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Invited review

Helminths and microbes within the vertebrate gut – not all studies are created equal

Alba Cortés[1], Laura E. Peachey [1,2], Timothy P. Jenkins[1], Riccardo Scotti[1] and Cinzia Cantacessi[1]

[1]*Department of Veterinary Medicine, University of Cambridge, Madingley Road CB3 0ES, Cambridge, United Kingdom*

[2]*Bristol Veterinary School, Faculty of Health Sciences, University of Bristol, Langford House, Langford, BS40 5DU, Bristol, United Kingdom*

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Corresponding author:
Cinzia Cantacessi
Department of Veterinary Medicine, University of Cambridge, Madingley Road CB3 0ES, Cambridge, United Kingdom
Tel. +44 (0) 1223 760541
Fax. +44 (0)1223 337610
E-mail: cc779@cam.ac.uk

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SUMMARY

The multifaceted interactions occurring between gastrointestinal (GI) parasitic helminths and the host gut microbiota are emerging as a key area of study within the broader research domain of host-pathogen relationships. Over the past few years, a wealth of investigations has demonstrated that GI helminths interact with the host gut flora, and that such interactions result in modifications of the host immune and metabolic statuses. Nevertheless, whilst selected changes in gut microbial composition are consistently observed in response to GI helminth infections across several host-parasite systems, research in this area to date is largely characterised by inconsistent findings. These discrepancies are particularly evident when data from studies of GI helminth-microbiota interactions conducted in humans from parasite-endemic regions are compared. In this review, we provide an overview of the main sources of variance that affect investigations on human-helminth-gut microbiota interactions and propose a series of methodological approaches that, whilst accounting for the inevitable constraints of human fieldwork, are aimed at minimising confounding factors and draw biologically meaningful interpretations from highly variable datasets.

1. INTRODUCTION

A plethora of experimental evidence supports a key role of infections by gastrointestinal (GI) helminth parasites in shaping the composition of the vertebrate gut microbiota, with significant implications for local and systemic host immunity (reviewed by Brosschot and Reynolds, 2018). For instance, recent studies have partly attributed the parasite-associated qualitative and/or quantitative alterations to host GI microbial profiles to the ability of GI helminths to stimulate the initial onset of T-regulatory (Treg) immune responses (cf. Cantacessi *et al.* 2014; Reynolds *et al.* 2014; Giacomin *et al.* 2015, 2016; Zaiss *et al.* 2016). On the other hand, other studies have reported associations between acute helminth infections and gut microbiota imbalances (= dysbiosis) characterised by significant expansion of populations of putative pro-inflammatory bacteria (e.g. Rausch *et al.* 2013; Jenkins *et al.* 2018a; Schneeberger *et al.* 2018a); these observations have lent credit to the hypothesis that helminth-associated alterations of gut microbiota composition may lead to both localised and systemic consequences for the host organism, that include immunopathology and exacerbated malnutrition in at-risk subjects from parasite-endemic areas (reviewed by Glendinning *et al.* 2014; Houlden *et al.* 2015; Cattadori *et al.* 2016).

Over the past decade, newly acquired knowledge of the impact that GI helminth infections exert on the vertebrate gut microbial composition and metabolism has contributed to a better understanding of parasite systems biology and host-pathogen interactions (reviewed by Peachey *et al.* 2017; Leung *et al.* 2018; Rapin and Harris *et al.* 2018), and has been proposed as a first step towards the identification and development of novel strategies of parasite control based on the targeted manipulation of the host gut microbiota (cf. Peachey *et al.* 2017). Nevertheless, for humans in particular, progress in this field of research is greatly impaired by the impact of several confounding factors that inevitably affect studies conducted in naturally infected individuals (Mutapi, 2015; Chabé *et al.* 2017). In this review, we summarise current knowledge of GI helminth-microbiome interactions in humans under natural conditions of infection, identify similarities and differences between datasets and provide an overview of the confounding factors that may affect the interpretation of findings.

2. HUMAN-HELMINTH-GUT MICROBIOTA INTERACTIONS IN REAL-WORLD SCENARIOS

In endemic areas for helminthiases, the vast majority of infected individuals harbour multiple helminth species, often occupying different niches of the host organism (Hotez *et al.* 2010). Whilst polyparasitism is often regarded as a major confounding factor in investigations of parasite-microbiota interactions conducted in humans under natural conditions of infection (Cooper *et al.* 2013; Jenkins *et al.* 2017; Martin *et al.* 2018; Rosa *et al.* 2018), findings from these studies are key to assessing the impact that GI helminths exert on gut microbiota homeostasis in a 'real-world' scenario. Nevertheless, several factors should be considered when interpreting results obtained from individuals infected by multiple helminth species. First, anthropometric (e.g. age and gender) and anthropologic variables (e.g. ethnicity, diet and occupation) are well known to profoundly impact the 'baseline' composition of the human gut microbiota (Sekirov *et al.* 2010; Yatsunenko *et al.* 2012) (cf. Fig. 1); therefore, the enrolment of large cohorts of individuals is often necessary in order to achieve sufficient statistical power and avoid uninformative and/or misleading results (Kelly *et al*. 2015). However, in many studies, the number of individuals enrolled and samples analysed is inevitably dictated by logistical and financial constraints. In these instances, population-related variables that impact gut microbiota composition may contribute substantially to inconsistencies among findings from different studies (cf. Fig. 1). For instance, a negative association between colonisation by the whipworm *Trichuris trichiura* and the abundance of bacteria belonging to the genus *Prevotella* in the faeces of infected individuals has been reported in two separate studies conducted in Malaysia (Lee *et al.* 2014; Ranaman *et al.* 2016), while other studies conducted in Ecuador, and Liberia and Indonesia, respectively, have failed to identify significant variations in faecal populations of *Prevotella* in individuals either solely infected by *T. trichiura* or co-infected with other species of soil-transmitted helminths (STHs) (Cooper *et al*. 2013; Martin *et al*. 2015; Rosa *et al*. 2018).

In addition, whilst Rosa and co-authors (2018) detected several distinctive features in the gut microbial profiles of helminth-harbouring individuals that were specifically associated to single

infections with the hookworm *Necator americanus*, the roundworm *Ascaris lumbricoides* or *T. trichiura*, such features were inconsistent between two independent cohorts of helminth-infected volunteers from Liberia and Indonesia; this discrepancy suggests that other yet undetermined environmental factors may contribute to qualitative and quantitative alterations of the gut microbial profiles of helminth-infected individuals from different geographical areas. In contrast, an association between the abundance of selected bacterial taxa and infections by one or more STHs could be consistently detected in samples from both Liberian and Indonesian cohorts (Rosa *et al.* 2018). These taxa included bacteria belonging to the genera *Olsenella* and *Allobaculum*, which were expanded in the gut microbiota of helminth-infected individuals when compared to that of uninfected controls. To the best of our knowledge, the study by Rosa *et al*. (2018) was the first to report a link between infections by STHs and the abundance of these bacterial genera in the human gut. Interestingly, in mice suffering from metabolic syndrome, administration of probiotics was followed by expansion of populations of *Olsenella* and/or *Allobaculum*, and a reduction in systemic and/or local gut inflammatory responses (Wang *et al.* 2015). Moreover, *Allobaculum* spp. are putative producers of anti-inflammatory short-chain fatty acids (Greetham *et al.* 2004), and are severely reduced in the gut of mice genetically predisposed to spontaneous colitis (Pérez-Muñoz *et al.* 2014). This knowledge led Rosa *et al.* (2018) to hypothesize that these bacteria may play a yet undetermined role in the anti-inflammatory properties of parasitic helminths, and reinforce the proposition that the interactions between hosts, parasites and gut microbiota are multidirectional and should be approached in a holistic manner (e.g. Cortés *et al*. 2018; Leung *et al*. 2018). Interestingly, in contrast to evidence acquired in human hosts, a negative association between the genus *Allobaculum* and colonisation by GI helminths has been observed in a mouse model of chronic trichuriasis (Holm *et al.* 2015), in which Th1-mediated immune responses are dominant (reviewed by Cliffe and Grencis, 2004), as well as in mice with patent infection by the blood fluke *Schistosoma mansoni* (Jenkins *et al.* 2018a), in which migrating eggs are responsible for the onset of marked Th2-mediated inflammatory responses (reviewed by Pearce and MacDonald, 2002). The immune-molecular mechanisms *via* which members of the genus *Allobaculum* may regulate local and systemic inflammation are still unclear (Greetham *et*

al. 2004; Pérez-Muñoz *et al.* 2014; Wang *et al.* 2015). Nonetheless, current data showing reductions in populations of *Allobaculum* alongside helminth-associated gut inflammation supports the hypothesis raised by Rosa *et al*. (2018); in the future, rodent models of GI helminth infections whose gut microbiota is deprived of, and subsequently recolonised with, the genus *Allobaculum* could be exploited to investigate the potential involvement of these bacteria in parasite-mediated immunomodulation.

Beside the intrinsic variability of the human gut microbiota, studies conducted under natural conditions of helminth colonisation are likely to be affected by factors linked to the different combinations of infecting species and their relative abundances. For instance, in a study conducted in a cohort of Ecuadorian children, the specific features detected in the gut microbial profiles of subjects co-infected with *T. trichiura* and *A. lumbricoides* could not be identified in the microbiota of *Trichuris*-only infected individuals (Cooper *et al.* 2013). Similarly, selected microbial features that were observed in studies conducted in human volunteers with mono-specific infections with, for instance, *A. lumbricoides*, could not be detected in the gut microbiota of subjects harbouring the same parasite alongside other helminth species (e.g. *T. trichiura* and *N. americanus*) (Rosa *et al.* 2018), thus suggesting that a complex interplay exists between the host gut and its macro- and microbiota, that might be difficult to replicate in experimental settings. Furthermore, current evidence obtained from animal models of helminth infections indicates that worm burdens can impact the nature and/or the magnitude of parasite-associated alterations in gut microbial composition (Wu *et al.* 2012; Peachey *et al.* 2018). Nevertheless, such evidence is not yet available for human infections, in which parasite burdens may range from low to very high in endemic areas (Barbour and Kafetzaki, 1991; Churcher *et al*. 2005).

Another frequent constraint of investigations conducted in cohorts of human subjects with natural helminth infections is the limited availability of 'genuine' negative controls, i.e. individuals from the same communities of parasite-infected subjects who lack previous exposure to infections by parasitic helminths. Instead, individuals with no evidence of patent helminth infections are inevitably enrolled as control subjects (e.g. Cooper *et al.* 2013; Lee *et al.* 2014; Jenkins *et al.*

2017; Rosa *et al.* 2018); nevertheless, studies in helminth-infected individuals subjected to anthelmintic treatment, as well as in primates and pigs exposed to *Trichuris* spp., have shown that parasite-associated alterations in gut microbial communities can persist, at least partly, in absence of active infections (Broadhurst *et al.* 2012; Wu *et al.* 2012; Cooper *et al.* 2013; Kay *et al.* 2015; Schneeberger *et al.* 2018a). These data call for caution when interpreting differences between the gut microbial profiles of helminth-infected and uninfected volunteers from the same communities. In addition, patent infections are often diagnosed using stool-based microscopic methods, that are known for their relatively low sensitivity and that may yield false negative results, e.g. in case of intermittent shedding of eggs and/or larvae (O'Connell and Nutman, 2016). Recently, Rosa *et al*. (2018) used quantitative real-time PCR to diagnose STH infections in individuals subjected to gut microbiota profiling, indicating that this technique may represent a robust and sensitive alternative to microscopic methods, since it provides users with the ability to semi-quantify burdens of different helminth species from minute amounts of DNA template. However, in spite of their higher sensitivity, molecular methods rely on the use of primers that selectively target the parasite species of interest, thus impairing the simultaneous detection of potential (asymptomatic or subclinical) co-infections with other helminth and/or non-helminth pathogens (O'Connell and Nutman, 2016). Indeed, the impact of protozoa on the gut microbial diversity and composition has been clearly demonstrated in humans and other vertebrates (reviewed by Chabé *et al.* 2017; Stensvold and van der Giezen, 2018). Furthermore, a recent study conducted in a cohort of Colombian schoolchildren reported common features in the faecal microbial composition of subjects co-infected with helminths and protozoans and mono-parasitized with the flagellate *Giardia intestinalis* compared to uninfected individuals (Toro-Londono *et al.* 2019). Whilst the mechanisms *via* which each group of parasites alters the host gut flora, as well as the nature of such alterations, are yet to be determined, these findings support the need to conduct additional diagnostic tests on stool samples from helminth-infected cohorts, as well as the corresponding uninfected subjects, in order to rule out the influence of concomitant bacterial, viral and/or protozoan infections that may be responsible for the changing gut microbial profiles of these individuals (cf. Chabé *et al.* 2017).

Nevertheless, in spite of the several confounding factors outlined above (cf. Fig. 1), observational studies in helminth endemic areas have proven useful for the identification of significant associations between parasite colonisation and the gut microbial profiles of humans under natural conditions of infection. Importantly, studies conducted in these communities provide excellent opportunities to evaluate the effect(s) that parasite removal (e.g. via the administration of broad-spectrum anthelmintics) exert(s) on the gut microbiota of previously infected individuals, thus contributing cues to understand the causality of helminth-microbiota relationships.

3. IMPACT OF DEWORMING ON THE HUMAN GUT MICROBIOTA

The implementation of mass drug administration programmes in endemic areas for STHs and schistosomiasis offers opportunities to elucidate potential mechanisms *via* which parasitic helminths modulate the host gut microbiota. For instance, qualitative and quantitative changes in gut microbial profiles that are caused by direct interactions between parasites and gut bacteria may be expected to rapidly reverse following parasite removal, whilst long-lasting alterations are likely to result from indirect interplay mediated by the host immune system (Houlden *et al*. 2015; Su *et al.* 2018). Nevertheless, such investigations are also generally constrained by the presence of several confounding factors that include not only the host- and parasite-dependent variables outlined above, but also variations linked to the use of different drugs and treatment regimes (Schneeberger *et al*. 2018b), as well as time of sampling post-anthelmintic treatment (Houlden *et al.* 2015) (Fig. 1). The latter in particular may profoundly affect findings from these studies, as the presence of tissue lesions caused by e.g. parasite feeding activity and location (e.g. blood-feeders *vs*. non blood-feeders and luminal *vs*. tissue dwellers) are likely to influence the timespan between helminth removal and microbiome recovery (reviewed by Leung *et al.* 2018). Moreover, for ethical reasons, data from these experiments is often biased by the lack of placebo-treated control groups. These limitations may be at least partially responsible for the differences between findings from studies aimed at elucidating the effect of deworming on the gut microbiota of helminth-infected volunteers; notwithstanding, it is worth noting that, in instances where

deworming-associated changes in human gut microbial profiles were detected, these were generally moderate (Ramanan *et al.* 2016; Martin *et al.* 2018; Schneeberger *et al.* 2018b).

Consistent with this, a recent study conducted on faecal samples collected from a rural community in Indonesia reported that the composition of the gut microbiota of individuals repeatedly treated with either albendazole or placebo (for 21 months) resembled that of samples collected from the same subjects prior to treatment, rather than that of uninfected controls (Rosa *et al.* 2018). Moreover, a parallel investigation conducted on the same cohort of individuals detected reduced populations of *Prevotella* in albendazole-treated subjects in which complete deworming did not occur, compared to placebo-treated individuals with patent helminth infections (Martin *et al.* 2018). Intriguingly, failure of albendazole treatment was accompanied by a dominance of *T. trichiura* (over other helminth species) in these subjects, while placebo-treated individuals maintained a diverse macrobiota (i.e. multiple helminth infections); hence, differences in the composition of the GI macrobiota (i.e. species present and their relative abundances) between albendazole- and placebo-treated individuals could account for variations in the composition of the intestinal microflora of these subjects (Martin *et al.* 2018). Significant associations between colonisation by *T. trichiura* and *Prevotella* abundance were not observed in the Indonesian cohort (Martin *et al.* 2018; Rosa *et al.* 2018). However, negative associations between whipworm infections and *Prevotella* abundance had been detected previously in two independent studies conducted in Malaysia (Lee *et al.* 2014; Ramanan *et al.* 2016). In particular, Ramanan and co-authors (2016) observed that, following albendazole treatment, expansion of *Prevotella* populations in the human faecal microbiota was related to reduced *T. trichiura* faecal egg counts. In contrast, no significant associations between helminth infection and abundance of bacteria belonging to the genus *Prevotella* was reported in a study investigating the impact of parasite colonisation and successful treatment with a combination of albendazole and ivermectin on the faecal microbial profiles of a cohort of *Trichuris*-infected children from Ecuador (Cooper *et al.* 2013), nor in a group of helminth-infected adults from Sri Lanka treated with pyrantel pamoate (Jenkins *et al.* 2017). Similarly, no qualitative or quantitative changes to faecal microbial

236 composition were observed in two cohorts of schoolchildren from Côte d'Ivoire and Zimbabwe
237 infected by *S. mansoni* and *S. haematobium*, respectively, following treatment with praziquantel
238 (Kay *et al*. 2014; Schneeberger *et al.* 2018a). However, successful elimination of *S. mansoni* was
239 associated with a higher abundance of *Fusobacterium* spp. pre-treatment, as well as 24 hrs post-
240 treatment (Schneeberger *et al.* 2018a).

241 Whilst drug administration in endemic regions may result in effective elimination of helminth
242 infections, potential co-infecting protozoan parasites are not susceptible to anthelmintic
243 treatment; this, together with the sub-standard hygienic and sanitary conditions that generally
244 characterise these areas and that result in continuous re-exposure to infective helminth
245 developmental stages (Campbell *et al*. 2018), impairs the full assessment of the consequences of
246 helminth removal on the composition of the human gut microbiota. To the best of our knowledge,
247 thus far, a single study has investigated the effects of chronic infections by a GI helminth,
248 *Strongyloides stercoralis*, and anthelmintic treatment on the composition of the faecal microbiota
249 and metabolome of humans from a non-endemic area of Europe, where parasite transmission had
250 been interrupted (Jenkins *et al.* 2018b). Treatment with ivermectin resulted in compositional
251 changes of the faecal microbiota (analysed 6 months post-treatment), which partially resembled
252 that of uninfected control subjects (Jenkins *et al.* 2018b); in particular, alpha diversity [= a
253 measure of the number of bacterial species present in a given microbial community (richness) and
254 their relative abundance (evenness)] was reduced in the microbiota of individuals post-treatment
255 (although statistical significance was not achieved) and accompanied by expanded populations of
256 potentially pathogenic bacteria (Jenkins *et al.* 2018b). In addition, the faecal metabolic profiles
257 obtained from samples collected post-ivermectin treatment shared features with both those
258 obtained from samples collected pre-treatment and from uninfected controls (Jenkins *et al.*
259 2018b); this observation led Jenkins *et al*. (2018b) to hypothesise that, following parasite removal
260 and over time, both gut microbiota and metabolome may revert to the original pre-infection state.
261 Multiple factors, including but not limited to those outlined above, may contribute to the
262 discrepancies observed between the findings from this work and those that reported no or minor

effects of anthelmintic treatment on the gut microbiome of helminth-infected humans (Cooper *et al.* 2013; Ramanan *et al.* 2016; Martin *et al.* 2018; Rosa *et al*. 2018; Schneeberger *et al.* 2018a,b).

Despite the limitations outlined above, studies of GI helminth-microbiota relationships conducted in endemic areas for helminthiases have provided repeated evidence of the perturbations that parasites and anthelmintic treatment exert on the equilibrium of resident populations of gut bacteria and on gut homeostasis. However, the identification of common signatures across studies remains key to designing future experiments, e.g. in animal models of helminth infections, that may assist the elucidation of the mechanisms that underpin the interactions between GI helminths, the gut microbiota and the host immune system.

4. DO COMMON SIGNATURES EXIST ACROSS STUDIES OF HOST-HELMINTH-MICROBIOTA INTERACTIONS?

The identification of gut microbial signatures that occur reproducibly across several host-GI helminth systems is crucial for designing novel anti-helminth intervention strategies based on the manipulation of the gut microbiota (Peachey *et al.* 2017). Studies conducted in animal models of helminth infections are expected to assist the identification of such signatures, as well as the direct (i.e. parasite-mediated) and/or indirect (i.e. immune-mediated) mechanisms that govern helminth-microbiota interactions (Cortés *et al.* 2018); nevertheless, the inconsistencies that characterise studies of helminth-microbiota relationships published to date make such a task highly challenging. Indeed, for patterns to be identified, fluctuations in selected populations of gut microbes must be interpreted in light of the physical and immunological alterations of the mucosal environment in which such alterations occur (Leung *et al.* 2018). For instance, expanded populations of *Lactobacillaceae* have been repeatedly detected following infection with several species of parasitic helminths in several host species (Reynolds *et al.* 2014; Duarte *et al*. 2015; Holm *et al.* 2015; Houlden *et al.* 2015; Cattadori *et al.* 2016; Jenkins *et al.* 2018a; Kim *et al*. 2018), and could thus be considered as a ‘consistent alteration’ in gut microbiota composition upon helminth colonisation. However, key differences exist between host-parasite pairs investigated in the studies that have reported such an outcome. Indeed, whilst populations of

290 *Lactobacillaceae* promote regulatory responses in mice infected by *Heligmosomoides polygyrus*
291 *bakeri* (Reynolds et al. 2014), a lack of correlation between *Lactobacillaceae* abundance and Treg
292 populations has been observed in other host-parasites systems, such as mice chronically infected
293 with *T. muris* and rabbits infected with *Trichostrongylus retortaeformis*, in which the expansion
294 of populations of gut *Lactobacillaceae* upon helminth infection occurs in an environment
295 dominated by Th1-mediated immune responses (Holm *et al.* 2015; Houlden *et al.* 2015; Cattadori
296 *et al.* 2016). These differences suggest that alternative mechanisms may regulate the
297 differentiation and development of adaptive immune responses in each host-parasite system
298 (Houlden *et al.* 2015), and thus that similar alterations in gut microbiota composition may result
299 in different consequences that are dependent on the microenvironment where these changes occur.
300 Notwithstanding, the interactions between hosts, helminths and the gut microbiota are likely
301 multifaceted and multidirectional, and therefore the potential consequences that selected
302 compositional changes in gut microbiota exert on host homeostasis are only one aspect of these
303 complex interplay. For instance, a common yet undetermined mechanism may determine the
304 expansion of *Lactobacillaceae* in the gut of helminth-infected hosts.

305 On the other hand, apparent 'contradictory' findings across studies may result from fundamental
306 differences between gut compartments under investigation. For instance, *Prevotella* spp. was
307 expanded in the abomasum and faeces of sheep infected by abomasal trichostrongyles (i.e.
308 *Haemonchus contortus* and *Teladorsagia circumcincta*; Li *et al.* 2016; Cortés *et al.* in
309 preparation), whilst the same taxa were reduced in the faeces of a range of host species, including
310 mice, humans and horses, infected by nematodes residing in the large intestine, i.e. *Trichuris* spp.
311 and cyathostomins, respectively (Lee *et al.* 2014; Houlden *et al.* 2015; Peachey *et al.* submitted).
312 It must be noted, however, that whilst increased abomasal pH favours *Prevotella* overgrowth in
313 the abomasum (De Nardi *et al.* 2016; Li *et al.* 2016), the same taxa are likely to be exposed to a
314 dramatically different microenvironment in the large intestine that may determine the contraction
315 of these bacterial groups. In addition, given the functional dissimilarities between the abomasal
316 and colonic microbiota, such alterations are expected to result in fundamentally different

outcomes for the homeostasis of each of these gut compartments (Ley *et al.* 2008), and hence comparisons are, in our opinion, unwarranted.

In parallel to species of bacteria with functions that may vary depending on the gut compartment, multiple taxa share the same functions in different microenvironments (Lozupone *et al.* 2012); therefore, it is plausible that, even though inconsistencies are detected across studies, these may result in similar functional alterations in the host-parasite pairs being compared. For instance, recent studies in mouse and humans infected with *S. mansoni* have reported the expansion of different genera of bacteria with pro-inflammatory functions in the gut microbiota of the respective hosts (Jenkins *et al.* 2018a; Schneeberger *et al.* 2018a). These observations lend credit to the hypothesis that the functional role of the gut microbiota in helminth infections could be far less 'diverse' than the taxonomic associations reported thus far. For this hypothesis to be confirmed or confuted, a better understanding of the function(s) of each bacterial taxon inhabiting the different gut compartments in a range of host species is needed. To this aim, the integration of metagenomic, metabolomic and metatranscriptomic technologies, alongside traditional microbiology and microscopy techniques, may assist to achieve a holistic picture of the impact of GI helminth infections on the functions of the human gut microbiota, and its significance for disease pathophysiology and overall host health (Wang *et al.* 2015).

5. CURRENT NEEDS AND FUTURE DIRECTIONS

Understanding the complex interactions between GI helminths and their vertebrate hosts is pivotal for advancing our knowledge of the fundamental biology of these parasites and the diseases they cause (see Peachey *et al.* 2017; Leung *et al.* 2018; Rapin and Harris *et al.* 2018 for reviews). Whilst the role of the gut microbiota in host-parasite relationships has long been overlooked, current knowledge of the key roles that resident bacteria play in host health and disease, together with recent technical advancements for microbiota profiling, have boosted research is this area. This is currently leading to increasing evidence of a role for the gut microbiota in the immune regulatory properties of helminth parasites (Cantacessi *et al.* 2014; Reynolds *et al.* 2014; Giacomin *et al.* 2015, 2016; Zaiss *et al.* 2016). In addition, data collected to date points towards

a likely role of the gut microflora in the immunopathology of selected GI helminth infections that awaits experimental validation. Trying to untangle the relevance of particular fluctuations of specific bacterial taxa on infection outcome is challenging; nevertheless, currently available data suggest that low-intensity, long-term helminth infections are commonly linked to high microbial diversity and predominance of bacteria typically associated with gut health. Conversely, high-intensity, acute infections are often associated to gut dysbiosis, characterised by reduced alpha diversity and an increase in pro-inflammatory and often opportunistic pathogens (Peachey *et al*. 2017). However, for this knowledge to be exploited in translational studies, further investigations in both natural and experimental settings are needed to distinguish spurious results from genuine helminth-microbiota associations (Peachey *et al*. 2017), and mechanistic studies in animal models of helminth infections are necessary to dissect the causality of these relationships (cf. Cortés *et al.* 2018). Importantly, minimising variations between studies is crucial to warrant meaningful comparisons between datasets.

Whilst reducing the variability amongst samples collected from naturally helminth-infected humans may be difficult to achieve, the enormous impact that differences in technical and experimental approaches (from sample collection to bioinformatics and biostatistical analyses) exert on the overall variation detected across studies can be reduced (Figs. 1 and 2; Lindgreen *et al*. 2017; Costea *et al.* 2017; Golob *et al*. 2017). In particular, a range of bioinformatics pipelines are available for the analysis of high-throughput amplicon and metagenomics sequence datasets that include, e.g., different sequence-processing tools and reference databases for sequence annotation that could yield slightly different results (Lindgreen *et al*. 2017; Golob *et al*. 2017). For instance, the use of validated open microbiome analysis packages such Multiplexed Analysis of Projections by Sequencing (MAPseq) (Matias Rodrigues *et al*. 2017) or QIIME2 (https://qiime2.org/) may assist accurate taxonomic classifications of bacterial 16S rRNA amplicon datasets; similarly, sequence annotation should rely on the use of regularly updated reference databases. Amongst these, SILVA (https://www.arb-silva.de/) (Quast *et al*. 2013) enables sensitive annotations of bacterial rRNA sequence data (Almeida *et al*. 2018). The use of

371 such standardized analysis workflows and reference databases for sequence annotation might
372 prove extremely useful to increase consistency across studies and enable researchers to identify
373 common and/or unique features between the gut microbiota of different host-parasite systems
374 which, in turn, might assist to better understand the mechanisms that regulate helminth-microbiota
375 relationships.

376 The consequences that elucidating such mechanisms may exert on future strategies of parasite
377 control are two-fold. First, disentangling the potential contribution of the gut flora to the
378 pathogenesis of the infection is necessary in order to discover and develop new strategies to
379 contrast helminth-associated pathology. Second, understanding the microbiota-dependent
380 mechanisms by which parasitic helminths are able to modulate host immune responses and
381 suppress inflammation may assist the discovery of novel immune-regulatory therapeutics against
382 chronic inflammatory disorders of the GI tract that may act in synergy with helminth-based
383 therapy (see Peachey *et al*. 2017 and Rapin and Harris, 2018 for reviews). However, in order for
384 this new knowledge to be fully exploited in translational research, further studies that thoroughly
385 consider inclusion/exclusion criteria for the selection of participants, include appropriate controls,
386 and follow standardised experimental and data analysis protocols are necessary, and will allow to
387 disentangle the potential influence of parasite-, drug- and/or population-dependent variables in
388 each setting (Fig. 2).

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586 FIGURE LEGENDS

587 Fig. 1 Sources of variation and confounding factors potentially impacting the outcome of studies
588 of human-helminth-gut microbiota interactions in helminth-endemic regions.

589 Fig. 2 Proposed approaches aimed at reducing the methodological sources of variation
590 surrounding investigations of human-helminth-gut microbiota interactions.

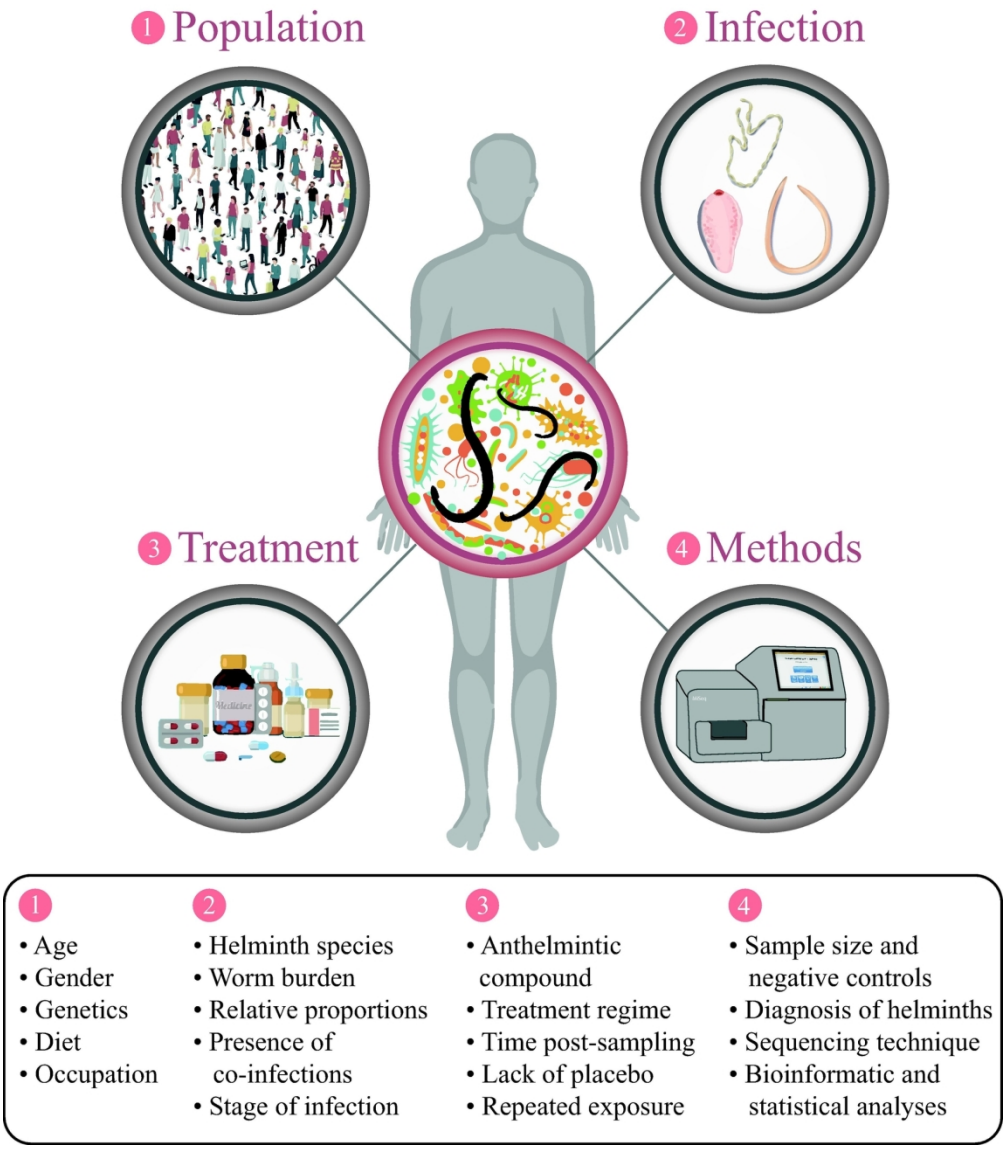

Figure 1. Sources of variation and confounding factors potentially impacting the outcome of studies of human-helminth-gut microbiota interactions in helminth-endemic regions.

158x180mm (300 x 300 DPI)

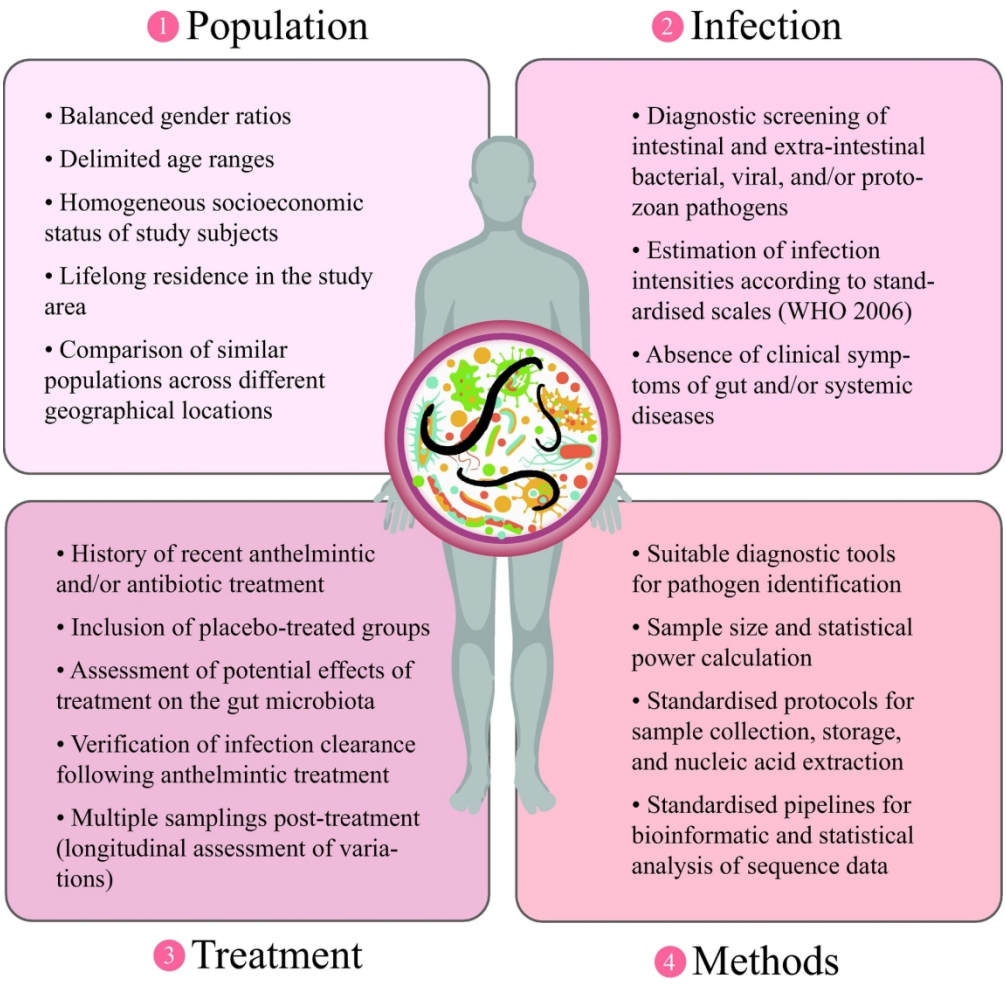

Figure 2. Proposed approaches aimed at reducing the methodological sources of variation surrounding investigations of human-helminth-gut microbiota interactions.

163x164mm (300 x 300 DPI)

Invited review

Helminths and microbes within the vertebrate gut – not all studies are created equal

Alba Cortés[1], Laura E. Peachey [1,2], Timothy P. Jenkins[1], Riccardo Scotti[1] and Cinzia Cantacessi[1]

[1]*Department of Veterinary Medicine, University of Cambridge, Madingley Road CB3 0ES, Cambridge, United Kingdom*

[2]*Bristol Veterinary School, Faculty of Health Sciences, University of Bristol, Langford House, Langford, BS40 5DU, Bristol, United Kingdom*

Running title: Helminth-microbiota interactions in the human gut

Corresponding author:
Cinzia Cantacessi
Department of Veterinary Medicine, University of Cambridge, Madingley Road CB3 0ES, Cambridge, United Kingdom
Tel. +44 (0) 1223 760541
Fax. +44 (0)1223 337610
E-mail: cc779@cam.ac.uk

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SUMMARY

The multifaceted interactions occurring between gastrointestinal (GI) parasitic helminths and the host gut microbiota are emerging as a key area of study within the broader research domain of host-pathogen relationships. Over the past few years, a wealth of investigations has demonstrated that GI helminths interact with the host gut flora, and that such interactions result in modifications of the host immune and metabolic statuses. Nevertheless, whilst selected changes in gut microbial composition are consistently observed in response to GI helminth infections across several host-parasite systems, research in this area to date is largely characterised by inconsistent findings. These discrepancies are particularly evident when data from studies of GI helminth-microbiota interactions conducted in humans from parasite-endemic regions are compared. In this review, we provide an overview of the main sources of variance that affect investigations on human-helminth-gut microbiota interactions and propose a series of methodological approaches that, whilst accounting for ~~taking into account~~ the inevitable constraints of human fieldwork, are aimed at minimising confounding factors and draw biologically meaningful interpretations from highly variable datasets.

1. INTRODUCTION

A plethora of experimental evidence supports a key role of infections by gastrointestinal (GI) helminth parasites in shaping the composition of the vertebrate gut microbiota, with significant implications for local and systemic host immunity (reviewed by Brosschot and Reynolds, 2018). For instance, recent studies have partly attributed the parasite-associated qualitative and/or quantitative alterations to host GI microbial profiles to the ability of GI helminths to stimulate the ~~initiate~~ initial ~~the~~ onset of T-regulatory (Treg) immune ~~mechanisms~~responses~~, which result in down-regulation of inflammatory responses and establishment of chronic infections, to helminthparasite-associated qualitative and/or quantitative alterations to GI microbial profiles the ability to initiate the onset of T-regulatory (Treg) immune mechanisms, that result in down-regulation of inflammatory responses and establishment of chronic infections~~ (cf. Cantacessi *et al.* 2014; Reynolds *et al.* 2014; Giacomin *et al.* 2015, 2016; Zaiss *et al.* 2016). On the other hand, other studies have reported associations between acute helminth infections and gut ~~microbiome~~ microbiota imbalances (= dysbiosis) characterised by ~~that involve~~ significant expansion of populations of putative pro-inflammatory bacteria (e.g. Rausch *et al.* 2013; Jenkins *et al.* 2018a; Schneeberger *et al.* 2018a); these observations have~~,~~ ~~thus lending~~ lent credit to the hypothesis that helminth-associated alterations of gut microbiota composition~~me~~ may lead to both localised and systemic consequences for the host organism, that includ~~eing~~ immunopathology and ~~(e.g. Rausch *et al.* 2013; Jenkins *et al.* 2018a; Schneeberger *et al.* 2018a), and as well as~~ exacerbated malnutrition in at-risk subjects from parasite-endemic areas (reviewed by Glendinning *et al.* 2014; Houlden *et al.* 2015; Cattadori *et al.* 2016).

Over the past decade, newly acquired knowledge of the impact that GI helminth infections exert on the vertebrate gut microbial~~ome~~ composition and metabolism has contributed to a better understanding of parasite systems biology and host-pathogen interactions (reviewed by Peachey *et al.* 2017; Leung *et al.* 2018; Rapin and Harris *et al.* 2018), and has been proposed as a first step towards the identification and development of novel strategies of parasite control based on the targeted manipulation of the host gut microbiota (cf. Peachey *et al.* 2017). Nevertheless, for humans in particular, progress in this field of research is greatly impaired by the impact of several

confounding factors that inevitably affect studies conducted in naturally infected individuals (Mutapi, 2015; Chabé *et al.* 2017). In this review, we summarise current knowledge of GI helminth-microbiome interactions in humans under natural conditions of infection, identify similarities and differences between datasets and provide an overview of the confounding factors that may affect the interpretation of findings.

2. HUMAN-HELMINTH-GUT MICROBIOTA INTERACTIONS IN REAL-WORLD SCENARIOS

In endemic areas for helminthiases, the vast majority of infected individuals harbour multiple helminth species, often occupying different niches of the host organism (Hotez *et al.* 2010). Whilst polyparasitism is often regarded as a major confounding factor in investigations of parasite-microbiota interactions conducted in humans under natural conditions of infection (Cooper *et al.* 2013; Jenkins *et al.* 2017; Martin *et al.* 2018; Rosa *et al.* 2018), findings from these studies are key to assessing the impact that GI helminths exert on gut microbiota homeostasis in a 'real-world' scenario. Nevertheless, several factors should be considered when interpreting results obtained from individuals infected by multiple helminth species. First, anthropometric (e.g. age and gender) and anthropologic variables (e.g. ethnicity, diet and occupation) are well known to profoundly impact the 'baseline' composition of the human gut microbiotame (Sekirov *et al.* 2010; Yatsunenko *et al.* 2012) (cf. Fig. 1); therefore, the enrolment of large cohorts of individuals is often necessary in order to achieve sufficient statistical power and avoid uninformative and/or misleading results (Kelly *et al*. 2015). However, in many studies, the number of individuals enrolled and samples analysed is inevitably dictated by logistical and financial constraints. In these instances, population-related variables that impact gut microbiota composition may contribute substantially to inconsistencies among findings from different studies (cf. Fig. 1). For instance, a negative association between colonisation by the whipworm *Trichuris trichiura* and the abundance of bacteria belonging to the genus *Prevotella* in the faeces of infected individuals has been reported in two separate studies conducted in Malaysia (Lee *et al.* 2014; Ranaman *et al.* 2016), while other studies conducted in Ecuador, and Liberia and Indonesia,

respectively, have failed to identify significant variations in faecal populations of *Prevotella* in individuals either solely infected by *T. trichiura* or co-infected with other species of soil-transmitted helminths (STHs) (Cooper *et al*. 2013; Martin *et al*. 2015; Rosa *et al*. 2018).

In addition, whilst Rosa and co-authors (2018) detected several distinctive features in the gut microbial profiles of helminth-harbouring individuals that were specifically associated to single infections with the hookworm *Necator americanus*, the roundworm *Ascaris lumbricoides* or *T. trichiura*, such features were inconsistent between two independent cohorts of helminth-infected volunteers from Liberia and Indonesia~~, respectively~~; this discrepancy suggests that other yet undetermined environmental factors may contribute to qualitative and quantitative alterations of the gut microbial profiles of helminth-infected individuals from different geographical areas. In contrast, an association between the abundance of selected bacterial taxa and infections by one or more STHs could be consistently detected in samples from both Liberian and Indonesian cohorts (Rosa *et al.* 2018). These taxa included bacteria belonging to the genera *Olsenella* and *Allobaculum*, which were expanded in the gut microbiota of helminth-infected individuals when compared to that of uninfected controls. To the best of our knowledge, the study by Rosa *et al*. (2018) was the first to report a link between infections by STHs and the abundance of these bacterial genera in the human gut. Interestingly, in mice suffering from metabolic syndrome, administration of probiotics was followed by expansion of populations of *Olsenella* and/or *Allobaculum*, and a reduction in systemic and/or local gut inflammatory responses (Wang *et al.* 2015). Moreover, *Allobaculum* spp. are putative producers of anti-inflammatory short-chain fatty acids (Greetham *et al.* 2004), and are severely reduced in the gut of mice genetically predisposed to spontaneous colitis (Pérez-Muñoz *et al.* 2014). This knowledge led Rosa *et al.* (2018) to hypothesize that these bacteria may play a yet undetermined role in the anti-inflammatory properties of parasitic helminths, and reinforce the proposition that the interactions between ~~thus underpinning the general idea that~~ hosts, ~~-~~parasites and ~~-~~gut microbiota are ~~interactions are~~ multidirectional and should be approached in ~~from~~ a holistic ~~perspective~~ manner (e.g. Cortés *et al*. 2018; Leung *et al*. 2018). Interestingly, i~~I~~n contrast to evidence acquired in human hosts, a

negative association between the genus *Allobaculum* and colonisation by GI helminths has been observed in a mouse model of chronic trichuriasis~~mice chronically infected with *T. muris*~~ (Holm *et al.* 2015), in which ~~is featured by a dominant~~ Th1-mediated immune responses are dominant (reviewed by Cliffe and Grencis, 2004), as well as in mice ~~and~~ with patent infection by the blood fluke *Schistosoma mansoni* (Jenkins *et al.* 2018a), in which migrating eggs are responsible for the onset of marked Th2-mediated inflammatory responses ~~are elicited to migrating eggs~~ (reviewed by Pearce and MacDonald, 2002). The immune-molecular mechanisms ~~*through*~~ *via* which members of the genus *Allobaculum* may regulate local and systemic inflammation are ~~yet to be elucidated~~still unclear (Greetham *et al.* 2004; Pérez-Muñoz *et al.* 2014; Wang *et al.* 2015). Nonetheless, current data ~~experimental evidence on~~ showing ~~concomitant~~ reductions in populations of *Allobaculum* ~~and~~alongside helminth-associated gut inflammation supports ~~seems consistent with~~ the hypothesis ~~of~~ raised by Rosa *et al.* (2018); in the future,~~,~~ ~~suggesting that laboratory~~ rodent models of GI ~~helminthiasis~~ helminth infections whose gut microbiota is deprived of, and subsequently recolonised with, the genus *Allobaculum* could be exploited to investigate the potential involvement of these bacteria in ~~the~~ parasite-mediated immunomodulation ~~mediated by helminth parasites (e.g. via exogenous recolonization with *Allobaculum* spp.).~~ ~~Notably, both models of helminth infection are characterised by the occurrence of severe intestinal inflammation involving different populations of T CD4+ cells (i.e. Th1 and Th2, respectively; Pearce and MacDonald, 2002; Cliffe and Grencis, 2004), and therefore, the observed reduction in populations of *Allobaculum* in these systems supports the immune regulatory role for this bacterial genus.~~

Beside the intrinsic variability of the human gut microbiota, studies conducted under natural conditions of helminth colonisation are likely to be affected by factors linked to the different combinations of infecting species and their relative abundances. For instance, in a study conducted in a cohort of Ecuadorian children, the specific features detected in the gut microbial profiles of subjects co-infected with *T. trichiura* and *A. lumbricoides* could not be identified in the microbiota of *Trichuris*-only infected individuals (Cooper *et al.* 2013). Similarly, selected

154 microbial features that were observed in studies conducted in human volunteers with mono-
155 specific infections with, for instance, *A. lumbricoides*, could not be detected in the gut microbiota
156 of subjects harbouring the same parasite alongside other helminth species (e.g. *T. trichiura* and
157 *N. americanus*) (Rosa *et al.* 2018), thus suggesting that a complex interplay exists between the
158 host gut and its macro- and microbiota, that might be difficult to replicate in experimental settings.
159 Furthermore, current evidence obtained from animal models of helminth infections indicates that
160 worm burdens can impact the nature and/or the magnitude of parasite-associated alterations in gut
161 microbial composition (Wu *et al.* 2012; Peachey *et al.* 2018).~~;~~ ~~nevertheless~~Nevertheless, such
162 evidence is not yet available for human infections, in which ~~whose burdens~~ parasite burdens ~~in~~
163 ~~endemic areas~~ may range from low to very high ~~due to overdispersion of parasite loads~~ in endemic
164 areas (Barbour and Kafetzaki, 1991; Churcher *et al.* 2005) ~~and, therefore, are likely to be an~~
165 ~~important confounding factor for studies of parasite-microbiota interactions in naturally infected~~
166 ~~individuals~~.

167 Another frequent constraint of investigations conducted in cohorts of human subjects with natural
168 helminth infections is the limited availability of 'genuine' negative controls, i.e. individuals from
169 the same communities of parasite-infected subjects who lack previous exposure to infections by
170 parasitic helminths. Instead, individuals with no evidence of patent helminth infections are
171 inevitably enrolled as control subjects (e.g. Cooper *et al.* 2013; Lee *et al.* 2014; Jenkins *et al.*
172 2017; Rosa *et al.* 2018); nevertheless, studies in helminth-infected individuals subjected to
173 anthelmintic treatment, as well as in primates and pigs exposed to *Trichuris* spp., have shown that
174 parasite-associated alterations in ~~the~~ gut microbial communities can persist, at least partly~~ially~~, in
175 absence of active infections (Broadhurst *et al.* 2012; Wu *et al.* 2012; Cooper *et al.* 2013; Kay *et*
176 *al.* 2015; Schneeberger *et al.* 2018a). These data call for caution when interpreting differences
177 between the gut microbial profiles of helminth-infected and uninfected volunteers from the same
178 communities. In addition, patent infections are often diagnosed using stool-based microscopic
179 methods, that are known for their relatively low sensitivity and that may yield false negative
180 results, e.g. in case of intermittent shedding of eggs and/or larvae (O'Connell and Nutman, 2016).

Recently, Rosa *et al*. (2018) used quantitative real-time PCR to diagnose STH infections in individuals subjected to gut microbiotame profiling, indicating that this technique may represent a robust and sensitive alternative to microscopic methods, since it provides users with the ability to semi-quantify burdens of different helminth species from minute amounts of DNA template. However, in spite of their higher sensitivity, molecular methods rely on the use of primers that selectively target the parasite species of interest, thus impairing the simultaneous detection of potential (asymptomatic or subclinical) co-infections with other helminth and/or non-helminth pathogens (O'Connell and Nutman, 2016). Indeed, the impact of protozoa on the gut microbial diversity and composition has been clearly demonstrated in humans and other vertebrates (reviewed by Chabé *et al.* 2017; Stensvold and van der Giezen, 2018). Furthermore, a recent study conducted in a cohort of Colombian schoolchildren reported common features in the faecal microbial composition of subjects co-infected with helminths and protozoans and mono-parasitized with the flagellate *Giardia intestinalis* compared to uninfected individuals (Toro-Londono *et al.* 2019). Whilst the mechanisms *via* which each group of parasites alters the host gut flora, as well as the nature of such alterations, are yet to be determined, these findings support the need to conduct additional diagnostic tests on stool samples from helminth-infected cohorts, as well as the corresponding uninfected subjects, in order to rule out the influence of concomitant bacterial, viral and/or protozoan infections that may be responsible for the changing gut microbial profiles of these individuals (cf. Chabé *et al.* 2017).

Nevertheless, in spite of the several confounding factors outlined above (cf. Fig. 1), observational studies in helminth endemic areas have proven useful for the identification of significant associations between parasite colonisation and the gut microbial profiles of humans under natural conditions of infection. Importantly, studies conducted in these communities provide excellent opportunities to evaluate the effect(s) that parasite removal (e.g. via the administration of broad-spectrum anthelmintics) exert(s) on the gut microbiota of previously infected individuals, thus contributing cues to understand the causality of helminth-microbiota relationships.

3. IMPACT OF DEWORMING ON THE HUMAN GUT MICROBIOTA

The implementation of mass drug administration programmes in endemic areas for STHs and schistosomiasis offers opportunities to elucidate potential mechanisms *via* which parasitic helminths modulate the host gut microbiota. For instance, qualitative and quantitative changes in gut microbial profiles that are caused by direct interactions between parasites and gut bacteria may be expected to rapidly reverse following parasite removal, whilst long-lasting alterations are likely to result from indirect interplay mediated by the host immune system (Houlden *et al*. 2015; Su *et al.* 2018). Nevertheless, such investigations are also generally constrained by the presence of several confounding factors that include not only the host- and parasite-dependent variables outlined above, but also variations linked to the use of different drugs and treatment regimes (Schneeberger *et al*. 2018b), as well as time of sampling post-anthelmintic treatment (Houlden *et al.* 2015) (Fig. 1). The latter in particular may profoundly affect findings from these studies, as the presence of tissue lesions caused by e.g. parasite feeding activity and location (e.g. blood-feeders *vs*. non blood-feeders and luminal *vs*. tissue dwellers) are likely to influence the timespan between helminth removal and microbiome recovery (reviewed by Leung *et al.* 2018). Moreover, for ethical reasons, data from these experiments is often biased by the lack of placebo-treated control groups. These limitations may be at least partially responsible for the differences between findings from studies aimed ~~to elucidate~~at elucidating the effect of deworming on the gut microbiota of helminth-infected volunteers; notwithstanding, it is worth noting that, in instances where deworming-associated changes in human gut microbial profiles were detected, these were generally moderate (Ramanan *et al.* 2016; Martin *et al.* 2018; Schneeberger *et al.* 2018b).

Consistent with this, a recent study conducted on faecal samples collected from a rural community in Indonesia reported that the composition of the gut microbiota~~me~~ of individuals repeatedly treated with either albendazole or placebo (for 21 months) resembled that of samples collected from the same subjects prior to treatment, rather than that of uninfected controls (Rosa *et al.* 2018). Moreover, a parallel investigation conducted on the same cohort of individuals detected reduced populations of *Prevotella* in albendazole-treated subjects in which complete deworming did not occur, compared to placebo-treated individuals with patent helminth infections (Martin *et*

al. 2018). Intriguingly, failure of albendazole treatment was accompanied by a dominance of *T. trichiura* (over other helminth species) in these subjects, while placebo-treated individuals maintained a diverse macrobiota (i.e. multiple helminth infections); hence, differences in the composition of the GI macrobiota (i.e. species present and their relative abundances) between albendazole- and placebo-treated individuals could account for variations in the composition of the intestinal microflora of these subjects (Martin *et al.* 2018). Significant associations between colonisation by *T. trichiura* and *Prevotella* abundance were not observed in the Indonesian cohort (Martin *et al.* 2018; Rosa *et al.* 2018). However, negative associations between whipworm infections and *Prevotella* abundance had been detected previously in two independent studies conducted in Malaysia (Lee *et al.* 2014; Ramanan *et al.* 2016). In particular, Ramanan and co-authors (2016) observed that, following albendazole treatment, expansion of *Prevotella* populations in the human faecal microbiota was related to reduced *T. trichiura* faecal egg counts. In contrast, no significant associations between helminth infection and abundance of bacteria belonging to the genus *Prevotella* was reported in a study investigating the impact of parasite colonisation and ~~effective~~ successful treatment with a combination of albendazole and ivermectin ~~treatment~~ on the faecal microbial profiles of a cohort of *Trichuris*-infected children from Ecuador (Cooper *et al.* 2013), nor in a group of helminth-infected adults from Sri Lanka treated with pyrantel pamoate (Jenkins *et al.* 2017). Similarly, no qualitative or quantitative changes to faecal microbial composition were observed in two cohorts of schoolchildren from Côte d'Ivoire and Zimbabwe infected by *S. mansoni* and *S. haematobium*, respectively, following treatment with praziquantel (Kay *et al.* 2014; Schneeberger *et al.* 2018a). However, successful elimination of *S. mansoni* was associated with a higher abundance of *Fusobacterium* spp. pre-treatment, as well as 24 hrs post-treatment (Schneeberger *et al.* 2018a).

Whilst drug administration in endemic regions may result in effective elimination of helminth infections, potential co-infecting protozoan parasites are not susceptible to anthelmintic treatment; this, together with the sub-standard hygienic and sanitary conditions that generally characterise these areas and that result in continuous re-exposure to infective helminth

developmental stages (Campbell *et al*. 2018), impairs the full assessment of the consequences of helminth removal on the composition of the human gut microbiota. To the best of our knowledge, thus far, a single study has investigated the effects of chronic infections by a GI helminth, *Strongyloides stercoralis*, and anthelmintic treatment on the composition of the faecal microbiotame and metabolome of humans from a non-endemic area of Europe, where parasite transmission had been interrupted (Jenkins *et al.* 2018b). Treatment with ivermectin resulted in compositional changes of the faecal microbiota (analysed 6 months post-treatment), which partially resembled that of uninfected control subjects (Jenkins *et al.* 2018b); in particular, alpha diversity [= a measure of the number of bacterial species present in a given microbial community (richness) and their relative abundance (evenness)] was reduced in the microbiota of the former group ofdewormed individuals post-treatment (although statistical significance was not achieved) and accompanied by expanded populations of potentially pathogenic bacteria (Jenkins *et al.* 2018b). In addition, the faecal metabolic profiles obtained from samples collected post-ivermectin treatment shared features with both appeared to fall somewhere in between those obtained from samples collected pre-treatment as well as fromand from uninfected controls (Jenkins *et al.* 2018b); this observation led Jenkins *et al*. (2018b), to hypothesise that, thus supporting the notion that, following parasite removal and over time,suggesting a (direct and/or indirect) effect of parasite infection and removal on both gut microbiotame and metabolome may revert to the original a pre-infection state. Multiple factors, including but not limited to those outlined above, may contribute to the discrepancies observed between the findings from this work and those that reported no or minor effects of anthelmintic treatment on the gut microbiome of helminth-infected humans (Cooper *et al.* 2013; Ramanan *et al.* 2016; Martin *et al.* 2018; Rosa *et al*. 2018; Schneeberger *et al.* 2018a,b).

Despite the limitations outlined above, studies of GI helminth-microbiota relationships conducted in endemic areas for helminthiases have provided repeated evidence of the perturbations that parasites and anthelmintic treatment exert on the equilibrium of resident populations of gut bacteria and on gut homeostasis. However, the identification of common signatures across studies

remains key to designing future experiments, e.g. in animal models of helminth infections, that may assist the elucidation of the mechanisms that underpin the interactions between GI helminths, the gut microbiota and the host immune system.

4. DO COMMON SIGNATURES EXIST ACROSS STUDIES OF HOST-HELMINTH-MICROBIOTA INTERACTIONS?

The identification of gut microbial signatures that occur reproducibly across several host-GI helminth systems is crucial for designing novel anti-helminth intervention strategies based on the manipulation of the gut microbiota (Peachey *et al.* 2017). Studies conducted in animal models of helminth infections are expected to assist the identification of such signatures, as well as the direct (i.e. parasite-mediated) and/or indirect (i.e. immune-mediated) mechanisms that govern helminth-microbiota interactions (Cortés *et al.* 2018); nevertheless, the inconsistencies that characterise studies of helminth-microbiota relationships published to date make such a task highly challenging. Indeed, for patterns to be identified, fluctuations in selected populations of gut microbes must be interpreted in light of the physical and immunological alterations of the mucosal environment in which such alterations occur (Leung *et al.* 2018). For instance, expanded populations of *Lactobacillaceae* have been repeatedly detected following infection with several species of parasitic helminths in several host species (Reynolds *et al.* 2014; Duarte *et al*. 2015; Holm *et al.* 2015; Houlden *et al.* 2015; Cattadori *et al.* 2016; Jenkins *et al.* 2018a; Kim *et al*. 2018), and could thus be considered as a 'consistent alteration' in gut microbiota composition upon helminth colonisation. However, key differences exist between host-parasite pairs investigated in the studies that have reported such an outcome. Indeed, whilst populations of *Lactobacillaceae* promote regulatory responses in mice infected by *Heligmosomoides polygyrus bakeri* (Reynolds et al. 2014), a lack of correlation between *Lactobacillaceae* abundance and Treg populations has been observed in other host-parasites systems, such as mice chronically infected with *T. muris* and rabbits infected with *Trichostrongylus retortaeformis*, in which the expansion of populations of gut *Lactobacillaceae* upon helminth infection occurs in an environment dominated by Th1-mediated immune responses (Holm *et al.* 2015; Houlden *et al.* 2015; Cattadori

316 *et al.* 2016). These differences suggest that alternative mechanisms may regulate the
317 differentiation and development of adaptive immune responses in each host-parasite system
318 (Houlden *et al.* 2015), and thus that similar alterations in gut microbiota composition may result
319 in different consequences that are dependent on the microenvironment where these changes occur.
320 Notwithstanding, the interactions between hosts, helminths and the gut microbiota are likely
321 multifaceted and multidirectional, and therefore the potential consequences that selected
322 compositional changes in gut microbiota exert on host homeostasis are only one aspect of these
323 complex interplay. For instance, a common yet undetermined mechanism may determine the
324 expansion of *Lactobacillaceae* in the gut of helminth-infected hosts.

325 On the other hand, apparent 'contradictory' findings across studies may result from fundamental
326 differences between gut compartments under investigation. For instance, *Prevotella* spp. was
327 expanded in the abomasum and faeces of sheep infected by abomasal trichostrongyles (i.e.
328 *Haemonchus contortus* and *Teladorsagia circumcincta*; Li *et al.* 2016; Cortés *et al.* in
329 preparation), whilst the same taxa were reduced in the faeces of a range of host species, including
330 mice, humans and horses, infected by nematodes residing in the large intestine, i.e. *Trichuris* spp.
331 and cyathostomins, respectively (Lee *et al.* 2014; Houlden *et al.* 2015; Peachey *et al.* submitted).
332 It must be noted, however, that whilst increased abomasal pH favours *Prevotella* overgrowth in
333 the abomasum (De Nardi *et al.* 2016; Li *et al.* 2016), the same taxa are likely to be exposed to a
334 dramatically different microenvironment in the large intestine that may determine the contraction
335 of these bacterial ~~taxa~~groups. In addition, given the functional dissimilarities between the
336 abomasal and colonic microbiota, such alterations are expected to result in fundamentally
337 different outcomes for the homeostasis of each of these gut compartments (Ley *et al.* 2008), and
338 hence comparisons are, in our opinion, unwarranted.

339 In parallel to species of bacteria with functions that may vary depending on the gut compartment,
340 multiple taxa share the same functions in different microenvironments (Lozupone *et al.* 2012);
341 therefore, it is plausible that, even though inconsistencies are detected across studies, these may
342 result in similar functional alterations in the host-parasite pairs being compared. For instance,

recent studies in mouse and humans infected with *S. mansoni* have reported the expansion of different genera of bacteria with pro-inflammatory functions in the gut microbiota of the respective hosts (Jenkins *et al.* 2018a; Schneeberger *et al.* 2018a). These observations lend credit to the hypothesis that the functional role of the gut microbiota in helminth infections could be far less 'diverse' than the taxonomic associations reported thus far. For this hypothesis to be confirmed or confuted, a better understanding of the function(s) of each bacterial taxon inhabiting the different gut compartments in a range of host species is needed. To this aim, the integration of metagenomic, metabolomic and metatranscriptomic technologies, alongside traditional microbiology and microscopy techniques, may assist to achieve a holistic picture of the impact of GI helminth infections on the functions of the human gut microbiota, and its significance for disease pathophysiology and overall host health (Wang *et al.* 2015).

5. CURRENT NEEDS AND FUTURE DIRECTIONS

Understanding the complex interactions between GI helminths and their vertebrate hosts is pivotal for advancing our knowledge of~~fn~~ the fundamental biology of these parasites and the diseases they cause (see Peachey *et al.* 2017; Leung *et al.* 2018; Rapin and Harris *et al.* 2018 for reviews). Whilst the role of the gut microbiota in host-parasite relationships has long been overlooked, current knowledge of the key roles that resident bacteria play in host health and disease, together with recent technical advancements for microbiota profiling, have boosted research is this area. This is currently leading to increasing evidence of ~~an active involvement of the gut microbiota in the immunopathology of GI helminth infections (e.g. Rausch *et al.* 2013; Jenkins *et al.* 2018a; Schneeberger *et al.* 2018a). Furthermore, several studies support~~ a role for the gut microbiota in the immune regulatory properties of helminth parasites (Cantacessi *et al.* 2014; Reynolds *et al.* 2014; Giacomin *et al.* 2015, 2016; Zaiss *et al.* 2016). ~~Furthermore~~In addition, data collected to date points towards a likely role ~~whether certain members~~ of the gut microflora in ~~are actively involved in~~ the immunopathology of ~~particular~~ selected GI helminth infections that awaits experimental validation~~is a currently outstanding question that awaits for a response~~. ~~Indeed, whilst t~~Trying to untangle the relevance of particular fluctuations of specific bacterial taxa on

infection outcome is challenging; nevertheless,~~,~~ currently available data suggest that low-intensity, long-term helminth infections are commonly linked to high microbial diversity and predominance of bacteria typically associated with gut health.~~,~~ Ce~~o~~nversely, high-intensity, acute infections are often associated to gut dysbiosis, characterised by reduced alpha diversity and an increase in pro-inflammatory and often opportunistic pathogens (Peachey *et al*. 2017). However, for this knowledge to be exploited in translational studies, further investigations in both natural and experimental settings are needed to distinguish spurious results from genuine helminth-microbiota associations (Peachey *et al*. 2017), and mechanistic studies in animal models of helminth infections are necessary to dissect the causality of these relationships (cf. Cortés *et al.* 2018). Importantly, minimising variations between studies is crucial to warrant meaningful comparisons between datasets.

Whilst reducing the variability amongst samples collected from naturally helminth-infected humans may be difficult to achieve, the enormous impact that differences in technical and experimental approaches (from sample collection to bioinformatics and biostatistical analyse~~i~~s) exert on the overall variation detected across studies can be reduced (Figs. 1 and 2; Lindgreen *et al*. 2017; Costea *et al.* 2017; Golob *et al*. 2017). In particular, a range of bioinformatics pipelines are available for the analysis of high-throughput amplicon and metagenomics sequence datasets that include, e.g., different sequence-processing tools and reference databases for sequence annotation that could yield slightly different results (Lindgreen *et al*. 2017; Golob *et al*. 2017). For instance, the use of validated open microbiome analysis packages such Multiplexed Analysis of Projections by Sequencing (MAPseq) (Matias Rodrigues *et al*. 2017) or QIIME2 (https://qiime2.org/) ~~taxonomy classification of 16S amplicon datasets, for instance, current trends indicate that optimised approaches should rely on open microbiome analysis packages such Multiplexed Analysis of Projections by Sequencing (MAPseq) (Matias Rodrigues *et al*. 2017) or QIIME2 (https://qiime2.org/), which have proven fast, accurate and specific in predicting taxonomic affiliations,~~ may assist accurate taxonomic classifications of bacterial 16S rRNA amplicon datasets; similarly, sequence annotation should rely on the use of ~~and the usage of~~

~~comprehensive, as well as~~ regularly updated reference databases. Amongst these, ~~, e.g.~~ SILVA (https://www.arb-silva.de/) (Quast *et al*. 2013)~~, that~~ enables ~~a~~ sensitive annotations of bacterial rRNA sequence data (Almeida *et al*. 2018). ~~Thus, t~~The use of such standardized analysis workflows and ~~continuously updated~~ reference databases for sequence annotation might prove extremely useful to increase consistency across studies and enable researchers to identify common and/or unique features between the gut microbiota of different host-parasite systems which, in turn, might assist to better understand the mechanisms that regulate helminth-microbiota relationships.

The consequences that elucidating such mechanisms may exert on future strategies of parasite control are two-fold. First, disentangling the potential contribution of the gut flora to the pathogenesis of the infection is necessary in order to discover and develop new strategies to contrast helminth-associated pathology. Second, understanding the microbiota-dependent mechanisms by which parasitic helminths are able to modulate host immune responses and suppress inflammation may assist the discovery of novel immune-regulatory therapeutics against chronic inflammatory disorders of the GI tract that may act in synergy with helminth-based therapy (see Peachey *et al*. 2017 and Rapin and Harris, 2018 for reviews). However, in order for this new knowledge to be fully exploited in translational research, further studies that thoroughly consider inclusion/exclusion criteria for the selection of participants, include appropriate controls, and follow standardised experimental and data analysis protocols~~,~~ are necessary, ~~thus allowing~~and will allow to disentangle the potential influence of parasite-, drug- and/or population-dependent variables in each setting (Fig. 2)~~, are necessary~~.

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FIGURE LEGENDS

Fig. 1 Sources of variation and confounding factors potentially impacting the outcome of studies of human-helminth-gut microbiota interactions in helminth-endemic regions.

Fig. 2 Proposed approaches aimed at reducing the methodological sources of variation surrounding investigations of human-helminth-gut microbiota interactions.