Probing the functional significance of wild animal microbiomes using omics data

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Abstract

Host-associated microbiomes are thought to play a key role in host physiology and fitness, but this conclusion mainly derives from studies of a handful of animal models and humans. To test the generality of this conclusion, studies in non-model and wild animals are needed. However, whilst microbiome taxonomic diversity has recently received much attention, characterization of its functional potential in wild animals is lagging behind. Functional “omics” approaches, such as metagenomics, metatranscriptomics, and metabolomics, represent promising techniques to probe the significance of host-associated microbiomes in the wild. In this review, we propose to (1) briefly define the main available functional omics tools along with their strengths and limitations, (2) summarise the key advances enabled by omics tools to understand microbiome function in human and animal models, (3) showcase examples of how these methods have already brought invaluable insights into wild host microbiomes and (4) provide guidelines on how to implement these tools to address outstanding questions in the field of wild animal microbiomes. To conclude, we suggest that functional omics tools represent a promising approach to probe the functional significance of wild host microbiomes once the presence of an abundant and resident microbiota has been established using more traditional (and less expensive) approaches such as qPCR and metabarcoding.

Introduction

Host-associated microbiomes represent complex ecosystems that often make fundamental contributions to host health (Sommer & Bäckhed, 2013, but see Hammer et al., 2019). The vast majority of research evidencing these contributions has been conducted using humans and laboratory animals. However, with the advent of affordable high-throughput sequencing methodologies and non-invasive sampling techniques, there has been a recent proliferation of studies investigating the microbiome of wild animals. Through the amplification of microbial marker genes, usually the bacterial 16S rRNA gene, such studies have uncovered immense microbial taxonomic diversity which varies dynamically across host species (Knowles et al., 2019; Song et al., 2020; Thomas et al., 2016), populations (Grieneisen et al., 2019; Uren Webster et al., 2018), and individuals (Björk et al., 2022; Risely et al., 2022; Worsley et al., 2021). Numerous factors have been shown to contribute to this variation, including habitat and dietary differences (Baniel et al., 2021; Michel et al., 2018), as well as variation in host genotype (Davies et al., 2022; Thomas et al., 2016), relatedness (Baniel et

al., 2022; Ren et al., 2017; Wanelik et al., 2023), social behaviour (Raulo et al., 2021; Tung et al., 2015), and age (Reese et al., 2021; Sadoughi et al., 2022).

However, aside from identifying which microbes are found in particular hosts and how their abundances vary, a key aim of microbiome research is to uncover the functional importance of microbial communities and their impact on host health and fitness. Despite this aim, few wildlife microbiome studies have integrated information on microbial function into their analyses. A variety of analytical ("omics") tools now exist that enable data on microbial functions to be collected (see Table 1), giving an additional layer of resolution and understanding to microbiome studies. That is, instead of asking "what is there?" we can now begin to ask questions like "what does it do?". While the gold standard for evaluating host-associated microbial function remains experimental manipulation in model systems, significant advances have been made by coupling non-invasive sampling with multi-omics approaches in non-experimental systems. For example, in the field of human microbiomes, it has been possible to identify specific microbial genes, transcripts, and metabolites that are linked to microbiome-related disease states (Heintz-Buschart & Wilmes, 2018). We believe that similar techniques could be applied to wild animal microbiomes to give a better understanding of their ecology and evolution, and the relevance of the huge microbial taxonomic diversity observed within and across wild systems.

In this review we have four main aims:

1) Highlight different types of functional data that can be generated (Table 1).
2) Illustrate how these have been applied to provide insight into the microbiomes of humans and model organisms.
3) Propose how these techniques might be transferred into the field of wild animal microbiomes to answer key knowledge gaps, and highlight examples of where such data have already provided additional resolution.
4) Provide a roadmap and guidelines to the use of omics tools and discuss the challenges and potential solutions associated with collecting and processing functional data from understudied, non-model animal systems.

Generating functional datasets

A variety of omics technologies now exist that enable characterisation of host-associated microbiomes (Table 1). Each of these methods can provide a different perspective on microbiome functionality. In Table 1 we provide an overview of the main technologies available, including their advantages and limitations.

Currently, the most widely used approach for characterising host-associated microbiomes, particularly in wild systems, is **metabarcoding** (Table 1). Here, marker genes (such as ribosomal RNA genes) are amplified and sequenced, then taxonomically assigned using microbial databases. This methodology provides information on the identity of microbes, and their relative abundances, within a sample. However, tools have also been developed that enable microbial functions to be inferred from these datasets (reviewed in Djemiel et al., 2022). Here, functions are estimated based on sequence homology to annotated microbial genomes in reference databases. For example, PiCRUST2 (Douglas et al., 2020) enables preliminary functional prediction from 16S rRNA gene metabarcoding datasets by providing rough estimates of KEGG pathways and the metabolic diversity present in the overall microbial community.

The accuracy of a metabarcoding approach can be limited, particularly if closely related microbial genomes are not present in reference databases. Thus, other omics methods have proved invaluable for evaluating the functional potential of microbial communities. For example, **shotgun metagenomics** (Table 1), whereby total genomic DNA is sequenced, can provide information on the taxonomic composition and presence of microbial genes within a sample (reviewed in Quince et al., 2017). This, in turn, enables detailed inference about the types of enzymes and metabolic pathways that may be expressed by the microbiome. High-coverage metagenomic sequencing can also enable metagenome-assembled genomes (MAGs) to be constructed, potentially allowing the identification of taxa (and their genes) that are currently uncharacterised (Quince et al., 2017).

However, the presence of a gene does not necessarily mean that it is expressed or translated *in situ* and other tools are needed to determine whether this is the case. **Metatranscriptomics** (Table 1) can be used to evaluate which genes are actually expressed by microbial members (at time of sampling), whilst **metaproteomics** (Table 1) and **metabolomics** (Table 1) enable the identification and quantification of microbial proteins and metabolites, respectively.

A final, often overlooked, approach that can be used to define microbial function is **culturomics** (Table 1). This technique involves isolating and culturing microbes associated with host animals and identifying them using sequencing approaches. Isolates can then be characterised using *in vitro* assays that directly test their functional capacity (e.g. their ability to inhibit the growth of pathogens) (Antwis & Harrison, 2018; Lagier et al., 2018). Assays can also be paired with whole-genome sequencing of isolates potentially allowing the identification of genes underlying functions of interest (e.g. Holmes et al., 2016; Kwong et al., 2014).

Whilst all methodologies come with limitations (Table 1), combining multiple omics tools (i.e. a “multi-omics” approach) can provide detailed information on microbiome functionality and be a powerful means to unpick complex host-microbe interactions. In the sections below we highlight examples of where functional approaches have provided additional insight and how similar methodologies could be applied in non-model systems to probe the functional significance of the microbiome.

Table 1. Functional "omics" approaches for studying the microbiome

Omic technology	Description	Pros	Cons	Examples of analytical tools	Key reviews
Metabarcoding Aim: Infer functional potential based on homology to annotated taxa	Marker gene amplicon sequencing (e.g. the 16S rRNA gene for bacteria) can be combined with taxonomic assignment using microbial databases (e.g. SILVA, Quast et al., 2012). This approach provides information on the identity and relative abundance of microbes in a sample. Some tools (see examples) also use information on the homology of marker gene sequences to functionally-annotated genomes in reference databases to provide rough estimates of the metabolic capabilities of microbial communities.	Cheap per-sample cost Samples can be multiplexed Less likely to be affected by host contamination or non-target reads compared to other omics methods as a specific microbial genomic region is amplified Easy to implement (standardised wet lab and bioinformatic procedures exist)	Low taxonomic resolution (microbes often only identified to family or genus level) Limited functional information, particularly if there are no close relatives in reference databases Difficult to compare studies that have sequenced different regions of the same marker gene, or used different bioinformatic workflows	Picrust2 (Douglas et al., 2020) FUNGuild (Nguyen et al., 2016) FungalTraits (Põlme et al., 2020) Tax4Fun2 (Wemheuer et al., 2020) MicFunPred (Mongad et al., 2021)	(Djemiel et al., 2022)
Metagenomics Aim: Infer functional potential based on gene content	Shotgun sequencing of total genomic DNA in a sample provides information on the genes, and potentially genomes, of the microbes present. Mapping sequences to reference databases ("reference-based profiling") provides information on the taxonomic identity of microbes (potentially to strain level), and the functional potential of the microbiome, such as the types of enzymes and metabolic pathways present. High-coverage metagenomic data may also allow metagenome-	Provides direct estimates of microbial gene content & 'functional potential' (e.g., KEGG & MetaCyc pathways, virulence factors, and antibiotic resistance genes) Possible to identify microbial taxa to species or even strain level with deep sequencing Can reconstruct microbial genomes if coverage is sufficient; this can identify	Expensive, particularly if deep sequencing Analytically more complex than metabarcoding Host contamination can be an issue for some sample types Pipelines for reconstruction of MAGs not yet standardised Presence of gene content does not imply genes are	**Reference based profiling:** Kraken 2 taxonomic profiler (Wood et al., 2019) MetaPhlAn 4 taxonomic profiler (Blanco-Míguez et al., 2023) HUMAnN 3 functional profiler (Beghini et al., 2021)	(Meyer et al., 2022; Quince et al., 2017; Sczyrba et al., 2017; Yang et al., 2021)

	assembled genomes (MAGs) to be constructed; this can enable the identification of genes carried by individual microbial strains, including those that are currently uncharacterised.	taxa that have not yet been isolated or functionally characterised Costs can be reduced by using shallow sequencing Potential to assay all microbial groups in a sample e.g. bacteria, fungi, viruses	transcribed and translated into functional products	**Genome assembly approaches (MAGs):** MEGAHIT, for assembly (Li et al., 2015) MetaBAT 2, for binning (Kang et al., 2019) Prokka, for gene prediction/annotation (Seemann, 2014)	
Metatranscriptomics Aim: Infer functional potential based on gene expression	RNA is converted to complementary DNA (cDNA) before subjecting it to shotgun sequencing. The resulting data provides information on the functional activity of the microbiome, including which genes are expressed by microbial members (at the time of sampling) and at what levels. It is also possible to use these data to identify genetic variation among microbial members.	Moves beyond descriptions of gene content by providing information on which genes are actively transcribed by microbes in a sample Can provide information on taxonomic composition Potential to assay all microbial groups in a sample e.g. bacteria, fungi, viruses Possible to examine host and microbial responses in tandem (e.g. to stressors/experimental manipulation) using dual RNA sequencing	Expensive, notably due to the higher abundance of ribosomal RNA than messenger RNA molecules, meaning high sequencing depth (or rRNA depletion) is needed Requires different sample storage and handling to DNA-based methods, and samples can be unstable due to the presence of RNases No consensus on standardised bioinformatic pipeline Host transcripts may overwhelm microbial signals when host and microbial RNA is co-extracted from samples When microbial taxonomic composition is unknown, pairing metatranscriptomics with	MetaTrans (Martinez et al., 2016) HUMAnN3 (Beghini et al., 2021) FMAP (Kim et al., 2016)	(Niu et al., 2017; Shakya et al., 2019; Y. Zhang et al., 2021)

			metagenomics is needed to determine whether differences in transcript abundance are driven by expression level changes or gene copy number differences (e.g. due to compositional change)		
Metaproteomics Aim: Infer functional potential based on protein content	High resolution mass spectrometry techniques can be used to identify and quantify the microbial proteins present in a microbiome sample. In addition, recently developed metaproteomic tools can allow per-species biomass to be quantified which provides information on community structure and carbon sources available for microbial members.	Information on which genes are translated by microbes in a sample, resulting in stronger evidence of function than transcription data Ability to measure both taxonomic composition and function Can examine both host and microbial responses (e.g. to stressors/experimental manipulation) Potential to assay all microbial groups in a sample e.g. bacteria, fungi, viruses	Expensive No consensus on standardised bioinformatic pipeline Extensive sample preparation and extraction steps Lower throughput than other omics methods due to long run times per sample Requires *a priori* knowledge of the functional properties of target proteins	Unipept (Verschaffelt et al., 2021) MetaProteomeAnalyzer (Muth et al., 2015) ProPHAnE (Schneider et al., 2011)	(Kleiner, 2019; Peters et al., 2019; Sajulga et al., 2020; Schiebenhoefer et al., 2020; Wilmes & Bond, 2006)
Metabolomics Aim: Infer functional potential based on metabolite content	Identifies and quantifies the small molecules (metabolites) present in a microbiome sample. Thus, provides information on which metabolites are being produced, or consumed, by microbes. Metabolomics is usually performed by coupling either gas chromatography or liquid	Provides information on microbial metabolites present in a sample which may have direct functional effects in hosts No *a priori* knowledge of metabolomic targets required	No consensus on standardised bioinformatic pipeline Expensive and can require sample-specific optimization prior to a full run	MetaboAnalyst (Chong et al., 2018) XCMS (Huan et al., 2017) MZmine 2 (Pluskal et al., 2010)	(Bhosle et al., 2022; Peters et al., 2019; Yasuda et al., 2020)

	chromatography to mass spectrometry. Signal peaks of different intensities are produced depending on metabolite mass to charge ratio (m/z); these peaks can sometimes be matched with those of known metabolites. Lipidomics, a subfield of metabolomics, involves the identification and quantification of lipid molecules (fatty acids). The functional activity of microbial lipids may reflect the community's ability to metabolise different nutrients, adapt to environmental stressors, or interact with host cells.	Imaging Mass Spectrometry (IMS) allows measurement of the spatial distribution of metabolites/ biomolecules	High numbers of unknown metabolites Analytically challenging to identify which microbe(s) produce sets of metabolites Distinguishing between host and microbial metabolites can be challenging No robust workflow for determining the taxonomic composition of the sample	iMAP (D. Zhou et al., 2021)	
Culturomics Aim: Characterising microbes through culturing, experimentation and functional assays.	For this technique, microbes are isolated from target environments (e.g. host, soil, water) and identified using sequencing - most often Sanger sequencing of the full-length 16S rRNA gene for bacteria. Isolates can then be tested using *in vitro* assays that directly assess their functional capacity e.g. inhibition of pathogen growth, ability to digest substrates, or antimicrobial resistance. Isolates can also be whole-genome sequenced to provide high quality genome sequences to serve as references.	Provides direct functional information for traits of interest Can differentiate isolates based on function even if they have the same taxonomic assignment Can scale up from single isolates to multi-isolate consortia to probe diversity-function relationships or test for interaction among microbes Cheap to identify microbes using Sanger sequencing e.g. via full-length sequencing of 16S rRNA gene for bacteria Full genome sequencing	Requires that microbes in a sample can be cultured Culturing and sequencing microbial isolates is time-intensive Assays of function *in vitro* may not represent the functional phenotype of that microbe within a community Difficult / time-intensive to track microbial dynamics in multi-isolate consortia Requires genome sequence to determine why taxonomically identical microbes have different functional profiles	NA	(Antwis & Harrison, 2018; Lagier et al., 2018; Renwick et al., 2021)

		of isolates can identify genes underpinning variation in functional assays Isolates with desirable functional properties *in vitro* can be validated *in vivo* with probiotic experiments Essential for developing high quality reference databases			

Functional insights from studies of model organisms and humans

Over the past decade, functional omics tools (Table 1) have been widely employed in a variety of research fields, particularly those aiming to understand how human gut microbiomes assemble and impact health (e.g. reviewed in Heintz-Buschart & Wilmes, 2018; Rojo et al., 2017; X. Zhang et al., 2019). Our goal here is not to give an exhaustive overview of this work. Instead, we aim to highlight key examples of how such datasets can provide a deeper understanding of host-microbiome interactions, beyond what could normally be achieved using traditional metabarcoding techniques. We suggest that many of the methods, as well as the key findings of this research, are likely to be applicable to the field of wild animal microbiomes.

Metabarcoding datasets (Table 1) remain an important part of characterising host-associated microbial communities and can provide key information about changes in the relative abundance of microbial taxa under different conditions. However, other omics tools have played a crucial role in advancing this research, by connecting changes in taxonomic structure to functional differences that impact host health and disease. For example, in human gut microbiome studies, metagenomic, metatranscriptomic, and metabolomic approaches have provided key insights into the gene pathways, proteins, and other metabolites that are altered under different disease states, as well as the specific components of the microbiome that are responsible for bringing about these changes (Heintz-Buschart et al., 2016; Lloyd-Price et al., 2019; W. Zhou et al., 2019). These studies have added to our understanding of the contribution of the gut microbiome to various diseases, including inflammatory bowel disease (IBD), diabetes, and cancer (X. Zhang et al., 2019). For example, a large-scale, longitudinal study of IBD patients used metagenomics, metatranscriptomics, metaproteomics, and metabolomics to determine functional changes in the gut microbiome associated with periods of increased disease activity (Lloyd-Price et al., 2019). Alongside a characteristic increase in the abundance of facultative anaerobic bacteria, this study also identified disrupted microbial transcription (particularly amongst *Clostridia*), as well as corresponding changes to the abundance of key metabolites (such as key bile acids and butyrate) that were potentially linked to gut inflammation (Lloyd-Price et al., 2019).

Whilst human microbiome studies have shown that metabarcoding and other omics datasets often correlate to some extent, with taxonomic shifts sometimes matching changes in functional profiles (e.g. Heintz-Buschart et al., 2016; Lloyd-Price et al., 2019), integration of

data from multiple omics tools has revealed some of the additional complexities of host-associated microbiomes.

This is partially due to the differences in resolution afforded by different methodologies. Metabarcoding is limited to coarse-scale taxonomic profiling, whereby microbial sequencing variants are assigned to genus or, more rarely, species-level taxonomy. However, shotgun metagenomics now enables more detailed, potentially strain-level, functional comparisons between samples through the reconstruction of microbial draft genomes (metagenome-assembled genomes or MAGs). The application of this methodology to human samples has revealed that microbiome-associated disease outcomes are often a consequence of specific microbial strains that, because of their unique gene content, perform different functions within the gut ecosystem (Hall et al., 2017; Ward et al., 2016). For example, studies have shown that specific bacterial strains are enriched in the gut microbiome of IBD patients (Hall et al., 2017). Despite belonging to the same bacterial species, these strains carry unique genes involved in bacterial stress responses and nutrient utilisation that may enable them to outcompete other microbes within the IBD gut (Hall et al., 2017). Other studies have shown that strain-level genetic diversity can contribute to the digestion of different dietary components (De Filippis et al., 2019) and enable microbes to resist antibiotics (Roodgar et al., 2021). Adding to this complexity, such genetic capabilities can rapidly evolve *in situ* without significant changes to a species' relative abundance (Dapa et al., 2023; Roodgar et al., 2021).

However, the presence of a gene does not necessarily imply that it is expressed in cells. As such, metatranscriptomics and metaproteomics have provided valuable information about alterations to the level of microbial transcripts and proteins under different conditions or disease states (Y. Zhang et al., 2021); studies have shown that these changes do not always correlate with differences in taxonomic or genetic composition (Franzosa et al., 2014; Heintz-Buschart & Wilmes, 2018).

The findings described above suggest that metabarcoding datasets rarely provide an accurate picture of the functional diversity present within a microbiome. For instance, a lack of taxonomic differences between samples in a metabarcoding experiment may not necessarily translate to functional similarity. This is because marker gene sequences that are targeted in metabarcoding studies may be identical amongst microbial strains that are highly divergent at other loci across their genome, or that exhibit different expression profiles under variable conditions. This also means that computational tools that make functional

inferences from metabarcoding data (see Table 1) may underestimate the functional capacity and plasticity of a microbiome. These findings are likely to extend beyond human and animal model studies to wild animal systems, but this has not been rigorously tested.

Taxonomic and functional diversity can also be decoupled in microbial communities because of functional redundancy (Doolittle & Booth, 2017; Louca et al., 2018). This is when different microbial taxa perform the same functional role within an ecosystem (Louca et al., 2018). Studies on various hosts, including humans (Turnbaugh et al., 2009; Vieira-Silva et al., 2016) and ruminants (Taxis et al., 2015), have provided evidence that taxonomically dissimilar gut microbes can share many metabolic pathways. Such redundancy has been hypothesised to increase community resilience in the face of perturbation (Lozupone et al., 2012; Vieira-Silva et al., 2016). These ideas have not been widely tested in the wild animal microbiome literature however, it is possible that at least some of the enormous microbiome variation observed across species, populations, and individuals could be explained by functional redundancy.

In summary, research from other fields has shown that the integration of multiple omics tools can be a powerful way to decipher the compositional and functional characteristics of host-associated microbiomes. Furthermore, these tools provide a means to better understand the mechanisms by which microbiome variation may impact host health. Crucially, the results of such studies also serve as an important hypothesis generating tool by identifying possible targets for future experimental studies and medical intervention (X. Zhang et al., 2019). Such tools could serve a similar purpose in studies of the wild animal microbiome. For example, the integration of multi-omics datasets could provide insight into the functional relevance of the taxonomic differences frequently identified between the microbiome of different host species, populations, and individuals. The results of these studies could also inform manipulative experiments to confirm the contribution of specific microbiome components to host health or fitness.

How and where have functional datasets been used to address questions in the field of wild animal microbiomes?

Microbiomes are usually assumed to play a key role in host physiology and fitness. However, the functional importance of host-associated microbes has only been causatively demonstrated in a handful of model organisms such as corals (Bourne et al., 2016), bees (Motta & Moran, 2023), ruminants (O'Hara et al., 2020), mice (Clavel et al., 2016), and

termites (Scharf & Peterson, 2021), although it can reasonably be assumed for many other animal taxa. In some cases, particularly in invertebrates, the microbiome might be of little importance to the host (reviewed in Hammer et al., 2019). This has been studied in caterpillar larvae which seem to lack an abundant, repeatable gut microbiome and instead rely on host-encoded digestive and detoxification mechanisms (Hammer et al., 2017). An important question thus asks: is it common that animal hosts rely on a microbiome for certain functions? Hammer et al. (2019) provide recommendations on how it may be possible to first quantify microbial load (e.g. using qPCR), eliminate the role of contaminant or transient microbes via controls, and establish that a resident (actively replicating) and potentially beneficial microbiome is stably associated with a host, without using expensive omics tools. Once a resident microbiome is confirmed, omics tools represent the natural next step to probe microbial functions. To highlight how this can be done in wild animals, we summarise some key examples of microbial functions that have been dissected using omics tools in different wild hosts.

The gut microbiome and host physiology: diet as a key example

Metabarcoding studies have made huge progress in characterising the taxonomy of microbial communities that interact with wild animals (e.g. Boscaro et al., 2022; Song et al., 2020). Comparatively few studies have used functional omics approaches to understand the potential contribution of these microbes to host physiology. However, studies are beginning to emerge that demonstrate the value of generating such datasets to gain mechanistic insights into the role of the microbiome (Table 2). This has been particularly exemplified by research aimed at understanding the contribution of microbes to host dietary adaptation. For example, in a recent study, Cabral et al (2022) used a combination of omics tools to elucidate the gut microbial community composition, enzymatic systems and metabolic pathways involved in depolymerization and utilisation of plant fibres in wild capybaras (*Hydrochoerus hydrochaeris*). In doing so, they elegantly linked microbiome composition to dietary processes. The study initially described microbial taxonomic community composition using metabarcoding data, identifying taxonomic novelties involved in plant fibre breakdown in the capybara gut. They then used metagenomic and metatranscriptomic data to investigate the genes and gene pathways associated with plant fibre degradation, and to identify the microbial agents involved in depolymerisation. Finally, using metabolomic data, they were able to confirm that these microbial taxa play a role in the conversion of free sugars into energy that is potentially usable by the host, focussing predominantly on short-chain fatty acids (SCFAs).

Similar functional approaches have been used to better understand the contribution of the microbiota to sanguivory (Zepeda Mendoza et al., 2018) and myrmecophagy (Teullet et al., 2023) in other mammalian hosts, as well as nitrogen cycling in ants (Hu et al., 2018). The broad finding of these studies is that host-associated microbial communities harbour some of the enzymatic toolkit needed to degrade host dietary molecules (e.g. chitin in insect-eaters) and/or synthesise key molecules unavailable from food (as in the nutrient poor sanguivorous diet of bats, or in the nitrogen-poor diet of turtle ants). A key outstanding question in many animal systems is the extent to which microbial degradation of the host diet actually contributes to host diet energy budget: is it relatively low as suggested for humans (1.8 - 12.1%) and mice (21.4%) (Arnoldini et al., 2024) or extremely high as in termites (Scharf & Peterson, 2021) or ruminants (Mizrahi et al., 2021)?

How does microbiome variation relate to host fitness differences?

Aside from providing mechanistic insight into host adaptations, functional datasets could also improve our understanding of the significance of the inter-individual microbiome variation present within wild populations. Several studies have found correlations between microbiome taxonomic variation and host fitness components in wild animals, including disease susceptibility (Bates et al., 2022; Risely et al., 2023), condition (Huang et al., 2022), survival (Davidson et al., 2021; Worsley et al., 2021), and reproductive success (Leclaire et al., 2022). In many cases, however, it is not known whether shifts in the microbiome play a causal role in fitness outcomes or are indirect consequences of changes to host physiology or health status. Functional omics tools applied on data collected *in situ* could provide (1) mechanistic insight into the relevance of microbial taxonomic differences in relation to host health and fitness outcomes, and (2) could also generate hypotheses about causal relationships that can be further tested in laboratory experiments.

Such an approach was recently used by Huang et al (2022) to better understand how seasonal gut microbiome variation in wild giant pandas (*Ailuropoda melanoleuca*) is associated with changes in host body mass. Using metagenomic and metaproteomic approaches, they showed that butyrate-producing microbes and metabolic pathways were differentially abundant between seasons, and that this correlated with butyrate concentrations in faecal samples. They then confirmed that these changes were the cause of the observed differences in body mass between seasons by using faecal transplants into gnotobiotic mice. They were also able to show that bacterially-derived butyrate, a key SCFA, influenced the expression of specific circadian host genes to bring about this effect.

In another example, Bates et al (2022) integrated metabarcoding, metagenomic and metabolomic analysis to better understand links between the skin microbiome and *Batrachochytrium dendrobatidis* (*Bd*) infection in wild amphibians. Using metabarcoding data they initially found differences in microbial taxonomic community composition associated with *Bd* infection. They confirmed this finding with metagenomic data and were then able to use microbe-metabolite integration to identify functionally-relevant taxa driving disease outcomes. This example demonstrates how using a multi-omics approach can shed light on the functional relevance of shifts in the microbiome for the health outcomes of hosts. Such insights could inform wildlife disease monitoring and conservation strategies (see Box 1 for more details about amphibians as a model for host-microbe-pathogen interactions).

Understanding how microbiome variation is associated with host fitness is essential if we are to understand the evolutionary and ecological importance of the microbiome. Furthermore, since hosts are likely to be under strong natural selection to shape their microbiome to be functionally beneficial, and microbial functions evolve to enable microbes to compete and survive within the host, studying microbial functions provides an important evolutionary perspective that may give insight into microbiome assembly mechanisms (Foster et al., 2017; Rühlemann et al., 2024). Though functional approaches have begun to yield valuable insights into the mechanism(s) by which microbes shape the life histories of their hosts, several outstanding questions remain to be addressed (see Box 2).

Table 2: Examples of wild animal studies which have applied functional omics tools and how they have added to our understanding of the wild animal microbiome. MGX= metagenomics, MTX= metatranscriptomics, MPX= metaproteomics, MBX= metabolomics.

Host Taxa	Function studied	Functional omics used	Added value of functional data	Reference
Capybara (*Hydrochoerus hydrochaeris*)	Host diet digestion	MGX, MTX, MBX	Identified microbial taxa involved in depolymerization and utilisation of plant fibres, as well as the enzymatic systems and metabolic pathways involved	(Cabral et al., 2022)
Midwife toad (*Alytes obstetricans)*	Host skin disease mitigation	MGX, MBX	Identified functionally-relevant taxa driving disease outcome (see also Box 1)	(Bates et al., 2022)
Giant panda (*Ailuropoda melanoleuca*)	Host diet digestion	MGX, MPX	Identified functionally-relevant taxa driving differences in body condition	(Huang et al., 2022)
Mammals	Host diet digestion	MGX, MTX	Showed that mammalian gut microbiota composition and functionality is partially shaped by host dietary differences	(Milani et al., 2020)

Ruddy Turnstone (*Arenaria interpres*)	Host metabolism	MTX	Showed that taxonomic composition of the microbiome and functional aspects of the metatranscriptome shift during weight gain	(Grond et al., 2023)
Common marmoset (*Callithrix jacchus*)	Various functions sensitive to environmental change	MGX, MTX	Found that differences in microbial gene expression were more dynamic and sensitive to environmental shifts than the abundances of microbial genes	(Uehara et al., 2022)
Wild (and captive) non-human primates	Host diet digestion	MGX, MTX	Identified that certain microbial taxa and functions were enriched in primate species with different diets	(M. Zhang et al., 2023)
Baleen whale (Mysticeti)	Host diet digestion	MGX	Showed that baleen whales, which prey on fish and crustaceans, harbour unique gut microbiomes with surprising parallels (in terms of functional capacity and taxonomy) to those of terrestrial herbivores	(Sanders et al., 2015)
Black howler monkeys (*Alouatta pigr*a)	Host diet shift	MBX	Showed that faecal metabolite profiles differed between seasons and were strongly associated with changes in plant metabolite consumption. However, faecal metabolite composition was not strongly associated with microbiome taxonomic composition	(Mallott et al., 2022)
Turtle ants (*Cephalotes)*	Nitrogen recycling (from diet and host excretions)	MGX, MBX	Used MBX to show that a specialised core gut microbiome provisions nitrogen for the host by recycling N-rich compounds (e.g. urea from diet). MGX then showed that this phenomenon is likely conserved across many ant species	(Hu et al., 2018)
Migratory bird species	Host metabolism and resistome	MGX	Showed that ten migratory bird species (predominantly waterfowl) vary greatly in gut bacterial composition but are similar in their microbiome metabolism and function	(Cao et al., 2020)
Wild vertebrates, including fish, birds, and mammals	Various functions including host diet digestion, toxin production and bioluminescence	MGX	Identified host traits that correlate with the composition, diversity, and functional content of their microbiota. For example, they identified proteases, some previously undescribed, from the gut of griffon vultures that degrade toxins present in their carrion diet	(Levin et al., 2021)
Vampire bats (*Desmodus rotundus*)	Host diet digestion	MGX	Compared the taxonomy and function of sanguivorous and non-sanguivorous bat gut microbiomes. The microbiome of vampire bats was functionally, but not taxonomically, distinct from non-sanguivorous bats suggesting that is highly specialised to its nutrient-poor diet	(Zepeda Mendoza et al., 2018)
Myrmecophagous mammals	Host diet digestion	MGX	Reconstructed bacterial genomes from the gut microbiome of nine myrmecophagous mammals. Many genomes carried chitinase genes and several were shared across host orders, suggesting convergent recruitment and highlighting their potential role in the digestion of insect prey	(Teullet et al., 2023)

Box 1: Amphibians as a Model for Host-Microbiome-Pathogen Interactions

Microbial communities associated with amphibians have been the focus of an enormous amount of research effort in the last decade, principally because of their role in defending their hosts from lethal pathogens such as *Batrachochytrium dendrobatidis* (*Bd*, reviewed in Rebollar et al., 2020) and ranavirus. For example, variation in the composition of skin-associated bacterial communities can predict the intensity of *Bd* outbreaks in natural systems (e.g. Bates et al., 2018). Furthermore, recent research has revealed the importance of the early-life gut bacterial microbiota for defence against infection by ranavirus in adulthood (Warne et al., 2019), and that skin bacterial diversity correlates with survival following ranavirus infection (Harrison et al., 2019). Though the majority of research to date has relied on metabarcoding approaches (see Table 1), combining multi-omic tools has yielded significant insight into the mechanism(s) by which amphibian hosts, resident microbes and wildlife pathogens interact.

A key component of amphibian immune defence is the production of antimicrobial peptides (AMPs) by the host, which can prevent infection by inactivating ranavirus virions (Chinchar et al., 2004) and *Bd* zoospores (reviewed in Varga et al., 2019) (Fig. 1A). Alongside these host derived defences, metabolites produced by resident bacteria and fungi (Kearns et al., 2017) on the skin can also prevent infection (Fig. 1B; see Woodhams et al., 2023). Indeed, experimental augmentation of the skin microbiome with a bacterium known to produce anti-*Bd* metabolites improved survival following *Bd* challenge by 40% (Kueneman et al., 2016). Furthermore, *in vitro* bacterial challenge assays have shown that more diverse bacterial communities can provide stronger or more consistent protection against a broad array of *Bd* genotypes (Harrison et al., 2020; Piovia-Scott et al., 2017). Other omics tools have provided further insight into these microbial defence mechanisms. Using a combination of metabarcoding & shotgun metagenomics, Rebollar et al (2018) quantified taxonomy-function associations in skin microbial communities of the amphibian host, *Craugastor fitzingerii.* They found that taxonomic similarity from metabarcoding data predicted similarity in functional profiles from metagenomics, and identified genes linked to the production of anti-*Bd* secondary metabolites in skin metagenomes (Fig. 1B). Bates et al (2022) integrated metabarcoding, metagenomics and metabolomics to investigate drivers of variation in *Bd* infection outbreak severity and dynamics in several populations of *Alytes obstetricans*; they found that both skin metagenome composition and metabolome varied depending on whether populations showed epizootic dynamics (associated with mass die offs) or more mild enzootic dynamics. Finally, metatranscriptomic data from a study investigating host transcriptional responses to viral infection in *Rana temporaria* have been used to discover a

novel virus, illustrating the utility of omic datasets for wildlife epidemiology (Parry et al., 2023).

Collectively these studies highlight the power of multi-omic studies for identifying the mechanisms by which host-associated microbes respond to pathogens. However, several outstanding questions remain. Chief amongst these is determining how and when host-derived AMPs and microbial metabolites interact to determine infection probability and disease severity (Fig. 1C). It has been hypothesised that amphibian hosts may 'select' for specific microbes through modulation of their AMP profiles, and that resident microbes may stimulate production of certain AMPs through cross-talk with the host immune system (Fig. 1C), though data for the latter are especially limited (Woodhams et al., 2023). Crucially, Rebollar et al (2018) found several genes involved in AMP resistance in metagenomes derived from the *C. fitzingerii* microbiome, illustrating a genomic mechanism by which symbiotic microbes can persist alongside host-derived immune defences. These insights would not be possible without functional multi-omic data. Unravelling complex interactions between host and microbial genomes, and how gene expression may shift in response to pathogens, requires that we continue to integrate omic approaches (Rebollar et al., 2016). Amphibians and their pathogens continue to provide a model system for understanding the interplay between hosts and their microbial symbionts. Using these systems, we can generate testable hypotheses about the mechanisms by which microbes drive variation in host life history in other wildlife species.

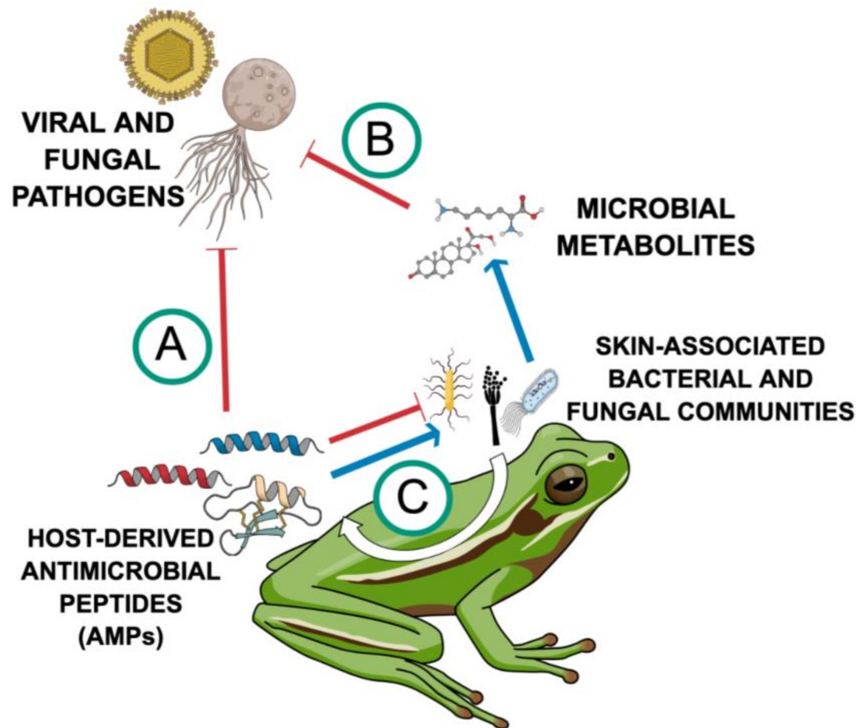

Figure 1. Simplified schematic of interactions between amphibian hosts, resident skin microbial communities, and pathogens. Both host-derived antimicrobial peptides (AMPs) **(A)** and metabolites from skin-associated microbes **(B)** can inactivate pathogens and either prevent infection or reduce burdens of infection. Though driven in part by host genomic variation, AMP composition in the 'mucosome' can select for particular suites of resident microbes, and microbe-host crosstalk may stimulate the production of AMPs **(C).** Red connecting lines represent inhibition, whilst blue lines represent promotion. Figure created in the Mind the Graph platform (mindthegraph.com)

Generating functional datasets for non-model organisms: challenges and potential solutions

Sample collection

A critical step in any microbiome workflow is preserving collected samples prior to further processing and analysis. Techniques that have been developed for laboratory systems or human samples may be helpful in some cases, however, collecting microbiome samples from wild animals comes with its own set of challenges. Often, sampling is undertaken in remote field locations without access to specialist equipment, and could take place days, or even months, before the samples are processed. As such, maintaining sample integrity can be a significant issue, particularly for functional studies that rely on biomolecules that are very sensitive to degradation, such as RNA or metabolites.

Freezing samples immediately (at -20°C or lower) is often considered to be the gold standard for preserving microbiome samples of all types (Cardona et al., 2012; Song et al., 2016). However, access to freezers is not always possible at remote field sites. As such, other alternatives have been extensively evaluated, particularly for DNA-based studies of faecal samples. For example, multiple studies suggest that storage in 95-100% ethanol or RNAlater can be an effective alternative if immediate freezing is not possible (Blekhman et al., 2016; Hale et al., 2015; Song et al., 2016; Vlčková et al., 2012). RNAlater has the added advantage that it can be used to preserve RNA, DNA, and proteins simultaneously (Mordant & Kleiner, 2021). Such buffers could be particularly useful if limited sample material is available and to improve cost-effectiveness. Similarly, pilot studies on human faecal samples have shown that buffers such as OMNImet-GUT and OMNIgene-GUT can simultaneously preserve both DNA and faecal metabolome profiles at room temperature for several days in a way that is comparable to flash-freezing (Lim et al., 2020; Ramamoorthy et al., 2021; Z. Wang et al., 2018). However, further studies are needed to establish the efficacy of these techniques in field-based studies.

The most appropriate method of sample storage will vary depending on the context, the availability of resources, and the research question. However, given that virtually all studies on sample storage have evidenced considerable variation in the ability of different methods to preserve sample integrity and information (Carruthers et al., 2019; Choo et al., 2015; Hale et al., 2015; Mordant & Kleiner, 2021; Vargas-Pellicer et al., 2019), using one method consistently within a study and/or system is an important consideration when planning a wild microbiome study to ensure results are comparable (Blekhman et al., 2016; Vargas-Pellicer et al., 2019).

Processing samples

Once samples have been preserved, the next step is to extract molecules of interest for sequencing or metabolic analysis. It is likely that extraction protocols will need to be optimised for each unique study system to ensure that the resulting material is representative of the original sample, and the quality is high enough for downstream processing.

Aside from high quality material, shotgun metagenomic sequencing pipelines also require high yields of DNA to be extracted (often tens of nanograms). This can be difficult for samples with low biomass, such as oral, cloacal, or skin swabs. Whilst studies have explored whole genome amplification methods to improve yield prior to sequencing such samples (Lasken, 2009; C. Wang et al., 2022), the extent to which these techniques impact upon subsequent community analysis remains unclear. As such, amplification methods are not widely applied in metagenomic library preparation pipelines. This means that certain microbiome sample types that yield high quantities of molecular material may be better suited for metagenomic sequencing.

Where adequate yields of starting material cannot be achieved, lower input methods such as metabarcoding, combined with tools such as PICRUST2 (see Table 1), could be used for coarse-scale functional inference. Whilst the accuracy and resolution of these functional predictions can be limited, there is some evidence that they enable coarse-level classification of microbiome functions that are comparable to results derived from shotgun metagenomic datasets (Toole et al., 2021).

A further consideration for methods that use untargeted shotgun sequencing approaches (e.g. metagenomics and metatranscriptomics) is the possibility of host contamination. Untargeted sequencing methods produce reads in proportion to the relative concentrations of DNA or RNA present within a sample; this means that, if co-extracted, host molecules can significantly overwhelm signals from the microbiome and result in poor sensitivity to detect low abundance microbial taxa due to poor coverage (Pereira-Marques et al., 2019). The amount of host contamination often varies depending on the sampling site. For example, in human studies, skin, saliva and vaginal samples suffer from greater levels of host contamination than faecal samples (Marotz et al., 2018). A high sequencing depth may be necessary to overcome host contamination issues, however, various chemical approaches have also been developed that can deplete host DNA sequences (Ahannach et al., 2021; Heravi et al., 2020; Marotz et al., 2018). Such depletion methods have mainly been applied

to human clinical samples and, whilst these may be transferable to other vertebrate systems, their general applicability to samples from non-mammalian hosts remains to be tested. It should also be noted that whilst it may be desirable to remove host molecules, these sequences could add valuable information to a study and dedicated tools have been developed for this purpose (Dylus et al., 2024). For example, it may be possible to reconstruct host genomes for phylogenetic analysis. This approach was recently demonstrated in a study on termites (*Isoptera*) whereby shotgun metagenomics enabled both functional profiling of the gut microbiome and reconstruction of near-complete host mitochondrial genomes across termite species (Arora et al., 2022).

Practicality of sequencing samples

Untargeted shotgun sequencing approaches can provide greater taxonomic resolution and functional insight compared to traditional metabarcoding techniques. However, the vast increase in sequencing depth required to achieve this resolution (tens to hundreds of millions of read pairs per sample, compared to just thousands of reads for metabarcoding studies) means that these approaches are also much more expensive (Ranjan et al., 2016; Stothart et al., 2022). In wild studies, where large sample sizes may be needed to detect small effects due to the noisy-nature of the data, such costs can be prohibitive. As such, less expensive alternatives could be used to gain coarse-level insight into the microbiome before carrying out more detailed functional analyses. For example, 16S rRNA gene metabarcoding, coupled with associated functional inference tools (Table 1), could be used to broadly characterise taxonomic and functional microbiome differences amongst groups of wild individuals. These results could be used to generate hypotheses to be further investigated in metagenomic studies on a more limited sample set.

As discussed, a disadvantage of the above approach is that metabarcoding datasets do not always reveal the full complexity of microbiome differences amongst samples. This was demonstrated in a recent study comparing the gut microbiota of two honey bee species; whilst 16S rRNA gene metabarcoding suggested that both host species carried the same, highly conserved bacterial phylotypes, metagenomic sequencing revealed that bacterial species and strains differed between the two hosts and that this had implications for polysaccharide degradation (Ellegaard et al., 2020).

Low coverage (“shallow”) metagenomic sequencing has been proposed as an economical alternative to deep metagenomic sequencing. Whilst this approach provides less resolution than deep metagenomic sequencing and does not enable the construction of MAGs, it provides the capability to accurately carry out species-level microbiome characterisation and

enables functional inference via information on microbial gene content (Hillmann et al., 2018; La Reau et al., 2023). A study using faecal samples from wild Sable Island horses (Stothart et al., 2022) showed that shallow metagenomic sequencing yielded near identical patterns of microbial alpha and beta diversity across samples when compared to a successively rarefied deep metagenomic sequencing dataset. This was despite read depths only reaching hundreds of thousands (compared to tens of millions) in the shallow dataset. Shallow shotgun sequencing also recapitulated microbiome taxonomic patterns found using a metabarcoding approach but yielded further functional insight than the metabarcoding dataset (Stothart et al., 2022). Thus, the accuracy and the comparative cost-effectiveness of shallow shotgun sequencing suggests that it may be a promising alternative to metabarcoding in wild animal studies. However, further studies are needed to validate shallow sequencing across other host taxa and sample types.

Analysis pipelines

In contrast to metabarcoding techniques where relatively well-standardised pipelines are in place (e.g. QIIME 2, Bolyen et al., 2019), numerous equivalent and competing bioinformatic pipelines for the analysis of more complex omic data have been developed. Choosing the most appropriate analysis for a given question and dataset is therefore not trivial. While we provide examples of such pipelines and tools for each omic data type in Table 1, we stress that the choice of pipeline requires a good understanding of the underlying methods, especially in non-model hosts where benchmarking of tools is usually lacking. Numerous standardised initiatives and studies have emerged to compare and benchmark tools and we refer readers to these to optimise their analytical tool choice (e.g. Jurado-Rueda et al., 2023; Meyer et al., 2022; Sczyrba et al., 2017; Ye et al., 2019). There are also a wealth of educational resources available (e.g. https://datacarpentry.org/, https://methods-in-microbiomics.readthedocs.io/en/latest/index.html) that can help the non-specialist to navigate through the jungle of non-standardized pipelines for omics data.

Taxonomic and functional profiling techniques rely on aligning reads, assembled genomes, or metabolic profiles to reference databases. This means that all omics tools are subject to database biases and incompleteness (Bundy et al., 2009; Jiang et al., 2016; Sun et al., 2020). Whilst this is a limitation facing all microbiome studies, it poses a particular issue for understudied wild taxa, given that databases are typically biased towards hosts of clinical or economic interest, such as humans, murine models, and ruminants (Sun et al., 2020). This is evidenced by the vast amounts of taxonomic novelty identified in recent large-scale metagenomics studies of wild animal microbiomes (e.g. Levin et al., 2021; Youngblut et al.,

2020). These results demonstrate that microbial databases lack information on the genetic diversity of microbes associated with most wild host species.

Certain bioinformatic approaches can be used to reduce the impact of database incompleteness on results. For example, in metagenomic studies, assembly-based (versus raw-read classification) approaches allow recovery of microbial sequences that have not yet been isolated or characterised and, thus, are absent from reference databases (Tamames et al., 2019). Assembly may also yield more accurate results because longer sequences are advantageous when assessing distant homology (Tamames et al., 2019). However, assembly-based methods are still influenced by database completeness to some extent and greater computational resources and higher coverage are required to achieve good assemblies. This, in turn, increases the costs per sample. Analytical tools are continuously being developed to improve the accuracy and resolution of taxonomic and functional profiling. For example, MetaPhlAn 4 now incorporates information on genome-sequenced isolates and metagenome assemblies from diverse environments (human and non-human hosts, as well as non-host associated) to enable more comprehensive microbiome taxonomic profiling (Blanco-Míguez et al., 2023).

Although databases are expanding exponentially, current biases do mean that some wild animals may be more amenable to functional studies than others. Host species that are closely related to those that are well-represented in databases may be better candidates. For example, the microbiotas of humans and murine models have been extensively characterised, potentially making it easier to functionally characterise the microbiomes of wild primates and wild mice.

Box 2: Outstanding questions for ecologists and evolutionary biologists in functional microbiome research

Adopting functional omics approaches to study microbial communities, and how they interact with both their wild animal hosts and the environment, unlocks a range of questions inaccessible to researchers using metabarcoding approaches alone. Here we list some key unanswered questions that should be addressed as an immediate priority by ecologists and evolutionary biologists studying host-microbe interactions, covering both fundamental and applied topics.

1. Is it common for wild animals to rely on a microbiome for a certain function?

2. How much of a given function is performed by each microbe under different abiotic or biotic conditions? For example, does a stressed or perturbed condition favour microbes which are metabolically independent (i.e. taxa that can independently complete all metabolic pathways defining a function)?
3. How do microbiome functions contribute to key host traits such as diet, cold tolerance, immunity and behaviour (e.g. via the gut-brain-axis)?
4. Is the microbiome causally related to variation in host fitness components (condition, survival, reproductive success) amongst individuals and which microbial functions contribute to this relationship?
5. How much functional redundancy is there in host-associated microbiotas, whereby taxonomically distinct communities converge on similar functional profiles?
6. Does functional redundancy contribute to microbiome resilience in the face of perturbation in the wild?
7. Is high functional diversity beneficial? For example, does it confer resistance against infection by pathogens, or greater tolerance of infection?
8. What does microbiome functional diversity represent in terms of host ecosystem services? Are microbiomes that are taxonomically rich more likely to contain the one species performing the beneficial function, or is microbiome function an emergent property of multiple synergistic interactions among microbes, including bacteria, viruses, and fungi?
9. How important is strain-level diversity for microbiome function in wild animals? How do strains evolve *in situ*?
10. How plastic is microbiome function? For example, do changes in microbial transcription enable hosts to rapidly respond to abiotic/biotic challenges? Or is compositional turnover and/or strain evolution more important?
11. How can the functional properties of a microbiota be manipulated, for example to improve host health outcomes and achieve conservation aims?

Conclusion

Huge progress has been made in characterising the taxonomic composition and diversity of wild animal microbiomes across many host species. However, despite this, we still have a relatively poor understanding of the functional characteristics, and importance, of the microbiome in wild hosts. This is largely because traditional marker gene profiling only provides limited taxonomic resolution and rough estimates of microbial functions.

To better understand if microbiomes play an important role in host physiology and fitness, their population size must be first quantified, at least in poorly characterised host taxa. Once it is clear that a resident and potentially beneficial microbiome colonises the host – which is the case in many animals – microbial functions can be then probed with more sophisticated omics tools. These tools represent a promising approach to move beyond descriptive studies, towards a deeper mechanistic understanding of host-microbiome interactions. The continued development of sample storage techniques, cheaper sequencing methods, analytical tools, and reference databases will further widen the applicability of these approaches to non-model organisms enabling key outstanding questions to be answered.

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Conflict of Interest

The authors declare no conflict of interest

Author Contributions

All authors were involved in developing ideas and concepts for the paper. SFW led the writing of the paper with significant input from FM, EV, XAH, JB, and KMW. All authors contributed critically to the drafts and gave final approval for publication.

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