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SYMPOSIUM

An Introductory "How-to" Guide for Incorporating Microbiome Research into Integrative and Comparative Biology

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Synopsis Research on host-associated microbial communities has grown rapidly. Despite the great body of work, inclusion of microbiota-related questions into integrative and comparative biology is still lagging behind other disciplines. The purpose of this paper is to offer an introduction into the basic tools and techniques of host-microbe research. Specifically, what considerations should be made before embarking on such projects (types of samples, types of controls)? How is microbiome data analyzed and integrated with data measured from the hosts? How can researchers experimentally manipulate the microbiome? With this information, integrative and comparative biologists should be able to include host-microbe studies into their research and push the boundaries of both fields.

Introduction

Overall, it is recognized that animals evolved in a microbial world, and so aspects of their biology should be tightly linked to the actions of their microbial partners (McFall-Ngai et al. 2013). These connections have been largely embraced in the field of human health, where the role of microbial communities in human nutrition and disease has been well studied (Kau et al. 2011; Cho and Blaser 2012). More recently, researchers have begun to incorporate microbiome research into the fields of integrative and comparative organismal biology (Kohl and Carey 2016). However, this area of research is still underdeveloped, and largely biased toward mammalian hosts (Colston and Jackson 2016). In this article I present tools and techniques for integrative and comparative biologists to incorporate microbiome research into their programs.

What is the microbiome?

Diverse microbial communities colonize various body sites of animal hosts. These communities contain bacteria, archaea, fungi, protozoans, and viruses. Although the terms "microbiome" and "microbiota" are largely used interchangeably, they do have some differences. The term "microbiome" has a number of definitions that range from specifying the collection of genes from a microbial community (Turnbaugh et al. 2008), to constituting a microbial community as well as their products and aspects of the host environment (Whiteside et al. 2015), to having both these meanings simultaneously (Huss 2014; Eisen 2015). For clarity, in this article I will use the term "microbiota" for describing the actual organisms or communities found within a sample, and the term "metagenome" for describing the collection of microbial genes contained in a community. Further, for the purposes of this paper I will largely focus on examples of gut bacterial communities, given that current research is highly biased toward these organisms. However, similar methods can be applied for other types of microbes (archaea [Gruninger et al. 2016], fungi [Miller et al. 2016], protozoans [Parfrey et al. 2014], viruses [Minot et al. 2011]) living on various body sites (skin [Kueneman et al. 2014], oral cavity [Stothart et al. 2016], scent glands [Theis et al. 2013], etc.).

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Considerations for designing studies on host-associated microbial communities

What to measure?

The most common method in the study of host-associated microbes is to conduct microbial inventories. This method involves the extraction of microbial DNA, followed by the amplification and sequencing of a marker gene (16S rRNA gene for bacteria and archaea; others for fungi, protozoa, etc.). These genes can be regarded as taxonomic markers and used to estimate taxonomic composition of a complex microbial community (Fig. 1). Additionally, if interested in microbial functions, one could conduct metagenomic sequencing of all genes of a complex microbial sample, or metatranscriptomic sequencing of all gene transcripts. The costs and benefits of these various methods have previously been discussed (Xu et al. 2014).

It should be noted that the most widely used technique (16S rRNA sequencing) provides information regarding the relative abundances of microbial taxa, but not the absolute abundances. The absolute abundances of bacteria can vary across host species (Cariveau et al. 2014; Sanders et al. 2017) and gut regions (Kohl et al. 2014a), which may have implications for the biological relevance of these communities. Absolute abundance of microbes can be measured several ways. For example, one could conduct qPCR of the 16S rRNA gene or other marker genes in a known amount of sample (Cariveau et al. 2014; Sanders et al. 2017), however, this method is unable to distinguish between live and dead microbes. Flow cytometry can differentially stain live and dead bacteria (Kohl et al. 2014a), though this method must be conducted in freshly collected samples. Integrating measures of absolute microbial abundance into experiments will further our understanding of the dynamics of these communities.

Sample collection and storage

A first consideration would be what types of samples to collect for a given study. For the gut microbiota, feces are a common source of gut contents. However, it should be recognized that microbial communities are structured spatially across the gut. Various gut chambers harbor distinct microbial communities in insects (Mikaelyan et al. 2017), rodents (Kohl et al. 2014a; Suzuki and Nachman 2016), reptiles (Colston et al. 2015; Kohl et al. 2016a), and likely other taxa as well. Additionally, microbial communities can vary between the gut lumen and the mucosa (Zoetendal et al. 2002). Sterile swabs have been used to sample the cloaca to characterize the gut microbial communities of reptiles and birds (Santos et al. 2012; Colston et al. 2015), and offer the benefit of controlling timing of sampling. In humans, fecal samples and rectal swabs exhibit similar microbial community structures (Bassis et al. 2017). While direct sampling of gut contents or tissue may be ideal for certain questions, this process

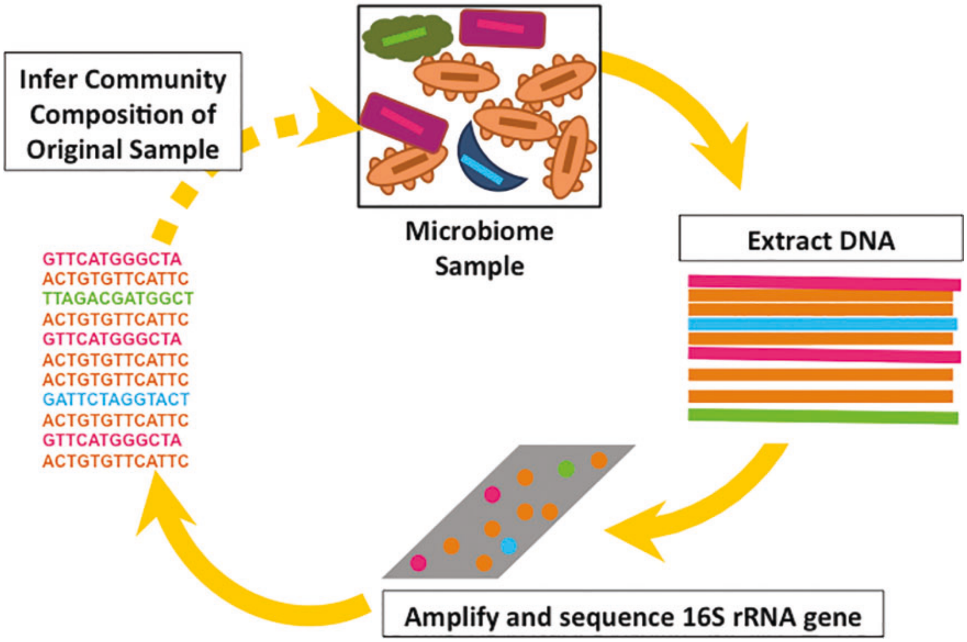

Fig. 1 A schematic of commonly conducted microbial inventories by high-throughput sequencing.

may be challenging as it often involves euthanizing and dissecting the animals. Feces or cloacal swabs may be more appropriate for repeated sampling and noninvasive/nonlethal sampling. Determining the appropriate sampling location will vary according to the questions and capabilities of various study systems.

When feces are non-invasively collected from the field, a number of environmental variables should be taken into account. For example, a large-scale study collecting feces from myrmecophagous (ant-eating) mammals from the field excluded 40% of their samples due to potential contamination from environmental sources (rain, soil, etc.; Delsuc et al. 2014). An additional detailed study into environmental contamination investigated how field conditions affect the microbial communities of primate feces (Hale et al. 2016). These researchers found that sunlight and contact with insects did not significantly alter microbial communities, though there was change in community structure over time (Hale et al. 2016). Last, rodent feces collected from Sherman traps are appropriate for microbiome studies, as the microbial communities are nearly identical to those collected under more sterile conditions (Kohl et al. 2015). Similar validation studies may be required for different sampling regimes depending on the study system.

Once samples are collected, they must be stored until DNA extraction. At field or room temperatures, the community composition of gut or fecal samples can change as certain microbial taxa bloom (Choo et al. 2015; Hale et al. 2016). Therefore, immediate freezing is often considered the “gold standard” for sample storage (Hale et al. 2016). However, immediate freezing may be difficult or impossible under field conditions. Numerous studies have demonstrated the utility of alternative storage methods for the preservation of microbial community structure, such as RNAlater (Vlčkova et al. 2012; Hale et al. 2015; Blekhman et al. 2016; Song et al. 2016), FTA cards (Fast Technology for Analysis of nucleic acids; Whatman Inc., Florham Park, NJ; Hale et al. 2015, 2016; Song et al. 2016), OMNIgene.GUT DNA Stabilization Kits (DNA Genotek; Choo et al. 2015; Song et al. 2016), and high percentage ethanol (Vlčkova et al. 2012; Hale et al. 2015; Blekhman et al. 2016). These preservation methods can result in slight alterations to microbial community structures, but these effects are typically smaller than the effects of individual variation (Blekhman et al. 2016; Song et al. 2016). Overall, the most important issue is that sample storage techniques are as consistent as possible across all samples in a given study.

Sample extraction and sequencing

Once in the laboratory, microbial DNA is extracted from samples. Differences in DNA extraction methods can impact the resulting microbial community structures (Yuan et al. 2012; Kennedy et al. 2014). Thus, similar to storage methods, it is important that all samples be extracted with the same protocols. An additional consideration is that extraction kits and reagents contain microbial DNA, which can influence sequencing results (Salter et al. 2014). This issue is especially problematic for low-biomass samples. Thus, it is recommended that researchers conduct “blank” extractions and submit these for sequencing. For example, many researchers had been investigating the potential symbiotic microbiota of the human placenta (Aagaard et al. 2014), but it was later revealed that these microbial communities did not differ significantly from contamination controls (Lauder et al. 2016). Studies may also benefit from the use of standard “mock communities”, mixtures of several known microbial members in known quantities, such as the ZymoBIOMICS Microbial Community Standard (Zymo Research, Irvine, CA). Researchers can extract and sequence the DNA from these mock communities to ensure that methods and results are consistent across experiments.

Extracted DNA then undergoes sequencing. Microbial 16S rRNA inventories can be conducted on a number of sequencing platforms, though the Illumina HiSeq and MiSeq platforms are most widely used (Caporaso et al. 2012), and more recently the PacBio platform (Schloss et al. 2016). A number of commercial services and university core facilities provide microbial inventories and sequencing services, and may provide some bioinformatics analyses. Samples can be sequenced to varying depths of coverage, which can depend on specific questions. If researchers are interested in rare microbial members, deep sequencing of few samples may be required. However, if comparing community structure across groups is the goal, it is generally thought that spending resources on more biological replicates is beneficial, or with increased spatial and temporal resolution (Caporaso et al. 2012; Knight et al. 2012).

Effect of captivity

The effects of captivity on microbial communities should also be considered for those who study animals in a laboratory setting. In captivity, animals may lose members of their native microbiota and gain foreign microbes from various sources. Differences in gut microbial community structures between wild and captive individuals have been demonstrated for insects (Hammer et al. 2014), fish (Dhanasiri et al. 2011), amphibians (Becker et al. 2014), reptiles (Kohl et al. 2016a), birds (Xenoulis et al. 2010; Wienemann et al. 2011), and mammals (Nelson et al. 2013; Kohl and Dearing 2014; Kohl et al. 2014b; Clayton et al. 2016). These differences in microbial communities are usually attributed to differences in diet between wild and captive settings, though other mechanisms may underlie these changes. The inclusion of soil from the field into cages acts as a microbial reservoir and increases the maintenance of microbial diversity on salamander skin (Loudon et al. 2014). We still lack an understanding of how other animal housing practices (regularity of cage-changes, efforts to minimize animal stress, etc.) might affect maintenance or loss of microbial diversity in captivity.

Understanding the role of environmental microbes

While animals host and maintain microbial populations, they are also constantly exposed to microbes in their diet and environment. For example, it is estimated that humans consume over a million microbes every day in their food (Lang et al. 2014). Ingested environmental microbes can sculpt the structures of resident microbial communities (Zhang et al. 2016). In natural settings, the gut communities of herbivorous woodrats (Kohl and Dearing 2014) and lizards (Kohl et al. 2016a) share >25% of their microbial members with the microbiota living on plant leaf surfaces. Researchers should collect samples of potential microbial sources (food items, soil, other environmental sources) and inventory the microbiota of these samples. The program SourceTracker (Knights et al. 2011) can be used to estimate the contribution of environmental sources to host-associated microbial communities. In the future, efforts could be made to understand the impact of environmental microbes on microbial community structure and function in ecological settings. Additionally, it would be interesting to understand how differences in host ecology might affect host-associated microbial communities. For example, are dietary generalists exposed to a greater diversity of environmental microbes compared with dietary specialists?

Analyzing microbial data

A large majority of studies inventory bacterial communities by amplifying and sequencing the 16S rRNA gene. Two major analysis pipelines are QIIME (Caporaso et al. 2010; Kuczynski et al. 2012) and mothur (Schloss et al. 2009). Depending on the experimental designs and specific questions, the statistical analysis of microbial data can be varied and complex. An online guide, GUide to STatistical Analysis in Microbial Ecology (GUSTA ME), allows researchers to explore different analyses and help them to choose the appropriate statistical techniques (Buttigieg and Ramette 2014). In integrative and comparative biology, researchers might be interested in connecting aspects of microbial community structure to other variables measured at the host level, such as host gene expression, hormone levels, or performance metrics. Multivariate Association with Linear Models (MaAsLin) can combine microbial data with information about the samples or hosts to find associations between these data types (Morgan et al. 2015).

For some questions, researchers may be interested in the functions encoded by microbial communities. The program PICRUSt allows researchers to use 16S rRNA sequencing inventories to predict the metagenomic content of a microbial community, based on previously sequenced genomes (Langille et al. 2013). However, researchers should exercise caution in using this technique on uncharacterized microbial communities, as microbes may have novel gene content that is not reflected in genomic databases (Langille et al. 2013). Overall, PICRUSt is better for generating hypotheses rather than drawing conclusions. Direct metagenomic and metatranscriptomic sequencing can better illuminate microbial functions, and the abundances of particular functions can be compared across treatment groups. Numerous programs have been developed for the assembly (Vollmers et al. 2017) and analysis of metagenomic (Lindgreen et al. 2016) datasets. Similarly, pipelines have been generated for metatranscriptomic datasets (Leimena et al. 2013). The field of data analysis moves quickly, and the citations here are by no means exhaustive. Collaboration with a bioinformatician may help to ensure that analyses of these more complex datasets are conducted correctly.

Upon publication, microbial data should be made accessible to other researchers, often through deposition in a repository, such as the NCBI SRA (Sequence Read Archive). Importantly, researchers should also include all relevant metadata, such as minimum information about a metagenome sequence (MIMS; Field et al. 2008), as well as other variables (host age, sex, diet, treatment, etc.) in their reports. These data are crucial for the repeatability of data analyses.

Experimental manipulation of microbial communities

Antibiotics have long been used as a method of experimentally manipulating microbial communities.

For example, understanding the nutritional roles of insect endosymbionts has been accomplished by removing endosymbionts with antibiotics and providing diets supplemented with essential amino acids or vitamins (Douglas and Prosser 1992; Hosokawa et al. 2010). Antibiotics have also been used to demonstrate the role that gut microbes play in metabolizing plant toxins for mammalian herbivores (Kohl et al. 2014c), or have been administered to penguin chicks in the field to investigate their effects on growth (Potti et al. 2002). It should be recognized though, that antibiotic compounds themselves could have confounding effects on animal physiology, and so studies should either incorporate proper controls or be interpreted cautiously.

Germ-free rearing of animals is another way to experimentally manipulate the microbiota. Here, animals are raised under completely sterile environments so that they do not harbor microbial communities. These techniques have been developed for several model systems such as mice (Arvidsson et al. 2012), fruit flies (Sang and King 1961), pigs (Meyer et al. 1964), and zebrafish (Pham et al. 2008). However, there are also methods to generate germ free *Nasonia* jewel wasps (Shropshire et al. 2016), desert locusts (Charnley et al. 1985), among other hosts. Germ-free systems offer the unique opportunity to investigate the effects of specific microbial members on host phenotypes. For example, mice inoculated with various microbial isolates exhibit differential gene expression (Hooper et al. 2001) and immune function (Geva-Zatorsky et al. 2017) in gut tissues. Additionally, locusts inoculated with varying combinations of gut bacteria have differential resistance to infection by a pathogen (Dillon et al. 2005).

Even though germ-free techniques were developed in model systems, they have much to offer the fields of integrative and comparative biology. First, some studies have inoculated the microbiota from wild animals into germ-free animals to investigate the effects on animal phenotypes. For example, germ-free mice inoculated with the feces from bears preparing for hibernation exhibit higher fat gain than mice inoculated with the feces from hibernating bears, suggesting that the summer gut microbiota aids bears in pre-hibernation weight gain (Sommer et al. 2016). Additionally, the techniques used to generate germ-free zebrafish have been adapted to generate populations of germ-free stickleback fish (Milligan-Myhre et al. 2016), and could be used to develop other germ-free systems.

In the absence of germ-free techniques, microbial transplants may offer another method to experimentally manipulate the microbiota. For example, transplantation of the feces of toxin-adapted woodrats (*Neotoma lepida*) into toxin-naïve woodrats or into laboratory rats conferred toxin tolerance in recipient animals (Kohl et al. 2014c, 2016b). Techniques have been developed to treat conventional mice with antibiotics and subsequently transfer the microbial communities from rats or humans (Manichanh et al. 2010; Hintze et al. 2014). These transplantation methods may be cheaper than germ-free techniques, though may not fully transfer the donor community. Moreover, while the effects of fecal transplants are largely attributed to bacteria, it should be recognized that feces contain many other organisms, host coloncytes, and metabolites which could underlie changes in the recipient (Bojanova and Bordenstein 2016). In the future, researchers could develop techniques to conduct microbial transplants in animal taxa other than rodents.

Conclusions

Animals live in many challenging environments, and their associated microbial communities may perform a wide array of functions, thus conferring ecological adaptation and the colonization of these habitats (Alberdi et al. 2016). Incorporating host–microbe interactions into our study of integrative and comparative biology will enhance our understanding of the roles microbes play in ecology and evolution. Additionally, the field of host–microbe interactions has much to gain from the fields of integrative and comparative biology. One example is the symbiosis between squid and microbes that reside in their light organs, which is now a widely used model system to investigate host–microbe interactions (McFall-Ngai 2014). Other animal systems may lend themselves to increase our understanding of the basic principles of host–microbe interactions. With the tools and techniques described above, integrative biologists can begin to investigate these connections in their own study systems, helping to bridge these fields.

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