compositions in Indigenous or "traditional" populations compared to industrialised groups. Some studies have also advanced evidence that lifestyle transitions from more "traditional" to more "industrialised" or "Westernised" have direct impacts on the gut microbiome (Vangay et al. 2018; Jha et al. 2018; Gomez et al. 2016; Smits et al. 2017). A study that compared gut microbiota from Cheyenne and Arapaho Native Americans living in the US to those of a non-Native US cohort and of non-industrialised Indigenous South American groups (Sankaranarayanan et al. 2015) found that the Cheyenne and Arapaho harboured microbial signatures distinct from the other industrialised cohort, yet overall resembled this group more

closely than they did the non-industrialised South Americans. Studies examining oral or skin microbiomes of Indigenous populations are fewer but have also reported differences between Indigenous and non-Indigenous participants, with the caveat that Indigeneity and "traditional" lifestyle are frequently confounded (Clemente et al. 2015; Nasidze et al. 2011; Lassalle et al. 2018; Abdul-Aziz 2018). The handful of oral microbiome studies that include Indigenous individuals living industrialised lifestyles have also identified systematic differences in diversity and composition between these individuals and industrialised non-Indigenous counterparts (Ozga et al. 2016; Handsley-Davis 2016).

Hence, current evidence indicates that Indigenous peoples around the world harbour microbiome signatures distinct from those of industrialised Euro-American populations who otherwise dominate human microbiota research (Nath et al. 2021; Rogers et al. 2019). Most studies supporting this conclusion have tied the diversity of microbes observed in Indigenous peoples to "traditional" aspects of their lifestyles, such as diets lower in industrially processed foods. These findings have been interpreted to support theories that humans in industrialised societies suffer from a depleted microbiome that drives observed increases in the incidence of non-communicable diseases (NCDs), particularly metabolic and immune-linked diseases. This line of reasoning draws on earlier hypotheses about the relationship between microbial or environmental exposures and NCDs. The Hygiene Hypothesis stands as the first recorded articulation of this link, proposing that reduced microbial exposure in early childhood increases children's susceptibility to allergens (Strachan 1989). Later evolutions of this theory included the Old Friends Hypothesis, in which reduced microbial exposure causes humans to lose specific beneficial microbes that protect against NCDs (Rook, Martinelli, and Brunet 2003), and the Biodiversity Hypothesis, which suggests that reduced contact between industrialised humans and biodiverse environments impacts the human skin microbiota and its capacity to modulate the immune system (Hanski et al. 2012). With a growing number of studies linking industrialised lifestyles to lower microbiota diversity, high-profile commentaries and reviews by leading researchers in the human microbiome field have posited that loss of microbiome diversity, or "microbiota insufficiency syndrome", underlies the increased incidence of NCDs in the industrialised world (Blaser and Falkow 2009; E. D. Sonnenburg and Sonnenburg 2014; Blaser 2016; 2018; Dominguez-Bello et al. 2018; E. D. Sonnenburg and Sonnenburg 2019; J. L. Sonnenburg and Sonnenburg 2019). By extension, "restoration" or "rewilding" of the

industrialised human microbiota to a more diverse and presumably healthier state has been proposed as a novel medical solution to protect against NCDs (Velasquez-Manoff 2016; Blaser 2018; Dominguez-Bello et al. 2018; E. D. Sonnenburg and Sonnenburg 2019; Kolata 2021).

As a result, discussions have emerged around the scientific and commercial value of microbial species, genes, functions, and communities that are found in Indigenous bodies. In this discourse, the microbiota of Indigenous or "traditional" peoples is framed as immensely valuable, perhaps the key to reversing the increase in chronic NCDs in the industrialised world. For example, prominent human microbiome researchers have called for scientists to "capture and preserve" unique gut microbes harboured by "traditional peoples in developing countries"— i.e., mostly Indigenous peoples (Dominguez-Bello et al. 2018; Blaser 2018). In anticipation of future health applications, researchers have even established a not-for-profit "microbiota vault" to support the collection and storage of human-associated microbial biodiversity ('The Microbiota Vault' 2021). The utility of this approach can be questioned on both technical and ethical grounds. While microbiota signatures plausibly linked to industrialisation have been identified, much work still remains to untangle causal relationships and the precise impacts of geography, body site, host ancestry, and cultural or lifestyle practices in shaping these microbial patterns and any related health effects. In addition, evolutionary experts have questioned the theoretical and empirical basis for microbiota "rewilding", arguing that there is insufficient evidence to suppose that shifting microbiota to a 'less industrialised' state would have beneficial health effects (Carmody, Sarkar, and Reese 2021; Kolata 2021). Here, we focus instead on some fundamental ethical issues raised by this research. We find it concerning that, amid the burgeoning interest in Indigenous microbiomes, there has been little mention of the agency, rights, or interests of the peoples at the centre of this proposed solution to rising NCDs.

On the contrary, some of the discourse surrounding microbiota diversity, restoration and rewilding can contain uncomfortable echoes of exploitation or at the very least unequal benefit. For example, the Microbiota Vault aims to collect and store microbiota samples from "globally diverse human populations", particularly "traditional" groups with minimal exposure to industrialisation, in pursuit of future health benefits (Dominguez-Bello et al. 2018). This framing of target populations is echoed in calls to "re-seed" the industrialised microbiota with "lost"

microbes maintained by “remote present-day peoples of traditional societies” (Blaser 2018). This goal is reminiscent of earlier projects that aimed to survey the global diversity of human DNA, including the Human Genome Diversity Project and the Genographic Project, which focused on gathering samples from so-called “isolated human populations”, including many Indigenous groups (Cavalli-Sforza et al. 1991). Indigenous people rallied together to oppose such efforts, termed “vampire projects”, including through the formation of the Indigenous Peoples Council on Biocolonialism (Indigenous Peoples Council on Biocolonialism 1995; Dodson and Williamson 1999). Given these parallels, it seems wise at this point to consider whether microbiota research initiatives are repeating the mistakes of the past. More targeted extractive behaviours can constitute biopiracy, defined as the enrichment of non-Indigenous actors using knowledge or resources that rightfully belong to an Indigenous community (ETC Group n.d.). A well-known example is the ‘San-Hoodia case’, wherein researchers and a pharmaceutical company patented and attempted to develop a commercial weight loss drug based on a plant traditionally used by the San people, without acknowledging the San’s knowledge or right to share in the potential benefits of commercialisation (Wynberg and Chennells 2009). Even where economic gain is not the primary motivation, approaching Indigenous peoples’ microbiomes as a resource to be mined for future health benefits supports an extractive logic that minimises the autonomy, benefit, and sovereignty of Indigenous peoples. Less than a decade ago, Reardon and TallBear analysed how science uses lofty goals to make inappropriate claims on Indigenous DNA:

> Native American DNA has emerged as a new natural resource that Native peoples possess but that the modern subject – the self-identified European – has the desire and ability to develop into knowledge. (Reardon and TallBear 2012)

Reardon and TallBear specifically link these claims on Indigenous DNA to earlier claims on Indigenous anthropological artifacts; it now seems that microbiomes could be added to this list of Indigenous resources claimed by scientists in the pursuit of useful knowledge. While we acknowledge that advocates for the potential benefits of “traditional” or “restored” microbiomes are likely motivated by a genuine desire to improve the health of people suffering from chronic NCDs, a problematic imbalance in the distribution of risks and benefits remains.

At the end of the day, this discourse primarily values Indigenous microbiomes for the benefit they are presumed to be capable of bringing to non-Indigenous communities, which can easily descend into treating Indigenous peoples as a means to an end: "to seek answers to current Western woes in the idealised purity of the past and primitive gut in turn instrumentalises brown and black bodies in the service of white health" (Benezra 2020). Therefore, any initiatives seeking to study Indigenous microbiota must be carefully scrutinised and measures taken to avoid risks of biopiracy and other exploitative or extractive practices.

Further social and ethical harms can arise from how Indigenous or "traditional" peoples are defined by microbiome researchers. Benezra has recently raised cautions about the presence of 'race' in human microbiome research, highlighting the use of poorly-defined and poorly-justified racial or lifestyle categories across multiple studies (Benezra 2020). In this way, terms that may have a social or anthropological basis, such as 'ethnicity', 'geography', or 'genetic ancestry', effectively become racially coded. Although these terms are likely chosen by researchers precisely with the intention of avoiding racial categories, they nevertheless become racialised in how they are used and subsequently embedded within investigator descriptions and the discipline as canon. Using broad, unexamined terminology can have the effect of collapsing and ignoring important economic, political, and cultural factors, which leaves vague, racialised categories as the presumed explanatory variable for microbiome differences (Benezra 2020). This is not only concerning from a social perspective, but also limits scientific understanding of mechanisms that shape the microbiome and human health. This lack of understanding promotes a sense of urgency in capturing 'wild' or 'vanishing' microbes from Indigenous populations for microbial "restoration" or "rewilding" efforts that disregard the rights and wishes of such populations. In analogy to salvage ethnography, which aims to record so-called 'disappearing' cultures, Benezra terms this approach "salvage microbiomics" (Benezra 2020). We should question why, rather than supporting Indigenous or "traditional" communities to maintain the heritage and environments that sustain their diverse microbiota, researchers are calling for microbiota to simply be dissociated from their human hosts and used for the benefit of others.

Overall, against the backdrop of increasing research interest in Indigenous peoples' microbiomes, insufficient attention has been paid to fundamental bioethical questions of

informed consent, data ownership and governance, and benefit-sharing. Why should Indigenous peoples participate in such projects? Who will own and control the data and products created through such research? How will the central role of Indigenous communities in microbiome research be recognised and bring direct economic and social benefit to Indigenous individuals and communities? And how will the risks of exploitation, instrumentalisation and racialisation be minimised or regulated? Very few publications currently address such questions or offer strategies to address potential ethical pitfalls, creating a disconnect between the perceived altruistic arguments employed by the scientific community in favour of such research and centuries of repeated scientific misconduct experienced by Indigenous communities. In this context, microbiome ownership rights could provide a mechanism to protect Indigenous microbiomes from exploitation and even enable Indigenous peoples to drive and benefit from commercially translatable research. However, the nature and scope of such rights have not yet been explored in relation to the microbiome. In the following section, we discuss ownership and governance of research data in the contemporary Indigenous microbiome space.

**Microbiome research and Indigenous data sovereignty**

Indigenous peoples around the world have expressed a strong political desire for control of data and biological samples originating from research involving their communities. These calls initially responded directly to high-profile controversies involving (mis)uses of Indigenous human DNA, such as the aforementioned Human Genome Diversity Project (HGDP) in the 1990s. Opponents from Indigenous communities around the world were concerned about the HGDP's overarching lack of consultation, cultural inappropriateness of blood sample collection, informed consent, and potential misuses of genetic data (Lock 1994; Liloqula 1996; Dukepoo 1998; Dodson and Williamson 1999; Indigenous Peoples Council on Biocolonialism 1995; *Green Left* 1994). In another prominent case, Havasupai tribal members launched a suit against Arizona State University and its Board of Regents after learning that blood samples collected for a research project on diabetes had been used for other research that was stigmatising and culturally harmful (Harmon 2010; R. Tsosie 2007; Garrison et al. 2019; Reardon and TallBear 2012). A growing presence of Indigenous geneticists and biomedical ethicists in academia has reshaped calls for norms regarding Indigenous consultation to shift from reactive to

continuous, in advance of an expected continuation of such initiatives. As a result of these proactive stances, the National Congress of American Indians (NCAI) adopted a resolution in October 2019 calling for the use of Native American samples and data in All of Us, a precision medicine project funded by the National Institutes of Health, to be halted until processes and guidelines for tribal oversight were adopted (National Congress of American Indians (NCAI) 2019). The All of Us project was similarly criticised for its lack of specific consultation with Indigenous communities on data access, intellectual property, and benefit-sharing (Fox 2020; Hudson et al. 2020). Because of these historical and ongoing negative experiences, many Indigenous people do not trust non-Indigenous researchers or governments to handle their data or samples appropriately (James et al. 2014; Claw et al. 2018; Garrison et al. 2019; Hudson et al. 2020).

Mechanisms for Indigenous data sovereignty have been proposed as a solution for repeated harmful uses of data and samples. Indigenous peoples have "inherent and inalienable rights and interests […] relating to the collection, ownership and application of data about their people, lifeways and territories" (Kukutai and Taylor 2016). Indigenous data sovereignty is therefore linked to self-determination as peoples and, in some cases, sovereign nations: "Indigenous data sovereignty thus refers to the proper locus of authority over the management of data about indigenous peoples, their territories and ways of life" (Kukutai and Taylor 2016). Articulations of data sovereignty do not simply reflect a right to control and manage, but also a responsibility to care for, Indigenous data:

> Information, data, and research about our peoples – collected about us, with us, or by us – belong to us and must be cared for by us.
>
> – Liz La quen náay Kat Saas Medicine Crow (United States Indigenous Data Sovereignty Network n.d.)

Calls for data sovereignty reflect a desire to protect Indigenous data from misuse and to ensure that benefits from the use of Indigenous samples and data flow back to Indigenous peoples (Global Indigenous Data Alliance (GIDA) n.d.). These goals can be supported by appropriate data governance mechanisms, such as the CARE principles: collective benefit, authority to control,

responsibility, and ethics (Global Indigenous Data Alliance (GIDA) 2019). Systems and mechanisms to support Indigenous data sovereignty will also vary according to the specific Indigenous people(s) concerned and the political and legislative systems in which they must operate (Kukutai and Taylor 2016).

The potential for data sovereignty principles to minimise the risks of inappropriate uses of Indigenous data, cultural harms, and exclusion of Indigenous peoples from research benefits has been strongly articulated by Indigenous scholars, particularly within the genomics realm (Claw et al. 2018; Garrison et al. 2019; Hudson et al. 2020). According to Hudson and colleagues:

> [T]he Indigenous data sovereignty movement […] asserts inherent Indigenous rights and interests in genomic data, expects Indigenous participation in the governance of genomic samples/data and anticipates Indigenous communities' involvement in research and policy that affects their lives and livelihoods. (Hudson et al. 2020)

However, data sovereignty claims are not limited to Indigenous human genetic data; genomic research that "draws on knowledge of [Indigenous] land, species and waters" are considered subject to the same considerations (Hudson et al. 2020). Tsosie and colleagues have also recently argued for the application of an Indigenous data sovereignty lens to data from biological anthropology research (K. S. Tsosie et al. 2020). However, there has been a notable lack of engagement with Indigenous data sovereignty in the microbiome context, despite a robust body of literature and discussion in closely related fields.

We argue that data sovereignty principles are applicable to, and should be required in, the microbiome field. Human microbiome data are intrinsically tied to someone's body and hence can give attributable information about an individual and potentially their health, environment, and other aspects of their lives. Microbiome data also have value to the community to which that individual belongs, since they have potential to inform on others within the community based on shared residence, environmental and interpersonal contacts, and general aspects of lifestyle. Importantly, many Indigenous communities view microbes as other-than-human kin to which they are obligated in maintaining right relationships with and preventing harm. While

important, the commercialisation potential of microbiota samples and data should not be used to justify seeing Indigenous peoples' microbiota as simply a resource to extract financial gain. Therefore, Indigenous data sovereignty principles should be implemented in microbiome research that involves Indigenous participants or stakeholders, whether research aims are basic or applied. The potential for translational products and commercialisation raises further specific issues that microbiome ownership rights could help to address.

**Property, intellectual property, and governance of Indigenous knowledge and resources**

In this section, we explore the legal landscape relevant to framing Indigenous microbiome ownership claims, primarily in the context of research that is translational or has commercialisation potential. The notion of "property" in the Anglo-American legal tradition has undergone several transformations through time, from the "absolute and indivisible" Roman *dominium*, to the contingent and relational 'guardianship' of feudal England, to the rise of liberal individualism and property as "private sovereignty" (Davies 2001). These transformations are not always complete, as the emergence of a new property concept does not necessarily extinguish the old. The political and social realities of whose property rights are recognised and protected have also changed over time, shaped by class, gender, and race (Davies 2001). Intellectual property (IP), or "the legal rights which result from intellectual activity in the industrial, scientific, literary and artistic fields" is a specific subfield of property and ownership (World Intellectual Property Organization 2008). The notion of IP first developed in a European cultural context beginning in the 15$^{th}$ century and evolved into the forms now predominant in Western countries and in international bodies, such as the World Intellectual Property Organisation (WIPO), that produce and enforce protection of IP rights.

Key goals of such dominant global IP protection systems include the expression of the moral and economic rights of creators, the expression of the rights of the public to access creations, and the promotion of creativity and fair trading leading to innovation and economic and social development (World Intellectual Property Organization 2008). Copyright, patents, and trademarks are examples of typical instruments of such IP protection systems. An important principle in these systems is the need to balance protection of the rights of creators against broader benefit in the form of the 'commons' or 'public domain'. Therefore, global dominant

IP systems generally protect creative works or inventions only for a limited period and include additional limitations to protect public access to works, such as fair use exemptions to copyright.

Such systems, shaped largely in a Western context, are not necessarily fit for the purpose of protecting Indigenous peoples, knowledge, and resources in a culturally appropriate manner. WIPO defines traditional knowledge (TK) as "knowledge, know-how, skills, innovations or practices […] passed between generations in a traditional context" (World Intellectual Property Organization (WIPO) n.d.). Similar terms to describe the broad concept of knowledge and practices developed and passed on by Indigenous peoples over time include Indigenous knowledge, traditional ecological knowledge, cultural heritage, and Indigenous cultural and intellectual property (ICIP). This type of knowledge challenges the dominant IP systems, which emphasize conditions of novelty, an inventive step, and a clearly identifiable creator for IP protection. Instead, Indigenous systems of managing and controlling TK may emphasise long-held knowledge rather than basing protection on the identification of a discrete inventive step, or collective rather than individual ownership of knowledge. These differing approaches create a disconnect between dominant global IP systems, where TK is typically situated in the public domain and free for anyone to use, and the reality that this system fails to protect TK from "misappropriation and misuse" (World Intellectual Property Organization (WIPO) n.d.).

Recognising this limitation, international bodies including the UN and WIPO have made some efforts towards recognising and protecting Indigenous peoples' interests in TK and other resources. Key international legislative instruments include the 1992 *UN Convention on Biological Diversity* (CBD), the 2007 *UN Declaration on the Rights of Indigenous Peoples* (UNDRIP), and the 2010 *Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from Their Utilization (ABS) to the Convention on Biological Diversity* (Nagoya Protocol). These documents present articulations of Indigenous rights and interests in knowledge and resources; for example, Article 31 of UNDRIP states that Indigenous peoples have IP rights over their "cultural heritage, traditional knowledge, and traditional cultural expressions", including "manifestations of their sciences, technologies and cultures, including human and genetic resources, seeds, medicines, knowledges of the properties of fauna and flora …" (United Nations 2007). The CBD and Nagoya Protocol respectively aim to

promote the conservation and sustainable use of global biodiversity and to provide clarity on this goal concerning genetic resources (GRs), which are defined as "any material of plant, animal, microbial or other origin containing functional units of heredity […] of actual or potential value" (United Nations 1992; 2010). These instruments highlight the importance of equitable sharing of benefits arising from the use of biodiversity and GRs; however, in places they also constrain Indigenous ownership claims. For example, the CBD generally places the locus of ownership for GRs and other biological resources at the level of the nation-state, rather than with Indigenous communities, and the Nagoya Protocol emphasises recognition of Indigenous ownership of TK "associated with GRs" rather than Indigenous ownership of GRs *per se*.

Moving to the national scale, several countries have now introduced legislation aiming to regulate and protect TK or cultural heritage (Okediji 2018). Janke (1998) offers an in-depth exploration of the concept of Indigenous cultural and intellectual property (ICIP) in the Australian context. Janke defines ICIP as "Indigenous Australians' rights to their heritage", where 'heritage' is in turn defined as:

> […] intangible and tangible aspects of the whole body of cultural practices, resources and knowledge systems that have been developed, nurtured and refined (and continue to be developed, nurtured and refined) by Indigenous people and passed on by Indigenous people as part of expressing their cultural identity. (Janke 1998)

Janke highlights that, while Indigenous Australians express a clear desire to define, own, control and protect ICIP, these rights have been inadequately protected under contemporary legal and policy systems (Janke 1998). She argues that notions of IP underlying existing Australian legislation are not commensurate with Indigenous Australian traditions of managing ICIP. For example, presumptions of a creative individual and time limitations on IP protection are not appropriate for governing ICIP, which is often held collectively and in perpetuity in accordance with specific cultural mechanisms (Janke 1998). Janke's work, therefore, further illustrates the challenges of protecting ICIP or TK in a system not designed with the appropriate cultural context in mind. Despite increasing awareness and discussion in recent years of the need for

protection of Indigenous knowledge and resources, this question has not yet been extensively considered in relation to the human microbiome.

**Microbiome ownership, patenting, and commercial applications**

Scope likely exists for the patenting and commercialisation of human-associated microbes and microbial products via conventional pathways within contemporary dominant IP systems. In *Diamond v Chakrabarty* (1980), the US Supreme Court upheld Chakrabarty's claim to patent a bacterium that he had genetically modified to give the bacterium additional functions (Diamond v. Chakrabarty 447 U.S. 303 (1980) 1980). This case established precedent that living things, including microbes, can be patentable IP if they have been substantially shaped or altered through human intervention. In Australia, naturally-occurring genes are excluded from patentability, but microorganisms, microbial products and microbial processes are all considered patentable (IP Australia 2016a; 2016b). This includes naturally-occurring microorganisms if they have been isolated from their natural environment and have a demonstrated new use or application (IP Australia 2016a). At the international level, WIPO also considers that genetic resources (GRs) cannot be patented because they are not creations of the human mind, but inventions developed using GRs may be patentable (World Intellectual Property Organization (WIPO) n.d.). Therefore, while naturally-occurring microbial genes or species may be excluded from patentability in some jurisdictions, it appears that patentable inventions, such as microbiome-based therapeutics, could be generated based on human microbiome samples or data. Yet, it is less certain that benefits of commercialisation would accrue to those who provided the samples or data in the first place. In *Moore v Regents of the University of California* (1990), the Supreme Court of California rejected the concept of *self*-ownership of the human body or products derived from it. The majority judgement held that consent for removal of Moore's tissues during medical treatment nullified his ownership interests in subsequent patenting and commercialisation of derived products (Davies and Naffine 2001). Although this principle has not yet been legally tested in relation to the microbiome, it is easy to imagine a similar argument being successfully deployed against claims to ownership of microbiome samples or derivative products by the individual or community from whom the sample was taken, provided that accepted consent processes for the initial sample collection are followed.

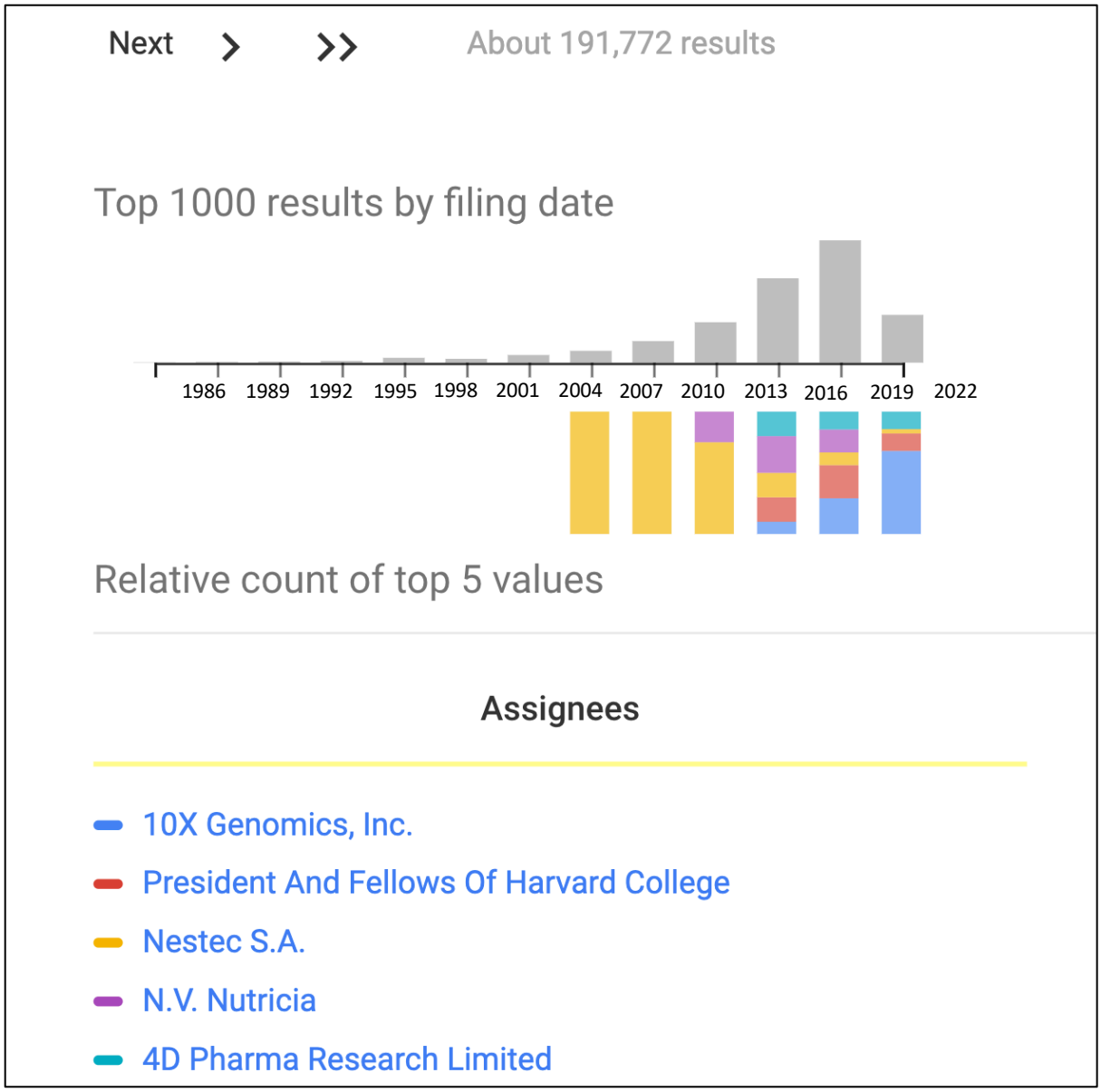


**Figure 1. Summary of results from Google Patents search for the key term "microbiome", 1983-2021.** *For the top 1,000 results, number of records filed (grey bars) are presented in three-year blocks ending in the year indicated on the timeline. Proportion of records associated with the top five patent assignees in each three-year period are indicated below the timeline by stacked coloured bars. Data are current as of September 2021.*

Furthermore, international corporations are currently focused on creating IP from nature, including microorganisms, at an industrial scale. In 2018, Blasiak and colleagues accessed 38 million records of genetic sequences associated with patents and created a database of 12,998 such sequences associated with 862 marine species, the majority of which were microbial (Blasiak et al. 2018). A single corporation had registered 47% of the associated patents, exceeding the combined share of 220 other companies (37%), while universities and their commercialisation partners had registered 12% of the identified claims (Blasiak et al. 2018). A

search of Google Patents, a publicly available IP and patent database, shows that the number of patent claims filed per year containing the term “microbiome” has clearly increased over the period from 1983 to 2021 (Figure 1). Of the 192,813 total results, approximately 3.5% were registered by a single private corporation, 10X Genomics, Inc., followed by another corporation, Nestec S.A., and the President and Fellows of Harvard College, each of whom registered approximately 2% of the claims. While not all of these records relate to directly patenting human-associated microbes or their derived products (as opposed to, for example, patenting a consumable product that may impact the microbiome), these figures clearly speak to the high level of commercial interest in microbiome-related IP.

However, describing the likelihood or feasibility of patenting does not answer the question of whether such actions are ethically desirable. Notably, previous literature on ethical, legal, and social issues in microbiome ownership has largely been centred on a Western context. Early work from Hawkins and O’Doherty highlighted the need to develop norms surrounding microbiome ownership in order to serve principles of justice and equitable distribution of health and material benefits and noted that “cultural identities” may influence research participants’ sense of microbiome ownership and the acceptability of research (Hawkins and O’Doherty 2011). Subsequent work by Schwab and colleagues reasoned that microbiome ownership and patenting are not in the best interests of either science or society (Schwab et al. 2013). These authors preferred a “science commons model” where contributing samples to scientific research is treated as “civic participation in a collective enterprise” rather than as a venue for claiming IP rights (Schwab et al. 2013). However, some arguments advanced by Schwab and colleagues rely on a fundamental framing of living things as private property, which cannot be assumed in all cultural contexts. For example, this view is directly contradicted by statements from the Indigenous Environmental Network: “within most Indigenous beliefs, no person can own living things or hold life forms as property” (Goldtooth 2008). Furthermore, such formulations of microbiome ownership ethics leave little room for notions of communal ownership or cultural obligations to treat human-associated materials in a certain way. As Reardon and TallBear previously argued in the context of human DNA, framing microbiome samples and data purely as an “objective neutral good that benefits all” may also effectively deny Indigenous peoples’ rights to control their own “resources and identity” (Reardon and TallBear 2012). Appeals to promote the common good as a rationale to supersede ownership

rights are unconvincing when applied to communities who have been parasitised by such claims for generations:

> Critical reflection upon the notion of 'the public' presumes a notion of inclusion and representativeness that is often at odds with Indigenous experiences within colonial contexts. (Hudson et al. 2020).

Against this backdrop, considering how to frame Indigenous microbiome ownership is a complex task. Microbiome ownership rights could include straightforward property rights over physical microbiome samples and materials derived from them, such as microbial cultures; IP rights under global dominant IP systems over data or inventions based on human-associated microorganisms, microbial communities, or microbial products; and determining whether human-associated microorganisms, microbial communities and products fall under notions of TK or ICIP – and what protection is available to them if so. Further, these concepts and questions are not necessarily independent of one another. For instance, while patenting microbes and microbial products and processes is generally considered acceptable under dominant IP regimes, patenting and commercialisation of microbiomes from Indigenous peoples may be restricted by international legal instruments such as UNDRIP, the CBD and the Nagoya Protocol. However, (legal) protection of microbiome data and samples under this framework likely hinges on whether human-associated microbes constitute a form of "cultural heritage, traditional knowledge or traditional cultural expressions" (United Nations 2007), or indeed a genetic resource (United Nations 1992; 2010) – questions to which there are currently no unequivocal answers.

As new tools to increase the sensitivity and specificity of microbiome analysis are deployed in new ecosystems (Clare et al. 2021), further claims to IP and profits by actors outside of communities who care for such ecosystems will likely continue. Indigenous communities have typically not been included or invited to share in the benefits of IP-generating processes. However, significant opportunities may exist for historically marginalised communities to create IP and, if desired, profit that could facilitate the development of circular economic systems to support community aspirations, such as land purchases. This discussion cannot address the question of whether microbiome ownership or patenting is desired by Indigenous

communities; communities will need to decide for themselves. Ownership and IP rights likely have the potential to both help and harm. On one side of the coin, Indigenous peoples may wish to use global dominant IP systems to block claims of ownership and commercialisation of their microbiomes by outside actors. On the other side, current dominant ethical and legal reasoning, which is rooted in Western cultural assumptions and demurs on ownership of genetic resources, may present a barrier to Indigenous claims to ownership and rights over the microbiome.

Nevertheless, we argue that avenues to promote and protect Indigenous microbiome ownership should be considered. The recent growth in patent claims filed relating to the microbiome, and the dominance of large corporations and wealthy academic institutions among these claimants (Figure 1), raises the stakes for protecting the rights of historically marginalised communities whose generations-long stewardship of microbial resources is currently going unrecognised. Recall that Janke defined cultural heritage as:

> cultural practices, resources and knowledge systems […] developed, nurtured and refined […] by Indigenous people and passed on by Indigenous people as part of expressing their cultural identity. (Janke 1998)

Furthermore, Article 32 of UNDRIP states that:

> Indigenous peoples have the right to determine and develop priorities for development or use of their lands or territories *and other resources* [emphasis added]. (United Nations 2007)

Human-associated microorganisms could arguably be understood as a "resource" "developed, nurtured and refined" by Indigenous peoples and passed on over generations. Many stakeholders already recognise the logic that peoples, or states, may claim some form of ownership over organisms to whose development they have disproportionately contributed, such as domesticated plants and animals that have been "influenced by humans to meet their needs" (United Nations 1992; Pullman and Arbour 2009). If traditional knowledge and practices have had a role in shaping living things, the owners or custodians of such knowledge and

practices can hold an ownership interest in these organisms. Under this lens, the distinction drawn, for example, in Australian patent law between (unpatentable) naturally-occurring genes and (patentable) microorganisms or microbial products becomes less clear. If, as current evidence indicates, human-associated microbial communities are influenced by host lifestyle, cultural factors, and vertical transmission across generations, might not Indigenous peoples' traditional knowledge and cultural practices that have shaped their microbiomes be translatable into an ownership claim? The best way forward may then lie in new, culturally-informed systems to recognise Indigenous rights and interests in the microbiome.

**Future directions**

Regardless of the forms and mechanisms that may eventually be developed in the microbiome ownership space, the priority should be to respect Indigenous sovereignty and to support the right of Indigenous individuals and communities to take the lead on this conversation. Questions of Indigenous engagement and sovereignty may be relatively novel to many non-Indigenous actors in the microbiome field. In the first instance, looking to guidelines for ethical conduct developed by Indigenous scholars and communities in related disciplines, such as human genomics and medical or pharmaceutical research, can provide a starting point (Bader et al. 2020; Bardill et al. 2018; Claw et al. 2018; National Health and Medical Research Council (NHMRC) 2018a; 2018b; Hudson et al. 2016). In the medium term, a small but growing workforce of Indigenous researchers with experience in the microbiome field are well-placed to lead the development of specific guidelines and recommendations for stakeholders seeking to work with Indigenous communities on microbiome projects. As a relatively new field, human microbiome research has an opportunity to avoid mistakes of the past and establish a positive legacy by adopting ethical frameworks that centre Indigenous sovereignty.

*Foregrounding Indigenous voices and perspectives*

Like other areas of genomic research, human microbiome research requires that Indigenous communities be made full partners in the research process. Collaboration with community members early in conception of a study ensures that community research priorities, knowledge, and values are reflected in the research design, as well as guarding against the

promulgation of misleading or problematic language or assumptions in the study. The aim of such consultation should not be to simply receive one-off authorisation, but to build relationships and consensus based on honest and good-faith exploration of the foreseeable benefits and risks. Furthermore, consultation and empirical research can be used to collate Indigenous views on the microbiome and microbiome ownership. For example, interviews, surveys, focus groups, or other consultation mechanisms may be employed to gather Indigenous perspectives and understand the rights and interests that communities wish to express in relation to the microbiome and the potential for derived IP and commercial products. These perspectives can then form the basis for policy or governance mechanisms that support aims such as data sovereignty and equitable benefit-sharing.

Currently, there are relatively few Indigenous researchers and community members who are active in the microbiome space. Therefore, addressing current deficits in access and training for microbiome research in Indigenous communities should be a priority for the field. Developing educational campaigns or resources that focus on the microbiome and attendant research governance and IP issues may be helpful for Indigenous stakeholders to support their engagement in these important conversations. Specific programs or workshops to build capacity for Indigenous individuals and communities to drive microbiome research and policy, akin to the model of the Summer Internship for Indigenous Peoples in Genomics (SING) program, should be considered. In a less formalised manner, research partnerships and inclusion of Indigenous community members in all stages of the research process will further increase the accessibility of the discipline to future potential trainees or independent researchers within the community. Concurrently, non-Indigenous researchers and other stakeholders need to work to develop their own cultural competency and strong relationships with partner communities in order to support ethical and mutually beneficial research.

*Supporting Indigenous data sovereignty in microbiome research*

As specific guidelines and discussions in the microbiome ownership space evolve, good practice for microbiome researchers includes respecting Indigenous data sovereignty and engaging with existing tools and guidelines to promote this goal. All Indigenous communities should be treated in ways consistent with their unique governing structures, independent of the nation-

states in which they are found. Indigenous data sovereignty is advanced through transparency and ownership of microbiome samples and the data derived from them. These samples and data can be considered contiguous with the Indigenous participants and, at a minimum, may represent kin to which the Indigenous community is tied. As such, projects need to establish Indigenous oversight and a bidirectional flow of information between community partners and the research team. Mechanisms to support such oversight and information flow can include community advisory groups consisting of Indigenous participants, knowledge keepers and Elders; or other Indigenous-controlled research governance bodies such as tribal institutional review boards. Plans for co-interpretation of results, review and co-authorship of publications, and the release and management of data and samples can be recorded in formalised agreements or memoranda of understanding (MOUs).

Researchers should develop long-term data and sample management plans in collaboration with their Indigenous research partners to ensure information obtained from Indigenous communities remains under Indigenous stewardship. Maintaining accurate records of data provenance and ensuring that information is correctly attributed to Indigenous communities supports ongoing management of data by the community (Hudson et al. 2020; Anderson and Hudson 2020). Although not all Indigenous communities have access to appropriate long-term storage facilities and secured server space for microbiome research, biobanks with Indigenous governance structures are located in the US, Canada, and Australia, and could operate as intermediaries if insufficient infrastructure exists in the partner community. Authorisation of sample or data release, including deposit of microbiome data in publicly accessible repositories, then lies with the partner community using the affiliated biobank as a control point for safeguarding and distribution. Hence, ownership and management of samples and data remains with the partner community, while researchers may be granted permission to access and, where appropriate, assist in stewardship of the data “on loan” (Arbour and Cook 2006). Release of materials and data for secondary use from the biobank should follow the CARE Principles (collective benefit, authority to control, responsibility, and ethics) recently laid out by Indigenous researchers that enhance the FAIR Principles of data management and stewardship (findable, accessible, interoperable, reusable) (Global Indigenous Data Alliance (GIDA) 2019). Communities and researchers may wish to explore the implementation of dynamic consent mechanisms, such as online portals, for approval of future sample and data

uses outside of the original study. Participants who do not wish for their sample or data should be re-used should have the option to have these resources returned or respectfully destroyed.

Looking to the future, the successful establishment of the Native BioData Consortium on the Cheyenne River reservation in the US highlights the feasibility of developing autonomous Indigenous research and biobanking entities on tribal lands. Even smaller infrastructure projects, such as server banks for data storage, dramatically improve access to microbiome research. Other opportunities for Indigenous data futures might include technologies such as high-performance computing services for data processing and analysis, or satellites for data transfer. Investment in such community capacity can be written into grant applications or achieved through partnerships with data security firms or academic institutions to support long-term data protection and sovereignty.

*Promoting IP opportunities and equitable benefit-sharing*

There is clear need in the human microbiome field for development and implementation of strategies to support equitable sharing of benefits from research and commercialisation with Indigenous participants and communities. Again, strong relationships and genuine partnering with communities will help to inform agreements about what benefits may be possible or desired and are relevant to community priorities and aspirations. Hence, the process needs to begin with two-way discussion that identifies potential benefits of research and how stakeholders will share them. For instance, non-commercial benefits might include knowledge about the microbiome, access to microbiome-based treatments or therapeutics, or opportunities for training and infrastructure support. The question of how any potential commercial applications will be handled for mutual benefit should also be discussed and agreed upon before starting a research project. As with other aspects of research, agreements regarding both commercial and non-commercial benefit-sharing may be formalised via an MOU. Options for sharing of financial benefits could include joint IP ownership and patent applications, discounts on downstream commercial products for community members whose samples and data aided development of a medication or therapy (James et al. 2014), or rights to 'resale' royalties where community members are compensated for each subsequent commercial use of their data or samples. At a more systemic level, recent discussions have

advanced suggestions for extending the Nagoya Protocol, which specifically focuses on equitable access and benefit-sharing, to explicitly include digital sequence information (DSI), including metagenomic sequence data (Ambler et al. 2021).

Building capacity for communities to manage their own IP claims and potential commercialisation is another mechanism for ensuring Indigenous peoples receive fair benefits from microbiome research. Currently, communities wishing to pursue IP claims may need to rely on assistance from non-Indigenous lawyers or university legal departments, which may not be ideal. Initiatives could include support for training and employment of Indigenous lawyers and patent clerks, or investment in institutions such as Indigenous-led IP think tanks or innovation accelerators. Scope for Indigenous IP clearing-houses or help centres should also be explored. Engagement with the ethical questions raised here and the development of systems to support ethical research and benefit-sharing will be crucial as research interest and commercialisation potential for the human microbiome continue to rapidly advance.

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# Chapter V: Researchers using environmental DNA must engage ethically with Indigenous communities

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# Researchers using environmental DNA must engage ethically with Indigenous communities

The study of environmental DNA can reveal information about the history and presence of Indigenous communities on their lands — potentially even inadvertently. Better engagement with the ethical aspects of environmental DNA research is required in the field as a whole, and especially for researchers working on Indigenous lands.

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Analysis of environmental DNA (eDNA) is a rapidly developing research area with broad applications for ecology and conservation biology[1–4]. Extraction of genetic material from environmental samples, such as water, soil, air or sediments, can provide current and historical information about people, their environment and their interactions with other species. Many environmental samples are legally collected from sediments, water and other materials on Indigenous lands, without extensive regard as to how the findings may affect Indigenous traditional owners and knowledge holders. Such considerations may be especially critical when considering that eDNA can be preserved in some environmental samples for thousands of years[5–7], which could be used to directly link certain human populations to specific locations in the past[4,8]. Indigenous peoples have often been excluded from genetic research, in part because of a history of ethical transgressions and a lack of trusting relationships with researchers[9–11]. Although collaborations between Indigenous communities and geneticists are now developing with enhanced ethical oversight, this has not been extended to the novel ethical, legal and social implications that arise from the use of eDNA.

Because environmental samples may contain DNA from many different organisms, it falls to researchers to choose how narrowly or broadly to target their sequencing efforts. Increasingly, eDNA research is moving away from metabarcoding and amplicon studies that target specific organismal groups and towards whole-genome or shotgun sequencing approaches that can reveal the total diversity of DNA present in a sample[8]. This indiscriminate approach raises key ethical questions, especially in Indigenous contexts. In settler-colonial contexts, such as Australia, Indigenous people have been marginalized, dispossessed and disadvantaged. Throughout this, however, many have maintained connection to culture and country, and exercise their cultural rights, albeit within a colonial framework which contains and constrains. Intergenerational trauma, and in particular the removal of children, has resulted in deep suspicion of and alienation from academic and scientific research. As such, it is critical that ethical questions arising from new approaches to genetic research, such as eDNA analysis, be carefully considered. For example, what are the potential risks and benefits for Indigenous peoples engaging with eDNA research? How should such research be classified, regulated and governed? How can the potential of this new technical approach be communicated without 'hype' or over-promising results?

Some of these questions can be illustrated using the example of the birthing trees that were regularly used by Aboriginal women in southeastern Australia before the expansion of European settlement in the mid-nineteenth century (Fig. 1). Women typically gave birth and buried placentas under a birthing tree, and contemporary Aboriginal researchers have used archival

**Fig. 1 | Birthing trees.** Gum trees, such as the one shown here, are integrated into Aboriginal Australian knowledge and practice. For example, an 800-year-old Djab Wurrung birthing tree (not pictured) in Victoria, Australia, was the site of perhaps 10,000 births of Aboriginal children and holds profound cultural and spiritual significance. The tree was slated for demolition in 2018 as part of a highway reconstruction project. After more than a year of on-site protesting by Djab Wurrung traditional owners and their supporters, protection for the birthing tree was secured in an agreement with the Victorian government. Efforts to secure protection for other culturally significant trees are ongoing yet not always successful, and the 350-year-old sacred Djab Wurrung Directions Tree was cut down in October 2020. Credit: THPStock.

records and oral testimony to show that the trees were associated with labour techniques, midwifery and ceremonies surrounding labour and birth[12]. Birthing trees also facilitated connections to land for the mother and baby, and aspects of the traditional practice are still known and used by Aboriginal groups in southeastern Australia. Acknowledging this historical and ongoing importance of birthing trees for Aboriginal women, we approach this topic as a team of Indigenous and non-Indigenous female researchers drawing collectively on several decades of engagement with Indigenous communities.

Environmental DNA research could provide insight into birthing trees via several mechanisms, investigating questions of mutual interest to researchers and Indigenous communities. For example, human DNA preserved in soil or sediment adjacent to a birthing tree could be revealed, potentially confirming a known birthing site, identifying a previously unknown birthing tree, or demonstrating a connection between a specific family or group and a specific tree. Further, identification of ancient human-associated microbes could provide information on past infections or birth complications. However, such research also presents risks for Indigenous peoples. For example, analysis of soil or sediment samples may not identify human DNA at a birthing tree site, which could potentially be used to argue against land rights claims or the protection of cultural heritage, or eDNA may identify microorganisms associated with potentially stigmatizing diseases, such as sexually transmitted infections. A further risk relates to the use of a Western scientific lens to assess eDNA use. In this example, we see Western science as complementary to Indigenous knowledge, although some may interpret the use of eDNA technology as an implication that Indigenous knowledge is insufficient to inform decision-making about the preservation of birthing trees.

Another potential discrepancy between Western and Indigenous viewpoints might arise from the way human and non-human research is distinguished. Human research is typically subject to more stringent ethical requirements and approval from institutional committees, but it is currently unclear if analysis of human DNA isolated from an environmental source would constitute human research. In Australia, for example, the National Statement on Ethical Conduct in Human Research (2015) states that "human research is… conducted with or about people, or their data or tissue"[13]. Our interpretation of these guidelines is that eDNA may not be considered human tissue, but human genome sequence data produced from eDNA may be, particularly if it is intended or possible to compare this with other human DNA to identify genetic relationships. If so, this would imply a requirement for human ethical review of eDNA research. The National Statement continues that any human research involving Aboriginal and Torres Strait Islander peoples must undergo ethical review. However, human ethical review is currently not typically required for the study of environmental samples, such as soil. We argue that the potential for eDNA analysis to intersect with human research means that, in certain circumstances, scientists working with environmental samples may need to engage with human research ethics.

It is also possible that relying on existing ethical frameworks is insufficient to regulate eDNA research in Indigenous contexts. Even if no human DNA is isolated from an environmental sample, or if researchers agree to disregard any human DNA detected, Indigenous stakeholders may still have ethical concerns. For example, the cultural significance of non-human animals, water, landscapes and natural phenomena differs between Indigenous and Western contexts[14–16], which may complicate existing distinctions between human and non-human research. Because the study of non-human eDNA, including that of animals or plants, could have culturally important implications for Indigenous people, ethical review of such research should implement Indigenous oversight from communities with connections to the land from where eDNA originates.

Like human genetics and human microbiome research before it, eDNA research faces a challenge in how to communicate its potential benefits without over-promising[17,18]. Research using eDNA offers a new technique for producing valuable knowledge about historical and current Indigenous practices, which may be particularly important to Indigenous groups who have experienced cultural dislocation. eDNA evidence could also be useful in influencing decisions on cultural preservation made by non-Indigenous government officials — for example, in obtaining protection for sacred trees (Fig. 1). However, the limitations of this technology must be appreciated and discussed in any consultation process. eDNA research is not infallible; technical limitations, such as DNA degradation due to age and environmental conditions[19], leaching of DNA between soil or sediment layers[20,21], or the difficulty of detecting false negative results, could all hamper eDNA research. This means, for example, that if the DNA of a particular species cannot be found in an eDNA dataset, its presence cannot be ruled out, as it may be in unsampled parts of the region or be a simple failure of DNA preservation in that environment. Furthermore, even if the technical work of DNA extraction, sequencing and analysis is successful, mistakes or uncertainties in the interpretation of genomic data are still possible. Engaging in multidisciplinary research that combines eDNA analysis with other lines of evidence represents the best opportunity to understand the past. Researchers have an ethical responsibility to avoid hype; hence, the limitations of eDNA research need to be clearly communicated to Indigenous groups who may have particular expectations of or interests in an eDNA research project.

The complex issues surrounding eDNA research illustrate how applying new technologies in novel contexts may modify our understanding of what counts as human research, and of what types of research need to recognize Indigenous interests. In addition to institutional mechanisms for human ethical review and Indigenous research governance, such as consultation with ethics review committees or Indigenous Advisory Committees, it is critical to include Indigenous communities and researchers in all stages of an eDNA study (design, sample collection, analysis and interpretation) to help minimize risks and maximize benefits of research. Ideally, this includes the establishment of long-term partnerships between researchers and Indigenous communities and the creation of Indigenous-led research governance structures. In the absence of dedicated structures, researchers should look to local Indigenous communities, regional and national Indigenous organizations, Indigenous scholars and/or regional and national guidelines for research with Indigenous people in the country in which they conduct their research. In the future, it may be beneficial to revisit current regulatory frameworks to formally incorporate these principles into ethical standards of conduct for eDNA research. Provided that these challenges can be adequately addressed, eDNA analysis offers an exciting new prospect for researchers and Indigenous communities to work together to generate knowledge. ❐

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## Competing interests

The authors declare no competing interests.

# Thesis discussion

This thesis has presented a multidisciplinary exploration of Indigenous microbiome research, defined as microbiome and metagenomic research that involves Indigenous people as research participants or stakeholders. To briefly summarise the preceding chapters, my research has investigated oral microbiota in Aboriginal Australians and Torres Strait Islanders and informed hypotheses about the roles of heritage, environment, and other factors in shaping the microbiome and oral health in these groups. Woven through the thesis, I present reasoning for the benefits that further microbiome research could bring for Indigenous peoples, while also highlighting the social and ethical risks and implications of this research and suggestions for how they may be addressed. My experiences of working on this PhD have reinforced my belief that cultural awareness and multidisciplinarity are essential to meaningful research in this field. In this final Discussion chapter, I address emergent themes and messages from my PhD research and experiences. These themes include the microbiome as a bridge between self and other and how this relates to the use of racial, ethnic, and lifestyle categories in human microbiome research; some major technical challenges in the microbiome field, including causal inference and hypothesis formation; and issues surrounding bias and representation of Indigenous peoples in microbiome research. Further, I use this Discussion to argue that scientists need to take seriously the potential risks and harms of Indigenous microbiome research and work to address them, and to reflect on the challenges of navigating ethical risks and multidisciplinary research in real life with examples from my own experience. I conclude this thesis with some suggestions for future directions in this complex yet promising field.

## Crossing physical and conceptual boundaries with the microbiome

Human microbiomes are fascinating because they can illustrate a lot about what it means to be human. Depending on your point of view, microbiomes are an intimate part of us, yet simultaneously foreign – separated from humans by billions of years of evolutionary divergence, living at a scale that we can barely conceptualise. The knowledge that all humans are fifty percent microbial at the cellular level (Sender, Fuchs, and Milo 2016) and rely on these

microorganisms to maintain our health has provoked some scholars to reconceptualise the human being from an individual to a "super-individual" (Hutter et al. 2015). What does our increasing knowledge of the human microbiome mean for concepts of human identity? Gligorov and colleagues have argued that the human microbiome is like an organ or body part: an individual does not become a different person if their microbiome changes, just as they do not become a different person when they receive an organ transplant (Gligorov et al. 2013). On the other hand, Rees and colleagues have argued that the microbiome deeply challenges our concept of self with the knowledge that microorganisms shape the "biological basis of self", ultimately contending that the traditional division between sciences and humanities is no longer tenable in the face of the microbiome (Rees, Bosch, and Douglas 2018). For Hutter and colleagues, while conventional human individuality remains useful in certain contexts, the "real *biological* individual is a *super-individual* defined as the sum of the organism + its microbiome: it is this *integrated symbiotic association* that is able to persist and survive" (Hutter et al. 2015) [emphases in original]. Of course, all of these authors are writing from a shared Western philosophical foundation, which tends to favour the centrality of the autonomous individual and the divide between humans and nature. Perhaps thinkers raised in a different culture would ask different questions about the microbiome and humanity (Redvers et al. 2020).

In my view, microbiomes sit at the intersection between humans and our environments. They can be a source of physical connection between ourselves and other host organisms or our surroundings (Song et al. 2013; Ross, Doxey, and Neufeld 2017; Rothschild et al. 2018; Finnicum et al. 2019; Selway et al. 2020). Current evidence indicates that human microbiomes are shaped by a combination of many factors, including genetics or ancestry, family and cultural practices, physical environment, and other life experiences. This echoes the anthropological concept of the "biosocial", or social relations shaping and being shaped by the physical body (Lock 2015; Palsson 2016). Scientific results in my thesis were consistent with this general picture of the complexity of factors that are understood to shape the microbiome. For instance, in Chapter III we found that factors including oral health behaviours, dental caries, and proxies for socioeconomic status (household size and number of people in the household who worked) were correlated with differences in oral microbiota diversity and composition (Handsley-Davis et al. 2021). In Chapter II, we identified significant differences in oral microbiota diversity and composition in Indigenous Australians and non-Indigenous individuals, independent of oral

disease status and of the large-scale 'lifestyle' (i.e. hunter-gatherer versus industrialised) and geographic differences present in many other studies of Indigenous peoples' microbiomes. As a result, we suggested that oral microbiota in Indigenous Australians may be shaped by 'heritage'. But what does this mean, exactly? In that chapter, I defined heritage as "both genetic (human and microbial) and cultural information passed on across generations, without privileging one or the other". Teasing apart whether one or several elements of this definition ultimately form the direct mechanism for microbiota differences will require further investigation. While there is currently mixed-to-limited evidence to support a direct influence of host genetics on the oral microbiota, heritage goes beyond simple 'ancestry' in the genetic sense. Heritage is also extending the idea that our own lifestyle, environmental and cultural context shape our microbiota to the idea that these factors also shaped our ancestors' microbiota and hence what could be passed down to us: how past environments, cultural practices, and evolutionary forces may continue to influence the microbiota of people living today, in addition to the somewhat better-understood influences that accumulate during an individual lifetime.

This framing may help us to grapple with the sometimes-troubling roles that concepts of race, ethnicity and Indigeneity[4] play in human microbiome research, which have been previously raised in the literature. In 2013, relatively early in the recent explosion of interest in the human microbiome, Fortenberry raised cautions about the uncritical use of racial and ethnic categories in this field (Fortenberry 2013). He noted that socially- and politically-defined racial and ethnic categories were commonly employed in microbiome studies "as if [they] defined inherent between-group differences that reliably transcend marked heterogeneity within each category", erroneously implying that such purported differences explain microbiota patterns. On the other hand, Fortenberry argued that while racial and ethnic categories do not describe inherent and reliable biological groupings, they do describe groupings linked to social, economic and political factors such as racism, colonisation, and immigration. These factors underpin real health disparities and hence are relevant to microbiome research. Therefore, he

[4] The remainder of this section is dominated by 'race', as this is the term most frequently used in the literature on this issue to date. However, the discussion is also relevant to other concepts that encompass socially-constructed human groupings that overlap with shared culture and ancestry, such as 'ethnicity', 'Indigeneity' and 'heritage''.

equally argued against ignoring race and ethnicity when attempting to understand the microbiome, calling on the field to "translate its insights into better understanding of [racial] health disparities without depending on the validity of the categories on which disparities are based" (Fortenberry 2013). Several years later, Benezra expanded similar concerns, incisively critiquing the role of race as a "ghost variable" in microbiome research: "one that is there and not there, hiding in shadows and jumping out when least expected" (Benezra 2020). Benezra showed how even terms intended to avoid race, such as 'ancestry' and 'geography', are employed in racialised ways when "microbiome differences are sought without corresponding investigations into existing economic, political, and health vulnerabilities" (Benezra 2020). Benezra further supported her arguments with detailed anthropological analysis of microbiome researchers seeking to understanding microbiota variation across humans, based on fieldwork in a human microbiome research lab, and raised additional ethical concerns regarding bioprospecting and the 'othering' of specific human groups in microbiome science (Benezra 2020).[5] She concluded her analysis by proposing "biosocial intersectionality", in which microbiomes are investigated by multidisciplinary research teams and interpreted as both biological and social phenomena, as a way forward for the human microbiome field (Benezra 2020). Hence, if concepts such as 'race' or 'ethnicity' are to be used in microbiome research, this must occur carefully and with clear acknowledgement and consideration of their complex biosocial nature.

Interestingly, a recent publication by Nieves Delgado and Baedke takes a very different approach, raising concerns about this very "biosocial" conception of race in human microbiome research (Nieves Delgado and Baedke 2021). Part of this paper draws on history in order to warn against the dangers of "biologizing social constructionist understanding of race in microbial ecology" (Nieves Delgado and Baedke 2021). The authors compare contemporary microbiome research in Indigenous and *mestizo* communities in Latin America to historical European beliefs that the "constitutions" of different human 'races' were shaped by the environment and climatic conditions. In this system, non-European bodies were framed as primitive, inefficient and inferior (Nieves Delgado and Baedke 2021). The point that 'environmentalist' (as opposed to biologically intrinsic) conceptions of embodied race can also

[5] Benezra's arguments are also touched on in Chapters II and IV of this thesis.

lead to exclusionary classifications and racist narratives is important and well-taken. Clearly, biosocial approaches are not in themselves a solution to racism in microbiome research.

However, some other arguments in this paper are less convincing. In particular, the critique of "conceptual problems" with a biosocial understanding of race in microbiome studies largely relies on juxtaposition against a "standard" view of human races as biological subspecies consisting of reproductive individuals who consistently pass on racial membership to their offspring – a view that is not adequately defended by the authors. At one point, Nieves Delgado and Baedke justifiably note that the reported observation of similar gut microbiota characteristics across geographically diverse hunter-gatherer populations "clearly indicate that microbiome composition could be informative about socio-cultural patterns certain groups share […], but not about their [biological] relatedness" (Nieves Delgado and Baedke 2021). However, they go on to argue that the idea of "race" affecting the microbiome should be rejected on this basis, which seems logical only if one believes that human 'races' have intrinsic biological meaning. In such discussions, it is important to distinguish between race having an inherent, immutable, and fundamentally biological *origin*, and the social construction of race having the ability to affect biology and create observable patterns. Furthermore, evidence of caregivers' role in transmitting commensal microorganisms to children negates the need for any evidence of microbiota inheritance to accept a "standard" view of biological race that treats racial categories as inherent and immutable. If race is primarily socio-culturally acquired, as most would agree, there is still scope for the socio-culturally-acquired race of parents to impact their microbiota and hence what they can pass on to their children.

Elsewhere in their conceptual critique, Nieves Delgado and Baedke cite a paper that generated oral microbiota data from individuals of four different ethnicities and presented a machine learning model that was moderately successful in classifying samples by ethnicity based on microbiota patterns (Mason et al. 2013). The original authors concluded that ethnicity shapes the subgingival oral microbiota, likely through host genetic mechanisms (Mason et al. 2013). Nieves-Delgado and Baedke claim that "[t]his and similar microbiome studies […] understand non-human species as central biological entities that allow racially grouping and distinguishing human individuals", which they criticise as taxonomically indefensible (Nieves Delgado and Baedke 2021). However, reporting that somewhat reliable correlations between microbiota

and ethnicity can be identified in a given dataset does not amount to claiming that non-human species are, or should be, the basis for defining ethnic categories. Further, Nieves Delgado and Baedke did not mention that the 2013 paper was publicly criticised by other scientists for over-interpreting the role of ethnicity and host genetics and failing to adequately consider alternative explanations for their results (Eisen 2013; Mason et al. 2014).[6] Hence, this single paper is hardly an accurate representation of understandings of race and ethnicity in the field as a whole. Overall, the suggestion that microbiome scientists are routinely "defining" human racial and ethnic categories based on the microbiome seems to be a straw man.

While conceptions of race and ethnicity and their uses in the microbiome field can absolutely be problematic and deserve close interrogation, the idea that *any* biosocial conception of these categories is inherently dangerous or problematic is less convincing. Rather, the microbiome may provide an illustration of how socially constructed concepts can be reified and interact to produce biological phenomena. Racialised factors or experiences, such as socioeconomic status, cultural practices, or exposure to racism, may also be inscribed in the microbiome; microbiome signatures, in turn, can tell a story about 'race' without supporting the notion that human 'race' is a biologically 'real' quality that resides in the human body independent of social and cultural influences. In some ways, then, microbiomes dissolve the nature:nurture dichotomy (Palsson 2016). This is part of what makes them so intriguing and at the same time extremely technically challenging to understand.

Making sense of the microbiome: causal inference and technical challenges

Many microbiome studies face challenges in defining or inferring causality, exacerbated by the complexity of biological, social and environmental factors at play that I have just discussed. For example, the dataset in Chapter II of this thesis showed patterns that could plausibly be explained by linking heritage to oral microbiota diversity and composition. However, as discussed above, there are several plausible exact mechanisms or relationships contained in the concept of 'heritage', any of which could be the direct cause. In Chapter III, the analysis revealed that the dataset contained many co-varying factors that were infeasible to disentangle

[6] See also the reader comments on the original 2013 publication (Mason et al. 2013).

without further studies (Handsley-Davis et al. 2021). In the course of my PhD, I also became interested in how researchers used the term 'lifestyle' in analyses of the human microbiome.[7] Like 'heritage', it seemed to illustrate our current imprecise understanding of the complexity of factors that can shape the microbiome. In studies discussing the impact of 'lifestyle' on the microbiome, human research participants are typically classified as either 'hunter-gatherer', 'traditional agriculturalist', or 'industrialised' (or alternatively, 'Westernised'). However, these groupings obscure a huge number of variables that might be considered aspects of lifestyle, including but not limited to dietary patterns, healthcare access, use of drugs and medications, antibiotic exposure, environmental exposures such as water quality and pollution, the built environment, greenspace exposure, physical activity, food security, and cultural practices such as grooming. Any or all of these factors could vary widely among individuals within the categories of industrialised, hunter-gatherer, and so forth. In practice, in the context of microbiome research 'lifestyle' is generally used to imply diet – particularly a divide between "traditional" and "industrialised" diets – but leaves room for other factors to be at play, despite these non-dietary factors rarely being directly measured or explicitly discussed.

Therefore, the challenge of causal inference might be partly addressed by using more precise language and measurement to define what is being investigated. Collecting more metadata might also help to disentangle covariance and pinpoint causation, but at what point does this become unacceptably invasive and burdensome for research participants? Furthermore, if equifinality[8] characterises the structure and function of human-associated microbial communities, this further increases the difficulty of straightforward causal inference. Hence, exploring analytical techniques used in social science disciplines that are designed for analysis of equifinal systems may be valuable in tackling the problem of causal inference in the microbiome field. Improved statistical methods may allow for better control of co-varying and confounding factors, but direct demonstration of a causal mechanism is likely required for definitive explanation. Elucidation of causal relationships is a key step towards harnessing the promise of microbiome research to improve human health and wellbeing.

[7] Including myself, as I have used the term elsewhere in this thesis.
[8] A situation where many systems, scenarios, or causes can lead to the same observed outcome.

This leads to the question of how best to work towards investigation and confirmation of such mechanisms. Classical philosophy of science describes two main types of reasoning: deductive reasoning moves from the general to the specific, or theory to observation; and inductive reasoning moves from the specific to the general, or observation to theory (Hepburn and Andersen 2021). In inductive research, data are collected and patterns in the data are identified, eventually leading to the generation and testing of specific hypotheses. By way of comparison, purely deductive research would begin with a specific hypothesis, then design experiments and collect data to test only that hypothesis. To date, the microbiome field has been dominated by inductive approaches, eliciting some criticism and concern. It is not uncommon to hear inductive studies dismissed as merely 'descriptive', a way of cutting intellectual corners by 'fishing' in large datasets for interesting patterns, rather than doing the hard work of formulating well-founded hypotheses and stringently testing them. In this vein, Prosser has argued that deductive hypothesis-testing is the only truly scientific approach to microbiome research, whereas descriptive and inductive research can lead only to knowledge, but not to understanding (Prosser 2020). In this view, too much time and money is being wasted on inductive work that does not meaningfully advance understanding of microbial communities (Prosser 2020). However, others argue for the value of inductive, exploratory, or otherwise non-hypothesis-driven work (Tripathi et al. 2018). Because microbiomes and the forces that shape them are so complicated, and so much about them still unknown, a strictly deductive approach is very difficult to implement. Since the underlying knowledge base from which to develop plausible hypotheses is relatively thin and patchy, we are limited in the usefulness and precision of hypotheses that can be made and our ability to properly test them. New tools and methods – laboratory techniques, programs, statistical approaches – will likely need to be developed in order to advance the precision of our understanding (Tripathi et al. 2018). In this view, jumping too quickly into strict hypothesis testing is also likely to waste time and resources.

Like the field at large, much of the scientific work in my thesis would fall in the category of the primarily inductive, or perhaps deductive-lite, in which hypotheses are formulated and tested but constrained by the data available, rather than using a precise hypothesis to guide the data collection. Hypotheses formulated in this context tend to be limited to the question of whether an association exists between some microbiome characteristic and some host or environmental characteristic – not to questions of whether and how one causes the other. In my experience,

a lack of explicit hypothesis formulation prior to data collection allows for the emergence of interesting patterns that can form the basis for future testable hypotheses; on the other hand, this approach can make data difficult to definitively interpret. Overall, I do sympathise more with the perspective that inductive research is useful and disagree with Prosser's assertions that inductive studies can almost invariably be replaced by deductive hypothesis-testing. However, it is worth reflecting on whether the field would benefit from shifting the balance more towards deductive approaches, although not necessarily because they are intrinsically more 'scientific' or valuable. Particularly for research promising tangible benefits to stakeholders, such as health benefits for Indigenous communities, perhaps there should be a stronger obligation to focus on direct hypothesis testing with greater potential for short-term translation. Conversations about improving microbiome research practice should also include the value of adopting multidisciplinary approaches, which are better equipped to make sense of complex biosocial causes and phenomena (Benezra 2020; Handsley-Davis et al. 2020). This can include research partnerships with Indigenous communities, who can often draw on contextual knowledge and expertise to inform hypothesis generation and the interpretation of research results.

Confronting benefit and risk in Indigenous microbiome research

Bias and representation in microbiome research emerged as another key theme from this thesis. Underrepresentation of specific groups, including Indigenous peoples, in microbiome research has been documented (Rogers et al. 2019; Nath et al. 2021). For example, in a survey of human oral microbiome studies available in MEDLINE as of February 2021, samples were dominated by individuals of European and Jewish (42%) and Asian (24%) ancestry, with Native peoples and South Pacific Islanders having the lowest representation at <1% of total samples (personal communications with S. Nath, 2021). Additionally, study populations within these two dominant ancestry groups were themselves dominated by the United States and China, demonstrating a profoundly unbalanced representation of human ancestry and lifestyle diversity within the field (personal communications with S. Nath, 2021). Exclusion from research leads to exclusion from benefits, whether they be knowledge about the microbiome and health, access to effective microbiome-based therapies, capacity-building for individuals or communities, or something less tangible; exclusion from research benefits then reinforces

health disparities (Nath et al. 2021). Less importantly, but also relevant, the exclusion of underrepresented groups from research is a lost opportunity to better understand human microbiomes in all their complexity. Hence, there are clear benefits to be gained from further Indigenous microbiome research.

However, there is good reason to be wary of the potential risks and harms of Indigenous microbiome research, many of which have previously been discussed in Chapters IV and V. Some of these risks are relatively straightforward to understand from a Western ethical perspective. Indigenous peoples around the world have experienced treatment in the name of science that is unequivocally unacceptable by current mainstream ethical standards, including lack of informed (or any) consent, exposure to physical harms, and the denial of access to medical treatment (Mosby 2013; Mosby and Swidrovich 2021; Pacheco et al. 2013; Kowal and Radin 2015; North and Jonscher 2019; Lewis 2019; Ladd 2020). Biopiracy, as the name implies, can be straightforwardly conceptualised as act of theft. It borders on trite to say that stealing or profiting from others' possessions is *a priori* unethical behaviour, and so is enriching oneself using resources or knowledge that rightfully belong to others. Many researchers would also be aware that research involving members of identifiable groups, such as Indigenous people, can have implications that affect the group as a whole, including group members who did not consent to or were not consulted on the research (Sharp and Foster 2007). Therefore, the question of how to grapple with risks of stigmatisation, discrimination and other harms that may arise from research on members of identifiable groups will likely be familiar even to researchers who do not typically interact with Indigenous communities.

Other risks and harms of Indigenous microbiome research may require a greater level of cultural and political awareness to comprehend. For example, Chapter V of this thesis highlights how metagenomic environmental DNA (eDNA) research may initially appear to non-Indigenous researchers and ethics committees as low-risk, yet actually raises several important ethical concerns for Indigenous communities (Handsley-Davis et al. 2020). Two key concepts that frequently arise in these discussions are Indigenous sovereignty and cultural harm. In brief, sovereignty refers to the authority to manage and make decisions about a people and/or territory. Articulations of, and protections afforded to, Indigenous sovereignties clearly vary across jurisdictions and contexts. For example, Native tribes in the United States are officially

designated as “domestic, dependent nations” and engage in government-to-government dealings with the US government (R. Tsosie 2007). In Australia, where British colonisation was justified on the basis of *terra nullius* (land belonging to no one), colonial governments have been much more circumspect in recognising Aboriginal and Torres Strait Islander jurisdictions and polities (Langton 2020). Nevertheless, rights consistent with sovereignty, including self-determination, self-government, practice of cultural traditions, and maintenance of political, cultural and economic systems, are affirmed in the United Nations Declaration on the Rights of Indigenous Peoples, indicating their global relevance (United Nations 2007). Hence, membership of an Indigenous group often entails both a cultural and a political identity. As touched on in Chapter IV, recognition of sovereignty is one key rationale underlying many widespread recommendations for ethical research with Indigenous peoples, including the ability to approve and lead research priorities, to manage and control data, and to access benefits of research.

According to Native American legal scholar Rebecca Tsosie, cultural harm comes in two main forms: (1) blocking access to the practice of one’s own culture, and (2) the control or appropriation of culture by outsiders (R. Tsosie 2007). Tsosie suggests that US law fails to adequately protect Native cultures from harm due to underlying differences in Western and Native conceptions of property rights and responsibilities (R. Tsosie 2007). This argument is mirrored in Terri Janke’s account of the failures of Western legal systems to protect Indigenous knowledge and cultural and intellectual property being partly due to different underlying conceptions of the purpose and appropriate uses of knowledge and property (Janke 1998), as referenced in Chapter IV. For Tsosie, tribes’ status as sovereign nations undergirds a collective right to cultural protection and survival (R. Tsosie 2007). Indigenous peoples’ concerns regarding cultural harms in the context of genetic research include the abuse or mishandling of biospecimens in a manner that conflicts with cultural beliefs about appropriate treatment of blood and tissues, and the use of research results to contradict traditional beliefs about the group’s origin and identity (R. Tsosie 2007). Such concerns about cultural loss or harm are also intimately linked with the experience of colonisation. After land, culture and other resources already being taken or otherwise denied them, “Native peoples fear that their genetic resources are the new ‘common property’ that researchers are laying claim to” (R. Tsosie 2007). Picking up on this thread, Reardon and TallBear argue that recent high-profile controversies

over Indigenous DNA are not simply instances of white researchers behaving unethically according to our own standards. Rather, they reveal how researchers use appeals to scientific importance and the pursuit of knowledge to disregard Indigenous sovereignty over their own biological samples and data, causing cultural harm in the process (Reardon and TallBear 2012). In Chapter V of this thesis, we discussed potential cultural harms that could arise from a theoretical environmental DNA study of soil from traditional Aboriginal birthing sites as one argument in support of better engagement with Indigenous communities by eDNA researchers (Handsley-Davis et al. 2020). As suggested by discussions of the differing normative frameworks underlying Indigenous and Western conceptions of property, the notion of cultural harm may not be immediately intuitive to non-Indigenous researchers. However, without an understanding of cultural harms and fundamental claims to sovereignty, self-determination and self-government, non-Indigenous researchers will likely continue to misunderstand and fail to address Indigenous peoples' ethical concerns.

Failure to consider Indigenous perspectives and priorities has serious negative consequences. It leads to bad or harmful research; favours the creation of culturally inappropriate guidelines, norms and behaviours; and reinforces a cycle of disincentive to engage in future research (James et al. 2014; Skewes and Lewis 2016; Guillemin et al. 2016). Within the relatively short history of modern human microbiome research, we can already see parallels to related fields that have previously generated unethical research practices and failed to engage with Indigenous communities and perspectives. Some medical and human genetic examples of such problematic research, including the Human Genome Diversity Project, the Havasupai vs Arizona State University case, and the All of Us initiative, have been discussed in Chapter IV. Pharmaceutical research on traditional medicinal plants also supplies some illustrative case studies, including the San hoodia case briefly described in Chapter IV (Wynberg and Chennells 2009) and a case in which patents were sought by a US government department for anti-HIV products derived from smokebush, a traditional medicinal plant used by Aboriginal people in Western Australia (Janke 2018), again without the consent of, or offer to share benefits with, traditional owners. As argued in Chapter IV, concerns surrounding data sovereignty, intellectual property and benefit-sharing are equally relevant to microbiome research. These examples clearly demonstrate the importance of slowing down the typical rush towards research and technical innovation while ethical practice is left to play catch-up. It was important to me that

my thesis should approach science and ELSI research questions simultaneously. Without carefully considering the ethical side, I could not do scientific research in a way I was happy with. Altogether, I hope my work encourages non-Indigenous scientists to deeply consider and respect Indigenous knowledge, views and perspectives when we are working with Indigenous peoples, Indigenous samples, or on Indigenous land.

Towards ethical and multidisciplinary microbiome research practice: a case study

Reflecting on my own experiences as a PhD candidate, I know that navigating the ethical and scientific dimensions of research together is never easy. While no project can be perfect or beyond reproach, it is still important for researchers to consider ethical issues seriously and act to address them in good faith. One example arising during my PhD has been increasing awareness of the growing Indigenous data sovereignty movement. As discussed in greater detail in Chapter IV, Indigenous data sovereignty principles fundamentally advocate for data about Indigenous "people, lifeways and territories" to remain under Indigenous control (Kukutai and Taylor 2016). This goal requires implementation of mechanisms that support the exercise of data sovereignty (Global Indigenous Data Alliance (GIDA) 2019; Hudson et al. 2020). In this context, models of 'tiered' or 'dynamic' consent have been discussed in positive terms as mechanisms to support Indigenous control of genomic or other data. Both these approaches represent an alternative to broad or blanket consent for use of research samples and data, which becomes problematic when these resources are shared or re-used outside of the initial research project without participants' knowledge or approval. In a tiered consent model, participants can select different types or 'tiers' of research uses that they do or do not consent to – for example, based on the level of expected risk to participants, or medical versus non-medical research (Tiffin 2018). In a dynamic consent model, a communication interface between participants and researchers allows participants to choose to grant or revoke consent for particular uses of their samples and data in real time (Kaye et al. 2015). While these mechanisms can present their own risks and challenges, such as 'consent fatigue' (Steinsbekk, Kåre Myskja, and Solberg 2013) or the difficulty of accessing technology required to interact with a dynamic consent interface, there is a growing interest in implementing some version of these consent models in the context of Indigenous research data (Hudson et al. 2020; Prictor

et al. 2020). These considerations do not apply solely to research with Indigenous peoples and data, but often hold particular weight in such contexts.

Currently, the norm in the wider microbiome field is to make sequence data and metadata publicly available, in order to facilitate meta-analysis and reproducibility.[9] However, these open data norms are in tension with Indigenous data sovereignty principles (Hudson et al. 2020; K. S. Tsosie et al. 2020). As such, microbiome researchers working with Indigenous data can face challenges in balancing ethical obligations regarding data storage and availability. In my case, most of the consultation, ethical approvals and sample collection had taken place some years before I began working on the projects. The participant information sheets and consent forms used for the projects followed the field standard and ethical priorities of the time. For example, analyses that would be conducted on the samples were described in general and non-technical terms – for example, to study or understand the bacteria or bugs in the mouth (Appendices II, III, V) – and the protection of participants' personal information, anonymity and individual privacy was emphasised (Appendices II, III, IV). Participants were informed that information from the study would be published (Appendix II) or that their data may be used to improve methods for diagnosis and treatment (Appendices II, III, V). However, the documents did not explicitly focus on what could be done with microbiome data (in contrast to "personal information") beyond the current study, or on the risks and benefits of data re-use in future research, particularly in the context of a public database. Yet, examples such as the Havasupai case referenced in Chapter IV demonstrate that inappropriate re-use of samples and data, even if not explicitly excluded by consent forms, can be a source of major harm and concern for Indigenous communities. In this context, it did not seem appropriate to upload the microbiota sequence data and patient metadata to public databases without more explicit informed consent from the Indigenous research participants to do so. Given the time that had passed, it would be prohibitive to track down every individual who had donated calculus or saliva to a study years before for further consultation. Furthermore, simply obtaining consent from individual patients to make their data publicly available might not be the ideal approach – what about group or community consent, or tiered or dynamic consent? Who ultimately had the

[9] For a representative example, see the editorial policies for the journal *Microbiome*: https://www.biomedcentral.com/getpublished/editorial-policies#availability+of+data+and+materials

right to approve what should be done with the data? It would have been easy to become permanently paralysed by uncertainty over these questions, but that would be doing no favours to anyone, including the people who had provided their samples and data for research.

Eventually, following reflection and consultation, the microbiota sequence data from Aboriginal and Torres Strait Islander patients were uploaded privately to a secure database, with access subject to review by the research project leaders and either an Aboriginal reference group or the Indigenous community involved, depending on the project. This outcome was intended to maintain reasonable protection against clearly inappropriate re-use of data (as experienced in the Havasupai case) and a layer of Indigenous control of the data, while not being unduly burdensome or restrictive towards future data uses that might be beneficial to research participants or their communities. Consultation and partnership with Indigenous stakeholders were also strengthened by inviting and collaborating with First Nations co-authors on all but one of my PhD chapters. This experience is offered as an example of working one's way towards an acceptable, if not necessarily perfect, solution to an ethical and logistical challenge in real-world circumstances. Often we have to do the best we can by acting in good faith with the options available at the time, and continuing to strive for better standards in the future. For example, drawing on this experience, future Indigenous microbiome research projects should include consultation on data storage, access and control, as well as outreach to support informed consent, as part of the research design from the start.

These lessons from personal experience also illustrate the value of education and training in cultural awareness, cultural competence, and bioethics for non-Indigenous researchers who work with Indigenous communities or data. Before and during my PhD, I participated in several formal cultural awareness and competency training activities, including a cultural awareness workshop run by an Aboriginal cultural consultancy, an Aurora internship focusing on cultural safety education with the Australian Indigenous Doctors' Association, and masterclasses in working on Country, Indigenous community engagement and Indigenous cultural heritage management offered through the ARC Centre of Excellence for Australian Biodiversity and Heritage (CABAH). I had further opportunities to learn from Indigenous perspectives in my role as a research assistant and Organising Committee member for the Summer Internship for Indigenous Peoples in Genomics (SING) Australia initiative, engagement with the international

SING Consortium, and working with Indigenous co-authors on publications. I continued to improve my understanding by, for example, reading and citing Indigenous scholars, or attending talks or public events showcasing Indigenous voices. I also benefited from attending an intensive summer school in bioethics in the first year of my PhD. This list is not intended to be self-congratulatory, but reflective of the time and effort non-Indigenous researchers should invest in building their own capacity to understand the cultural, social, and ethical implications of their work with Indigenous individuals and communities. Without this investment, I may not have recognised, or been able to adequately work through, the issues outlined in the preceding paragraphs. Of course, there will always be more to learn, but this training helped me at least begin to understand the key concepts, such as sovereignty and cultural harm, that were crucial to informing my ethical navigation of this project. Making this kind of training standard for trainees engaging in microbiome research is another important priority for the future of the field.

## Conclusion

To conclude, advancing the field of Indigenous microbiome research is both a technical and an ethical challenge. A more robust understanding of how microbiomes across the body (and indeed the wider environment) are shaped, and how they interact with Indigenous health and wellbeing, will require considerable effort and creativity. The design of rigorous experiments and development of appropriate statistical approaches to deal with highly complex systems rife with confounding factors remain major challenges for the human microbiome field as a whole. Navigating ethical data sharing and control in Indigenous contexts is another key issue that does not currently receive adequate attention. However, the first and overarching priority for this field should be supporting Indigenous peoples to formulate their own priorities for microbiome research. This may include outreach and capacity-building initiatives that support greater familiarity with the microbiome and all the technical and ethical questions that stem from it. While structural incentives maintained by funding bodies, academic publishing, and the precarity of science careers all promote a drive towards ever more rapid generation, analysis and publication of scientific results, both individual researchers and the scientific community as a whole need to push back against this temptation to race ahead. Instead, the field should focus on involving Indigenous individuals and communities and equipping them with the

knowledge and relationships to guide research priorities. The Summer Internship for Indigenous Peoples in Genomics (SING) model has made important strides towards similar goals in the field of Indigenous human genomics, supporting Indigenous scholars and community members to lead conversations about the direction and practice of genomic research (Bardill et al. 2018; Claw et al. 2018; Hudson et al. 2020). Complementary efforts could be made towards improving the cultural competence of microbiome researchers working outside their own communities, raising awareness of the issues specific to Indigenous microbiome research, and prompting microbiome scientists to reflect on who is included in their studies and why. Finally, further exploration of the systems and actions that are needed from all stakeholders – including communities, researchers, governments, funding bodies, and other institutions – to promote the rights and wellbeing of Indigenous communities in relation to the microbiome is an important area for future work. In this way, we can hopefully draw closer to the goal of realising the exciting potential of this field.

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# Appendices

# Appendix I: Diversity and bias in oral microbiome research: a commentary

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Commentary

# Diversity and bias in oral microbiome research: A commentary

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The oral cavity has the second largest and most diverse microbiota of the human body, harbouring over 700 microbial species [1]. Oral microbial communities are dominated by bacteria, but also contain archaea, viruses, and eukaryotes, whose roles in oral health and disease are less well understood [2]. Lifestyle, diet, and other host-related factors, such as ethnicity or ancestry, are associated with the composition of these microbial communities, and oral microbiome variation may affect the assessment, response, and effectiveness of disease interventions [3,4]. Therefore, it is necessary to understand how oral microbiome traits are associated with oral health in diverse human populations. Currently, microbiome research is dominated by gut microbiome studies and is strongly biased towards populations of European descent [5]. Such populations, by definition, provide a poor basis from which to understand microbiome-health relationships in under-studied populations, including groups who carry the highest burdens of disease.

A similar bias is present in the oral microbiome research field. Many oral microbiome studies are conducted primarily on people living in industrialized countries, such as the United States and China. These countries maintain large funding allocations for biomedical research; for example, the United States National Institutes of Health (NIH) invested approximately USD $728 M in human microbiome research over a five-year period (2012-2016), of which $48 M was utilized for oral microbiome research [6]. Nevertheless, oral microbiome research within the United States has produced relatively few studies that include people from non-European backgrounds (e.g. African Americans or people of Asian or Indigenous ancestry), and even fewer of these specifically investigate non-bacterial members of the oral microbiota. The NIH Revitalization Act mandates the inclusion of racial and ethnic minorities in federally funded biomedical research, but the implementation of this mandate has been problematic [7]. In the global context, it is not unusual to observe oral microbiome studies using inconsistent or problematic racial and ethnic categories, or failing to mention participants' race, ethnicity or ancestry entirely [4]. Hence, studies do not reflect the diversity of ancestries even within industrialized nations that dominate the field, nor those who are most likely to benefit from improvements in oral health therapies based on microbiome research. If this pattern continues, oral microbiome research is likely to reinforce existing oral health disparities, which often fall along racial lines [8]. While racial categories do not represent biological reality, they intersect with factors relevant to the microbiome and oral health, such as ancestry, experience of racism, and socioeconomic status. It follows that more research should focus on underrepresented groups who experience poor oral health and could benefit most from new therapeutics. Increasing diversity in oral microbiome research could also benefit groups who are currently well-represented. For example, transitions from hunter-gatherer to agricultural or industrialized lifeways have been linked to oral health deterioration [9], so understanding the mechanisms that shift oral microbiomes more broadly could provide insights that improve oral health in industrialized societies.

Several barriers likely contribute to the underrepresentation of minority groups in oral microbiome research. From a research perspective, including diverse communities in the study design can pose cultural and linguistic obstacles, as researchers may be insufficiently trained to design and implement studies in these communities. Studies may also take longer to complete, and resources to recruit and retain a sufficient number of participants across different backgrounds may be limited [7]. From the participant perspective, participants may be justifiably reluctant to participate in biomedical research studies due to fear of exploitation, based on histories or personal experience of unethical practices, and may feel distrust toward field researchers or recruiters [10]. Hence, the goal of increasing diversity in oral microbiome research can only be pursued with the full consent and appropriate involvement of all stakeholders and should include equal sharing of financial and non-financial benefits arising from research.

There is no quick fix or single solution to these disparities. As a general principle, underrepresented communities or stakeholder groups should be involved in decision making, planning and

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conducting the research wherever possible, to help guard against inappropriate research practices and align research projects with community priorities. Some practical approaches to improve participation of currently underrepresented groups in oral microbiome research include positioning study sites in areas of diverse residents, employing recruitment staff with whom participants can communicate in their own language, providing travel support for participants who lack access to transportation, and creating culturally sensitive resources describing how the samples and data will be collected and stored. Researchers also need to be aware of the importance of recording race and ethnicity when planning a study [4]. In the longer term, a more systematic approach to tackling this bias could be increasing the diversity in investigators/researchers, as well as grant reviewers. By drawing on these approaches, researchers and communities can find ways to redress inequalities and ensure that everyone benefits from oral microbiome research.

## Declaration of Competing Interest

The authors have nothing to disclose.

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# Appendix II: Participant information sheet and consent form for participants in the PerioCardio project

**Note:** applies to dental calculus samples from Aboriginal and Torres Strait Islander participants in Chapter II

ARCPOH
Australian Research Centre for POPULATION ORAL HEALTH

THE UNIVERSITY OF ADELAIDE AUSTRALIA

SUB CRUCE LUMEN

**SCHOOL OF DENTISTRY**
FACULTY OF HEALTH SCIENCES
122 Frome St, SA 5005
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# Information sheet for participants

"Calculus study for participants in the perio-cardio study"

*This is for you to keep*

**Investigator**
Lisa Jamieson
Australian Research Centre for Population Oral Health
The University of Adelaide
Ph: 08 8303 4611
Fax: 08 8303 4858
E-mail: lisa.jamieson@adelaide.edu.au

You are invited to take part in this study. The investigator would like to understand more about the role of bugs involved in periodontal disease (gum disease) that are stored in calculus (tartar). Calculus is hardened plaque which sits around the gum line of teeth.

**What is this study about?**
The link between bugs in dental calculus and diet is something that is not well understood. Understanding these links may help researchers learn more about the role of calculus and gum disease, which then has a role in heart disease.

**Who is doing this project?**
There are six different partners in this research project; the University of Adelaide, Menzies School of Health Research, Baker IDI Heart and Diabetes Institute, University of South Australia, University of Sydney and the University of North Carolina.

**What is involved, including time frame?**
As a study participant, you will be asked to have a dental examiner take a small sample of calculus from the lower teeth of your mouth. It will take approximately five seconds to remove this calculus and it will not be painful. The calculus sample will then be stored in a sterile container and sent to the University of Adelaide for analysis. The calculus will be kept at the University of Adelaide for 5 years, after which time it will be destroyed.

**Are there any risks?**
There is a very slight risk of bleeding when we take a calculus sample. This bleeding should stop within five minutes.

**Participation**
You do not have to take part in this study, and if you choose not to take part, this will not affect any dental health care. If you do agree to take part, you are free to withdraw from the study at any time, without having to give a reason. This will also not effect your future health care.

**Will it cost anything?**
No, participating in the study will not cost anything.

**Confidentiality and Privacy**
A study number will be assigned to your data to ensure your personal identity is protected. All data will be stored securely at the Menzies School of Health Research for 10 years. No material that could identify you will be used in any reports of the study.

**Results**
If you wish to receive a copy of the results, please write your address in the space provided on the consent form.

Please feel free to contact the researcher if you have any questions about this study.

**Independent complaints**
If you have any concerns or complaints regarding the ethics conduct of this study, you are invited to contact the Ethics Administrator of the Human Research Ethics Committee of the NT Department of Health and Families and Menzies School of Health Research on 89227922 or email ethics@menzies.edu.au

Thank you very much, your assistance is greatly appreciated.

THE UNIVERSITY OF ADELAIDE HUMAN RESEARCH ETHICS COMMITTEE

**STANDARD CONSENT FORM**
**FOR PEOPLE WHO ARE PARTICIPANTS IN A RESEARCH PROJECT**

***<u>This means you can say 'NO'</u>***

1. I, …… *(please print name)*

   consent to take part in the research project entitled: **'**Calculus study for participants in the perio-cardio study"

2. I acknowledge that I have read the attached Information Sheet entitled: 'Calculus study for participants in the perio-cardio study"

3. I have had the project, so far as it affects me, fully explained to my satisfaction by the research worker. My consent is given freely.

4. Although I understand that the purpose of this research project is to improve the quality of medical care, it has also been explained that my involvement may not be of any benefit to me.

5. I have been given the opportunity to have a member of my family or a friend present while the project was explained to me.

6. I have been informed that, while information gained during the study may be published, I will not be identified and my personal results will not be divulged.

7. I understand that I am free to withdraw from the project at any time and that this will not affect medical advice in the management of my health, now or in the future.

8. I am aware that I should retain a copy of this Consent Form, when completed, and the attached Information Sheet.

   ……...
   *(signature)* *(date)*

**WITNESS:** I have described to …….. *(name of subject)*

the nature of the research to be carried out. In my opinion she/he understood the explanation.

Name: ……Status in Project……

…….....
(signature) (date)

**INTERPRETER** (if required)**:**

Name: ……..….

……...
*(signature)* *(date)*

| I agree to the project workers doing the following tests on me at the beginning of the project: | **Please** **YES** | **circle** **NO** |
|---|---|---|
| • Taking a calculus sample | | |

Please provide your name and address if you would like a copy of the results sent to you.

_____
_____
_____
_____
_____

**Thank you very much for your participation, your assistance is greatly appreciated.**

# Appendix III: Participant information sheet for the project 'Using plaque and calculus to help us understand oral disease'

**Note:** applies to calculus samples from non-Indigenous participants in Chapter II

# PARTICIPANT INFORMATION SHEET

## PROJECT TITLE: Using Plaque and calculus to help us understand oral disease
## HUMAN RESEARCH ETHICS COMMITTEE APPROVAL NUMBER: H-2012-108
## PRINCIPAL INVESTIGATOR: Dr Laura Weyrich

Dear Participant,

You are invited to participate in the research project described below.

**What is the project about?**

- To see what types of bacteria are in the mouths of people in our population. This is done by identifying bacteria in plaque (the soft film cleaned off your teeth) and calculus (the hard calcified plaque that collects on teeth). This is done by looking at the DNA of the bacteria only, and no human DNA will be analyzed.

- To compare the bacteria of people with the bacteria of past populations.

- This will help identify the changes in the diversity of bacteria over many hundreds of generations.

- This will give us some insight into why common oral diseases such as decay are so common today

**Who is undertaking the project?**
This project is being conducted by:

- Dr Laura Weyrich (The University of Adelaide)
- A/Prof John Kaidonis (The University of Adelaide)
- Prof Alan Cooper (The University of Adelaide)
- Prof Grant Townsend (The University of Adelaide)

**Why am I being invited to participate?**
We are collecting plaque and calculus from patients randomly attending this clinic for general dental procedures.

**What will I be asked to do?**
Simply allow us to take a small amount of plaque and calculus after it has been removed by the dentist and before it is discarded.
**How much time will the project take?**
No more than one minute.

**Are there any risks associated with participating in this project?**
There are no risks in acquiring the plaque and calculus. The procedure will normally be done by your dentist anyway.

**What are the benefits of the research project?**
Understanding how and why bacterial diversity has changed over many generations. This will give us some insight on how and why oral diseases (i.e. tooth decay) are so prevalent in our current populations. This research may help us determine better ways in preventing oral diseases.

**Can I withdraw from the project?**
Participation in this project is completely voluntary. If you agree to participate, you can withdraw from the study at any time. You can also withdraw your sample from the study at any time.

**What will happen to my information?**
The samples taken from you will be de-identified (ie. Your personal details will not be attached to the samples), and no-one will be able to associate you with the research and any publications. The DNA in the sample will be removed, and any remaining sample tissue will be destroyed after ten years.

**Who do I contact if I have questions about the project?**

- Dr Laura Weyrich (The University of Adelaide)
  - Ph: 83135565, Email: laura.weyrich@adelaide.edu.au
- A/Prof John Kaidonis (The University of Adelaide)
  - Ph: 83133297, Email: john.kaidonis@adelaide.edu.au
- Prof Alan Cooper (The University of Adelaide)
  - Ph: 83135950, Email: alan.cooper@adelaide.edu.au
- Prof Grant Townsend (The University of Adelaide)
  - Ph: 83135968, Email: grant.townsend@adelaide.edu.au

**What if I have a complaint or any concerns?**
The study has been approved by the Human Research Ethics Committee at the University of Adelaide (approval number H-2015-108). If you have questions or problems associated with the practical aspects of your participation in the project, or wish to raise a concern or complaint about the project, then you should consult the Principal Investigator. Contact the Human Research Ethics Committee's Secretariat on phone +61 8 8313 6028 or by email to hrec@adelaide.edu.au. if you wish to speak with an independent person regarding concerns or a complaint, the University's policy on research involving human participants, or your rights as a participant. Any complaint or concern will be treated in confidence and fully investigated. You will be informed of the outcome.

**If I want to participate, what do I do?**
You will be asked to sign a consent form and to keep a copy along with the participation sheet.

Yours sincerely,

- Dr Laura Weyrich (PhD)
- A/Prof John Kaidonis (PhD)
- Prof Alan Cooper (PhD)
- Prof Grant Townsend (PhD)

# Appendix IV: Participant information sheets for parents and children for the project 'Effectiveness, cost-effectiveness and cost-benefit of a single annual professional intervention for the prevention of childhood dental caries in a remote rural Indigenous community'

**Note:** applies to saliva samples collected from children and adolescents for Chapter III

## INFORMATION SHEET: PARENTS/GUARDIANS

**Project title:**
**Effectiveness, cost-effectiveness and cost-benefit of a single annual professional intervention for the prevention of childhood dental caries in a remote rural Indigenous community**

**Project team:**

- **Chief Investigator:**
  Professor Newell Johnson (Griffith University)
  Contact Phone: 07-5552 9306 ; Mobile: 0448 954 344
  Contact E-mail: n.johnson@griffith.edu.au

- **Principal investigators:**
  Professor Ratilal Lalloo (Griffith University), Professor Jeroen Kroon (Griffith University), Mrs Valda Wallace (James Cook University), Associate Professor Lisa Jamieson (University of Adelaide), Dr Ohnmar Tut (Griffith University), Dr Sanjeewa Kularatna (Griffith University)

**Why is the project being undertaken?**
To reduce tooth decay in school-going children in the Northern Peninsula Area by applying some simple and safe solutions to your child's teeth once a year. This will prevent pain and infection now and in the future, and improve overall health.

**Who is funding the project?**
The project is paid for by the Australian Government through a grant from the National Health and Medical Research Council.

**What will you and your child be asked to do?**
Your child will receive a separate Information Sheet to explain what we want to do. Please make sure that your child understands this and that he/she does not have to take part if he/she does not want to.
**If you agree to their participation, please sign the Consent Form and hand it back to our liaison officer who has given you this form.**
Your child's involvement will be a simple examination of his/her mouth and teeth and the completion of a questionnaire. We will be collecting a small amount of your child's saliva (your child's spit) by asking you to spit into a small bottle. Your child's saliva will be taken to test how many bacteria (germs) there are and we will hold onto the saliva for future tests. Only the head of this project and scientists who work for him will have access to the samples. Planned tests will only be done with the approval of Griffith University Ethics Committee. Individual results will never be told to anyone else. The findings will only be described across the whole group of children. We will give each child an appointment to fill any deep grooves on the back teeth with plastic, disinfect the mouth and paint a fluoride varnish on the teeth. This will be done in the dental clinic at Bamaga. If any teeth are decayed, these will be fixed at the same time.
We will do this again after one year and after two years.

**How has your child been selected?**
This project has full permission from the Northern Peninsula Area State College, the Queensland Department of Education and The Queensland Health Torres and Cape Hospital

and Health Service. We will try to see every child attending each of the three school in the NPA on the days the dentists come, and whose parents/guardians have given consent.

**Expected benefits**
Our work will improve the dental and general health of the children. If your child needs any treatment we will tell you. We will learn about how much this kind of dental care costs.

**Risks to your child**
There are no risks in the examination of your child's mouth. If your child needs to have fillings this will be done in the QH clinic under proper hygienic conditions.

**Confidentiality**
The information we collect about your child's mouth will be kept strictly confidential. If he/she needs treatment we will tell the dental clinic at Bamaga and refer you to Community Health or the hospital for any other problem. This will be done in private.
Personal information is confidential and will not be disclosed to others without your consent, except to meet government, legal or other regulatory authority requirements. Your child's anonymity will at all times be safeguarded, except where you have consented otherwise. You can consult the University's Privacy Plan at http://www.griffith.edu.au/about-griffith/plans-publications/griffith-university-privacy-plan or tele-phone 07 3735 4375.

**Participation is voluntary**
Participation is completely voluntary and if you have any questions or concerns feel free to ask any member of the survey team. Your child is free to withdraw without comment or penalty.

**Mechanism for distribution and return**
Kindly ensure that you return the form to our liaison officer who has given it to you.

**Questions / further information**
Should you have any questions or require further information, please do not hesitate to ask a member of the survey team or contact the Chief Investigator.

**The ethical conduct of this project**
Griffith University conduct research in accordance with the *National Statement on Ethical Conduct in Human Research*. If potential participants have any concerns or complaints about the ethical conduct of the research project they should contact the Manager, Research Ethics on 07 3735 4375 or research-ethics@griffith.edu.au. **You may also contact the** Far North Queensland Ethics Committee on 07 4226 5513 or Cairns_Ethics@health.qld.gov.au.

**Feedback to you**
The results of the project will be shared with the Bamaga community, Queensland Department of Education and The Queensland Health Torres and Cape Hospital and Health Service. You may ask Professor Johnson to see the results if you wish. No participant will be identified in the final report.

**Expressing Consent**
By signing, completing the front page of the attached Consent Form and returning it to our Liaison Officer, you confirm that you have read and understood the Information Sheet and wish for your child to participate in this survey.

**PLEASE DETACH THIS INFORMATION SHEET AND RETAIN IT FOR YOUR LATER REFERENCE**

## INFORMATION SHEET: STUDENTS

**Project title:**
**Effectiveness, cost-effectiveness and cost-benefit of a single annual professional intervention for the prevention of childhood dental caries in a remote rural Indigenous community**

**Project team:**

- **Chief Investigator:**
  Professor Newell Johnson (Griffith University)
  Contact Phone: 07-5552 9306 ; Mobile: 0448 954 344
  Contact E-mail: n.johnson@griffith.edu.au

- **Principal investigators:**
  Professor Ratilal Lalloo (Griffith University), Professor Jeroen Kroon (Griffith University), Mrs Valda Wallace (James Cook University), Associate Professor Lisa Jamieson (University of Adelaide), Dr Ohnmar Tut (Griffith University), Dr Sanjeewa Kularatna (Griffith University)

**Why are we doing this project?**
We wish to treat any tooth decay and prevent this from happening again in future. It is important for us to see if this has made a difference to the health of your teeth.

**What you will be asked to do?**
Please read this paper and ask your parent/guardian to explain to you if you do not understand anything it says.
On the day of our visit we will as you a few questions, have a quick look at your teeth and get you to spit in a bottle. This should not take longer than 20 minutes.
Should we find that you need any treatment we will ask the dental clinic to fix your teeth and you will receive a preventive treatment (filling up the grooves on the back teeth with plastic, disinfect the mouth and paint a fluoride varnish on the teeth).
We will do this again after one year and two years.

**Who will take part?**
We will look at the teeth of all children from the Northern Peninsula Area State College

**Have we been given permission to do this project?**
Yes, this project has the full permission of Queensland Health and Education Queensland and we have asked your parent/guardian to give us permission to look at your teeth.

**Do I have to take part?**
You can tell us at any time if you do not want to take part. It is very important to us that you do. It will also be important to you to know if your teeth are healthy and if you will need to have anything done to fix them.

**What if I have any questions?**
If you are not sure about anything please ask us when we visit the school or ask your parent guardian to contact us.

# Appendix V: Consent form for the project 'Effectiveness, cost-effectiveness and cost-benefit of a single annual professional intervention for the prevention of childhood dental caries in a remote rural Indigenous community'

**Note:** applies to saliva samples collected from children and adolescents for Chapter III

## CONSENT FORM

**Project Title:**
**Effectiveness, cost-effectiveness and cost-benefit of a single annual professional intervention for the prevention of childhood dental caries in a remote rural Indigenous community**

By signing and returning this form I confirm that I have read and understood the information sheet and:

- I understand that my child's participation in this project is voluntary and that all information provided will be confidential;
- I understand that my child's involvement will include an initial simple examination of his/her mouth and teeth, treatment if required and application of a preventive intervention, as well as examinations 1 and 2 years after the initial examination;
- I understand that that my child's involvement will include providing a saliva sample as part of each dental examination.
- I understand that the information gained from this research may result in improved methods for diagnosis or treatment, but neither I nor my child owns the results, research records, or the sample that he/she gives.
  I have had any questions answered to my satisfaction and understand that if I have any additional questions I can contact the survey team and/or Chief Investigator (Mobile: 0448 954 344; Contact E0mail: n.johnson@griffith.edu.au);
- I understand that my child is free to withdraw at any time, without comment or penalty;
- I understand that I can contact the Manager, Research Ethics, at Griffith University Human Research Ethics Committee on 07 3735 4375 or research-ethics@griffith.edu.au. I may also contact the Far North Queensland Ethics Committee on 07 4226 5513 or Cairns_Ethics@health.qld.gov.au) if I have any concerns about the ethical conduct of the project; and
- **I agree for my child to participate**.

| | | | |
|---|---|---|---|
| Parent/Guardian First and Last Name | | | |
| Parent/Guardian Signature | | | |
| I agree to use of my child's data/sample in future research projects that are an extension of, or closely related to, this research (please circle) | | Yes | No |
| Date | | | |

**Please complete the following detail on this page only for your child:**

| | | | | |
|---|---|---|---|---|
| Child's First and Last Name | | | | |
| Child's Signature (if able to sign) | | | | |
| Child's Date of Birth | | | | |
| Child's actual age | | Child's Sex (circle) | Male | Female |

**Kindly return this form to our Liaison Officer who has given it to you**

| | |
|---|---|
| Researcher/Witness Signature/Date on sighting this form | |