Population genetics and the microbiome: solving conservation problems

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Statement of Originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

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Authorship Attribution

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I carried out the research required for this manuscript and wrote the initial draft of the text. My co-author and supervisor (C. E., Grueber) provided notes on the content and structure of the manuscript and carried out the process of submitting the manuscript to the journal.

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As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

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Contents

Abstract

Conservation science has long focussed on how a target species interacts with its biotic and abiotic environment and how these relationships can be manipulated to generate better conservation outcomes. Interventions may include removing predator species, increasing food species, providing more suitable habitat, or protecting species from anthropogenic disturbance such as land clearing or development. However, to conserve a species it is important to consider its biotic interactions in their entirety, including the microbes living both on and within it. Microbial communities are vital to the survival of arguably all multicellular life on Earth, and should be a key consideration when planning conservation or population management interventions. In particular, gut microbiome communities in the digestive tract of animals play a key role in digestive capabilities, as well as immune function and behavioural responses. This thesis examines how sequencing gut microbiomes of threatened species can inform conservation strategy. This objective is achieved through first a review of the approaches available for answering conservation questions with microbiome data. Second, I present an empirical example of how the gut microbiome can provide insight into the biology of a species with a unique ecology and how population demographic processes, such as inbreeding, can influence the structure of the gut microbiome.

Chapter Two reviews the tools available to a researcher beginning a microbiome investigation in wildlife, with emphasis on how the different methods can be used to solve specific conservation problems. The gut microbiome can be studied in two broad ways: assessing either the taxonomic or functional diversity of the microbial community. Taxonomic approaches can provide an excellent starting point for wildlife studies, especially in host species where there is little known about their microbiome diversity. Functional approaches can then be useful in asking specific questions about microbiome function and applying these to solving conservation problems. For example, taxonomic approaches have identified microbial taxa involved in immune responses to fungal pathogens in frogs. I found that functional approaches are now being used to understand how microbes interact with the host immune system and how this relationship can be manipulated to improve conservation outcomes.

Chapter Three then uses the taxonomic methods described in Chapter Two and applies them to a free-ranging population of long-nosed potoroos (*Potorous tridactylus*), a marsupial in the family Potoroidae. This work provides the first description of both the bacterial and fungal communities present in the potoroo gut microbiome, as well as functional inference to provide preliminary data on the microbiome's functional capabilities. The diet of potoroos consists primarily of the underground fruiting bodies of hypogeal fungi, making the family Potoroidae one of the few vertebrate taxa that are specialist fungivores (mycophages). As a result, they play a key role in the dispersal of fungal spores and soil engineering through the digging behaviours involved in foraging for fungi. Both spore dispersal and soil perturbation play a key role in aiding the post-fire recovery of the eucalypt forests where potoroos are found, although the increased severity and frequency of bushfires in eastern Australia risks further fragmenting populations of potoroos, potentially leading to the loss of genetic diversity and increased inbreeding. The study population for this analysis is a closed, free-ranging population of potoroos at Tidbinbilla Nature Reserve, where previous population genetic analysis has revealed variance in individual inbreeding.

We found that the potoroo gut microbiome is dominated by the bacterial phyla Firmicutes and Bacteroidota, with a mean Firmicutes to Bacteroidota ratio (F:B) of 2.4. These observations are similar to reports from members of the family Macropodidae, which contains kangaroos and wallabies. We found no evidence for an effect of inbreeding, sex or breeding status on the alpha diversity of the potoroo gut microbiome (fungal nor bacterial). However, we found a higher abundance of both the bacterial phylum Actinobacteriota and bacterial genus *Parabacteroides* in male potoroo gut microbiomes, relative to females, and a higher abundance of fatty acid and lipid biosynthesis pathways within the gut microbiome of male potoroos compared to females. We further observed that increased individual inbreeding (measured as internal relatedness calculated from 6893 SNPs) was associated with an increase in the abundance of the bacterial genus *Parabacteroides*, and a decrease in the abundance in the *Lachnospiraceae NK4A136 group* (equivalent to genus). These observations suggest the possibility that inbreeding depression in a small population could feasibly manifest through changes in the microbiome, although the fitness ramifications cannot be determined from our data. Taken together, our microbiome findings inform the hypothesis that the microbiome may be a source of adaptive potential within the host, and is influenced by host-level factors such as inbreeding. Our work builds on previous reports that the gut microbiome can be affected by population demographics and management interventions within a captive, free ranging population, and highlights the importance to considering the microbiome when making management decisions.

Overall, this thesis advances the discussion around microbiome research in conservation and demonstrates the role of the microbiome in the biology of a unique host species. The majority of current microbiome evidence comes from model organisms and this study demonstrates the value of captive, free-roaming populations in applying microbiome science to small, wild populations. The gut microbiome of potoroos likely plays a key role in both their unique biology as fungivores and the ecosystem processes they contribute to, particularly regarding post-fire forest recovery, but may be influenced by inbreeding. Going forward it is important that the potential of inbreeding depression to manifest via the gut microbiome is considered during population management strategies, through application of a wide variety of analytical tools, especially in species with unique or highly specialised diets.

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Chapter One: Introduction

There is a growing appreciation in conservation about the importance of conserving microbial diversity and the ecosystem functions they provide (Trevelline et al., 2019). Microbes provide a wide range of environmental functions both in free-living communities such as those in soil (Dubey et al., 2019), and in conjunction with multicellular organisms as part of a microbiome community (Bahrndorff et al., 2016). As a result, the conservation of microbes and microbial processes will be essential for future conservation efforts targeting larger organisms and even whole ecosystems (Bahrndorff et al., 2016; Trevelline et al., 2019). Historically, microbiome research relied on culture-dependant methods which were complex and time consuming, restricting their application outside of a controlled lab environment (Lagier et al., 2012). The reliance on culturing also severely limited the types of microbes that could be studied and made community-level microbiome analyses very challenging (Cocolin et al., 2011; Lagier et al., 2012). The development of advanced sequencing methods has allowed for the characterisation of entire microbiome communities regardless of whether they were culturable, and next-generation, high-throughput sequencing methods have now made microbiome research cheaper, quicker, and far more accessible to a range of research fields (Maljkovic Berry et al., 2020; Trevelline et al., 2019). These technological advances have made using microbiome research to answer ecology and conservation questions much more feasible and has led to increased interest in the field (Trevelline et al., 2019). This thesis demonstrates how microbiome research can be used to answer conservation questions by first providing an overview of the tools available to conservation microbiome researchers and how they can best be used (Chapter Two) and then using these tools to assess how the gut microbiome can be shaped by population demographics and inbreeding in a captive, free-ranging population of long-nosed potoroos (*P. tridactylus*) (Chapter Three).

The microbiome

A microbiome is any community of microbes which inhabit a defined area of the environment or a host species (Berg et al., 2020). In animals, microbiomes are present at every host-environment juncture, be that on external surfaces such as the skin, feathers, or scales (Woodhams et al., 2015); or on internal mucus membranes in the respiratory, digestive or reproductive tracts (Kinross et al., 2011). These microbial communities act to mediate the interactions between a host and its environment and can have substantial effects on how both the host responds to environmental stimuli and how the biotic environment interacts with the host (McKenney et al., 2018). For example, within the life cycle of the malaria-causing *Plasmodium* parasite, almost all interactions between the parasite and its two hosts (mosquitoes and mammals) are mediated in some way by microbial communities (Ippolito et al., 2018). These microbiome-mediated interactions can influence host immune responses (Mooney et al., 2015), mosquito behaviour (Leyden et al., 1981) and host digestive function (Taniguchi et al., 2015). The gut microbiome is a particularly key community in the biology of many animals as it provides crucial digestive functions which have may have aided dietary expansion (Ley et al., 2008; Nishida & Ochman, 2018) and feeding niche partitioning (Greene et al., 2020). This is particularly pertinent for animals with unique or highly specialised diets, who often rely heavily on the gut microbiome to aid in digestion, e.g. giant pandas (*Ailuropoda melanoleuca*) (Zhu et al., 2011) and koalas (*Phascolarctos cinereus*) (Barker et al., 2013), or to supplement dietary

compounds absent from their specialised diet, e.g vampire bats (*Desmodus rotundus*) (Zepeda Mendoza et al., 2018). Specialists are also generally more vulnerable to environmental changes than generalists (Gallagher et al., 2015) given their reliance on specific food sources or habitats, and so it is important to understand the role the microbiome plays in these species and how it can be manipulated for conservation purposes (Bahrndorff et al., 2016; Trevelline et al., 2019).

The gut microbiome consists off all forms of microbial life present in the digestive tract of the host. This includes well studied taxa such as bacteria and archaea, as well as often overlooked groups such as unicellular eukaryotes (fungi and protists) (Laforest-Lapointe & Arrieta, 2018), viruses (Chong, Shi, et al., 2019) and mobile genetic elements (Broaders et al., 2013). This is in addition to multicellular parasites such as platyhelminths or nematodes which contribute their own gut microbiomes to the complex ecosystem (Klomkliew et al., 2022). Even if a study is focussing on bacterial functions alone, it is important to consider how the broader microbial community interacts with the bacteria present, with nematodes (Midha et al., 2018), bacteriophages (De Paepe et al., 2014) and fungi (Castagliuolo et al., 1999) all known to interact with bacterial communities in the gut.

The bacterial community within the mammalian gut microbiome tends to be dominated by the phyla Firmicutes and Bacteroidota (de Jonge et al., 2022), with other common phyla in vertebrate gut microbiomes including Proteobacteria, Fusobacteria and Spirochaetes (Youngblut et al., 2020). The ratio of Firmicutes to Bacteroidota (F:B ratio) is a commonly used marker for gut microbiome health and function as it has been associated with increased risk of obesity in humans and mice due to an increase in energy harvesting efficiency from the diet (Ley et al., 2006; Turnbaugh et al., 2006). In wildlife species, many hyper carnivores show low levels of Bacteroidota, leading to a high F:B ratio, e.g. cheetah (*Acinonyx jubatus*) (Wasimuddin et al., 2017), Tasmanian devils (*Sarcophilus harisii*) (Y. Cheng et al., 2015), and polar bears (*Ursus maritimus*) (Ley et al., 2008), with herbivores tending to have a much lower F:B ratio, e.g. cows (*Bos taurus*) (Myer et al., 2015), lorises (*Nycticebus javanicus*) (Cabana et al., 2019) and koalas (*Phascolarctos cinereus*) (Barker et al., 2013). The F:B ratio can also vary within species according to age (Mariat et al., 2009) and diet (De Filippo et al., 2010), as well as displaying variation across the digestive tract (Barker et al., 2013), so should be interpreted in the wider context of the study population rather than as a stand-alone metric.

Many microbiome studies focus primarily on bacterial or archaeal diversity, however the gut microbiome also can host a huge diversity of fungal diversity (Laforest-Lapointe & Arrieta, 2018). The human gut mycobiome (fungal microbiome community) is less diverse than the bacterial gut microbiome communities (Nash et al., 2017; Strati et al., 2016), and yet shows more variance within individuals over time (Nash et al., 2017). Common genera in the human gut mycobiome include *Saccharomyces*, *Candida*, *Malassezia* and *Aspergillus* (Nash et al., 2017; Strati et al., 2016). Studies in ruminants and non-ruminant herbivores have identified the phylum Neocallimastigomycota as a key taxon involved in the digestion of plant material (Ljungdahl, 2008; Orpin, 1975). Neocallimastigomycota possess a wide range of biomass degrading enzymes (Ljungdahl, 2008; Youssef et al., 2013), and appear to play a key role in cellulose digestion in multiple mammalian taxa (Liggenstoffer et al., 2010; Nicholson et al., 2010). The phylum is also the only obligately anaerobic group of fungi (Hanafy et al., 2022; Orpin, 1975), making culturing methods and research into this unique group challenging.

For species that rely heavily on their microbiome to effectively digest their natural food sources, it may be pertinent to consider how the gut microbiome contributes to host fitness and can influence the adaptive potential of the using the hologenome framework. The concept of the hologenome recognises the adaptive potential of the microbiome and considers the holobiont (a host and its microbiome(s)) as an independent level upon which selection can act (Rosenberg & Zilber-Rosenberg, 2018). For example, adaptation to blood-feeding (sanguivory) in vampire bats (*Desmodus rotundus*) is facilitated by changes in both the bat genome and the gut microbiome (Zepeda Mendoza et al., 2018). Many of the adaptive challenges of a blood-only diet, such as high dietary nitrogen and iron, low dietary vitamins and high exposure to blood-born parasites, are solved through a combination of both gut microbial and host-genomic traits, demonstrating how selection has acted upon the hologenome to enable adaptation to sanguivory in *D. rotundus* and its holobiont (Zepeda Mendoza et al., 2018).

Microbes are the foundation of all life on Earth, playing essential roles in every ecosystem and providing vital roles as part of host associated microbiomes. Microbes are biodiversity and there is no other biodiversity without microbes (McLaren & Callahan, 2018), as a result it is essential that biodiversity conservation programs closely examine both how best to conserve microbial diversity and how microbial diversity can be utilised to maintain macro-level biodiversity.

The microbiome in biodiversity conservation

There are two key ways in which the microbiome can be relevant to conservation management practices: the microbiome itself may be directly affected by threats facing wild populations, such as disease (Harris et al., 2006) or climate change (Le Sage et al., 2021; Maurice et al., 2015), or management practices may inadvertently alter the gut microbiome in negative ways (Clayton et al., 2016; Kueneman et al., 2022; McKenzie et al., 2017). As a result, it is important to understand both the natural processes affecting the gut microbiome of wild populations, and the conservation-induced processes that affect managed populations. Any level of human intervention has the potential to influence the biology of the target species in some way, including possible changes in the microbiome (Clayton et al., 2016; Kueneman et al., 2022; McKenzie et al., 2017). For example, bringing animals into a captive breeding facility (such as a zoo) exposes individuals to a drastically different habitat, reduced in many of the bacteria they would be naturally exposed to in soil or on other host species they interact with in the wild, and increased in novel bacterial species (Clayton et al., 2016; Kueneman et al., 2022; McKenzie et al., 2017). As a result, it is not surprising that captivity broadly alters the microbiome of many species compared to wild conspecifics (Dallas & Warne, 2022). Even relatively minor interventions such as providing supplementary food to wild populations can still lead to a change in gut microbiome composition (Couch et al., 2021).

Many microbiome communities play an important role in host immune function, and disruption of this function can increase susceptibility to disease. In these instances, preserving or manipulating microbial diversity can aid conservation programs targeting the host species (Denton et al., 2005; Estrada et al., 2022; Kueneman et al., 2016, 2022). For example, there are microbes present in the skin microbiome of many amphibians, which

play an important role in providing resistance to *Batrachochytrium dendrobatidis* (*Bd*) (Harris et al., 2006), a fungal pathogen and major cause of many declines in amphibian populations worldwide (T. L. Cheng et al., 2011). The prevalence of these anti-*Bd* microbes varies seasonally (Le Sage et al., 2021), and their effectiveness in aiding host immunity can be impaired by cold temperatures (Robak et al., 2019). Global patterns of seasonality and ambient temperatures will be affected by climate change over the coming decades, so understanding exactly how these changes will affect the amphibian skin microbiome will be essential for effective population management of many amphibian species (Jiménez & Sommer, 2017). In addition, the inoculation of amphibians with anti-*Bd* probiotics gives protection from infection in lab populations (Becker et al., 2011; Kueneman et al., 2016), giving hope for the development of treatments for the disease. Studies have also shown that while captivity can alter the composition of the microbiome of managed amphibians (Kueneman et al., 2022), the use of soft-release strategies can successfully rewild the microbiome (Estrada et al., 2022; Kueneman et al., 2022), increasing *Bd* resistance in the process (Estrada et al., 2022).

Captive management is commonly used as a conservation tool to protect populations from threats such as predation (Kraaijeveld-Smit et al., 2006), disease (Farquharson et al., 2017) or anthropogenic disturbances (Havmøller et al., 2016). Captive management has been successful in aiding the recovery of wild populations such as the California condor (*Gymnogyps californianus*) (Toone & Wallace, 1994) and black-footed ferret (*Mustela nigripes*) (Dobson & Lyles, 2000) through breed-to-release programs and providing conditions in which to study the biology of threatened species (Tripovich et al., 2021). However, captive management necessarily alters the environment of the target species in some way, which can alter selection pressures on both the host and its microbiome and may lead to changes in microbiome diversity and structure (Clayton et al., 2016; Kueneman et al., 2022; McKenzie et al., 2017). The effect of captivity on the gut microbiome can be highly variable. One study of 41 mammal species found trends associated with certain taxa and feeding ecologies, for example primates, equids and canids tend to have lower gut microbiome alpha diversity in captivity (McKenzie et al., 2017) while myrmecophagous (ant-eating) species maintain alpha diversity but experience major changes in beta diversity (McKenzie et al., 2017). In contrast, a study of 18 amphibian species found consistently significant changes associated with captivity, but with effects varying substantially between species even of the same genus (Kueneman et al., 2022). The variability of the effects of captivity on the microbiome makes predicting how these changes influence host fitness very challenging (Kueneman et al., 2022). This is especially true given that many studies also opt for a purely taxonomic approach to assess the effects of captivity, using methods such as amplicon sequencing (McKenzie et al., 2017), as opposed to a functionality informed approach (Jovel et al., 2016). Taxonomic microbiome methods can be extremely useful for identifying microbial taxa that may play a key role in microbiome function but provide no information of the functions explicitly being carried out by the microbiome at any point in time. Instead, taxonomic methods rely on inferring microbiome function based on previously published data. Inferring microbiome function from taxonomic data involves matching taxa to previously reported functions in databases using programs like PICRUSt2 (Douglas et al., 2020). For some well-studied use-cases, such as investigating *Bd* in amphibians, taxonomic approaches are likely sufficient as there has been extensive work to identify bacterial species with antifungal properties (Woodhams et al., 2015). This has led to the creation of well curated sequence databases which allow for accurate inference of a

microbiome's antifungal function from taxonomic data alone (Woodhams et al., 2015). However, for most other wildlife contexts, where little is known about the microbiome and its role, functional inference methods rely on generic tools such as PICRUSt2, for which reference databases have been developed from model species (Sun et al., 2020). The publication of large-scale comparative microbiome datasets, e.g. (Youngblut et al., 2020), is improving database coverage, but for many host species there is still a great deal of uncertainty as to how taxonomic changes in the microbiome, and corresponding functional shifts, influence host fitness.

If captivity-induced gut dysbiosis (disruption to the microbiome) is detrimental to captive populations, and these changes in the microbiome are easily re-established when captive individuals are released into the wild it may not be detrimental to wild populations. Tasmanian devils released from the breeding program quickly re-established their wild-type gut microbiome (Chong, Grueber, et al., 2019) and soft-release programmes using captive-bred frogs from a *Bd*-impacted species (*Atelopus limosus*) found that the skin microbiome shifted it its 'wild-type' composition within 27 days of the release (Kueneman et al., 2022). The pattern of Bd infection was also similar between reintroduced and wild frogs (Estrada et al., 2022). There have been some attempts to inoculate captive amphibians with probiotics to prevent *Bd* infection (Becker et al., 2011; Kueneman et al., 2016), however the quick reversion of released individuals back to their wild-type microbiome may make these efforts ineffective (Kueneman et al., 2022). As a result, it will be important that any microbiome manipulation approaches to management are monitored via long-term field studies to ensure that any beneficial effects are maintained long enough in the released individuals to support conservation objectives.

When looking at the gut microbiome specifically, diet is the most important factor which influences the composition and structure of the microbiome (Ley et al., 2008), with associations between diet and microbiome composition being found in large-scale metanalyses (Muegge et al., 2011), wild populations (Sullam et al., 2015) and lab populations (J. A. Chandler et al., 2011). This is because the food an animal consumes acts as the medium though which its digestive system interacts with its wider environment (Goto & Kiyono, 2012; Statovci et al., 2017). This is important as the diets of captive animals can vary considerably to their natural diet (Matsuda et al., 2018), potentially leading to a dysbiosis that may impede their ability to digest their natural diet.

Another important consideration is how gut microbiome composition can dictate the ecological niche of a host individual and how this may influence their ability to respond to environmental changes such as those experienced when captive individuals are released to the wild. One such species where this is very apparent is the koala (*Phascolarctos cinereus*), an Australian folivore which specialises on *Eucalyptus* trees (Cork et al., 1983). Koalas with specific gut-microbiome profiles exhibit strong dietary preferences for specific *Eucalyptus* species (Brice et al., 2019), and have even been known to starve when their preferred species is absent even if other known food species are available (Whisson et al., 2016). This suggests that in koalas, the gut microbiome dictates dietary preference, rather than certain food species favouring the development of specific gut microbiome signatures (Blyton et al., 2023). While there are differences between the gut microbiomes of captive and wild koalas (Eisenhofer et al., 2023), and antibiotic use in captivity has been shown to alter the gut microbiome composition (Dahlhausen et al., 2018), the koala gut microbiome is relatively resilient to changes in diet (Blyton et al., 2023; Eisenhofer et al., 2023). As a result it is

important that captive release programs carefully consider the gut microbiome composition of released koalas and how this may limit their ability to respond to changes in diet brought about by wild release.

Overall, microbiome communities provide essential functions for their hosts in many wildlife species and the impact of threats to wild populations and management actions upon the microbiome should be considered in species where the microbiome is of particular importance.

Inbreeding depression and the microbiome

Inbreeding depression is a phenomenon where the offspring of closely related individuals tend to have lower fitness on average than the offspring of more distantly related parents (Charlesworth & Willis, 2009). Evolutionary fitness is defined as lifetime reproductive success, but in wildlife inbreeding depression is often measured via fitness proxies, such as litter size, first-year survival, body condition, or health scores (Grueber, Waters, et al., 2011). Extension of these fitness proxies to encompass all hologenomic traits may allow for unique insight into how inbreeding depression manifests in small populations. The microbiome can play a key role in facilitating the evolutionary adaptation of the host species, particularly in the gut where the microbiome acts as a key mediator between a host and its external environment (Zepeda Mendoza et al., 2018). Studies in *Drosophila* have shown that both host genetic diversity and microbiome diversity contribute to host fitness, suggesting that changes in the gut microbiome have the potential to influence host fitness (Ørsted et al., 2022). As host genetic background can influence the composition of the gut microbiome (Zhao et al., 2013) and host-level population bottlenecks constrain the richness and diversity of the gut microbiome (Ørsted et al., 2022) it is plausible that inter-individual variation in host inbreeding depression could manifest as variation in microbiome composition.

A number of studies have investigated the influence of inbreeding on the diversity of the gut microbiome. Differences in gut microbiome richness, or differences in the abundance of certain taxa, have been observed between highly inbred and non-inbred populations of house mice (*Mus musculus*) (Kreisinger et al., 2014), Indian bison (*Bos gaurus*) (Prabhu et al., 2020) and banna minipigs (*Sus domesticus*) (Wei et al., 2020). Kreisinger et al. (2014) and Wei et al. (2020) both compared experimentally highly inbred populations to randomly mating populations under similar rearing conditions. Prabhu et al. (2020) compared domesticated individuals to wild individuals, and while they controlled for sampling location and diet, this study encompassed all of the effects of domestication and artificial selection by humans, not just the increased levels of inbreeding. While these studies show how the gut microbiome can be affected by very high levels of inbreeding, it is also useful to understand how conservation-relevant levels of inbreeding can influence the microbiome within a natural population. In a wild population of the threatened gopher tortoise (*Gopherus polyphemus*), gut microbiome alpha diversity showed a negative association with host inbreeding level (Yuan et al., 2015). Yuan et al. (2015) also found that full-sibling and half-sibling pairs tended to be more similar to one-another in their gut microbiome structure than unrelated pairs however found no significant associations between inbreeding and the abundance of any individual bacterial taxa. Thus while host inbreeding

may influence gut microbiome diversity, it is unclear how these changes contribute to host fitness and whether these limited findings extend generally to species of conservation concern.

Fenced wildlife sanctuaries

There are different extents to which a population of animals can be managed, acting along a continuum of management intensity with highly managed zoo or lab populations at one end and completely wild populations at the other receiving little or no human intervention (Grueber et al., 2019). One important type of management strategy which lies between these two is the use of fenced wildlife sanctuaries. A fenced wildlife sanctuary is an area where an important threat to a species is absent, either naturally or through human intervention (Grueber et al., 2019; Legge et al., 2018). These sanctuaries can be literally fenced, as many constructed mainland sanctuaries are, or on offshore islands, which provide similar isolation and protection (Grueber et al., 2019). In Australia, domestic cats (*Felis catus*) and European red foxes (*Vulpus vulpus*) have been the main drivers of at least 20 mammal extinctions since European settlement (Woinarski et al., 2015b), and are often one of the threats removed from fenced wildlife sanctuaries (Legge et al., 2018). Sanctuaries can also be used to protect populations from infectious diseases, such as in Tasmanian Devils (*Sarcophilus harrisii*), where offshore islands provide havens free from devil facial tumour disease (Grueber et al., 2019). Fenced wildlife sanctuaries provide an excellent opportunity to study the microbiome of threatened species, because such populations experience natural social dynamics and can forage for their own food while still being regularly monitored, protected from invasive predators, and given supplementary food if required (Legge et al., 2018). Fenced wildlife sanctuaries experience the same evolutionary dynamics as small, isolated wild populations, such as increased levels of inbreeding and inbreeding depression as well as population bottlenecks and founder effects (Gooley et al., 2020). This makes fenced wildlife sanctuaries suitable for investigating how naturally occurring variation in inbreeding can be associated with changes in the gut microbiome, and for generating conservation recommendations to improve species management outcomes.

Microbiome analysis in a free-ranging population of long-nosed potoroos

Human development, land clearing and an increase in the severity and frequency of wildfires has led to the fragmentation of many small mammal populations in eastern Australia, leading to individual populations becoming small and isolated (Legge et al., 2022; Woinarski et al., 2015b). This increases the risk of a loss of genetic diversity through founder effects and genetic drift in individual populations as well as increased levels of individual inbreeding (Charlesworth & Willis, 2009). Many species are now being protected within fenced wildlife sanctuaries (Legge et al., 2018), which not only provide protection from invasive predators, but also provide an opportunity to study the population genetic dynamics of small, isolated populations in a more controlled, informed way (Grueber et al., 2019). One species that was particularly badly affected by the devastation 2019-2020

bushfire season in Eastern Australia was the long-nosed potoroo (*Potorous tridactylus*) (family Potoroidae) (Legge et al., 2022).

The family Potoroidae is of particular conservation interest due to their unique role in fire ecology. Potoroidae are one of the few mammalian taxa which rely primarily on the fruiting bodies (sporocarps) of hypogeal (underground) fungi as a food source (Guiler, 1971). Fire stimulates both increased sporocarp production and increased digging (foraging) behaviours in potoroids (C. N. Johnson, 1995). In doing so, potoroids provide the soil perturbation and dispersion of ectomycorrhizal spores essential for post-fire plant recolonisation and ecosystem recovery (Elliott et al., 2022; Nguyen et al., 2016). The potoroo gut microbiome is thought to play a key role in their ability to survive on a primarily fungivorous diet (Kinnear et al., 1979; Wallis, 1994; Wallis & Hume, 1992). As a result, maintaining the diversity and function of the potoroo gut microbiome may be important to both the survival of potoroo populations, and wider ecosystem health and functions. As described above, population bottlenecks can constrain gut microbiome diversity (Ørsted et al., 2022), and inbreeding may influence gut microbiome diversity and structure (Kreisinger et al., 2014; Prabhu et al., 2020; Wei et al., 2020; Yuan et al., 2015). Thus, it is important the population fragmentation brought about by more intense fire regimens and anthropogenic development does not alter the functional capabilities of the potoroo gut microbiome.

The Tidbinbilla Nature Reserve (TNR) in the Australian Capital Territory manages a free-roaming population of potoroos. This population is habituated to humans, regularly monitored, and a recent study has shown that the population maintains a high level of genetic diversity (Mulvena et al., 2020). This makes the population an excellent model for assessing how variation in individual inbreeding may influence microbiome structure and function in a small wild population of potoroos, while being much easier to study, sample from and manipulate than a wild population. This study will assess the diversity of the gut microbiome using fecal samples as a proxy for gut samples. Fecal sampling will allow us to sample the gut microbes in a non-invasive way, minimising the stress involved in anaesthetising animals to collect gut samples (Ingala et al., 2018). Previous studies using amplicon sequencing (Liggenstoffer et al., 2010) and culturing methods have identified members of the fungal phylum Neocallimastigomycota in macropods. Given this and the fungivorous ecology of potoroids, I will investigate both the bacterial and fungal communities present in the potoroo gut microbiome.

This thesis aims to show how microbiome research can be used to answer important conservation questions. This is achieved in two ways. Chapter Two provides an overview of the techniques and approaches available for studying the microbiomes of wild animals. This aims to be a useful resource to show conservation biologists how incorporating microbiome research into their work can aid in solving conservation problems, particularly in regard to species with specialised or unique feeding ecologies. Chapter Three contextualises some of these approaches by using them to understand the role the gut microbiome plays in the biology of long-nosed potoroos. This empirical study also investigates how population demographics and individual inbreeding can influence the diversity and composition of the long-nosed potoroo gut microbiome.

Chapter Two: Functional Diversity within Gut Microbiomes: Implications for Conserving Biodiversity

Note: This chapter was published in the journal *Conservation* (reference below). The content is unchanged, but the text has been reformatted for consistency within this thesis

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Abstract

Conservation research has historically been conducted at the macro level; focusing on animals and plants and their role in the wider ecosystem. However, there is a growing appreciation of the importance of microbial communities in conservation, both in the relationships they form with their hosts and the roles they play in important ecosystem processes. Most wildlife microbiome research thus far has used amplicon sequencing methods to assess the taxonomic composition of microbial communities. This approach is invaluable in identifying factors that may lead to major shifts in a host species' microbiome and predicting those host species likely to be most affected. However, as manipulation of the microbiome as a conservation tool becomes more and more feasible, there is a growing need to understand the direct functional consequences of shifts in the microbiome composition. This review outlines the latest advances in approaching microbiome research from a functional perspective and how these data can be used to inform conservation strategies. This review will also consider some of the challenges faced when studying the microbiome of wildlife and how best these can be accounted for. In summary, human actions and climate change have the potential to disrupt key host-microbiome dynamics and understanding the functional implications of this will be essential for preserving and restoring these relationships.

Introduction

The Earth is currently in the midst of its sixth mass extinction (Ceballos et al., 2015) largely due to human-induced changes to the environment and climate change (Thomas et al., 2004). Conservation biology focusses primarily on preventing the loss of animal or plant species, however preserving the biodiversity of key microbial communities can be just as important (Bahrndorff et al., 2016). Microbial communities form relationships crucial the wider ecosystem, including vital ecological processes such as nitrogen cycling (Lladó et al., 2017), as well as contributing to the health of a range of host taxa (McFall-Ngai et al., 2013; McKenney et al., 2018). Climate change can reduce diversity in natural microbial communities (Maestre et al., 2015) potentially putting essential ecological processes at risk. For example, a recent study in a frog species (*Ololygon perpusilla*) found that changes in gut microbiome brought on by increased temperatures stunted growth in tadpoles (Greenspan et al., 2020). In that study, temperature itself had no detectable effect on tadpole growth, but acted indirectly by changing environmental bacterial composition (Greenspan et al., 2020). Understanding how microbial communities will respond to climate change and how their preservation will benefit the ecosystem as a whole will be vital for future conservation efforts. There is also a concern that the microbiome of humans and domestic animals is encroaching into natural wildlife communities (Fackelmann et al., 2021). For example, captive primates have much higher levels of human-associated microbes than is seen in their wild counterparts (Clayton et al., 2016).

Although climate change and other anthropogenic ecological disturbances can have major effects on microbial communities, actions intended to restore biodiversity can themselves also alter the composition and diversity of environmental and host-associated microbial communities (Bahrndorff et al., 2016; Trevelline et al., 2019). For example, captive breeding is an essential component of the conservation programmes of thousands of species (CPSG,

2018), but bringing individuals from the wild to controlled conditions can perturb the microbiome. The gut microbiome of captive western capercaillie (*Tetrao urogallus*) is enriched for microbial taxa associated with diarrhoea in mammals (Wienemann et al., 2011) and captive cheetah (*Acinonyx jubatus*) gut microbiomes are enriched for disease-causing bacteria relative to their wild counterparts (Wasimuddin et al., 2017). The use of antibiotics as part of routine veterinary care for captive individuals has also been shown to decrease the abundance key digestive microbes in koalas (*Phascolarctos cinereus*) (Dahlhausen et al., 2018). Captive breeding is only one avenue by which conservation action can influence microbiomes. Supplementary feeding of wild elk (*Cervus canadensis*) populations led to a significant shift in gut microbiome composition (Couch et al., 2021) and translocation of captive Tasmanian devils (*Sarcophilus harisii*) saw them quickly re-establish their wild-type microbiome (Chong, Grueber, et al., 2019). Thus, human actions through both neglect of the environment and active conservation of it can lead to major changes in microbial communities. Such changes may have wide-ranging effects and therefore potentially important implications for species and ecosystem survival.

There is a growing appreciation of the importance of preserving microbial diversity (Bahrndorff et al., 2016; Trevelline et al., 2019; Zhu et al., 2021). By conserving natural microbial communities, we protect the functions they provide, thus helping to conserve biodiversity at other levels of the ecosystem, including plants and animals (Bahrndorff et al., 2016; Dubey et al., 2019; Trevelline et al., 2019). The concept of functional diversity in a microbiome incorporates the diversity in functions a community can carry out, rather than just the microbial taxa present (Carmona et al., 2016). Escalas et al. (2019) provide a list of over 400 genotypic functional traits carried out by microbes, varying from carbon and nitrogen cycling to virulence and antibiotic resistance, demonstrating their roles in a huge variety of processes. In macro-organisms like plants or animals, these traits are often continuously expressed and relatively easy to observe (Escalas et al., 2019). However, many microbial traits are highly environmentally dependant, making it much harder to characterise the entire functional capabilities of any individual microbe, let alone an entire community (Spor et al., 2011). This highlights the importance of a focus on the conservation of functional diversity in microbiomes as opposed to a focus on taxonomic diversity.

As for multicellular species, microbial biodiversity can be quantified at both the species and genic level. For bacteria and archaea however, a “species” is less well defined (Gevers et al., 2005): processes such as horizontal gene transfer (Mourkas et al., 2019) and greater capacity for genome hybridisation (Sheppard et al., 2008) make defining taxonomic units with common functions more challenging than for plants or animals. As a result, it can sometimes be useful to consider a bacterial community as a collection of functions and processes, with individual microbes acting as vectors for genes to carry out these functions (Escalas et al., 2019; McLaren & Callahan, 2018). This is especially useful in conservation, where we are often interested in how microbial functions benefit their hosts or an ecosystem as a whole. For example, obligate blood-feeding invertebrates all rely on unique bacterial taxa to help cope with the near absence of B-vitamins in their diet (Manzano-Marín et al., 2015). Comparisons of the genome of key gut microbes belonging to a phyloglosssid leech (*Haementeria officinalis*), a tsetse fly (*Wigglesworthia sp.*), a tick (*Amblyomma americanum*), and a louse (*Pediculus humanus corporis*) found microbial taxa in all four hosts demonstrated remarkable convergence in their retention of genes associated with B-vitamin metabolism, despite both the bacteria and their host taxa being

distantly related to each other. Distantly related microbial taxa can thus carry out similar functions in distantly related hosts, suggesting that the functional capability of a community, not just its taxonomic makeup, is an important consideration.

On top of their relevance in the wider ecosystem, conserving microbial diversity is important due to the tools they can provide the medical and biotechnology industries. Challenges like antibiotic resistance and global energy shortages may have ready-made solutions waiting to be discovered in environmental (Abhilash et al., 2012; Ling et al., 2015) and host-associated (Imai et al., 2019) microbial populations and to lose these would be to starve future generations of the tools needed to address these problems. We have seen dramatic advances in the technologies available to survey taxonomic and functional diversity of microbial communities, particularly their associations with characteristics pertinent to human health (Integrative HMP (iHMP) Research Network Consortium et al., 2019). Many of these tools can be translated to benefit conservation biology too, although doing so is not without challenges.

This review describes how recent developments in functional microbiome research can be used to advance research into the microbiome of wild animals and how the results can help preserve microbial biodiversity and the important ecological functions of microbial communities. Understanding the functional capabilities of a microbiome is essential for determining the role it plays in host fitness and allows us to identify those host species that might suffer most from changes to their microbiome. Throughout this review, we focus primarily on the gut microbiome of animals. The gut microbiome is of particular interest in conservation biology because of its close association with host health (Lee & Hase, 2014). It can also be altered significantly by changes in diet (Kennedy et al., 2020), ambient temperature (Ramsby et al., 2018), and ingestion of chemical contaminants (Xue et al., 2021), all of which are likely consequences of human actions and climate change. The gut microbiome also has the practical advantage of being able to be studied non-invasively through opportunistic fecal sampling (Gibson et al., 2019). This allows individuals to be sampled without the stress of being physically handled, which is particularly important when studying vulnerable populations. We first summarise major methodological approaches to microbiome research, and their contributions to our understanding of microbial biodiversity, with special focus on those methods that have led to studies of functional diversity. In the second part of the review, we examine how each of these approaches can generate knowledge to support biodiversity conservation, and some of the considerations in doing so. In summary, we outline challenges and opportunities in extending microbiome research in wild animals to target functional microbial diversity. We refer to wildlife microbiomes being those associated with non-domesticated animal species.

Broad approaches for studying wildlife microbiomes

There are three common approaches to characterising a microbiome: taxonomic profiling, which involves amplifying marker genes such as 16S rRNA to canvas the taxa present in a sample (Janda & Abbott, 2007); functional profiling predicted from taxonomic profiles (Aßhauer et al., 2015; Douglas et al., 2020; Langille et al., 2013; Nguyen et al., 2016; Ward et al., 2017); and functional profiling inferred from functional data such as a shotgun sequenced metagenome (Gill et al., 2006) or proteome.

Taxonomic profiles

Taxonomic profiling involves quantifying the identity and abundance of the microbial taxa present in a sample (Janda & Abbott, 2007). By comparing taxonomic profiles of microbiomes sampled from individuals, populations or species subject to different conditions, such as climatic shifts (Greenspan et al., 2020), pollution (Xue et al., 2021), and captive management actions (Couch et al., 2021), it is possible to determine how these factors affect the microbiome and which host species may be most negatively affected. Comparing the taxonomic profiles of communities can also help explain how an individual's microbiome develops in response to environmental conditions and external microbial communities.

An individual's microbiome is initially derived from the maternal microbiome, a phenomenon almost universal across the animal kingdom (Funkhouser & Bordenstein, 2013), but can change seasonally (Maurice et al., 2015), with age (Li et al., 2020) or with reproductive status (Y. Cheng & Belov, 2017; Weiss et al., 2021). Understanding the dynamic nature of the microbiome is valuable for implementing effective conservation strategies that accommodate a species' commensal microbiome and avoid dysbiosis and/or pathogens. For example, the abundance of key microbes associated with resistance to the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) changes seasonally within the skin microbiome of southern leopard frogs (*Rana sphenocephala*) (Le Sage et al., 2021). Understanding the natural seasonal variation in host defences will aid researchers in predicting the effects of climate-induced changes in seasonality on pathogen resistance in amphibians. Thus, taxonomic profiles can provide both a baseline "normal" microbiome state, as well as helping identify environmental drivers that cause perturbations from that baseline.

Taxonomic profiles can be constructed on various evolutionary scales, such as comparing bacterial phyla, genera or species. For example, comparisons at the phylum level have suggested that a high *Firmicutes* to *Bacteroidota* ratio (*F*:*B* ratio) is associated with increased energy uptake efficiency, leading to an increased obesity risk in humans and mice (Ley et al., 2006; Turnbaugh et al., 2006). Microbial profiles at the species level have been used to identify individual species that carry out key functions for their host (Manzano-Marín et al., 2015). Thus, taxonomic profiles constructed to different degrees of taxonomic resolution are useful depending on the questions a study wishes to answer. Studies comparing very distinct microbiomes can identify patterns at the phylum level, whereas more subtle differences between similar microbiomes may equally be identified by comparing species-level taxonomic profiles.

To characterise the taxonomic makeup of a microbiome, the most common method is to amplify and sequence marker genes such as 16S rRNA present in a mixed DNA sample (such as DNA extracted from a scat sample). This gene is commonly used as the functional RNA it encodes is essential for cellular protein synthesis, as a result it is under strong purifying selection and is highly conserved across all bacteria and archaea (Tringe & Hugenholtz, 2008). Related amplicon methods are available that target protists (18S rRNA sequencing) (Hugerth et al., 2014), and fungi (ITS sequencing) (Schoch et al., 2012), which may be used in combination with 16S rRNA sequencing to capture a greater portion of the microbiome

taxonomy. In this review we will refer to this approach as “amplicon microbiome sequencing”.

Next generation sequencing platforms such as Illumina Miseq are the most used in amplicon microbiome sequencing studies due to their low cost and high-quality sequence output (Segerman, 2020). The protocol uses universal primers that anneal to the conserved stem regions of the 16S rRNA gene to sequence the variable loop regions containing the most informative phylogenetic signal (Tringe & Hugenholtz, 2008). This technology generates relatively small read lengths, targeting specific variable regions of the 16S rRNA gene. No individual region perfectly reflects the evolutionary history of the entire gene (Schloss, 2010), however regions V4, V5 and V6 are reportedly the most reliable (Yang et al., 2016). Sequencing of the entire gene gives more accurate phylogenetic inference (J. S. Johnson et al., 2019), however emerging third-generation sequencing platforms that allow for the required long read lengths have much higher error rates (Laver et al., 2015) and are therefore not as widely used as Illumina sequencing (Segerman, 2020).

Bioinformatic processing of the sequencing reads is then used to quantify taxonomic identity and diversity of bacterial taxa within a sample. Sequence reads are cross referenced with reference catalogues such as SILVA (Quast et al., 2013) or Greengenes (DeSantis et al., 2006) to determine the taxa present. The relative abundance of amplified sequences can also be used to estimate the relative abundances of their respective taxa. Pipelines, such as QIIME 2 (Caporaso et al., 2010) and mothur (Schloss et al., 2009), have been developed to process raw sequencing reads into a taxonomic profile and calculate summary statistics to compare among samples or groups of samples. When comparing taxonomic profiles, the relative abundances of bacterial taxa is a good starting point for analyses as this can help identify similar communities – be that from different host species (Lemieux-Labonté et al., 2016), or conspecifics living in different environments (McKenzie et al., 2017). Identification of certain genera or species that are highly represented in a sample can also give an indication of those which may play a key role in the community dynamics of the microbiome (Delsuc et al., 2014; Song et al., 2019).

Taxonomic diversity of microbiome samples can be quantified via measures of alpha and beta diversity. Alpha diversity is a measure of the species-level diversity present in an individual microbiome, while beta diversity quantifies differences among samples and can therefore quantify changes in microbiome composition (Jost, 2007). Taxonomic profiles can be compared using similarity metrics, e.g. Bray-Curtis dissimilarity, or distance metrics, e.g. UniFrac distances (Lozupone & Knight, 2005; McDonald et al., 2018). Similarity metrics treat taxonomic profiles as lists of species and do not account for phylogenetic similarities between the taxa present. For comparisons between similarity metrics see Jost et al. (2010). Distance metrics measure the phylogenetic distance between two sets of taxa, meaning that taxa that are more distinct are given greater weighting (Lozupone & Knight, 2005). The results of this approach can enable broader inferences into the functional consequences of microbiome shifts due to the positive correlation between phenotype and 16S rRNA richness in microbial communities (Nübel et al., 1999).

A taxonomic approach to microbiome analysis has been used in a wide array of wildlife applications. For example, changes in water temperature were found to alter the relative abundance of key nitrogen-processing taxa in the microbiome of the sponge *Clino arientalis*, leading to bleaching (Ramsby et al., 2018). These methods have also been used to identify

specific taxa that inhibit the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) in toads (Harris et al., 2006). These findings enabled researchers to successfully reverse the captivity-induced loss of anti-*Bd* activity in the amphibian skin microbiome, increasing survival rates by 40% (Kueneman et al., 2016). Anti-*Bd* microbes are also sensitive to temperature changes (Le Sage et al., 2021), meaning that understanding the role they play in host immunity will be essential to negating the potential effects of climate change. This example demonstrates how effective microbe-targeted conservation efforts can be at preserving biodiversity on a macro level. A study in elk (*Cervus canadensis*) also used this approach to show that supplementary feeding of populations with processed alfalfa pellets led to a shift in gut microbiome composition, whereas supplementation with unprocessed loose hay had no effect (Couch et al., 2021).

Overall, amplicon microbiome sequencing is the cheapest and most straightforward method for characterising microbiomes and is especially useful for initial studies of as-yet unstudied microbiomes. It can be a very useful tool for generating hypotheses about microbiomes and how they respond to different conditions. However, given the incredible functional diversity seen in bacteria, care must be taken in inferring functional capabilities from taxonomic data alone.

Functional profiles inferred from taxonomic data

Once a taxonomic profile has been generated, it can be used to predict the functional capabilities of a microbial community by integrating the phylogeny with published data on the functional capabilities of the constituent taxa. This approach layers functional insights over the phylogenetic data, to infer the functional potential of a microbiome sample, although with some important caveats (see below). Several bioinformatic tools have been developed to achieve these goals, including PICRUSt (Langille et al., 2013), PiCRUSt2 (Douglas et al., 2020), Tax4Fun (Aßhauer et al., 2015), BugBase (Ward et al., 2017) and FUNGuild (Nguyen et al., 2016). These methods give an estimation of the abundance of genes falling into various functional categories; two common classification schemes are KEGG Orthology (KOs) (Kanehisa et al., 2012) and Clusters of Orthologous Groups (COGs) (Tatusov et al., 1997).

Inferring functional profiles from taxonomic data enables researchers to predict the potential consequences of an observed shift in microbiome taxonomic composition to inform strategies to mitigate or even reverse the negative effects of such a change in microbiome. For example, 16S rRNA sequencing revealed that 51 bacterial genera were differentially abundant in the microbiome of captive slow lorises (*Nycticebus spp.*) relative to their wild counterparts (Cabana et al., 2019) Extrapolating functional profiles from these data suggested that this shift may result from the lower levels of plant secondary metabolites in the captive diet (Cabana et al., 2019). The indication that diet was responsible for the microbiome shift was supported by further work by Ni et al. (2021) who used 16S rRNA sequencing to discover a comparable shift in the abundance of certain bacterial taxa when the captive diet of Bengal Slow Lorises (*Nycticebus bengalensis*) was changed. This example shows the utility of predicting functional potential from taxonomic data, as the suggestion that diet may be responsible for the patterns observed laid the groundwork for development of a captive diet capable of maintaining a gut microbiome more closely resembling that of wild individuals.

Inferring functional profiles from taxonomic data has potential to predict the functional capabilities of microbiome, particularly when only amplicon sequencing data is available. However, one challenge in applying this approach to conservation problems is a lack of specificity and accuracy in functional prediction. Functional profiles inferred in this way are presented as a list of broad functional categories as defined by frameworks such as KOs or COGs. These are useful for giving a broad assessment of the sort of processes undertaken by a microbiome, but are unable to identify specific genes or pathways present in the community. Functions in the KO category "environmental information processing" are the least accurately predicted by PICRUSt, as these functions typically vary considerably between closely related communities (Langille et al., 2013). Functions in this category act in response to environmental stimuli, suggesting these methods may be limited in their ability to predict the effects of climate change and human-caused environmental changes on host-associated microbiomes. The accuracy of functional inferences from taxonomy alone also varies according to region of the target gene studied (Rausch et al., 2019) and host species (Rausch et al., 2019; Sun et al., 2020), because databases used by these methods are dominated by human data, and perform much better in human studies as a result (Sun et al., 2020). However, taxon-specific tools have been developed to improve the performance of inferring microbiome functional capabilities in certain groups e.g. CowPi for bovids (T. J. Wilkinson et al., 2018) and may therefore be the most useful for related threatened taxa.

Overall, inferring the functional capabilities of a microbiome from taxonomic data alone can be useful to identify broad-scale patterns, although specifics details may be overlooked. Nevertheless, such data can provide a platform for further studies investigating specific genes or gene pathways that carry out key functions for their host, helping to inform wildlife conservation strategies that mitigate the negative effects of climate change on microbiome and host diversity.

Functional profiles inferred from metagenomic data

Beyond inferring functional microbiome profiles from taxonomic data, microbial functions can also be characterised directly by assessing all the genes present of expressed in a microbial community using methods such as metagenomic sequencing (Liu et al., 2021). This approach involves sequencing all genes present within a microbiome and predicting how they interact to carry out community-level functions (Takami et al., 2012). The results can be incredibly useful in identifying functions that are key to host fitness, and how these may be impacted by human actions and environmental changes. Characterising microbiomes using metagenome sequencing is also more consistent between replicate samples than amplicon sequencing methods (Huttenhower et al., 2012).

The most common approach to assess functional profiles is to sequence the metagenome of a microbiome sample (Gill et al., 2006). This involves examining all DNA present in a sample, rather than just specific marker genes, as is the case for amplicon sequencing methods. Sequencing may employ short-read platforms such as Illumina, or long-read sequencing technologies such as Oxford Nanopore to reconstruct complete bacterial genomes. Functional profiles are then developed by comparing the metagenome to functional databases such as the KO database (Kanehisa et al., 2012) to characterise microbiome function at the community level. This approach treats genes as the functional unit rather than species, avoiding uncertainty surrounding species classification in bacteria (Gevers et al., 2005). Due to the large amount of data produced by metagenome sequencing it is

considerably more expensive and computationally demanding than amplicon methods (Kuczynski et al., 2011). Taxonomic insights are not excluded from this approach, as it is also possible to use the sequence data to reconstruct marker gene sequences (such as 16S rRNA) and infer the taxonomic makeup of a community. (Darling et al., 2014).

Metagenome sequencing has so far been sparingly used in conservation research, with most studies relying on amplicon approaches to infer functional capabilities, likely due to the comparatively high cost of metagenomic sequencing. However, high-throughput sequencing is only getting cheaper and more accessible (Maljkovic Berry et al., 2020) so these methods will undoubtably play a major role in future studies. Metagenome sequencing in conservation has been effective in establishing differences in the functional capabilities of the gut microbiomes of captive individuals compared to their wild counterparts. For example, this method was used to discover that in the Amur tiger (*Panthera tigris tigris*), 13 gene families associated with carbohydrate metabolism were differentially abundant in captive individuals relative to wild tigers (Ning et al., 2020). This suggests that the tiger captive diet differs in its carbohydrate composition, and that correcting this discrepancy might be an important consideration when transitioning individuals for wild release (Ning et al., 2020). Likewise, metagenomics has also been used to show that captive black rhinos (*Diceros bicornis*) also exhibit functional shifts in their microbiomes, apparently due to dietary change (Gibson et al., 2019).

In humans, functional profiling of the microbiome has extended beyond metagenome sequencing, to include RNA (transcriptomics) (Pérez-Losada et al., 2015), proteins (proteomics) (Grassl et al., 2016) and metabolites (metabolomics) (Rojo et al., 2017). These tools will provide a real asset to future conservation studies by allowing researchers to characterise not just the genes present in a microbiome, but when and how they are expressed. These methods can generate a snapshot of the functions a microbiome is carrying out at a given time, as opposed to the functional potential obtained by metagenome sequencing. High precision functional data can be useful given how environmentally-dependant many bacterial phenotypes can be (Spor et al., 2011). In a wildlife context, metabolomic studies found that routine parasite treatments such as ivermectin can alter the metabolites present in the Amur tiger gut, suggesting a functional change within the microbiome (He et al., 2018).

In addition to expanding our tools to provide more nuanced assessment of the role bacteria play in the microbiome, metagenome sequencing is also able to capture the functional diversity of non-bacterial taxa (Marcelino et al., 2020). Fungi and protists, for example, have been implicated as contributing to gut microbiome function in both cows (Terry et al., 2019) and humans (Laforest-Lapointe & Arrieta, 2018). Studies in Tasmanian devils have shown that the gut microbiome hosts a rich diversity of viruses which can play a key role in individual health and future conservation efforts (Chong, Shi, et al., 2019). Insights into these other types of microorganisms may be completely ignored by amplicon microbiome sequencing methods if only a single gene (such as 16S rRNA) is targeted, potentially losing vital information about microbiome structure and function.

Overall, metagenome data allows to researchers to predict the functional capabilities of a microbiome to a much higher degree of precision and accuracy than relying on amplicon methods alone. As a result, the approach has great potential in conservation research to identify host species being negatively affected by microbiome changes and to inform the

design of intervention measures to mitigate or reverse these negative effects. However, this method is far more expensive and computationally demanding than amplicon sequencing methods and might therefore be best suited for cases where specific hypotheses are invoked (e.g. dietary impacts) and interventions are available (i.e. change to the diet).

Functional microbiome insights in conservation

Among the most common questions in conservation microbiome research are whether certain threats to a species or conservation interventions have any effect on the microbiome and whether these microbiome effects impact the viability of threatened populations. The microbiome plays a key role in a range of essential processes for its host such as digestion, immune responses and even behaviour (McFall-Ngai et al., 2013). Functional changes in the microbiome due to human actions might therefore have fitness implications that threaten individual or population survival. The consequences of such changes on host fitness are ultimately determined by how the microbial functions provided to the host by the microbiome are affected. For example, only a few microbial taxa in amphibian skin microbiomes aid in immune responses to the fungal pathogen *Bd* (Harris et al., 2006). In order to protect and even restore these functions it is essential we understand which microbes provide which functions and how they achieve this.

For many managed species in conservation, captivity provides a vital refuge for safe breeding and the preservation of biodiversity that is under threat in the wild. However, captivity has been shown to alter the taxonomic and functional profiles of the microbiome of a wide range of species (Delsuc et al., 2014; Trevelline et al., 2019). Understanding the consequences of these changes is important for both animal health and welfare in captivity, and the success of breed-for-release (e.g. reintroduction) programmes. Where captive animals are released to supplement wild populations, microbial dysbiosis might leave individuals susceptible to disease (as seen in cheetah [14]) or unable to obtain the required nutrients from their wild food sources (as seen in capercaillie [13]). There is a capacity for some released species to regain their wild-type microbiome (as seen in Tasmanian devils (Chong, Grueber, et al., 2019)), however identifying which species would benefit most from strategic efforts to re-establish a wild-type microbiome pre-release will be vital in ensuring the long-term success of released individuals (West et al., 2019). Diet is considered a major cause of gut microbiome changes both in captivity (McKenzie et al., 2017) and the wild (Suzuki, 2017) and quantifying how diet manipulation can improve microbiome function may be one way to improve the success of reintroduction programmes (Allan et al., 2018). For some species, the microbiome can be manipulated even more directly using probiotics (McKenzie et al., 2018). This has shown particular promise in aiding resistance to fungal pathogens in wildlife such as *Bd* in amphibians (Kueneman et al., 2016; Rebollar et al., 2016) and *Psuedogymnoascus destructans* (which causes white-nose syndrome) in bats (T. L. Cheng et al., 2017).

Changes in microbiome function might not only affect host fitness directly, but also disrupt the important ecosystem functions their hosts perform. For example, corals and sponges are essential components of biodiverse reef ecosystems and play a vital role as oxygen producers and carbon fixers (C. R. Wilkinson, 1983). The photosynthetic cyanobacteria and dinoflagellates in their microbiomes are essential for these functions and are sensitive to

the pressures of climate change (Botté et al., 2019; Hoegh-Guldberg et al., 2007). Changes to these microbiomes will not just impact their host species but have a huge range of ecological consequences (Brown, 1997).

Challenges faced when studying the microbiome of threatened species

Wildlife microbiome studies, and especially those of threatened populations, can present unique challenges not faced when studying model organisms. Studies of dwindling or vulnerable populations often have small sample sizes, may occur in remote locations, and present limited opportunities for experimental manipulation. Resources are often limited (Wiedenfeld et al., 2021), and problems are time sensitive (Isaac et al., 2007). As a result, it is essential that the potential benefits of a study are balanced against the cost and time needed to answer questions appropriately. Further, for many wildlife taxa, especially those without domesticated relatives, reference datasets may be limited or non-existent (Youngblut et al., 2020), restricting the types of inferences that can be achieved, and/or the amount of research effort required to obtain deep insights. Given the vast differences in costs and quantity of data produced by methods such as amplicon versus metagenome sequencing respectively, access to previous research and reference data can have a huge influence on which method is the most cost effective. In all, factors of cost-effectiveness, logistical and technical feasibility, and the need to obtain rapid insights all contribute to the ranking of costs and benefits of alternate microbiome analysis methods. Below we explore some of these issues in the context of functional microbiomics for threatened wildlife.

Using pre-existing genomic resources to support wildlife studies

One of the most important considerations in deciding the methods to use in wildlife microbiome research is the genomic resources available for your study species. This can help determine the most effective approach to take in characterising the microbiome. In the past, the vast majority of our knowledge of microbiomes came from human studies alone (Gill et al., 2006). However, the last few years have seen a major increase in non-human microbiome studies, providing many more resources and knowledge for studies in a wide range of socially, economically, and ecologically important species (Youngblut et al., 2020). For example, gut microbiomes have been characterised for many commercially important species such as Atlantic cod (Riiser et al., 2019), cows (Stewart et al., 2018) and chickens (Huang et al., 2018) as well as laboratory model organisms including mice (Lagkouvardos et al., 2016) and fruit flies (Bost et al., 2018). Large-scale comparative studies on zoo-housed species (Muegge et al., 2011), and more recently wildlife (Youngblut et al., 2020), provide a wealth of reference data that can aid in future conservation programmes. These datasets also represent a point of comparison for targeted studies of related threatened species, and inform the generation of broad hypotheses for unrelated threatened species.

Nevertheless, many conservation studies are conducted on species with poorly known ecology and life history. For example, 18% of described animal species are listed by the IUCN as ‘data deficient’, almost as many as the number of species threatened by extinction (19%) (IUCN, 2021). It is reasonable to presume that for species so poorly studied that their population trend cannot be determined, the chance that their microbiome contains unique microbes is undoubtedly very high. Microbiome characterisation using amplicon methods – and matching sequences against reference databases to infer their taxonomy relies heavily on identifying microbes known to science to infer their potential importance in the

community. In cases where species identification is likely to be imprecise due to the presence of novel species or genera, metagenome studies can be very useful in that they can infer the microbial roles based on the putative functions of microbial gene products, regardless of whether the microbes themselves have been previously classified.

For many conservation studies, taxonomic profiling using amplicon sequencing is nevertheless an excellent place to start as it is much cheaper and easier than methods like metagenome sequencing. Beta diversity metrics allow for straightforward comparisons between microbiomes to identify changes in microbiome structure or similarities to other microbiomes. Identifying a change in the taxonomic makeup of a microbiome due to environmental changes or direct human actions using amplicon sequencing can be an indication of a shift in microbiome functional capabilities, which could affect host fitness. Examining the taxa present in a microbiome, and how the taxonomic profile differs from related communities, can also suggest the functional consequences of changes to the microbiome. This inference can be aided by algorithms such as PICRUSt (Langille et al., 2013) to compare community-level functions between samples. Once a factor is shown to alter the microbiome, it might then be useful to use methods such as metagenome sequencing to provide more detail into the microbiome functions how changes in these may affect host fitness.

Study design considerations and sample collection

The sampling method used in microbiome studies depends on the nature of the microbiome being studied. Oral, skin or cloacal microbiomes can often be collected with nonlethal sampling, while internal communities such as in the gut are much more difficult to access directly. Samples can be taken post-mortem (Kohl et al., 2014) or under sedation (Dill-McFarland et al., 2016), however there are obvious animal welfare implications for these, something that is especially relevant in studies of protected species. Opportunistic sampling from deceased individuals can be useful if internal sampling is of particular importance (Wan et al., 2021). Scat sampling is an invaluable tool for sampling the gut microbiome as it is non-invasive and can, in many cases, be done without trapping or handling the host animal. It is important to note that the gut microbiome varies in composition and function along the digestive tract (Suzuki & Nachman, 2016), and that fecal samples can be distinct from gut samples taken post-mortem (Ingala et al., 2018). Nevertheless, provided samples are collected and processed in a consistent manner, they can still be an excellent tool in identifying changes in the gut microbiome while minimising the exposure of host individuals to handling stress.

The logistical demands of occasionally remote fieldwork also dictate the types of samples that can be reliably collected and stored prior to microbiome analysis. For example, fecal samples should ideally be either processed immediately or stored at -80^{o}C to obtain the most accurate results (Fouhy et al., 2015). However, many researchers may not have immediate access to an ultralow freezer at or near the sight of sample collection, and samples often need to be stored long term for transport before being processed. Improper storage of fecal samples can lead to DNA degradation (Cardona et al., 2012) and fungal growth (Lauber et al., 2010) so can have a major effect on microbiome inference (Lauber et al., 2010). If freezing of fecal samples is not possible, chemical preservation using 95%

ethanol, OMNIgene Gut or FTA cards keeps samples comparable to fresh samples after eight weeks in terms of taxonomic composition (Song et al., 2016). For metagenomic or metatranscriptomic analyses however, samples must be processed within 24 hours of collection or frozen at -20 immediately to prevent DNA degradation (Cardona et al., 2012). Collecting fresh fecal samples may be fairly easy from larger host species that are easy to track, e.g. rhinos (Gibson et al., 2019), however for more cryptic species this is not possible.

Another important consideration is controlling for environmental contamination when sampling microbiomes. Skin microbiomes or fecal samples are exposed to a plethora of microbial communities in the surrounding environment, and it is important that these potential sources of contamination are controlled for when assessing the composition of the microbiome. Eisenhofer et al. (2019) developed a sampling framework to help reduce the risk of contamination in microbiome studies and help account for any contamination that does occur. Important considerations they propose include standardisation of all sampling methods to aid in comparability and the processing of negative controls at each stage from collection to sequencing to identify contamination as it occurs. Sampling blank controls can be particularly useful to identify contamination in the field at the point of sampling. Algorithms like decontam (Davis et al., 2018) can then be used to filter contaminant taxa out of final sequence data. These approaches were used in a study of the pouch microbiome in wild southern hairy-nosed wombats *(Lasiorhinus latifrons)* (Weiss et al., 2021). The researchers collected negative control samples at the start of each sampling day by holding a swab in the air for 30 seconds and in doing so were able to exclude 60 contaminant sequence features from their final analyses.

Sample size constraints

Many studies of managed species are restricted in their ability to maximise sample sizes. This may be because of small population sizes or populations being difficult to access and sample in the wild. Human microbiome studies are easily able to resample from the same individuals to assess patterns in microbiome composition over time (A. J. Johnson et al., 2019), however this near impossible for many wild animal populations. The gut microbiome has been known to change seasonally (Le Sage et al., 2021; Maurice et al., 2015) and understanding this natural variation can be important for identifying changes which may negatively affect the host. However, sampling from the same wild individual at different time points is very challenging in some species. As a result, it can be helpful to use studies of species with high recapture rates to help inform the biology of more cryptic species (Le Sage et al., 2021; Maurice et al., 2015). It is also important to be creative with how samples are obtained, especially when working with these more cryptic species. For example, cetaceans are incredibly hard to sample due to their vast ranges and the inaccessibility of many feeding grounds to researchers. As a result, many samples are opportunistically collected from necropsies of beached individuals (Sanders et al., 2015; Wan et al., 2021). This opportunistic sampling can be an excellent tool to supplement traditional sampling of wild populations and help overcome the challenges of small samples sizes. Captive populations can also be invaluable tool to address these challenges in some species as they give researchers access to more statistical power through larger sample sizes (Sanders et al., 2015; Zhu et al., 2011) and allowing for experimental manipulation of diet (Allan et al., 2018; Ni et al., 2021) and environmental conditions (Greenspan et al., 2020).

Conclusion

Studying the functional profile of microbiomes in conservation contexts can provide answers to practical questions that improve biodiversity management, whether via improving the status of threatened wildlife themselves, or providing greater means to monitor and maintain microbial diversity generally. Extensive studies on humans and model organisms have driven rapid development of new methods and protocols in microbiome research (Costea et al., 2017; A. J. Johnson et al., 2020), which will likely find application in wildlife studies too. Depending on the study species, questions to be answered and funding availability, the ease with which these methods can translate is variable. Nevertheless, methods and protocols developed in model species and any genomic resources available for a study's focal species should be closely considered when designing wildlife microbiome studies to provide useful, reliable answers to conservation challenges. Although conservation research may often have limited resources, thinking creatively in study design and approach can produce novel functional insights into wildlife microbiomes and help preserve biodiversity at both the micro and macro level.

Chapter Three: Relationship between individual genetic diversity and microbiome composition in a captive, free-ranging population of long-nosed potoroos (*Potorous tridactylus*)

Abstract

The world is in the midst of a biodiversity crisis, with climate change, habitat destruction and interactions with humans threatening all most all forms of biodiversity. Many conservation efforts target biodiversity at the macro-level, focusing primarily on animals and plants, but microorganisms also play a crucial role in the ecology of many species and ecosystem processes. Microbes form close relationships with host species in the form of microbiome and can be involved in a diverse range of functions that benefit the host. One such species is the long-nosed potoroo (*Potorous tridactylus*), an Australian marsupial which is one of the few mammalian species that is highly adapted for a diet of primarily fungi. Here we investigate the bacterial and fungal communities of the long-nosed potoroo gut microbiome using amplicon sequencing of scat samples taken from a population in a fenced wildlife sanctuary at the Tidbinbilla Nature Reserve in the Australian Capital Territory, Australia. We test how host population demographics and individual host inbreeding influence the diversity and structure of the gut microbiome. We found that the potoroo gut microbiome is largely similar to those of closely related macropods (kangaroos and wallabies) and that the bacterial genera *Lachnospiraceae NK4A136 group* and *Parabacteroides* are associated with individual inbreeding in our population. We failed to detect any gut-associated species of fungi in our analyses, suggesting that further work is needed to determine to what degree resident fungal communities aid in the function of the potoroo gut microbiome. Taken together, these results add to the growing number of species for which gut microbiome data is available and demonstrate the potential for levels of individual host inbreeding to influence the composition of the gut microbiome. We found evidence that host inbreeding can be associated with functional changes in the gut microbiome using functional profiles predicted from taxonomic profiles. Additional work is needed using metagenomic microbiome data to determine how these changes could influence host biology, however our data supports the idea that host-population genetic processes, such as variation in inbreeding, and potentially inbreeding depression, could manifest through changes in microbiome function. We suggest that the health and function of the microbiome is closely considered in future conservation work both in this species and others for which the microbiome plays a key functional role.

Introduction

Australia has the highest rate of mammal extinctions globally (Woinarski et al., 2015a). One taxon that has been particularly impacted is the family Potoroidae. The main threats facing potoroids are predation by invasive predators and population destruction/fragmentation as a result of human development (Woinarski et al., 2015b). The recent 2019-2020 Australian bushfire season had a large impact on wild populations of long-nosed potoroos (*Potorous tridactylus*) specifically, with the fires estimated to have led to a 24-33% reduction in population size (Legge et al., 2022). Populations have also become highly fragmented, risking local extinctions occurring and risking the disruption to the important ecological roles potoroos play. An important tool in the conservation of potoroids is the use of fenced wildlife sanctuaries. These allow threatened populations to experience natural wild social dynamics and foraging behaviour, while being protected from threats such as invasive predators and human development (Legge et al., 2018). Sanctuaries also offer a unique

opportunity to study the dynamics of wild, small, isolated populations in a setting where fieldwork is logistically feasible, enabling conservation researchers to generate insights that can be more broadly applied to other threatened populations.

Potoroos are one of the few groups of mammals that primarily feed on fungi. Fungi-eating (mycophagy or fungivory) has been recorded in a range of Australian mammals including macropods, bandicoots and native rodents (Claridge & May, 1994), however these taxa will only utilise fungi as a food source at certain times of the year, particularly in winter when other food sources are less abundant (Elliott et al., 2022). Potoroos on the other hand feeds on fungi all year round with scat dissection studies suggesting that fungal material makes up 20-90% of their diet, varying seasonally and by location (Figure 1) (Bennett & Baxter, 1989; Claridge et al., 1993; Guiler, 1971; Tory et al., 1997).

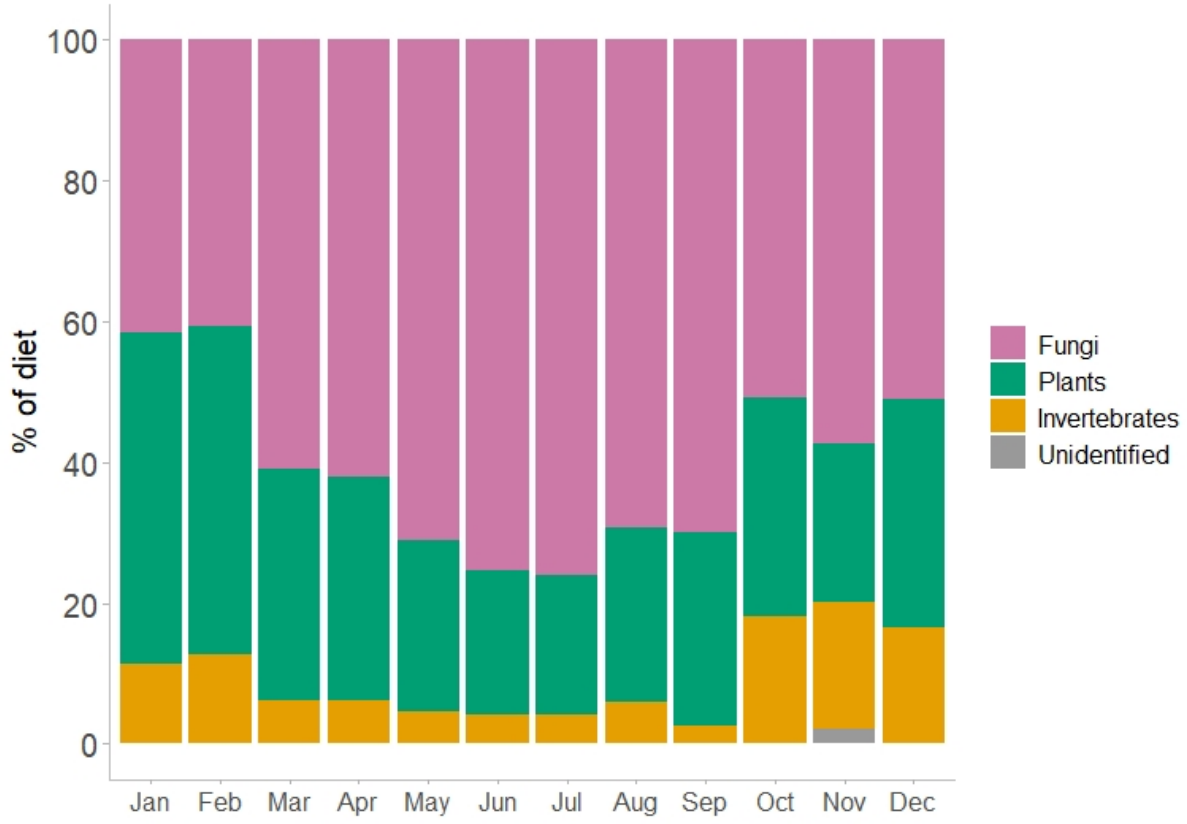

Figure 1 – breakdown of the diet of the long-nosed potoroo (*Potorous tridactylus*) based on scat dissection studies and its seasonal variation. Data compiled from reports of studies conducted at various sites in New South Wales and Victoria, Australia (Bennett & Baxter, 1989; Claridge et al., 1993; Guiler, 1971; Tory et al., 1997).

Other fungivores, such as some North American rodents, are unable to efficiently digest fungal tissue, limiting the nutritional value of fungi as a food (Cork & Kenagy, 1989). Feeding trials in potoroos on the other hand have shown they are able to maintain a positive nitrogen balance on a diet of fungi alone and are able to digest a much higher percentage of available energy and nitrogen in fungal tissue than is seen in rodents (Claridge & Cork, 1994). This suggests the digestive biology of potoroos is highly adapted to a fungivorous diet, with their gut microbiome likely playing a key role in this (Kinnear et al., 1979; Wallis, 1994; Wallis & Hume, 1992). It has been further suggested that microbial communities

present in the digestive tract of potoroos are able to ferment fungal tissue, allowing for more efficient digestion of the material by the host (Kinnear et al., 1979; Wallis, 1994; Wallis & Hume, 1992).

The family Macropodidae, containing kangaroos and wallabies, is closely related to Potoroidae (Duchene et al., 2018) with both families sharing many similarities in physiology and ecology, as a result, the potoroos gut microbiome may be expected to be broadly similar in its gut microbiome diversity, with possible exceptions related to mycophagy. The three Macopodidae species whose bacterial gut microbiomes have been studied [tammar wallaby (*Notamacropus eugenii*) (Chhour et al., 2008; Pope et al., 2010), red kangaroo (*Osphranter rufus*) (Gulino et al., 2013) and eastern grey kangaroo (*Macropus giganteus*) (Gulino et al., 2013)] are all broadly similar to each other at the phylum level, with Firmicutes and Bacteroidota making up >90% of the community and F:B ratios being approximately 1 (Chhour et al., 2008; Gulino et al., 2013; Pope et al., 2010). Resident gut fungi in potoroos may also play an important role in the digestion of fungal sporocarp material, particularly members of the fungal phylum Neocallimastigomycota, which have been implicated as important biomass digesters in many mammalian herbivores (Liggenstoffer et al., 2010; Nicholson et al., 2010). One study of fungal gut microbiome diversity in whiptail wallabies (*Notamacropus parryi*) and red kangaroos (*Osphranter rufus*) identified two genera of Neocallimastigomycota: *Piromyces* and Anaeromyces, as well as several genera novel at the time of publication (Liggenstoffer et al., 2010) (described genera in this group has since increased from six to twenty (Hanafy et al., 2022)). An additional Neocallimastigomycota genus (*Testudinimyces*) has also been isolated from red kangaroo feces using culturing methods (E. E. Chandler et al., 2022).

While sex differences have not been reported in marsupial gut microbiomes, studies have identified shifts in pouch microbiome communities associated with female reproductive. The marsupial pouch microbiome experiences a compositional shift during lactation (Charlick et al., 1981; Old & Deane, 1998; Peel et al., 2016) caused by antimicrobial compounds produced in the pouch to protect immunologically naïve pouch young (Y. Cheng & Belov, 2017). The relationship between the pouch and gut microbiomes is not well understood, but given marsupials have a cloaca, meaning young are born in close proximity to the digestive tract (Sharman, 1970), it is possible that the gut microbiome is in some way affected by reproductive condition, either through the production of antimicrobials or some other mechanism (Y. Cheng & Belov, 2017). As a result, we will examine the effect of sex and breeding status on the gut microbiome of our population of potoroos.

Host population bottlenecks restrict the diversity of gut microbiome communities (Ørsted et al., 2022) and individual inbreeding can also impact the diversity and composition of the gut microbiome (Kreisinger et al., 2014; Prabhu et al., 2020; Wei et al., 2020; Yuan et al., 2015). Compared to less-inbred individuals, more-inbred individuals have lower gut microbiome alpha diversity in tortoises (Yuan et al., 2015) and pigs (Wei et al., 2020). In addition, the bacterial phylum Firmicutes shows lower abundance in inbred populations of domesticated pigs (Wei et al., 2020) and bison (Prabhu et al., 2020) vs. respective wild non-inbred populations of the same species. As a result, it can be useful to consider the entire holobiont (host and its associated microbiomes) (Rosenberg & Zilber-Rosenberg, 2018) when assessing the adaptive capabilities of a host species, encompassing the role of the microbiome. For potoroos, changes to the gut microbiome due to population bottlenecks and increased inbreeding brought about by population fragmentation have the potential to limit the

functional potential of their gut microbiome, potentially lowering host fitness as a consequence.

This study focusses on a population of potoroos living in a fenced wildlife sanctuary at the Tidbinbilla Nature Reserve (TNR), Australia. The potoroos can roam freely around the sanctuary and experience natural social dynamics and foraging behaviours. The sanctuary is also free from species' two major invasive predator threats: domestic/feral cats (*Felis catus*) and red foxes (*Vulpus vulpus*). This population is an excellent model for wild, small, isolated populations of potoroos that allows us to investigate how levels of host demographics and inbreeding could affect the gut microbiome in wild populations. Here we investigate how variation in individual inbreeding within the TNR population influences the diversity, composition, and functional potential of the potoroos gut microbiome. This population has low levels of individual inbreeding and maintains a high level of genetic diversity, with no evidence of inbreeding depression found in this population using six measures of fitness (Mulvena et al., 2020). However, the effect of inbreeding on the gut microbiome in this species has not been studied so the potential for inbreeding depression to manifest through changes in the gut microbiome has not yet been investigated.

Methods

Study population

This study focusses on a population of potoroos living in the Eucalypt Forest Enclosure, a 17-hectare fenced wildlife sanctuary within the Tidbinbilla Nature Reserve, Australian Capital Territory, Australia. At the time of sampling (March 2019), this population was estimated to consist of 71 individuals and was established in 2008 from around 17 individuals (Mulvena et al., 2020).

Sampling

This analysis used samples that had been collected previously, under approval from The University of Sydney Animal Ethics Committee (authority 2019/1470), and Australian Capital Territory (licence LT20193). Full sampling methods are detailed in Mulvena et al. (2020). In short, trapping was carried out in March 2019 across two days and used Sheffield wire traps baited with a peanut butter and oat mixture. New individuals were microchipped and had an ear biopsy taken for DNA analysis. Individual microchip ID, sex and reproductive status (females), body condition and weight were recorded for each trapped individual. Reproductive status was determined by whether pouch young were present, or teats were elongated, indicating a joey at foot. Fecal sampled were opportunistically obtained from scats left in traps after animals had been removed and processed. Fully intact scats were collected using clean gloves and stored at -20°C in the field and then stored at -80°C upon returning to the lab.

Re-trapped individuals over the two trapping days were not reprocessed and were immediately released, however traps were still checked for scats, allowing for resampling of some individuals, giving a total of 40 scat samples from 30 individual potoroos. Of these, 21 individual potoroos are represented by 1 scat sample, 8 potoroos by 2 scat samples and 1 potoroo by 3 scat samples.

Potoroo SNP genotyping

SNP genotyping was carried out through to Diversity Arrays Technology Pty Ltd (DArT), Canberra, and data has previously been published by Mulvena et al. (2020). We did not have microbiome data for all of the individuals for which we had genetic data, however all SNP diversity analyses were carried out using the entire population dataset, with microbiome-data-individuals then selected for further analyses. Minor inconsistencies with microchip ID numbers were identified in the Mulvena et al. (2020) dataset, so the raw SNP data was reprocessed to check if these errors were consequential using similar methods as this previous study. Our methods produced virtually identical results to Mulvena et al. (2020).

DaRT's in-house pipeline returned 22,898 SNPs in total present across 67 individual samples. We then filtered further using the R package dartR v1.9.9.1 (Gruber et al., 2018) for R v4.2.0 (R Core Team, 2022). We filtered out all SNPs with a call rate of < 1, a minimum minor allele frequency of < 0.05 and a reproducibility of < 1. We also removed SNPs which shared sequence tags as these are likely to exhibit strong linkage disequilibrium. Two samples were identified as duplicates and removed; another sample was removed after massive variation between technical replicates. This left us with 6893 SNPs from 63 individual potoroos. Individual multi-locus heterozygosity was quantified using individual internal relatedness (IR) as a measure of level of individual inbreeding, calculated using the R package Rhh v1.0 (Alho et al., 2010).

Scat processing + extractions

Scats were cut in half and a sample was taken from their centre to minimise contamination from soil or other materials which may be present on the outer layers of the scat. DNA was then extracted using the Bioline Isolate II Fecal DNA Kit according to the manufacturer's instructions. Extractions were performed in duplicate and then pooled in equimolar amounts for amplification and sequencing.

All amplification and sequencing was carried out by the Ramaciotti Centre for Genomics (University of New South Wales, Sydney, Australia). Bacterial diversity was assessed using the V3-V4 region of the 16S rRNA gene which was amplified using the 341F and 805R primer pair, and fungal diversity was characterised using the V9 region of the 18S rRNA gene which was amplified using the 1391F and EukBr primer pair. PCR was carried out in triplicate before being pooled for sequencing using the Illumina MiSeq platform (2 x 300 bp).

Bioinformatic processing of amplicon sequencing data

Sequencing reads were processed using the R package DADA2 (v1.16) (Callahan et al., 2016). This approach identifies unique amplicon sequence variants (ASVs) present in the dataset, using a statistical model to determine the likelihood of sequence variants being present due to sequencing error alone (Callahan et al., 2016). This is in contrast to operational taxonomic unit methods (OTUs), for example as employed by QIIME (Caporaso et al., 2010), where sequences are grouped according to sequence similarity to determine the number of OTUs present. The DADA2 (v1.16) pipeline is available at: https://github.com/benjjneb/dada2.

Quality profiles of sequence reads for each sample were examined to determine the appropriate trimming parameters. For 16S sequences, forward reads were truncated at position 270, with reverse reads truncated at position 220. For 18S sequences, forward reads were truncated at position 150, with reverse reads also truncated at position 150.

Unless stated, all further parameters were the same for both the 16S and 18S datasets. Other filtering parameters were as recommended by DADA2 (2 expected errors allowed per read, 'maxEE=2'). Reads were then dereplicated using DADA2 default parameters, meaning identical sequences were pooled to reduce computation time. Error rates were determined for our dataset using default parameters and the DADA2 algorithm was used to remove likely sequencing errors from the dataset and determine the number of ASVs present. Forward and reverse reads were then combined, chimeras were removed and a final ASV table was generated, all functions within DADA2. Taxonomic classification of each ASV was then determined by training the 16S sequences against the SILVA 16S database (v138.1) (Quast et al., 2013) and training the 18s sequences against the SILVA 18S database (v132) (Quast et al., 2013). A multiple-sequence alignment of the processed 16S sequences was generated using the AlignSeqs function in the R package DECIPHER (v2.22) (Wright, 2015) and this was used to generate a phylogenetic tree of sequences using FastTree (v2.1.11) (Price et al., 2010). Data visualisation and manipulation was done using the Phyloseq R package (McMurdie & Holmes, 2013).

Diversity metrics

Alpha and beta diversity metrics were calculated using the R package Phyloseq (v3.17) (McMurdie & Holmes, 2013). Alpha diversity of microbial communities was quantified using total ASV richness and Shannon diversity. Beta diversity was quantified using Bray-Curtis distances (Bray & Curtis, 1957), weighted and unweighted Unifrac distances (Lozupone & Knight, 2005). Unifrac distances evaluate community similarity while considering the level of sequence similarity between individual sequences (Lozupone & Knight, 2005). Unweighted Unifrac treats ASV abundance as binary (present or absent), whereas weighted Unifrac considers the abundance of each ASV when determining sample similarities (Lozupone & Knight, 2005). This can be useful in microbiome research as 16S diversity of certain microbial taxa can correlate with microbiome function and phenotype (Nübel et al., 1999), whereby phylogenetically similar communities are hypothesised to carry out similar functions (Lozupone & Knight, 2005).

Bacterial functional inference

The functional potential of our bacterial gut microbiome communities was predicted using PICRUSt2 v2.5 (Douglas et al., 2020), implemented using python3.6 (Van Rossum & Drake, 2009) within Miniconda3 (Anaconda Inc., 2020). PICRUSt2 takes the 16S bacterial ASV sequences from a dataset and aligns them to a reference tree generated using the Integrated Microbial Genomes (IMG) database (Markowitz et al., 2012). From this combined tree of reference sequences and ASVs, individual gene copy numbers for each ASV are inferred based on the known gene content of closely related taxa on the tree (Douglas et al., 2020). Pathways were then grouped by MetaCyc functional pathway superclass to allow for more data to be represented in our information theory modelling analysis using the MetaCyc database v26.5 (Caspi et al., 2014).

Linear modelling

Of the 63 individuals for which we had SNP data, 30 were represented in the microbiome dataset, however the breeding status of one individual was not noted in the field, so this individual was excluded, leaving 29 individual potoroos to be included in our modelling. As only 9 individuals (30%) were represented by more than one microbiome dataset (retrapped

individuals with multiple scat samples), we decided to randomly choose a microbiome dataset from each of these individuals to use for our modelling.

We tested the effect of IR, sex and breeding status on bacterial alpha diversity, bacterial phyla relative abundance, bacterial genera relative abundance and functional pathway relative abundance by fitting linear models using the glm function in base R (R Core Team, 2022). Sex was treated as a binary predictor (female = 0, male = 1) and breeding status was a categorical variable with three levels: 'male', breeding female' or 'non-breeding female'. The relative abundance of each microbial taxon was calculated using dividing the raw abundance by the total ASV richness within each sample dataset and multiplying by 100. Model formulae for each dataset and each response variable are detailed in Table 1.

Table 1 – summary of the global models generated for each response variable describing the long-nosed potoroo (*Potorous tridactylus*) gut microbiome within the population at the Tidbinbilla Nature Reserve (TNR).

Response variable type	Global model formula + response variables
Bacterial alpha diversity	$alpha_i = IR + Sex + Breeding$ *i* = bacterial ASV richness, bacterial Shannon diversity, fungal ASV richness or fungal Shannon diversity
Bacterial phylum relative abundance	$phylum_i = IR + Sex + Breeding$ *i* = Actinobacteriota, Bacteroidota, Firmicutes, Proteobacteria or Verrucomicrobiota
Bacterial genus relative abundance	$genus_i = IR + Sex + Breeding$ *i* = *Oscillospiraceae UCG 005*, *Rikenellaceae RC9*, *[Eubacterium] ventriosum group*, *Bacteroides*, *Christensenellaceae R-7 group*, *Prevotella*, *Parabacteroides*, *Lachnospiraceae NK4A136 group*, *Ruminococcus* or *Prevotellaceae UCG-003*
Functional pathway relative abundance	$pathway_i = IR + Sex + Breeding$ *i* = Amino Acid Biosynthesis, Nucleoside and Nucleotide Biosynthesis, Cofactor, Carrier, and Vitamin Biosynthesis, Fatty Acid and Lipid Biosynthesis, Carbohydrate Biosynthesis, Cell Structure Biosynthesis, Secondary Metabolite Biosynthesis, Carbohydrate Degradation, Nucleoside and Nucleotide Degradation, Carboxylate Degradation or Fermentation

All predictors were standardised to 2SD to allow for comparisons between the effect magnitudes of each variable (Gelman, 2008). Model selection proceeded under information theory following Burnham and Anderson (1998) and Grueber (2011). Briefly, all submodels of the global model were generated using the glm function in base R (R Core Team, 2022) and the top $2AIC_C$ models were retained and combines via model selection averaging using R package MuMIn (Barton et al., 2023). This approach incorporates both parameter variance and model selection uncertainty into our final model outputs (Grueber, Nakagawa, et al.,

2011). Inference is based on parameter effect sizes, the magnitude of their errors (confidence intervals), and the relative importance (RI) of each variable. RI is calculated as the sum of the AIC weights of all submodels in which a parameter appears within the final model set and only values of >0.9 are considered strong support for this parameter affecting the response variable (Grueber, Nakagawa, et al., 2011). As the some of the variance attributable to breeding status would also be attributable to sex differences, models containing both variables were removed from each model set to avoid collinearity.

Results

SNP genotyping

After filtering and quality control, we had 6893 SNPs representing our population of 63 individual long-nosed potoroos (*Potorous tridactylus*). The population had a mean internal relatedness (IR) of -0.0443 ± 0.0406 SD, ranging from -0.1037 to 0.1388.

Bacterial microbiome community

16S amplicon sequencing of our 40 gut microbiome samples generated 6,464,287 sequence reads in the raw dataset, ranging from 96,763 to 201,977 sequences per sample (mean = 161,607.2, sd = 23,507.3). Trimming and filtering using DADA2 generated 17,435 unique ASVs in our dataset, with filtered coverage ranging from 36,957 to 71,991 sequences (mean = 58132.3, sd = 8017.3). Samples were rarefied to 36,957 sequences per sample for downstream analyses. Of the 17,435 unique ASVs, 17,395 were assigned to bacterial taxa.

A total of 18 phyla were identified in the potoroo gut microbiome (Supplementary Table 1), with the vast majority of sequences belonging to the two dominant phyla: Firmicutes, which ranged from 86.2% to 43.2%, with a mean of 64.7%; and Bacteroidota which ranged from 51.7% to 11.9%, with a mean of 30.3%. Other phyla identified included Verrucomicrobiota (mean = 1.8%), Actinobacteriota (0.8%) and Proteobacteria (0.8%) (Figure 2a). The mean F:B ratio was 2.4, ranging from 7.3 to 0.8 between samples.

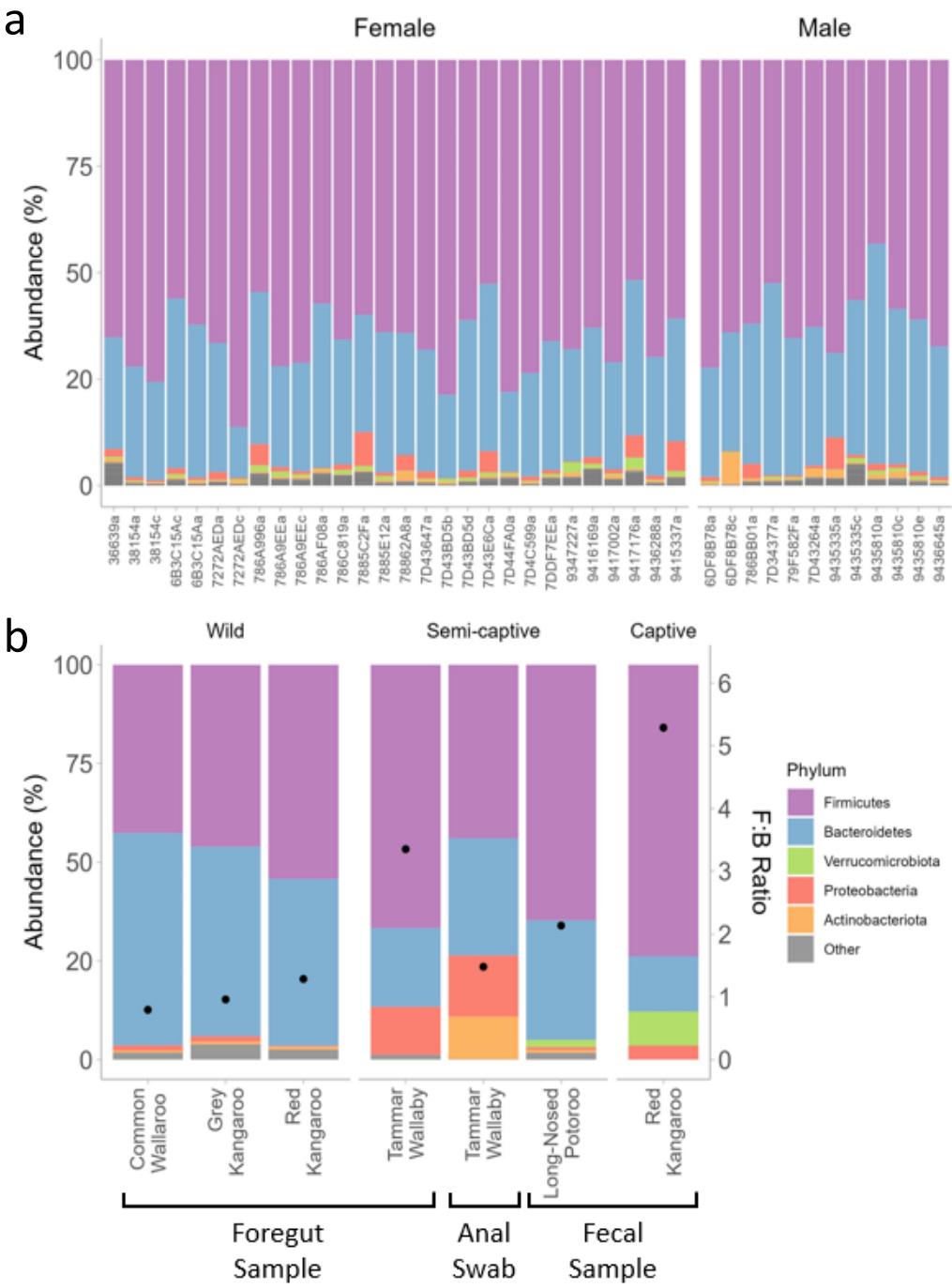

Figure 2 – a) abundance of bacterial phyla in the long-nosed potoroo (*Potorous tridactylus*) gut microbiome within the population at the Tidbinbilla Nature Reserve (TNR). Each stacked bar represents an individual sample from the population, with some individuals represented more than once (shown by IDs which differ by only the final digit). b) abundance of bacterial phyla in the long-nosed potoroo (*Potorous tridactylus*) gut microbiome compared to other Macropodiformes species: common wallaroo (*Osphranter robustus*), eastern grey kangaroo (*Macropus giganteus*), red kangaroo (*Osphranter rufus*) and tammar wallaby (*Notamacropus eugenii*). Firmicutes: Bacteroidota ratio (F:B ratio) represented by points on bars. Data for the three wild samples was obtained from foregut samples taken from deceased individuals, data in this study and the captive populations is derived from fecal samples. 'Other' refers to any phylum with a mean abundance of <0.5% in the potoroo gut microbiome. Phyla were assigned using the SILVA 16S database (v138.1) (Quast et al., 2013).

The potoroo gut microbiome consists of 251 unique bacterial genera. The most abundant was *Oscillospiraceae UCG-005* (mean = 18.7%), followed by *Rikenellaceae RC9 gut group* (5.0%), *Bacteroides* (4.7%), *Christensenellaceae R-7 group* (4.2%), *[Eubacterium] ventriosum group* (3.2%), *Parabacteroides* (2.2%), *Prevotella* (2.2%), *Lachnospiraceae NK4A136 group* (2.0%) and *Prevotellaceae UCG-003* (1.5%) (Figure 3). 67.4% of OTUs were identified down to genus level and 34% of OTUs were assigned to named and cultured genera.

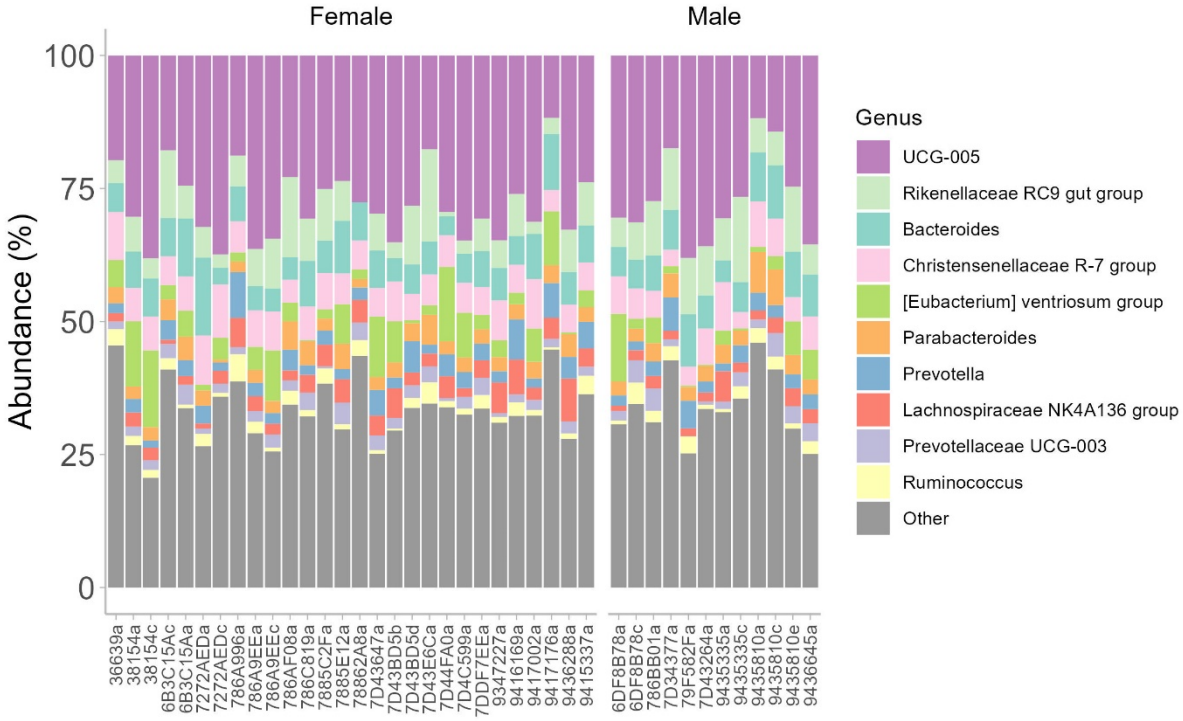

Figure 3 – abundance of bacterial genera in the long-nosed potoroo (*Potorous tridactylus*) gut microbiome within the population at the Tidbinbilla Nature Reserve (TNR). Each stacked bar represents an individual sample from the population, with some individuals represented more than once (shown by IDs which differ by only the final digit). Phyla were assigned using the SILVA 16S database (v138.1) (Quast et al., 2013). 'Other' refers to all genera with a median relative abundance of <1.2%.

Fungal microbiome community

18S amplicon sequencing of our 40 gut microbiome samples generated 6,563,638 sequence reads in the raw dataset, ranging from 9,934 to 289,988 sequences per sample (mean = 164,091, sd = 92,086.3). Trimming and filtering using DADA2 generated 1799 unique ASVs, with filtered coverage ranging from 4,844 to 263,088 sequences (mean = 143,343.3, sd = 85,970.3). To include all samples in the analyses and control for sequencing depth, samples were rarefied to 4,844 sequences per sample.

Analysis of the 18S amplicon data identified a much lower diversity of ASVs in the fungal community compared to the bacterial. Of the 5,733,733 filtered sequences, 5,199,422 of the filtered sequences (90.68%) were assigned to a single species: *Bannoa ogasawarensis*. Only two other species were identified, and both made up less than 0.01% of sequences: *Parafabraea caliginosa* (339 sequences) and *Exophiala salmonis* (164 sequences). Of the 1,799 ASVs, 792 were identified as *Bannoa ogasawarensis*, 5 as *Parafabraea caliginosa* and 2 as *Exophiala salmonis* (Figure 4). The remaining 1,000 ASVs were not resolved to the species level. Of these, the majority (907) were classified as Basidiomycota at the Phylum level and no further.

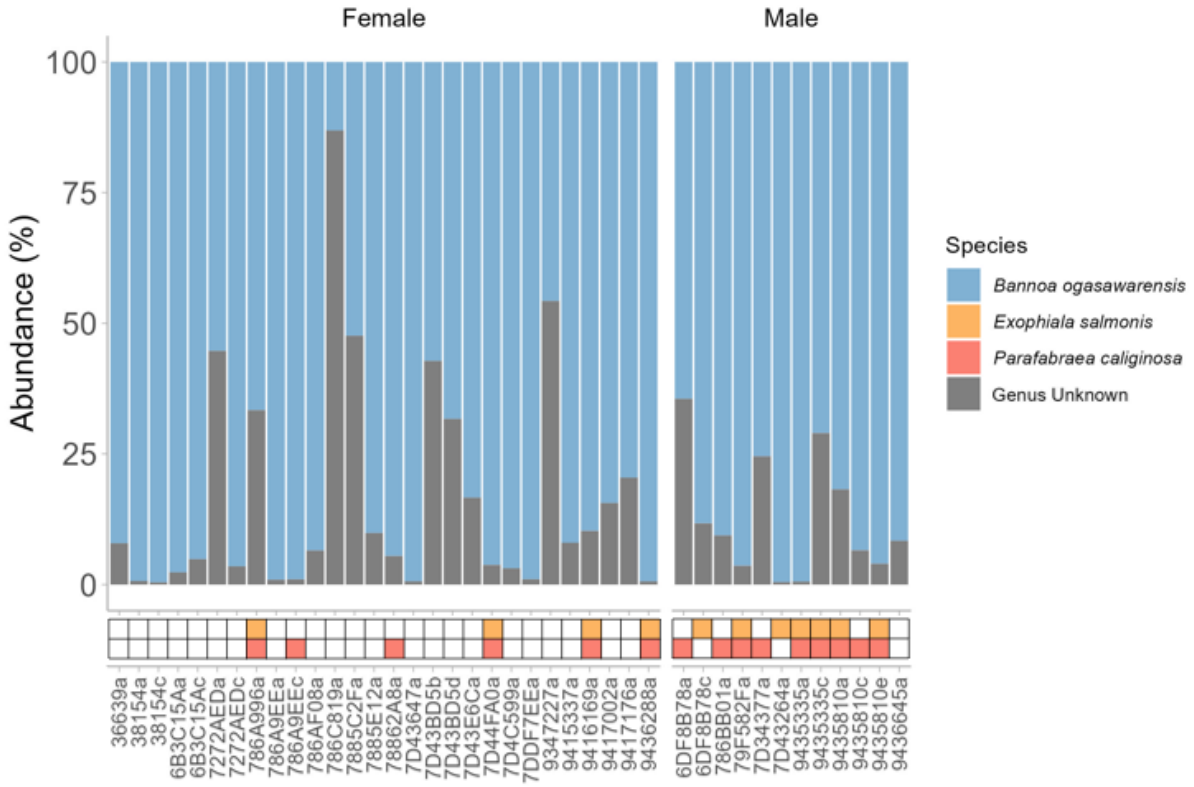

Figure 4 – abundance of fungal species in the long-nosed potoroo (*Potorous tridactylus*) gut microbiome within the population at the Tidbinbilla Nature Reserve (TNR). Each stacked bar represents an individual sample from the population, with some individuals represented more than once (shown by IDs which differ by only the final digit). Taxa were assigned using the SILVA 18S database (v132) (Quast et al., 2013). Abundances of *E. salmonis* and *P. caliginosa* are very low, so are not visible on the bar chart, however presence/absence of these species in gut microbiome samples is noted by the coloured squares below the graph.

Effects of inbreeding and population demographics on the gut microbiome

Alpha diversity

The fungal community was substantially less diverse than the bacterial community both in terms of ASV richness (mean bacterial ASV richness = 1644.6, mean fungal ASV richness = 72.0) and Shannon diversity (7.0 vs 2.1) (Table 2).

Table 2 – linear mixed effect models modelling the effect of microbial dataset (fungal vs bacterial) on the alpha diversity of the long-nosed potoroo (*Potorous tridactylus*) gut microbiome. The reference category for both models is the fungal (18S) dataset. Models were generated using the glm function in base R (R Core Team, 2022).

Alpha Diversity Metric	Parameter	Estimate	Unconditional SE	95% Confidence interval
ASV richness	(Intercept)	1649	30.29	1589, 1708
	Bacterial dataset	1576	41.89	1494, 1658
Shannon diversity	(Intercept)	7.01	0.08	6.85, 7.17
	Bacterial dataset	4.86	0.10	4.66, 5.07

Abbr: SE: Standard error

Considering our demographic parameters of IR, sex and breeding status, none were found to be reliable predictors of our four alpha diversity metrics (all relative importance values [RI] were <0.58) (Table 3). As a result there is no compelling evidence for an effect of IR, sex or breeding status on the alpha diversity of the potoroo gut microbiome.

Table 3 – final averaged models modelling the effect of IR (internal relatedness), sex and breeding status on Shannon diversity and ASV richness in the long-nosed potoroo (*Potorous tridactylus*) gut microbiome within the population at the Tidbinbilla Nature Reserve (TNR). Models were generated using the glm function in base R (R Core Team, 2022) and model selection and averaging were carried out using the R package MuMIn (Barton et al., 2023). Full model sets are detailed in Supplementary Table 2.

Dataset	Response variable	Parameter	Estimate	Unconditional SE	95% Confidence interval	Relative Importance
Bacteria	Shannon Diversity	(Intercept)	7.0142	0.03393	6.95, 7.08	
	ASV Richness	(Intercept)	1684.83	42.73	1601.08, 1768.58	
		IR	-19.33	55.78	-128.66, 90.00	0.28
Fungi	Shannon Diversity	(Intercept)	2.15	0.109	1.94, 2.37	
		IR	-0.21	0.249	-0.70, 0.28	0.56
		Sex* (male)	0.12	0.217	-0.30, 0.55	0.4
	ASV Richness	(Intercept)	72.71	3.889	65.09, 80.34	
		Sex* (male)	8.75	9.981	-10.81, 28.32	0.58
		IR	-4.92	7.667	-19.95, 10.11	0.44

Where only the intercept parameter is specified, the final model set for this diversity metric consisted solely of the null model
Effect sizes have been standardised according to Gelman (2008)
*For sex, the reference category is female
Abbr: IR: internal relatedness, SE: Standard error

Bacterial phyla

Considering our demographic parameters (IR, sex and breeding status) on the relative abundance of the five most common bacterial phyla in the potoroo microbiome, only the effect of sex on the relative abundance of Actinobacteriota was supported by our modelling (RI = 1). The confidence intervals for this effect exclude zero, meaning there is strong confidence that male potoroos tend to have a higher relative abundance of Actinobacteriota

than female potoroos (Figure 5). Our modelling suggests that males tend to have 1.17% more Actinobacteria in their gut microbiome (Table 4).

Table 4 - final averaged models modelling the effect of IR (Internal Relatedness), sex and breeding status on the relative abundance of the five most common bacterial phyla present in the long-nosed potoroo (*Potorous tridactylus*) gut microbiome within the population at the Tidbinbilla Nature Reserve (TNR). Models were generated using the glm function in base R (R Core Team, 2022) and model selection and averaging were carried out using the R package MuMIn (Barton et al., 2023). Full model sets are detailed in Supplementary Table 3.

Phylum	Parameter	Estimate	Unconditional SE	95% Confidence interval	Relative Importance
Actinobacteriota	(Intercept)	0.889	0.240	0.42, 1.36	
	Sex* (male)	1.171	0.532	0.13, 2.21	1
Bacteroidota	(Intercept)	30.326	1.462	27.46, 33.19	
	Sex* (male)	3.018	3.614	-4.07, 10.1	0.55
	IR	1.085	2.315	-3.45, 5.62	0.34
Firmicutes	(Intercept)	64.023	1.635	60.82, 67.23	
	Sex* (male)	-1.862	3.215	-8.16, 4.44	0.41
Proteobacteria	(Intercept)	0.680	0.115	0.45, 0.91	
	Sex* (male)	-0.28	0.298	-0.86, 0.3	0.61
Verrucomicrobiota	(Intercept)	2.275	0.443	1.41, 3.14	
	IR	-0.379	0.738	-1.82, 1.07	0.39

Effect sizes have been standardised according to Gelman (2008)
*For sex, the reference category is female
Abbr: IR: internal relatedness, SE: Standard error

The effect of sex on Actinobacteriota relative abundance may be driven by the very high abundance of this bacterial genus in male potoroo 6DF8B78, 7.26%, which is substantially higher than all other samples (mean = 0.89%). Excluding this sample produced a final model in which sex had a relative importance of 0.57 which is interpreted as poor evidence. As a result, as is plausible that this result is driven by a single individual, we are reluctant to draw firm conclusions on this relationship without additional sampling.

Bacterial genera

Modelling of the effects of IR, sex and breeding status on the relative abundance of the ten most common bacterial genera showed compelling evidence for an effect of IR on the relative abundance of *Lachnospiraceae NK4A136 group*, as well as compelling evidence for an effect of both sex and IR on the relative abundance of *Parabacteroides* (Figure 5).

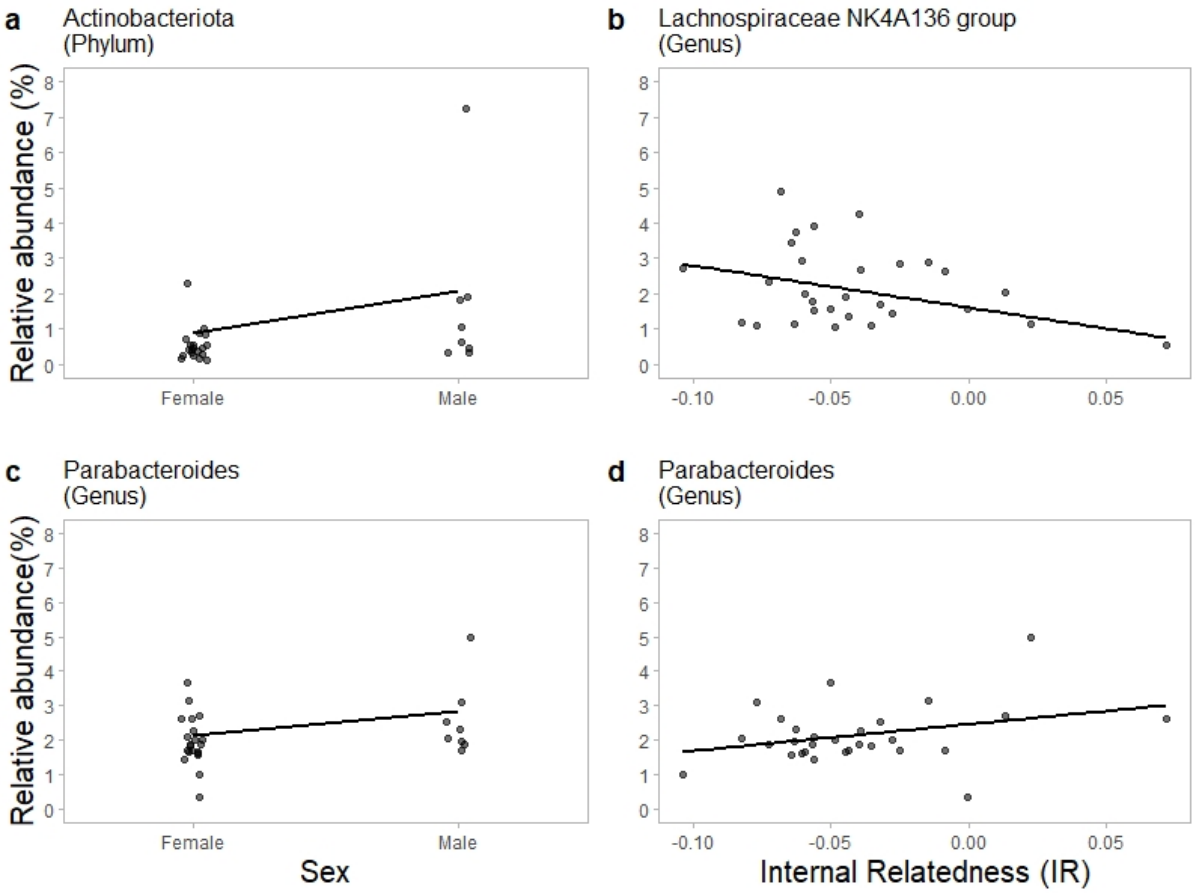

Figure 5 – effects of sex (a,c) and internal relatedness (IR) (b,d) on the relative abundance of bacterial taxa within the long-nosed potoroo (*Potorous tridactylus*) gut microbiome within a population at the Tidbinbilla Nature Reserve (TNR). Final averaged models are detailed in Table 4 for phyla and Table 5 for genera, with fitted lines generated using those model coefficients.

We observed a negative effect of IR on the relative abundance of *Lachnospiraceae NK4A136 group*. This confidence intervals of this effect size excludes zero (-1.58, -0.11), so there is strong evidence for this negative relationship between IR and *Lachnospiraceae NK4A136 group* relative abundance (Table 5).

Table 5 – final averaged models modelling the effect of IR (Internal Relatedness), sex and breeding status and on the relative abundance of the ten most common bacterial genera phyla present in the long-nosed potoroo (*Potorous tridactylus*) gut microbiome within the population at the Tidbinbilla Nature Reserve (TNR). Models were generated using the glm function in base R (R Core Team, 2022) and model selection and averaging were carried out using the R package MuMIn (Barton et al., 2023). Full model sets are detailed in Supplementary Table 4.

Genus	Parameter	Estimate	Unconditional SE	95% Confidence interval	Relative Importance
Oscillospiraceae UCG 005	(Intercept)	18.2283	0.9351	16.4, 20.06	
	IR	-1.4016	1.9551	-5.23, 2.43	0.48
	Sex* (male)	0.7216	1.5913	-2.4, 3.84	0.33
Rikenellaceae RC9	(Intercept)	4.7423	0.4677	3.83, 5.66	
	Sex* (male)	1.7922	1.1961	-0.55, 4.14	0.64
	IR	0.9102	0.5487	-0.17, 1.99	0.19
[Eubacterium] ventriosum group	(Intercept)	2.9604	0.7071	1.57, 4.35	
	Sex* (male)	-0.7132	1.1028	-2.87, 1.45	0.42
	IR	0.2753	0.698	-1.09, 1.64	0.27
	Breeding**	-0.1811	0.6235	-1.4, 1.04	0.14
	Breeding Male**	-0.3561	0.9927	-2.3, 1.59	0.14
Bacteroides	(Intercept)	4.5385	0.2401	4.07, 5.01	
	Sex* (male)	0.2137	0.4298	-0.63, 1.06	0.36
Christensenellaceae R-7 group	(Intercept)	4.1111	0.2042	3.71, 4.51	
	IR	0.1100	0.2836	-0.45, 0.67	0.3
Prevotella	(Intercept)	2.1972	0.4177	1.38, 3.02	
	Breeding**	0.3325	0.5953	-0.83, 1.5	0.31
	Breeding Male**	0.1355	0.4139	-0.68, 0.95	0.31
Parabacteroides	(Intercept)	2.1335	0.1437	1.85, 2.42	
	Sex* (male)	0.6968	0.3252	0.06, 1.33	1
	IR	0.6438	0.2958	0.06, 1.22	1
Lachnospiraceae NK4A136 group	(Intercept)	2.9234	0.2829	2.35, 3.45	
	Breeding**	0.4172	0.4901	-0.54, 1.38	0.56
	Breeding Male**	-0.2628	0.4196	-1.09, 0.56	0.56
	IR	-0.8446	0.3773	-1.58, -0.11	1
	Sex* (male)	-0.3945	0.5218	-1.42, 0.63	0.44
Ruminococcus	(Intercept)	1.4066	0.1574	1.1, 1.72	
	IR	-0.2973	0.3553	-0.99, 0.4	0.55
Prevotellaceae UCG-003	(Intercept)	1.3848	0.1447	1.1, 1.67	

Where only the intercept parameter is specified, the final model set for this genus consisted solely of the null model
Effect sizes have been standardised according to Gelman (2008)
*For sex, the reference category is female
**For breeding status, the reference category is non-breeding females
Abbr: IR: internal relatedness, SE: Standard error

We found an effect of both sex and IR on *Parabacteroides* relative abundance (Table 5). Males and more inbred individuals (high IR) tend to have a higher relative abundance of *Parabacteroides*, with the effect of sex (0.697) similar to the effect of a one standard deviation increase in IR (0.643) (Figure 5).

Microbiome function

Analysis of our bacterial ASVs using the PICRUSt2 pathway generated 389 MetaCyc functional pathways present in the potoroo gut microbiome. These were grouped into 50 functional classes based on the MetaCyc hierarchical classification system. 17 of these classes were associated with biosynthesis functions, 16 with degradation/utilisation/ assimilation functions, 12 with generation of precursor metabolites and energy functions, 2 with glycan pathways, 2 with macromolecule modification and 1 with detoxification functions (Table 6) We investigated the effects of IR, sex, and breeding status on the 11 classes with mean relative abundance of >2%. Of these classes, 7 were associated with biosynthesis pathways, 3 with degradation/utilisation/assimilation functions and 1 with generation of precursor metabolites and energy functions (Table 6).

Table 6 – MetaCyc functional pathways present in the long-nosed potoroo (Potorous tridactylus) gut microbiome within the population at the Tidbinbilla Nature Reserve (TNR). Classes are grouped by superclass and listed in descending order within each superclass. Assignment of functional pathways to the potoroo microbiome were carried out using PICRUSt2 (Douglas et al., 2020).

MetaCyc Functional Superclass	MetaCyc Functional Class	Mean abundance (%)
Biosynthesis	Amino Acid Biosynthesis*	17.93
	Nucleoside and Nucleotide Biosynthesis*	16.85
	Cofactor, Carrier, and Vitamin Biosynthesis*	11.04
	Fatty Acid and Lipid Biosynthesis*	8.26
	Carbohydrate Biosynthesis*	6.06
	Cell Structure Biosynthesis*	4.46
	Secondary Metabolite Biosynthesis*	2.60
	Aromatic Compound Biosynthesis	1.66
	Polyprenyl Biosynthesis	1.23
	Tetrapyrrole Biosynthesis	0.89
	Inosine-5'-phosphate Biosynthesis	0.88
	Adenosine Deoxyribonucleotide De Novo Biosynthesis	0.80
	Guanosine Deoxyribonucleotide De Novo Biosynthesis	0.80
	Aminoacyl-tRNA Charging	0.79
	Other Biosynthesis	0.26
	Amine and Polyamine Biosynthesis	0.18
	Chorismate Biosynthesis	<0.01
Degradation/Utilization/ Assimilation	Carbohydrate Degradation*	3.53
	Nucleoside and Nucleotide Degradation*	2.66
	Carboxylate Degradation*	2.43

	Polymeric Compound Degradation	1.83
	Secondary Metabolite Degradation	1.82
	C1 Compound Utilization and Assimilation	1.08
	Inorganic Nutrient Metabolism	0.39
	Amino Acid Degradation	0.31
	Amine and Polyamine Degradation	0.25
	Hexuronide and Hexuronate Degradation	0.20
	Fatty Acid and Lipid Degradation	0.03
	Alcohol Degradation	0.02
	Aromatic Compound Degradation	0.01
	Degradation/Utilization/Assimilation - Other	<0.01
	Aldehyde Degradation	<0.01
	Chlorinated Compound Degradation	<0.01
Detoxification	Antibiotic Resistance	0.08
Generation of Precursor	Fermentation*	4.19
Metabolites and Energy	Glycolysis	1.98
	Pentose Phosphate Pathways	1.27
	TCA Cycle	0.74
	Respiration	0.25
	Electron Transfer Chains	0.20
	Glycoxylate Cycle	0.01
	Photosynthesis	0.01
	Ethylmalonyl Pathway	<0.01
	Methylaspartate Cycle	<0.01
	Isopropanol Biosynthesis	<0.01
	Methyl Ketone Biosynthesis	<0.01
Glycan Pathways	Glycan Biosynthesis	0.87
	Glycan Degradation	0.83
Macromolecule	Nucleic Acid Processing	0.35
Modification	Protein Modification	<0.01

*functional pathway classes with a relative abundance of >2.0% which were used to model the effect of internal relatedness (IR), sex and breeding status on the functional capabilities of the potoroo gut microbiome.

IR, sex and breeding status did not show strong effects on the relative abundance of the most abundant functional pathway classes, with a possible exception of a relationship of an effect of sex on between fatty acid and lipid biosynthesis relative abundance (Table 7). The gut microbiomes of male potoroos had higher relative abundance of fatty acid biosynthesis pathways compared to females (Figure 6).

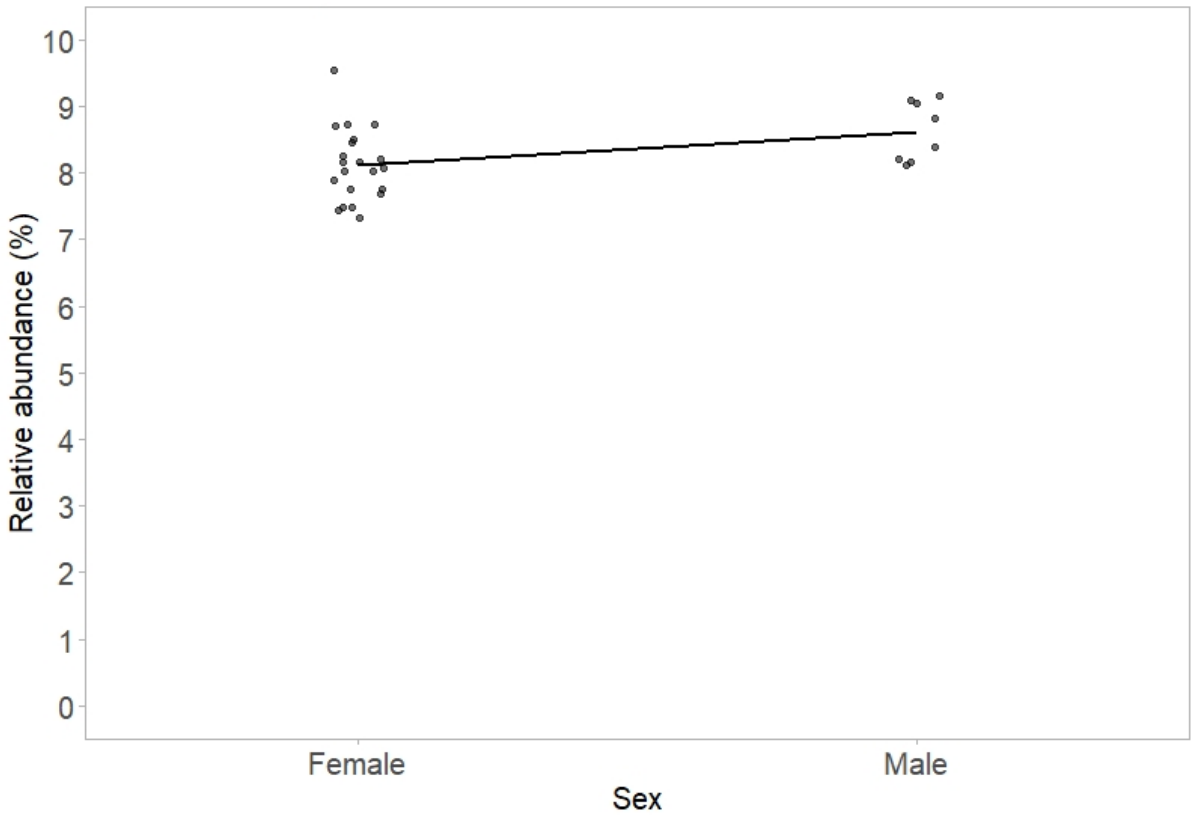

Figure 6 – effect of sex on the relative abundance of the fatty acid and lipid biosynthesis MetaCyc pathway within the long-nosed potoroo (*Potorous tridactylus*) gut microbiome within a population at the Tidbinbilla Nature Reserve (TNR). Final averaged models are detailed in Table 7 with fitted lines generated using those model coefficients.

Table 7 - final averaged models modelling the effect of IR (Internal Relatedness), sex and breeding status on the relative abundance of the eleven most abundant functional pathway classes in the long-nosed potoroo (*Potorous tridactylus*) gut microbiome. Models were generated using the glm function in base R (R Core Team, 2022) and model selection and averaging were carried out using the R package MuMIn (Barton et al., 2023). Full model sets are detailed in Supplementary Table 5.

Functional Category	Parameter	Estimate	Unconditional SE	95% Confidence interval	Relative Importance
Amino Acid Biosynthesis	(Intercept)	17.9354	0.1754	17.59, 18.28	
	Sex* (male)	-0.1049	0.2544	-0.6, 0.39	0.31
Nucleoside and Nucleotide Biosynthesis	(Intercept)	16.8422	0.0712	16.7, 16.98	
Cofactor, Carrier, and Vitamin Biosynthesis	(Intercept)	11.0954	0.1157	10.87, 11.32	
	Breeding**	-0.0883	0.1655	-0.41, 0.24	0.3
	Breeding Male**	-0.0250	0.1101	-0.24, 0.19	0.3
Fatty Acid and Lipid Biosynthesis	(Intercept)	8.1178	0.1129	7.9, 8.34	
	Sex* (male)	0.5061	0.2149	0.08, 0.93	1
Carbohydrate Biosynthesis	(Intercept)	6.0390	0.0332	5.97, 6.1	
	Sex* (male)	-0.3636	0.5942	-1.53, 0.8	0.42
Cell Structure Biosynthesis	(Intercept)	4.4696	0.0547	4.36, 4.58	
	Breeding**	-0.1234	0.0754	-0.27, 0.02	0.46
	Breeding Male**	0.0208	0.0831	-0.14, 0.18	0.46
	Sex* (male)	0.0913	0.0732	-0.05, 0.23	0.44
Secondary Metabolite Biosynthesis	(Intercept)	2.5928	0.0145	2.56, 2.62	
	Sex male*	0.0066	0.0193	-0.03, 0.04	0.28
Carbohydrate Degradation	(Intercept)	3.5384	0.0393	3.46, 3.62	
Nucleoside and Nucleotide Degradation	(Intercept)	2.7103	0.0852	2.54, 2.88	
	Sex* (male)	-0.1767	0.1629	-0.5, 0.14	0.64
	Breeding**	0.0540	0.1032	-0.15, 0.26	0.36
	Breeding Male**	-0.0696	0.1234	-0.31, 0.17	0.36
Carboxylate Degradation	(Intercept)	2.4481	0.0383	2.37, 2.52	
	Sex* (male)	-0.0368	0.0657	-0.17, 0.09	0.39
Fermentation	(Intercept)	4.2101	0.0552	4.32, 4.1	
	Breeding**	-0.0478	0.0805	-0.21, 0.11	0.33
	Breeding Male**	-0.0179	0.0548	-0.13, 0.09	0.33

Where only the intercept parameter is specified, the final model set for this pathway class consisted solely of the null model

Effect sizes have been standardised according to Gelman (2008)

*For sex, the reference category is female

**For breeding status, the reference category is non-breeding females

Abbr: IR: internal relatedness, SE: Standard error

Beta diversity

Visualisation of population structure using beta diversity metrics showed no clear discrimination between scat microbiomes collected from potoroos varying in IR, sex or breeding status, either in the bacterial or fungal communities, based on the three measures of beta diversity. (Figure 7). PCoAs further showed no obvious clustering of repeat samples from the same individual (joined by lines) (Figure 7). This suggests high variation in gut microbiome structure between samples from the same individual.

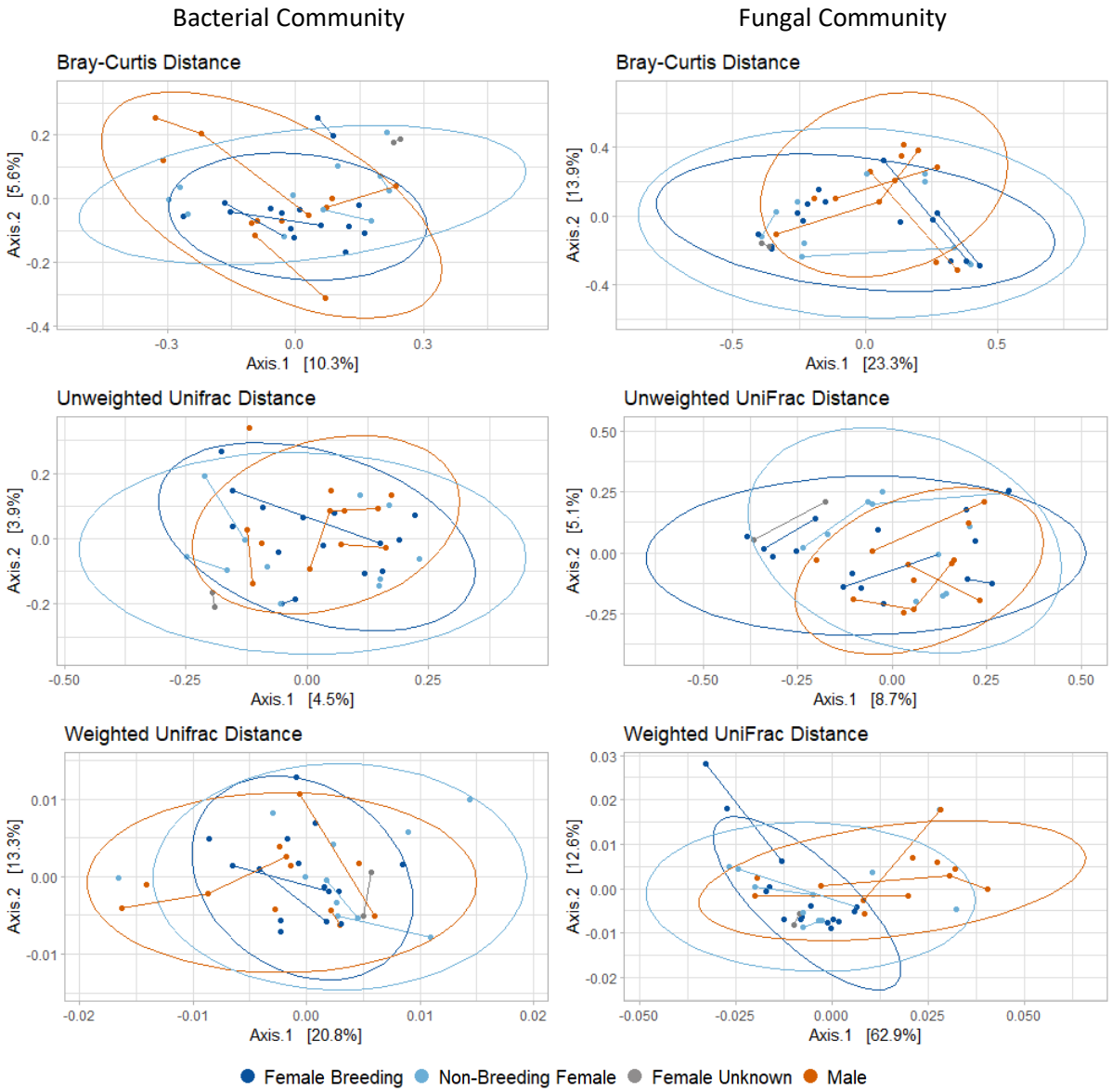

Figure 7 – PCoA of beta diversity within the gut microbiome of a population of long-nosed potoroos (*Potorous tridactylus*). Diversity metrics are calculated using amplicon sequence variant (ASV)-level diversity generating using DADA2 (Callahan et al., 2016). Each point represents a fecal sample, with samples from the sample individual potoroo joined by a soild line, ellipses are generated using that 'stat_ellipse' in the R package ggplot (Wickham, 2011).

Discussion

This study aimed to provide the first gut microbiome characterisation from the marsupial family Potoroidae and investigate how population demographics can influence the composition and structure of the potoroo gut microbiome. We have found that the bacterial portion of the potoroo gut microbiome is composed primarily of phyla Firmicutes (mean = 64.7%) and Bacteroidota (30.3%), with a mean F:B ratio of 2.4. We also showed that sex and inbreeding levels were associated with changes in the taxonomic and functional composition of the potoroo gut microbiome. We conclude that it is possible for inbreeding depression to manifest as changes in the gut microbiome, however we cannot determine whether there are fitness consequences of these changes in our study population. Taken together, this study highlights the importance considering multiple fitness correlates when determining inbreeding depression in small populations.

Bacterial diversity

The potoroo bacterial gut microbiome was primarily composed Firmicutes and Bacteroidota, with these two phyla making up 95% of the sequences from each sample on average (Figure 2). This is consistent with studies in many other mammalian herbivores where these two phyla tend to dominate the bacterial gut microbiome (Muegge et al., 2011; Stewart et al., 2018). This suggests that the gut microbiome of mammalian fungivores is broadly similar to that of mammalian herbivores. The mean F:B ratio (2.4, range 0.8-7.3) found in the gut microbiome of potoroos at TNR was lower than the F:B ratio found in captive macropods (5:1 in *O. rufus* faecal samples (Ley et al., 2008) and 3:1 in captive *N. eugenii* foregut samples (Pope et al., 2010)) and higher than observations from wild macropods (1:1 in foregut samples of wild *O. rufus*, *O. robustus* and *M. giganteus* (Gulino et al., 2013). Higher F:B ratios have been associated with increased energy harvesting efficiency from the diet (Turnbaugh et al., 2006), and further work comparing the diets of wild and captive potoroids and macropods may help determine how F:B ratio and diet contribute to host phenotype.

Of the ten most common bacterial genera in the potoroo gut microbiome, only four have been previously cultured (*Bacteroides*, *Parabacteroides*, *Prevotella* and *Ruminococcus*), the other six were uncultured, theoretical genera based on their high sequence similarity (Figure 3) (Quast et al., 2013). One of these, *Oscillospiraceae UCG-005*, was by far the most abundant bacterial genus in the potoroo gut microbiome, with a mean abundance of 18.7%. No other genera had a mean abundance of >5%. *Oscillospiraceae UCG-005* abundance has been associated with increased fibre digestibility in pigs (*Sus scrofa*) (Li et al., 2021) and gut microbiome health in calves (*Bos taurus*) (Fan et al., 2021), suggesting the genus could also play a key role in the digestive capabilities of potoroos. Other observed genera, *Bacteroides*, *Parabacteroides*, *Prevotella* and *Ruminococcus*, are all commonly found in mammalian gut microbiomes (Arumugam et al., 2011; Wu et al., 2011) so also may play a key role in the potoroo gut microbiome.

Fungal diversity

The vast majority of the fungal diversity in our potoroo fecal samples was attributed to the species *Bannoa ogasawarensis*, making up 90.68% of all 18S sequences recovered. *Bannoa ogasawarensis* [formerly *Sporobolomyces ogasawarensis* (Q. M. Wang et al., 2015)] is a species of basidiomycetous yeast (Hamamoto et al., 2002) that are often associated with infected by rust fungi (James et al., 2016; Parra & Aime, 2019), a group of obligate plant

parasites (order Pucciniales) (Ordonez et al., 2009). It is possible that the presence of *B. ogasawarensis* sequences in the potoroo gut microbiome is as the result of consumption of plant matter infected with rust fungi, however we do not have data on the prevalence of rust fungi at TNR, so cannot confirm this. Only two other species of fungi were identified in the potoroo fecal samples: *Exophiala salmonis* and *Parafabrea caliginosa*. The genus *Exophiala* is generally associated with soil and decaying wood (Yazdanparast et al., 2017), however, can be opportunistic pathogens of a range of species, including humans (Woo et al., 2013) and fish (Madan et al., 2006). *Parafabraea caliginosa* (also *Coleophoma caliginosa*) is a known pathogen of eucalypts (Crous et al., 2019). As none of these species are typically considered gut microbes, we cannot determine how their presence influences gut microbiome function or host health/fitness without further work.

Despite our predictions, we did not identify any members of Neocallimastigomycota in our fungal dataset. This is surprising as the phylum has been recovered in a wide range of mammalian herbivores, including macropods (Liggenstoffer et al., 2010; Nicholson et al., 2010). Some studies have suggested using the ITS ribosome spacer as an amplicon target when studying Neocallimastigomycota as the 18S gene in this group is highly invariable (Dore & Stahl, 2011). However, the same study notes that 18S amplicon methods are still very useful in identifying phylum-level Neocallimastigomycota abundance (Dore & Stahl, 2011), so this does not explain no Neocallimastigomycota sequences were recovered in our dataset. It is possible that the potentially dietary sources of fungi were so large that sequences from these fungal species dominate potoroo fecal matter, making it harder to detect sequences from gut fungi. This could be especially possible if there is a high incidence of rust fungi infection in plant matter available at TNR. *B. ogasawarensis* readily infects the sori of rust fungi-infected leaves and our samples were taken during the summer when plant matter consumption is close to its highest in potoroos (Figure 1). Fungal gut communities also tend to be less diverse than bacterial (Nash et al., 2017; Strati et al., 2016), which would make it more likely that dietary fungal sequences could swamp gut-derived ones in our datasets.

Sex and the gut microbiome

We found an effect of sex on the relative abundance of two bacterial taxa within the *P. tridactylus* gut microbiome: the phylum Actinobacteria and the genus *Parabacteroides* (Figure 5), although this pattern may be driven by one particular sample. Actinobacteria are one of the main phyla of bacteria in the human gut microbiome, alongside Bacteroides, Firmicutes and Proteobacteria (Binda et al., 2018) and is also commonly found in soil (Araujo et al., 2020).

The other bacterial taxon associated with sex was *Parabacteroides*, with males having 0.70% higher relative abundance of Parabacteroides than females (Figure 5). Higher abundance of *Parabacteroides* in males has also been observed in lab mice (*Mus musculus*) (Markle et al., 2013), and in humans the *Bacteroides-Prevotella* group (which contains *Parabacteroides*) is more abundant in males than females. While there is precedence for increased abundance of *Parabacteroides* in males, *Parabacteroides* is one of the most abundant bacterial genera in the human gut microbiome, alongside and both high and low abundance of certain species of *Parabacteroides* have been associated with a range of human conditions and diseases including [reviewed in (Cui et al., 2022)]. The wide range of positive and negative traits the genus has been associated with makes it difficult to determine the effects of

higher *Parabacteroides* abundance in male potoroos without further data. Re-examining our scat samples using metagenomic methods would be an excellent way to investigate this further as it would allow us to determine which bacterial genes are associated with sex difference, and thus allowing more confident inferences of the potential functional consequences.

Inbreeding and the gut microbiome

The effect of factors such as level of captive management on microbiome alpha diversity may be species specific (Kueneman et al., 2022), and the effect of inbreeding on the microbiome also seems to vary considerably by context. We found no association between individual host inbreeding and alpha diversity in the potoroo gut microbiome with regard to our bacterial or fungal datasets, consistent with work in mice (Kreisinger et al., 2014) and bison (Prabhu et al., 2020). Yuan et al. (2015) found a negative association between Simpson's index and individual inbreeding within a population of gopher tortoises, however only observed Simpson's index values of between 0.90 and 0.98 so any effect of inbreeding would be minimal. Wei et al. (2020) is the only study to find consistently higher levels of alpha diversity in a non-inbred population of domestic pigs compared to an inbred population, with all four alpha diversity indices reported being significantly higher in non-inbred individuals. As a result, individual inbreeding may be associated with gut microbiome alpha diversity in certain contexts, but this is does not appear to be a universal pattern.

Some previous studies have identified lower abundance of the bacterial phylum Firmicutes in inbred populations compared to non-inbred populations (Kreisinger et al., 2014; Yuan et al., 2015). We found no association between IR and the abundance of Firmicutes or any of the other five most abundant phyla in the potoroo gut microbiome. However, our study did find a negative relationship between IR and the relative abundance of *Lachnospiraceae NK4A136 group*, a genus within the Firmicutes. Prabhu et al. (2020) also identified several OTUs within Firmicutes with decreased abundance in the gut microbiome of domesticated (presumed inbred) bison (*Bos frontalis*) compared to wild individuals. However, Prabhu et al. (2020) found similar patterns in a range of non-Firmicutes taxa, suggesting the effects of domestication in bison are not limited to Firmicutes. *Lachnospiraceae NK4A136 group* is a member of the Firmicutes family Lachnospiraceae, a common gut microbiome family capable of fermenting plant polysaccharides (Biddle et al., 2013). Kreisinger et al. (2014) found that the Lachnospiraceae family was more abundant in wild-type mice vs. inbred populations, however they did not look at specific genera within the family. Taxa within Lachnospiraceae have been associated with a range of positive and negative effects in humans (Vacca et al., 2020), so further work characterising the functional capabilities of within the potoroo gut microbiome would be needed to determine the effects of higher abundance in more inbred individuals. As well as a negative association between IR and *Lachnospiraceae NK4A136 group*, we also found a positive association between IR and genus *Parabacteroides*, however, like with our pattern between sex and *Parabacteroides* abundance, we do not have the data available to determine the consequences of this relationship for the host. We did not measure individual fitness as part of our analyses, therefore we cannot determine whether the gut microbiome variation we have observed affects host health or evolutionary fitness in potoroos. We can however conclude that both sex and level of inbreeding have the capacity to influence the composition of the potoroo gut microbiome. Given that changes in the gut microbiome have been associated with negative effects for the host in humans and other wildlife species (Cui et al., 2022; Gallardo-

Becerra et al., 2020; Ørsted et al., 2022; Vacca et al., 2020; K. Wang et al., 2019; Wasimuddin et al., 2017), we show that it is possible for inbreeding depression to manifest via changes in an animal's gut microbiome. As a result, it is important that the health of the gut microbiome is closely considered when assessing the presence of inbreeding depression in small populations and that microbiome-directed conservation interventions could potentially be used to alleviate the effect of inbreeding depression.

Inferring microbiome function

We found that male potoroo gut microbiomes had 0.51% higher abundance of functions associated with fatty acid and lipid biosynthesis pathways than females. This pattern could be due to a range of factors including differences in the physiology of the two sexes, or dietary differences. Male potoroos tend to be larger than females (Norton et al., 2011), however the sexes have similar metabolic rates (Wallis et al., 1997). Females are also perpetual breeders, so near-constantly bear the energetic and nutritional demands of rearing pouch young, perhaps leading to differential nutritional requirements compared to males. Differences in microbiome function may also reflect differences in diet (Ni et al., 2021). It is suspected that not all individual potoroos at TNR consume the supplementary food provided to them (J Pierson pers. comm.), so further work determining the dietary preferences of male and female potoroos could explain the functional differences observed in our study.

Replicate sampling (beta diversity)

Considering bacterial beta diversity, we observed that replicate samples from the same individual host rarely clustered together compared to samples from other individuals. This suggests that either our sampling and processing methods introduce a substantial degree of variance to our dataset, or that the gut microbiome of individual potoroos varied considerably between trapping nights (trapping nights spanned 16 days). The nature field sampling means that some variance may have been introduced as a result of the actual time of trapping and defecation relative to the collection of samples, or the time taken to place samples into storage (Fouhy et al., 2015; Lauber et al., 2010). Alternatively, this result may reflect true variation in the potoroo gut microbiome. Studies in humans have found daily diet changes can lead to considerable changes in gut microbiome composition at the genus level (A. J. Johnson et al., 2019; Vandeputte et al., 2021) and while human diets can vary much more than the natural diet of our potoroo population, the provision of supplementary foods in the TNR population gives them access to broader nutritional space than wild populations would. Further research involving daily sampling of the potoroo gut microbiome, likely in fully captive populations, would help determine whether the variation we observe is biological or methodological.

Conclusion

This study provides the first description of a microbiome community in the mammalian family Potoroidae and act as an excellent addition to the growing number of gut microbiome communities being characterised given the family's unique ecology. This study also provides evidence that the abundance of certain taxa within the gut microbiome can be associated with levels of individual host inbreeding. The ability for host inbreeding to

influence the microbiome suggests the possibility that processes such as inbreeding depression could theoretically manifest through changes in gut microbiome structure which, in turn, alter host fitness. Further work is required to determine the fitness consequences of changes to the potoroo gut microbiome, but there is precedent for the gut microbiome to directly contribute to host fitness (Ørsted et al., 2022) and for host-level population bottlenecks to influence the functional diversity of their gut microbiome (Ørsted et al., 2022). We recommend that for host species where there is evidence that the gut microbiome is directly affected by threats to the hosts or where there is potential for the microbiome to be manipulated to aid conservation goals, the effect host population genetics on the gut microbiome should be closely considered in future conservation programs to ensure host population conservation goals can be met.

Chapter Four: Discussion

This project aimed to advance the knowledge of wildlife gut microbiomes by providing a summary of the tools available to microbiome researchers and demonstrating how these tools can be used to answer conservation questions and inform conservation outcomes. This was achieved by reviewing how microbiome research has been used in a conservation context, how this work can be built upon, and how projects that target new, poorly studied host species can be initiated (Chapter Two) (Dodd & Grueber, 2021). This thesis next demonstrated how these tools can be used on a wildlife species for which the microbiome is hypothesised to play a key role in their ecology, but for which no prior data on this topic existed – the long-nosed potoroo (*Potorous tridactylus*) (Chapter Three). Potoroos are specialist fungivores and are able to digest fungal material much more efficiently than other mammal taxa – even those which are themselves fungivores (Claridge & Cork, 1994; Cork & Kenagy, 1989). This study shows that the potoroo gut microbiome is largely similar to that of the closely related macropods and demonstrates how gut microbiome structure can be associated with levels of host inbreeding. This result provides further evidence to support the idea that the gut microbiome acts a reservoir of adaptive potential for the host (Ørsted et al., 2022; Zepeda Mendoza et al., 2018) and should be an important consideration when determining the evolutionary fitness of host individuals and the adaptability of hosts to environmental changes or management regimens.

Advancing the study of wildlife microbiomes

The review presented in Chapter Two of this thesis was written during 2021 and was published towards the end of that year. Even in the short time since that work was conducted, wildlife microbiome studies have progressed significantly and our knowledge of the subject has increased considerably. The number of articles published yearly containing the words “wildlife” and “microbiome” has more than doubled, increasing from 1,630 in 2018, to 3,780 in 2022 according to Google Scholar (as of 04/05/2023). The recent publication of articles such as Ørsted et al. (2022), have provided experimental evidence to show that host-population-level processes can have similar effects on the diversity of the collective meta-population of gut microbiomes as is it can on host population genetics. Such studies have been instrumental in showing how important microbiome communities can be for the adaptive potential of the host and how the hologenome framework (Rosenberg & Zilber-Rosenberg, 2018) can enrich how an organism’s fitness and evolutionary history can be contextualised. Studies such as Couch et al. (2022) have provided useful frameworks for applying these host-population-level processes to the meta-populations of microbiome communities, which will enable the development of more novel and interesting applications of host-population-level processes to microbiome communities. Despite the advances in the field, Chapter Two (Dodd & Grueber, 2021) remains a useful resource to show how microbiome research can be in conservation and how best to initiate a conservation microbiome project. My brief review could be used in conjunction with comprehensive reviews, such as Combrink et al. (2023) who have provided an excellent overview of best practice in 16S rRNA microbiome studies in wildlife, addressing many of the limitations of studying animal microbiomes outside of the lab.

Lessons for conservation from fenced wildlife sanctuaries

Fenced wildlife sanctuaries play a crucial role in a range of conservation programs, particularly in Australia where the exclusion of invasive pest species (Legge et al., 2018) and

infectious diseases (Grueber et al., 2019) are proving extremely effective for maintaining populations in a wild-adjacent setting. These sanctuaries also provide an excellent opportunity to study the dynamics of small, isolated populations, similar to many wild populations, with more capacity for comprehensive data collection and environmental manipulation.

The long-nosed potoroo population at TNR as a model system

Captive management is a crucial tool for many conservation programs, be that in an intensive zoo-based setting, or less intensive management in the form of fenced wildlife sanctuaries. Some within-species comparisons have identified an increase in Firmicutes:Bacteroidota (F:B) ratios associated with captive management, e.g. (Bensch et al., 2023; Eisenhofer et al., 2021). Within the marsupial sub-order Macropodiformes, samples from wild populations have tended to have lower F:B ratios of close to 1:1 (Gulino et al., 2013), whereas samples from managed populations have tended to be higher (Figure 2b). This is particularly true for the only macropodiform for which wild and captive samples have been analysed, the red kangaroo (*Osphranter rufus*), where wild populations have an F:B ratio of around 1:1 (Gulino et al., 2013) and captive samples have an F:B ratio of around 5:1 (Ley et al., 2008). Our population of potoroos, as well as other semi-captive populations of Tammar wallaby (*Notamacropus eugenii*) sit between the wild and captive populations with F:B ratios of 2.4 and 3 respectively (Chhour et al., 2008). The existence of these semi-captive populations gives us the opportunity to treat captivity as a spectrum and look at how different levels of management intensity can influence the biology of managed species. F:B ratio has been used as a biomarker for obesity (Turnbaugh et al., 2006) and low fibre diets have been associated with high F:B ratios in humans (De Filippo et al., 2010) and mice (Sonnenburg et al., 2016). The diets of captive herbivores tend to be deficient in in fibre compared to natural diets (Diereweld, 1997; Matsuda et al., 2018; Nijboer & Dierenfeld, 1996), which could potentially explain the pattern within Macropodiformes. Populations such as the potoroo population at the Tidbinbilla Nature Reserve are an excellent tool to investigate the potential effects of captive management programs on the gut microbiome of Macropodiformes as they provide a closed population exposed to largely natural conditions that is able to be easy manipulated experimentally. As a result, diet manipulation of the TNR potoroo population could be an excellent tool to investigate the effects of supplementary feeding and captive diets on the F:B ratio of Macropodiformes. There are also differences in sampling method within the current literature on Macropodiformes gut microbiomes, with some studies using fecal samples (Ley et al., 2008; Pope et al., 2010), one using anal swabs (Chhour et al., 2008) and another directly sampling from the foregut of deceased individuals (Gulino et al., 2013). The TNR population would provide an excellent opportunity to utilise each of these approaches and identify differences among them.

Observations on microbial reference databases

In the current dataset, only 67.4% of bacterial OTUs were identified to genus level, and only 34% were assigned to named and cultured genera. This highlights a wide knowledge gap in the taxonomic identities of bacteria present in the gut microbiomes of wild animals, particularly in species with unique ecologies such *as* potoroos. Amplicon sequencing methods, as used here, rely on publicly available databases to characterise the taxonomic diversity present in microbiomes and are therefore limited in their application to poorly studied species. Large scale studies of non-model gut microbiomes consistently discover

thousands of new bacterial species and novel metagenomic diversity not previously characterised. For example, Youngblut et al. (2020) used metagenome sequencing of the gut microbiome of 180 phylogenetically and ecologically diverse animal hosts to reconstruct 5596 bacterial genomes attributable to 1522 bacterial species, 78% of which were previously undescribed. Similarly, Levin et al. (2021) analysed the gut microbiome of 184 wild animals and constructed 5080 bacterial genomes, attributable to 1209 bacterial species, 75% of which were previously undescribed. Large scale studies like Youngblut et al. (2020) and Levin et al. (2021) as well as more targeted studies such as ours provide invaluable additions to existing datasets concerning microbial diversity in the gut microbiome of wild animals. As more and more knowledge is accumulated, the ability to answer specific conservation questions using microbiome data will become more feasible for a wider range of species.

Unlike in our bacterial dataset, none of the fungal species identified in the potoroo fecal samples were known gut microbes and were instead more associated with soil and dead/decaying plant matter. There are several potential explanations for this. Firstly, the fungal sequences recovered may not be residents of the potoroo gut microbiome and may have been ingested by potoroos either along with their food or in soil. All three fungal species identified are found in soil or plant matter (Crous et al., 2019; Raghavendra et al., 2017; Yazdanparast et al., 2017) and the effect of the soil microbiome on host gut microbiome is comparable to the effect of diet in mice (Zhou et al., 2018). It is also possible that *B. ogasawarensis* can colonise some macro-fungal species that potoroos eat, given *B. ogasawarensis* is known to colonise the sori of rust fungi (James et al., 2016; Parra & Aime, 2019), however no data currently exist to support this. I was unable to obtain data on the presence of rust fungi on the plants at TNR, but the consumption of infected plant matter by potoroos could also explain the presence of *B. ogasawarensis* in our fecal samples given 29% of the potoroo diet is plant matter (Figure 1). Given *P. tridactylus* forages by digging in the soil for underground fungi (Bennett & Baxter, 1989), it is very plausible that the presence of these soil fungi in our fecal samples is due to potoroos consuming them via soil or food items and the fungal species then passing though the potoroo's gut. Experimental work in humans has showed that this is possible, with several fungal species commonly identified in stool samples not detected when subjects were fed fungi-free diets, suggesting that detection of fungi in species does not necessarily indicate gut microbiome (Auchtung et al., 2018). Further work looking at the soil microbiome at TNR and how it can influence animal microbiomes would help confirm this.

Sampling recommendations

Sampling methods used herein aimed to minimise the chance of environmental contamination by collecting fresh pellets from traps during processing of trapped individuals. Scats were collected with clean gloves, stored in individual vials and quickly moved to -20^{o}C for transport and then -80^{o}C in the lab. Samples taken for extraction were collected from the centre of fully intact scat pellets to minimise contamination by material on the outer surface of the scat. However environmental control swabs from the traps, soil or surface of the scats were not collected. Doing so would have enabled us to eliminate some environmental contaminant sequences using pipelines such as decontam (Davis et al., 2018) as has been done in other studies e.g., Weiss et al. (2021). Without these environmental swabs, it is not possible to rule out contamination of the analysed scats by fungi or bacteria not present in the potoroo gut microbiome (Eisenhofer et al., 2019; Salter

et al., 2014). Best practice for scat sampling should always be to take control samples or any possible contaminant including media such as soil, water or air and surfaces such as gloves, storage vials and lab benches (Weiss et al. 2021). This allows for the removal of as many potential contaminant sequences as possible and gives more confidence in interpreting gut microbiome data. The final possibility is that the fungal species recovered here actually are resident taxa within the potoroo gut microbiome. This could be investigated by determining whether these fungal species are present in the gut microbiome of a population of potoroos not exposed to environmental sources of these fungi, such as a zoo population, similar to experimental approaches in humans (Auchtung et al., 2018). However, given that no known gut fungi were identified at all and only soil-associated fungi, whereas other studies in macropods have identified known gut fungi, the most likely explanation is that large fungal loads, particularly of *B. ogasawarensis*, are ingested by individuals in our population, leading to the presence of these species in the analysed scat samples.

Conclusion

This thesis adds to the rapidly growing field of wildlife microbiome research by providing both an accessible review aimed at prospective microbiome researchers and an empirical example of how these tools can be used in a poorly studied host species. For some wildlife microbiome systems, such as amphibian skin microbiomes (Jiménez & Sommer, 2017), the microbiome is sufficiently well understood that manipulation of it to achieve conservation outcomes is the subject of much active research (Kueneman et al., 2022). However, for most host-microbiome systems, there is precious little understood about the functions the microbiome can carry out on behalf of its host and how these can contribute to, and be manipulated to solve, conservation problems. I recommend that all conservation researchers consider the value of incorporating microbiome data into their projects, supported by the evidence presented in this thesis and the broader body of knowledge herein cited. Microbiome research can be of value to the conservation of a range of species and in an ever-changing world, it is important that this field continues its proactive approach to conservation to ensure that future conservation challenges can be effectively managed before becoming major concerns.

Supplementary material

Supplementary Table 1 – mean relative abundance of bacterial phyla in the long-nosed potoroo (*Potorous tridactylus*) gut microbiome.

Phylum	Mean abundance (%)	Standard Error	95% Confidence interval
Firmicutes	64.70	1.317	62.12, 67.28
Bacteroidota	30.31	1.172	28.02, 32.61
Verrucomicrobiota	1.83	0.329	1.19, 2.48
Actinobacteriota	0.79	0.183	0.43, 1.15
Proteobacteria	0.65	0.089	0.48, 0.82
Planctomycetota	0.41	0.104	0.20, 0.61
Cyanobacteria	0.30	0.081	0.14, 0.46
Spirochaetota	0.27	0.067	0.14, 0.40
Patescibacteria	0.23	0.041	0.15, 0.31
Campylobacterota	0.21	0.046	0.12, 0.3
Elusimicrobiota	0.11	0.029	0.17, 0.06
Desulfobacterota	0.06	0.009	0.08, 0.04
Synergistota	0.05	0.013	0.08, 0.03
Fibrobacterota	0.01	0.006	0.02, 0.00
Chloroflexi	<0.01		
Fusobacteriota	<0.01		
Acidobacteriota	<0.01		
Deferribacterota	<0.01		

Supplementary Table 2 – Full model sets for modelling of all gut microbiome alpha diversity metrics. Models were generated using the glm function in base R (R Core Team, 2022).

Bacterial Shannon diversity ~ IR + Breeding Status + Sex								
(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
7.014	NA	NA	NA	2	8.654	-12.85	0.000	0.53
7.014	NA	NA	-0.034	3	8.780	-10.60	2.246	0.17
7.014	NA	-0.015	NA	3	8.675	-10.39	2.457	0.16
6.981	+	NA	NA	4	9.288	-8.91	3.937	0.07
7.014	NA	-0.021	-0.037	4	8.821	-7.97	4.872	0.05
6.984	+	NA	-0.027	5	9.368	-6.13	6.720	0.02
Bacterial richness ~ IR + Breeding Status + Sex								
(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
1649	NA	NA	NA	2	-198.3	401.1	0.000	0.45
1649	NA	NA	-68.15	3	-198.0	402.9	1.853	0.18
1649	NA	-55.01	NA	3	-198.1	403.2	2.152	0.15
1601	+	NA	NA	4	-197.0	403.6	2.542	0.13
1649	NA	-67.69	-77.33	4	-197.7	405.1	4.034	0.06
1608	+	NA	-60.72	5	-196.7	406.0	4.947	0.04
Fungal Shannon diversity ~ IR + Breeding Status + Sex								
(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
2.152	NA	NA	-0.390	3	-24.36	55.68	0.000	0.38
2.152	NA	NA	NA	2	-25.96	56.39	0.705	0.26
2.152	NA	0.342	NA	3	-24.96	56.89	1.207	0.21
2.051	+	NA	NA	4	-24.96	59.59	3.909	0.05
2.051	+	NA	NA	4	-24.96	59.59	3.909	0.05
2.091	+	NA	-0.356	5	-23.60	59.81	4.126	0.05
Fungal richness ~ IR + Breeding Status + Sex								
(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
72.71	NA	15.89	NA	3	-128.0	263.0	0.000	0.34
72.71	NA	14.20	-10.32	4	-127.1	263.9	0.826	0.22
72.71	NA	NA	NA	2	-129.8	264.0	0.942	0.21
69.21	+	NA	NA	4	-128.0	265.7	2.652	0.09
69.21	+	NA	NA	4	-128.0	265.7	2.652	0.09
70.44	+	NA	-10.78	5	-127.0	266.6	3.574	0.06

Supplementary Table 3 – Full model sets for modelling of the relative abundance of the five most abundant bacterial phyla in the long-nosed potoroo (*Potorous tridactylus*) gut microbiome. Models were generated using the glm function in base R (R Core Team, 2022).

Relative Actinobacteriota abundance ~ IR + Breeding Status + Sex								
(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
0.8895	NA	1.171	NA	3	-45.43	97.87	0	0.48
0.8895	NA	NA	NA	2	-47.82	100.13	2.257	0.16
0.4347	+	NA	NA	4	-45.29	100.32	2.448	0.14
0.8895	NA	1.145	-0.171	4	-45.37	100.48	2.609	0.13
0.8895	NA	NA	-0.314	3	-47.64	102.27	4.402	0.05
0.4511	+	NA	-0.130	5	-45.25	103.23	5.364	0.03
Relative Bacteriodota abundance ~ IR + Breeding Status + Sex								
(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
30.33	NA	5.278	NA	3	-95.63	198.3	0.000	0.31
30.33	NA	NA	NA	2	-97.02	198.5	0.273	0.27
30.33	NA	5.796	3.530	4	-94.84	199.4	1.173	0.17
30.33	NA	NA	2.810	3	-96.57	200.1	1.889	0.12
29.69	+	NA	NA	4	-95.42	200.6	2.320	0.10
29.27	+	NA	3.305	5	-94.74	202.2	3.964	0.04
Relative Firmicutes abundance ~ IR + Breeding Status + Sex								
(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
64.02	NA	NA	NA	2	-99.78	204.0	0.000	0.41
64.02	NA	-4.624	NA	3	-98.91	204.8	0.792	0.28
64.02	NA	NA	-1.418	3	-99.68	206.4	2.333	0.13
64.02	NA	-4.922	-2.029	4	-98.71	207.2	3.130	0.09
65.14	+	NA	NA	4	-98.90	207.5	3.512	0.07
65.39	+	NA	-2.014	5	-98.71	210.2	6.117	0.02
Relative Proteobacteria abundance ~ IR + Breeding Status + Sex								
(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
0.6797	NA	-0.460	NA	3	-24.40	55.80	0.000	0.41
0.6797	NA	NA	NA	2	-26.10	56.68	0.881	0.26
0.8598	+	NA	NA	4	-24.29	58.33	2.527	0.12
0.6797	NA	-0.456	0.029	4	-24.39	58.52	2.722	0.11
0.6797	NA	NA	0.086	3	-26.03	59.07	3.270	0.08
0.8584	+	NA	0.011	5	-24.29	61.31	5.513	0.03
Relative Verrucomicrobiota abundance ~ IR + Breeding Status + Sex								
(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
2.2747	NA	NA	NA	2	-63.14	130.8	0.000	0.42
2.2747	NA	NA	-1.040	3	-62.44	131.9	1.115	0.24
2.2747	NA	-0.5749	NA	3	-62.96	132.9	2.163	0.14
2.2747	NA	-0.7409	-1.132	4	-62.13	134.0	3.239	0.08
1.9074	+	NA	NA	4	-62.13	134.0	3.242	0.08
2.0284	+	NA	-0.960	5	-61.52	135.8	5.010	0.03

Supplementary Table 4 – Full model sets for modelling of the relative abundance of the ten most abundant bacterial genera in the long-nosed potoroo (*Potorous tridactylus*) gut microbiome. Models were generated using the glm function in base R (R Core Team, 2022).

Relative *Oscillospiraceae UCG-005* abundance ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
18.23	NA	NA	-2.972	3	-86.62	180.2	0.000	0.31
18.23	NA	NA	NA	2	-87.90	180.3	0.072	0.30
18.23	NA	2.392	NA	3	-87.23	181.4	1.220	0.17
18.23	NA	1.949	-2.708	4	-86.14	181.9	1.756	0.13
18.00	+	NA	NA	4	-87.12	183.9	3.714	0.05
18.32	+	NA	-2.883	5	-85.90	184.4	4.212	0.04

Relative *Rikenellaceae RC9 gut group* abundance ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
4.742	NA	1.752	NA	3	-66.55	140.1	0.000	0.34
4.742	NA	NA	NA	2	-68.01	140.5	0.434	0.28
4.742	NA	1.886	0.910	4	-66.04	141.8	1.701	0.15
4.742	NA	NA	0.680	3	-67.76	142.5	2.420	0.10
4.444	+	NA	NA	4	-66.46	142.6	2.540	0.10
4.359	+	NA	0.879	5	-66.00	144.6	4.552	0.04

Relative *[Eubacterium] ventriosum group* abundance ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
2.787	NA	-1.743	NA	3	-68.29	143.5	0.000	0.28
2.787	NA	NA	NA	2	-69.58	143.6	0.091	0.27
2.787	NA	NA	1.105	3	-68.97	144.9	1.361	0.14
3.982	+	NA	NA	4	-67.66	145.0	1.446	0.14
2.787	NA	-1.607	0.904	4	-67.85	145.4	1.830	0.11
4.008	+	NA	1.026	5	-67.07	146.7	3.216	0.06

Relative *Bacteroides* abundance ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
4.538	NA	NA	NA	2	-48.13	100.7	0.000	0.46
4.538	NA	0.600	NA	3	-47.48	101.9	1.182	0.25
4.538	NA	NA	-0.021	3	-48.13	103.2	2.497	0.13
4.244	+	NA	NA	4	-47.32	104.3	3.584	0.08
4.538	NA	0.610	0.062	4	-47.47	104.6	3.872	0.07
4.232	+	NA	0.100	5	-47.30	107.2	6.482	0.02

Relative *Christensenellaceae R-7 group* abundance ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
4.111	NA	NA	NA	2	-43.37	91.2	0.000	0.50
4.111	NA	NA	0.365	3	-42.96	92.9	1.685	0.21
4.111	NA	0.022	NA	3	-43.36	93.7	2.496	0.14
3.889	+	NA	NA	4	-42.79	95.3	4.060	0.07
4.111	NA	0.083	0.377	4	-42.94	95.5	4.357	0.06
3.838	+	NA	0.446	5	-42.18	97.0	5.784	0.03

Relative *Prevotella* abundance ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
2.372	NA	NA	NA	2	-49.77	104.0	0.000	0.44

2.372	NA	NA	-0.181	3	-49.70	106.4	2.372	0.13
1.806	+	NA	NA	4	-47.97	105.6	1.608	0.20
2.372	NA	-0.176	NA	3	-49.72	106.4	2.399	0.13
1.768	+	NA	-0.409	5	-47.62	107.8	3.853	0.06
2.372	NA	-0.206	-0.205	4	-49.64	108.9	4.945	0.04

Relative *Parabacteroides* abundance ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
2.134	NA	0.697	0.643	4	-32.13	73.93	0.000	0.44
2.134	NA	NA	NA	2	-36.07	76.60	2.667	0.11
2.134	NA	0.591	NA	3	-34.55	76.07	2.136	0.15
1.912	+	NA	0.651	5	-32.11	76.83	2.900	0.10
1.986	+	NA	NA	4	-34.55	78.76	4.831	0.04
2.134	NA	NA	0.548	3	-34.49	75.94	2.010	0.16

Relative *Lachnospiraceae NK4A136 group* abundance ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
2.010	+	NA	-0.885	5	-37.75	88.11	0.000	0.34
2.190	NA	-0.757	NA	3	-41.69	90.34	2.230	0.11
2.190	NA	-0.887	-0.794	4	-39.44	88.55	0.446	0.28
2.190	NA	NA	-0.674	3	-41.76	90.47	2.366	0.11
2.051	+	NA	NA	4	-40.74	91.15	3.039	0.08
2.190	NA	NA	NA	2	-43.21	90.87	2.767	0.09

Relative *Ruminococcus* abundance ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
1.407	NA	NA	-0.536	3	-34.90	76.76	0.000	0.38
1.407	NA	0.225	-0.499	4	-34.68	79.02	2.265	0.12
1.407	NA	0.323	NA	3	-35.94	78.84	2.087	0.13
1.308	+	NA	NA	4	-35.94	81.55	4.790	0.03
1.381	+	NA	-0.512	5	-34.65	81.91	5.153	0.03
1.407	NA	NA	NA	2	-36.37	77.20	0.441	0.30

Relative *Prevotellaceae UCG-003* abundance ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
1.385	NA	NA	NA	2	-33.40	71.3	0.000	0.57
1.311	+	NA	NA	4	-33.31	76.3	5.013	0.05
1.385	NA	NA	-0.039	3	-33.39	73.7	2.480	0.16
1.311	+	NA	NA	4	-33.31	76.3	5.013	0.05
1.312	+	NA	-0.027	5	-33.30	79.2	7.946	0.01
1.385	NA	0.077	NA	3	-33.37	73.7	2.440	0.17

Supplementary Table 5 – Full model sets for modelling of the relative abundance of the eleven most abundant MetaCyc functional pathway classes long-nosed potoroo (*Potorous tridactylus*) gut microbiome. Models were generated using the glm function in base R (R Core Team, 2022).

Amino Acid Biosynthesis ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
17.91	NA	NA	NA	2	-36.43	77.33	0.000	0.49
18.00	NA	+	NA	3	-35.97	78.90	1.579	0.22
17.85	NA	NA	-1.387	3	-36.38	79.73	2.404	0.15
17.92	NA	+	-2.074	4	-35.87	81.40	4.072	0.06
18.09	+	NA	NA	4	-35.88	81.42	4.092	0.06
18.01	+	NA	-1.835	5	-35.79	84.19	6.869	0.02

Nucleoside and Nucleotide Biosynthesis ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
16.84	NA	NA	NA	2	-12.85	30.16	0.000	0.56
16.84	NA	NA	+	3	-12.85	32.65	2.497	0.16
16.84	NA	-0.082	NA	3	-12.85	32.65	2.497	0.16
16.75	+	NA	NA	4	-12.40	34.46	4.303	0.06
16.84	NA	-0.071	+	4	-12.85	35.36	5.202	0.04
16.74	+	-0.342	NA	5	-12.38	37.37	7.217	0.02

Cofactor, Carrier, and Vitamin Biosynthesis ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
11.05	NA	NA	NA	2	-14.03	32.53	0.000	0.45
11.20	+	NA	NA	4	-12.30	34.27	1.740	0.19
11.03	NA	+	NA	3	-13.89	34.75	2.218	0.15
11.06	NA	NA	0.105	3	-14.03	35.03	2.496	0.13
11.23	+	NA	0.798	5	-12.22	37.05	4.524	0.05
11.04	NA	+	0.276	4	-13.89	37.44	4.907	0.04

Fatty Acid and Lipid Biosynthesis ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
8.118	NA	+	NA	3	-20.99	48.94	0.000	0.53
8.147	NA	+	0.787	4	-20.95	51.56	2.620	0.14
8.112	+	NA	NA	4	-20.99	51.64	2.705	0.14
8.257	NA	NA	NA	2	-23.70	51.86	2.921	0.12
8.249	NA	NA	-0.195	3	-23.70	54.35	5.415	0.04
8.147	+	NA	0.787	5	-20.95	54.50	5.562	0.03

Carbohydrate Biosynthesis ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
6.054	NA	NA	NA	2	20.37	-36.28	0.000	0.42
6.019	NA	NA	-0.859	3	21.31	-35.66	0.620	0.31
6.049	NA	+	NA	3	20.45	-33.94	2.343	0.13
6.017	NA	+	-0.841	4	21.33	-32.99	3.286	0.08
6.031	+	NA	NA	4	20.62	-31.56	4.715	0.04
5.991	+	NA	-0.910	5	21.65	-30.68	5.597	0.03

Cell Structure Biosynthesis ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
2.372	NA	NA	NA	2	-49.77	104.0	0.000	0.44

2.372	NA	NA	-0.181	3	-49.70	106.4	2.372	0.13
1.806	+	NA	NA	4	-47.97	105.6	1.608	0.20
2.372	NA	-0.176	NA	3	-49.72	106.4	2.399	0.13
1.768	+	NA	-0.409	5	-47.62	107.8	3.853	0.06
2.372	NA	-0.206	-0.205	4	-49.64	108.9	4.945	0.04

Fermentation ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
4.185	NA	NA	NA	2	9.44	-14.42	0	0.43
4.259	+	NA	NA	4	11.36	-13.04	1.375	0.22
4.178	NA	+	NA	3	9.52	-12.07	2.348	0.13
4.172	NA	NA	-0.338	3	9.51	-12.05	2.366	0.13
4.257	+	NA	-0.049	5	11.36	-10.11	4.314	0.05
4.167	NA	+	-0.290	4	9.56	-9.46	4.959	0.04

Carbohydrate Degradation ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
3.538	NA	NA	NA	2	4.37	-4.29	0.000	0.56
3.544	NA	NA	0.139	3	4.38	-1.81	2.483	0.16
3.540	NA	+	NA	3	4.38	-1.79	2.496	0.16
3.580	+	NA	NA	4	4.68	0.30	4.593	0.06
3.545	NA	+	0.134	4	4.38	0.90	5.189	0.04
3.592	+	NA	0.260	5	4.71	3.19	7.480	0.01

Nucleoside and Nucleotide Degradation ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
2.741	NA	+	NA	3	-3.45	13.86	0.000	0.44
2.656	+	NA	NA	4	-2.66	14.99	1.130	0.25
2.753	NA	+	0.311	4	-3.43	16.52	2.662	0.12
2.665	NA	NA	NA	2	-6.17	16.81	2.945	0.10
2.659	+	NA	0.060	5	-2.66	17.93	4.070	0.06
2.699	NA	NA	0.832	3	-6.04	19.03	5.168	0.03

Secondary Metabolite Biosynthesis ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
2.595	NA	NA	NA	2	35.39	-66.31	0.000	0.51
2.588	NA	+	NA	3	35.70	-64.44	1.872	0.20
2.587	NA	NA	-0.176	3	35.50	-64.03	2.283	0.16
2.583	NA	+	-0.134	4	35.76	-61.86	4.453	0.06
2.588	+	NA	NA	4	35.70	-61.74	4.578	0.05
2.582	+	NA	-0.138	5	35.77	-58.92	7.391	0.01

Carboxylate Degradation ~ IR + Breeding Status + Sex

(Intercept)	Breeding Status	Sex	IR	df	logLik	AICc	delta	weight
2.438	NA	NA	NA	2	8.659	-12.86	0.000	0.42
2.464	NA	+	NA	3	9.470	-11.98	0.875	0.27
2.449	NA	NA	0.282	3	8.702	-10.44	2.411	0.13
2.437	+	NA	NA	4	9.660	-9.65	3.202	0.09
2.468	NA	+	0.106	4	9.477	-9.29	3.569	0.07
2.438	+	NA	0.026	5	9.661	-6.71	6.143	0.02

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