



Review

# Functional Diversity within Gut Microbiomes: Implications for Conserving Biodiversity

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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. Most microbiome research in conservation thus far has used amplicon sequencing methods to assess the taxonomic composition of microbial communities and inferred functional capabilities from these data. 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 microbiome composition. This review outlines the latest advances in 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 microbiomes of wild animals and how they can be overcome by careful study design and sampling methods. Environmental changes brought about by climate change or direct human actions have the potential to alter the taxonomic composition of microbiomes in wild populations. Understanding how taxonomic shifts affect the function of microbial communities is important for identifying species most threatened by potential disruption to their microbiome. Preservation or even restoration of these functions has the potential to be a powerful tool in conservation biology and a shift towards functional characterisation of gut microbiome diversity will be an important first step.

**Keywords:** bacteria; environmental change; genetic diversity; health; taxonomic profiling; threatened species



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## 1. Introduction

The Earth is currently in the midst of its sixth mass extinction [1], largely due to human-induced changes to the environment and climate change [2]. 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 [3]. Microbial communities form crucial relationships across the ecosystem, including ecological processes such as nitrogen cycling [4], as well as contributing to the health of a range of host taxa [5,6]. Climate change can reduce diversity in natural microbial communities [7] potentially putting crucial 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 [8]. In that study, temperature itself had no detectable effect on tadpole growth, but acted indirectly by changing environmental bacterial composition [8]. 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 concern that the microbiome of humans and domestic animals is encroaching into wild animal populations [9]. For example, captive primates have much higher levels of human-associated microbes than are seen in their wild counterparts [10].

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 [3,11]. For example, captive breeding is a key component of the conservation programmes of thousands of species [12], 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 [13] and captive cheetah (*Acinonyx jubatus*) gut microbiomes are enriched for disease-causing bacteria relative to their wild counterparts [14]. 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*) [15]. 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 [16] and translocation of captive Tasmanian devils (*Sarcophilus harrisi*) saw them quickly re-establish their wild-type microbiome [17]. 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 [3,11,18]. 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 [3,11,19]. The concept of functional diversity in a microbiome incorporates the diversity of functions that a microbial community can carry out, rather than just the microbial taxa present [20]. Escalas et al. [21] 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, including many plants or animals, these traits are often continuously expressed and relatively easy to observe [21]. However, many microbial traits are highly environmentally dependent, making it much harder to characterise the entire functional capabilities of any individual microbe, let alone an entire community [22]. This challenge highlights the importance of a focus on the conservation of functional diversity in microbiomes as opposed to a focus on solely taxonomic diversity.

As for multicellular species, microbial biodiversity can be quantified at both the species and the genic level. For bacteria and archaea, however, a “species” is less well defined [23]: processes such as horizontal gene transfer [24] and greater capacity for genome hybridisation [25] 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 [21,26]. 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 [27]. Comparisons of the genome of key gut microbes belonging to a phyloglossid 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 with 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 [27]. 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 such as antibiotic resistance and global energy shortages may have ready-made solutions waiting to be discovered in environmental [28,29] and host-associated [30] 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 [31]. 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 enables researcher to determine 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 [32]. It can also be altered significantly by changes in diet [33], ambient temperature [34], and ingestion of chemical contaminants [35], 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 [36]. 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 this 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. Here, we refer to wildlife microbiomes being those associated with non-domesticated animal species.

## 2. 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 [37]; functional profiling predicted from taxonomic profiles [38–42]; and functional profiling inferred from functional data, such as a shotgun sequenced metagenome [43] or proteome.

### 2.1. Taxonomic Profiles

Taxonomic profiling involves quantifying the identity and abundance of the microbial taxa present in a sample [37]. By comparing taxonomic profiles of microbiomes sampled from individuals, populations or species subject to different conditions, such as climatic shifts [8], pollution [35], and captive management actions [16], it is possible to infer 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 [44], but can change seasonally [45], with age [46] or with reproductive status [47,48]. 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 sphenoccephala*) [49]. 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 Bacteroidetes ratio (F:B ratio) is associated with increased energy uptake efficiency, leading to an increased obesity risk in humans and mice [50,51]. Microbial profiles at the species level have been used to identify individual species that carry out key functions for their host [27]. Thus, 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 because 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 [52]. Related amplicon methods are available that target protists (18S rRNA sequencing) [53], and fungi (ITS sequencing) [54], 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 these methods 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 [55]. No individual region of the 16S rRNA gene perfectly reflects the evolutionary history of the entire gene [56]; however, regions V4, V5 and V6 are reportedly the most reliable [57]. Sequencing of the entire gene gives more accurate phylogenetic inference [58]; however, emerging third-generation sequencing platforms that allow for the required long-read lengths have much higher error rates [59] and are therefore not as widely used as Illumina sequencing [55].

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 [60] or Greengenes [61] to determine the taxa present. Pipelines, such as QIIME 2 [62] and mothur [63], have been developed to process raw sequencing reads into a taxonomic profile and calculate summary statistics to compare among samples or groups of samples. The relative abundance of amplified sequences can be used to estimate the relative abundances of their respective taxa. This can be a good starting point for analyses as this can help identify similar communities—be that from different host species [64], or conspecifics living in different environments [65]. Identification of certain genera or species that are highly represented in a sample can also give an indication of those that may play a key role in the community dynamics of the microbiome [66,67].

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 be used to quantify changes in microbiome composition [68]. Taxonomic profiles can be compared using similarity metrics, e.g., Bray–Curtis dissimilarity, or distance metrics, e.g., UniFrac distances [69,70]. 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. [71]. Distance metrics measure the phylogenetic distance between two sets of taxa, meaning that taxa that are more distinct are given greater weighting [69]. 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 [72].

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*

*orientalis*, leading to bleaching [34]. These methods have also been used to identify taxa that inhibit the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*) in toads [73]. 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% [74]. Anti-*Bd* microbes are also sensitive to temperature changes [49], meaning that understanding the role they play in host immunity will be valuable for 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 [16].

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 observations alone.

## 2.2. 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 [38], PICRUSt2 [39], Tax4Fun [40], BugBase [41] and FUNGuild [42]. These methods give an estimation of the abundance of genes falling into various functional categories; two common classification schemes are KEGG Orthology (KOs) [75] and Clusters of Orthologous Groups (COGs) [76].

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 [77]. Extrapolating functional profiles from these data suggested that this shift may result from the lower levels of plant secondary metabolites in the captive diet [77]. The indication that diet was responsible for the microbiome shift was supported by further work by Ni et al. [78], 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 are 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 [38]. Functions in this category includes responses 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 [79] and host species [79,80], because databases used by these methods are dominated by human data, and perform much better in human studies as a result [80]. However, taxon-specific tools have been developed to improve the performance of inferring microbiome functional capabilities in certain groups, e.g., CowPi for bovids [81], 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 specific 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.

### 2.3. 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 or expressed in a microbial community using methods such as metagenomic sequencing [82]. This approach involves sequencing all genes present within a microbiome and predicting how they interact to carry out community-level functions [83]. 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 [84].

The most common approach to assess functional profiles is to sequence the metagenome of a microbiome sample [43]. 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 [75] 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 [23]. Due to the large amount of data produced by metagenome sequencing, the approach is considerably more expensive and computationally demanding than amplicon methods [85]. 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 [86].

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 [87], so these methods will undoubtedly play a growing 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 [88]. 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 [88]. 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 [36].

In humans, functional profiling of the microbiome has extended beyond metagenome sequencing, to include RNA (transcriptomics) [89], proteins (proteomics) [90] and metabolites (metabolomics) [91]. These tools will provide an 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 dependent many bacterial phenotypes can be [22]. 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 [92].

In addition to expanding our tools to provide more nuanced assessment of the roles bacteria play in the microbiome, metagenome sequencing is also able to capture the functional diversity of non-bacterial taxa [93]. Fungi and protists, for example, have been implicated as contributing to gut microbiome function in both cows [94] and humans [95]. 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 [96]. 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 overlooking essential information about microbiome structure and function.

Overall, metagenome data allow 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).

### 3. 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 processes for its host such as digestion, immune responses and even behaviour [5]. 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* [73]. In order to protect and even restore these functions, it is necessary to identify which microbes provide which functions and how they achieve this.

For many managed species in conservation, captivity provides a 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 [11,66]. 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 [17]); so, identifying which species would benefit most from strategic efforts to re-establish a wild-type microbiome pre-release will be useful in ensuring the long-term success of released individuals [97]. Diet is considered a major cause of gut microbiome changes both in captivity [63] and the wild [98] and quantifying how diet manipulation can improve microbiome function may be one way to improve the success of reintroduction programmes [99]. For some species, the microbiome can be manipulated more directly using probiotics [100]. This approach

has shown particular promise in aiding resistance to fungal pathogens in wildlife such as *Bd* in amphibians [72,101] and *Pseudogymnoascus destructans* (which causes white-nose syndrome) in bats [102].

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 major components of biodiverse reef ecosystems and play a vital role as oxygen producers and carbon fixers [103]. The photosynthetic cyanobacteria and dinoflagellates in their microbiomes are necessary for these functions and are sensitive to the pressures of climate change [104,105]. Changes to these microbiomes will not just impact their host species but have a huge range of ecological consequences [106].

### 3.1. 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 [107], and problems are time sensitive [108]. As a result, the potential benefits of a study must be 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 [109], 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.

### 3.2. 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 a given 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 [43]. However, there has been a major increase in non-human microbiome studies in recent years, providing many more resources for studies in a wide range of socially, economically, and ecologically important species [109]. For example, gut microbiomes have been characterised for many commercially important species such as Atlantic cod [110], cows [111] and chickens [112] as well as laboratory model organisms including mice [113] and fruit flies [114]. Large-scale comparative studies on zoo-housed species [115], and more recently wildlife [109], provide a wealth of reference data that can aid in future conservation programmes. These datasets 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%) [116]. 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 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 such as 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 change 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 [38] 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 how changes in microbiome functions may affect host fitness.

### 3.3. 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 [117] or under sedation [118]; 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 [119]. 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 [120], and that fecal samples can be distinct from gut samples taken post-mortem [121]. 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  $-80\text{ }^{\circ}\text{C}$  to obtain the most accurate results [122]. However, many researchers may not have immediate access to an ultralow freezer at or near the site 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 [123] and fungal growth [124] so can have a major effect on microbiome inference [124]. 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 [125]. For metagenomic or metatranscriptomic analyses, however, samples must be processed within 24 h of collection or frozen at  $-20\text{ }^{\circ}\text{C}$  immediately to prevent DNA degradation [123]. Collecting fresh fecal samples may be fairly easy from larger host species that are easy to track, e.g., rhinos [36]; but much harder for more cryptic species.

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. [126] 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 such as decontam [127] 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 (*Lasiiorhinus latifrons*) [48]. The researchers collected negative control samples at the start of each sampling day by holding a swab in the air for 30 s and in doing so were able to exclude 60 contaminant sequence features from their final analyses.

#### 3.4. 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 can relatively easily resample from the same individuals to assess patterns in microbiome composition over time [128]; however, this is near impossible for many wild animal populations. The gut microbiome has been known to change seasonally [45,49] 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 multiple 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 [45,49]. It is also useful 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 [119,129]. This opportunistic sampling can be an excellent tool to supplement traditional sampling of wild populations and help overcome the challenges of small sample 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 [129,130] and allow for experimental manipulation of diet [78,99] and environmental conditions [8].

#### 4. Conclusions

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 [131,132], 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 focal species should be considered when designing wildlife microbiome studies that 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.

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