A bug’s life: Delving into the challenges of helminth microbiome studies

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The body of vertebrates is inhabited by trillions of microorganisms, i.e., viruses, archaea, bacteria, and unicellular eukaryotes, together referred to as the “microbiota.” Similarly, vertebrates also host a plethora of parasitic worms (the “macrobioota”), some of which share their environment with the microbiota inhabiting the gastrointestinal (GI) tract [1]. Complex interactions between the helminths and the gut microbiota have been associated with establishment of parasite infection, disease manifestations, and host immune-modulation [2, 3]. Remarkably, not only GI helminths alter the gut microbiome composition [4], but also the infections with blood flukes of the genus Schistosoma have been associated with intestinal dysbiosis, that even occurs before the onset of egg laying [5, 6]. Comparably, over the last decade, evidence has emerged of the contribution(s) of the resident microbiota to several physiological and reproductive processes of invertebrate hosts, including insects, arachnids, worms, and snails [7, 8]. These noteworthy discoveries, coupled with the recent expansion of high-throughput microbiota- and microbiome-profiling approaches (the former referring to the community of microorganisms themselves and the latter to the microorganisms and their genomes, within a given ecological niche), are rapidly leading to a much better understanding of the composition and functions of microbial communities inhabiting parasitic worms of major public health and socioeconomic significance. This basic knowledge might expose exploitable vulnerabilities of parasites, thus paving the way to the development of novel control strategies [9].

In this Viewpoint, we consider the challenges associated with the study of the helminth microbiota/-me, spanning not only bacteria transiently associated with parasites in which the life cycle includes free-living and parasitic stages, but also putative helminth endosymbionts. Indeed, endosymbionts have been described in both roundworms and flatworms [10, 11]. In nematodes, the most notable example of a mutualistic relationship between worms and bacteria is represented by filarial parasites [12], i.e., Onchocerca volvulus, Wuchereria bancrofti, and Brugia malayi, agents of human lymphatic filariasis. In particular, the fitness, propagation, and survival of these worms depend on endosymbiotic bacteria of the genus Wolbachia that have thus become the target of intense research aimed to develop novel filaricidal compounds [11, 13]. On the other hand, bacteria of the genus Neorickettsia have been identified in the endoparasitic digeneans, i.e., trematodes [10]. These intracellular bacteria inhabit the worm reproductive tissues and are vertically-transmitted to the next generation of parasites via the eggs [10]. In addition, horizontal transmission of Neorickettsia from the fluke to the fluke-infected vertebrate host, where the bacteria colonize macrophages among other cell types, is a determinant
for the pathogenesis of severe disease in, for example, horses, dogs, and humans [14]. Recently, we have sequenced and characterized the whole genome of a Neorickettsia endobacterium in an isolate of adult Fasciola hepatica liver flukes [15]. The Neorickettsia, related to the etiological agents of human Sennetsu and Potomac horse fevers, was localized in the gonads of the liver fluke and its DNA detected by PCR in eggs, thus supporting a germline transmission [15].

To decipher the role of parasite-associated microbiota on the pathophysiology of helminthiases, the Parasite Microbiome Project (PMP) was launched in January 2019 [9]. Importantly, the PMP encourages best practices for experimental designs to ensure robust and reliable comparisons between datasets and promotes the inclusion of appropriate controls to correctly identify environmental microbial contaminants [9]. These practices are particularly important in experiments in which microbiota profiling is conducted using next generation sequencing (NGS) (i.e., high-throughput) technologies that are particularly prone to exogenous bacterial contamination, such as bacterial 16S rRNA-amplicon sequencing on low-biomass samples, e.g., helminths [16, 17]. Therefore, given the potential confounders in helminth-associated microbiome studies, we propose that four elements, outlined below, must be considered in order to generate reliable and reproducible data (Fig 1).

1: Appropriate controls

The identification and characterization of the helminth-associated microbiota/me includes several experimental steps from sample collection to library preparation and sequencing, each of which is exposed to different sources of contamination. Therefore, the inclusion of matching negative controls (“blanks”) in each step of the experiment is critical. The sample collection should ideally be carried out under clean conditions by using disposable sterile consumables and autoclaved instruments to minimize the risk of sample contamination with environmental bacteria. In addition, controls for each tentative source of environmental contaminants should be included. Following thorough screening, the sequence data generated from these negative controls from each experimental step can be subtracted from the datasets under consideration.

2: Microscopical visualisation of helminth-associated bacteria

Following in silico subtraction of putative contaminant sequences, unequivocal identification and characterization of worm microbiomes can rely on microscopical techniques aimed to localize bacteria of interest across different helminth tissues and developmental stages. Widely used approaches to localize specific groups of microorganisms are based on fluorochromes conjugated to either antibodies or nuclei acid probes that bind to specific bacterial proteins or nuclei acids, respectively. Neorickettsia bacteria were identified within the reproductive tissue of the liver fluke F. hepatica via fluorescent immunohistochemistry [15], whereas a recent report characterized the “core microbiome” associated with the ovine GI nematode Haemonchus contortus using fluorescence in situ hybridization and light and transmission electron microscopy [18].

3: “Core microbiome” versus transiently associated bacteria

The localization of microorganisms in helminth tissues is a robust indication of the occurrence of a worm microbiome, and may provide clues on its function(s); for instance, Wolbachia localized in the reproductive tissues of filarial parasites have been shown to be involved in sexual differentiation [19] and worm survival [20]. However, the distinction between bacteria that might be transiently associated with the parasite, e.g., coating the surface of free-living larval stages or transported within the alimentary tract of the parasite among different host niches, and those that might belong to the worm “core microbiome” is crucial.
Protocols to eliminate bacteria contaminating the external tegument of GI worms have been implemented. Treatment of *Trichuris muris* worms with sodium hypochlorite allowed the identification of a specific parasite intestinal microbiota distinct from that of its host [2]. Whether transiently associated bacteria have direct effects on parasite biology still needs to be investigated.

Fig 1. Key elements for a reliable and reproducible characterization of the helminth-associated microbiome. NGS: next generation sequencing.

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ascertained; notwithstanding, they might contribute to the pathophysiology of the infection and comorbidity in the host. The Asian liver fluke *Opisthorchis viverrini* provides a signal example in this regard; accumulating evidence suggests that the juvenile form of the parasite excysted in the duodenum of the human host might ingest *Helicobacter pylori* and/or related species of bacteria and transport the bacillus to the bile duct, where this fluke establishes [21, 22]. Chronic infection with either *H. pylori* bacteria or *O. viverrini* is classified by the International Agency for Research on Cancer as a Group 1 carcinogen, leading to gastric adenocarcinoma or cholangiocarcinoma (CCA), respectively [23]. We have previously reported a synergistic association between the liver fluke and *Helicobacter* bacteria in the development of the opisthorchiasis-associated CCA [21], which may result from an eventual horizontal transmission of bacteria from the parasite to host tissue. On the other hand, a worm “core microbiome” (particularly, if associated with the parasite gonads) may be vertically-transmitted to the next generation and, hence, detected across different developmental stages. Therefore, screening for the presence of bacteria in different developmental stages of the parasite, either by PCR, qPCR, and/or 16S-rRNA amplicon NGS [15, 18] is recommended to define the “core microbiome” that might serve as a foundation to explore novel strategies for transmission control. In addition to bacteria, the “core microbiome” may comprise microeukaryotes, such as fungi and protozoa, and viruses that can be detected by shotgun metagenomic approaches [9]. Although these methods are not inexpensive and generate complex data (which require advanced bioinformatics analyses that include the identification and in silico subtraction of helminth-derived sequences), their application is recommended to gain an overall picture of the helminth microbiome and enable the prediction of bacterial metabolic pathways that might be essential for worm biology and (patho)physiology associated with the infection [24]. Subsequently, the use of functional approaches to investigate the roles of this “core microbiome” in worm biology, helminth infection, establishment, and host–parasite interactions becomes critical.

4: Functional studies of the helminth-associated microbiome

The follow-on step after the identification of both transiently associated bacteria and the worm “core microbiome” is to understand the biological relevance of these interactions. The use of broad- or narrow-spectrum antibiotics to alter the worm microbiome might assist the determination of the essentiality of these bacteria for worm survival, fitness, and/or reproduction [25]. In addition, optimization of protocols for in vitro and *ex vivo* culturing of parasitic developmental stages [26–28], and the use of organoids to simulate interactions between parasites, host cells, and selected bacteria [29], in tandem with functional genomic tools currently under development for helminths (e.g., genome editing [30–32]) and bacteria [33] will assist the setup of controlled experiments to address hypotheses on mechanisms underlying worm–microbes interactions. Similar approaches have been employed for model organisms such as *Caenorhabditis elegans*. The *C. elegans*–associated microbiome has been analyzed in laboratory settings by culturing worms on individual bacterial strains and evaluating helminth growth rate and responses of stress and immune related genes. The majority of the bacterial strains investigated were found to be beneficial for worm fitness [34]. Finally, where feasible, *in vivo* studies using rodent models of helminth infection might provide invaluable functional insights on transmission of bacteria across parasitic developmental stages, microbial horizontal translocation to host tissues, and bacteria-mediated pathologies associated with helminthiases. Germ-free and gnotobiotic mice (i.e., animals exclusively colonised by known microbes) are extensively used in microbiome studies [35]. However, the dysfunctional immune response of these animals might add several confounders to the infection model. On the other hand, the
use of antibiotics in well-established murine models of helminthiases might allow to target specific groups of host- and/or worm-associated bacteria.

To conclude, similarly to the human microbiome, bacteria associated with helminth parasites likely represent an intrinsic part of these organisms, so much so that parasite biology might not be completely understood in its absence. However, the characterization of the genuine helminth microbiome may turn out to be complex, or even daunting, due to several technical challenges. In our view, rigorous hygiene to exclude or at least minimize contaminants, together with bacterial localization in helminth tissues and across developmental stages, and their functional characterization are essential steps to unequivocally identify bacteria associated with parasitic worms and ascertain their roles in the dynamic crosstalk among the parasite, the host, and the host microbiome. Ultimately, this will contribute to the current incomplete understanding of the biology and pathogenicity of helminths.

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