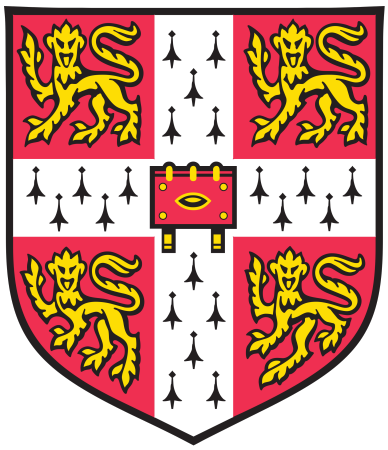
Exploring the impact of gastrointestinal parasitic helminths on the human microbiome using advanced biomolecular and bioinformatics technologies



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This dissertation is submitted for the degree of
Doctor of Philosophy (PhD) in Molecular Parasitology

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DECLARATION

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text

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Timothy P. Jenkins

May 2019

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By Timothy P. Jenkins

SUMMARY

Our understanding of the biology of human gastrointestinal (GI) parasitic helminths is greater than ever before. However, so far, the research has focused on gene expression profiling, immune- and protein-protein interactions in host-parasite systems. The importance of parasite-microbiota interactions has, so far, been largely overlooked. The microbiome is key to host health and it has been demonstrated that the balance between the gut microbiota and the host is crucial for health maintenance and that a disturbance of this balance can result in a range of diseases. Hence, given that GI nematodes and the gut microbiota share the same ecological niche within the human host, it is plausible that GI helminths and the host microbiota interact, and that this could significantly impact on the health and homeostasis of the parasite-infected hosts. Fortunately, the availability and affordability of next generation sequencing now enables us to investigate such host-parasite-microbiota interactions in depth and at high throughput. Therefore, the aims of this thesis were to explore the impact of such helminth infections in various systems, ranging from natural multi-species infections in a developing country to highly controlled and experimental infections involving a single species of parasitic helminth. This would allow the identification of microbiome changes that are consistent across different settings, as well as help detect alterations that are specific to a certain host-helminth system. Thus, the main aims of the thesis were: (i) to investigate the consequences of natural multi- or mono-species infections by helminth parasites on the composition of the human gut microbiota (Chapters 2 and 3), (ii) to elucidate the longitudinal impact of experimentally controlled mono-species helminth infections on the human gut microbiota (Chapter 4), (iii) and to examine whether an extra-intestinal (EI) helminth infection has an impact on the host microbiome in a murine model of human schistosomiasis (Chapter 5).

Overall, I found that GI and EI helminths have a substantial impact on the host gut

microbiota, both on individual taxa and on a community level. Many of the observed changes appeared to be specific to the host-helminth system that was being investigated, yet some consistencies emerged. Firstly, low level, long term, and single species infections that were not accompanied by pathology appeared to increase the gut microbial diversity within their host and promote a stable and healthy gut microbial composition (Chapters 3 and 4). Contrarily, acute heavy burden infections, associated with pathology, appeared to have the opposite effect, i.e. reducing the overall diversity of the host's gut microbiome and promoting the proliferation of opportunistic pathogens (Chapters 2 and 5). This suggests that parasitic helminth infections could detrimentally impact the hosts they infect besides the direct pathology they induce, but also adds further weight to the idea of a therapeutic use of helminths in the context of helminth therapy. Indeed, the beneficial effect helminths can have on the host gut microbiota, together with the mounting evidence towards an intrinsic link between autoimmune diseases and the microbiome, might present a mechanism through which helminths could exert a therapeutic effect on patients suffering from such conditions.

In, conclusion the present thesis has contributed significantly by providing entirely new insights into the impact of natural and experimental parasitic helminth infections on the human gut microbiome (Chapter 6). The findings provide a sound basis for future fundamental investigations of, for example, the relationship of helminth species, abundance, and host to microbiome changes in the context of infection. However, the results also act as a stepping stone for studies exploring the translational potential of helminth-microbiota interactions, such as the role that helminth induced microbiome modulations play in infection pathology, or whether such changes play a key role in the therapeutic potential of helminth therapy.

ACKNOWLEDGEMENTS

"It is the long history of humankind that those who learned to collaborate and improvise most effectively have prevailed." – Charles Darwin

Foremost, I would like to express my sincere gratitude to my advisor Dr Cinzia Cantacessi for her continuous support, patience, motivation, enthusiasm, and immense knowledge throughout my PhD. Her guidance helped me improve in countless ways and provided me with the key skills I require to become an independent researcher. I cannot imagine receiving comparable opportunities to improve and progress both on an academic and personal level elsewhere. For that I will be forever grateful.

I would also like to take this opportunity to thank Dr Terrence Miller, since it was he, who first introduced me to the field of parasitology and the practicalities of research. His contagious enthusiasm and endless encouragement were pivotal in my decision to pursue a PhD. At such an early career stage, such confidence in my skills and abilities was the greatest motivation I could ask for.

Many thanks to my colleagues Dr Laura Peachey, Dr Alba Cortes-Carbonell, Dr Riccardo Scotti, and Xiaopei Su for their helpful discussions, valuable advice, the sleepless nights we were working together before deadlines, and for all the fun we have had over the past four years.

My deepest and sincerest thanks to my friends in Cambridge, all of whom, transformed my time here into arguably the best experience of my life. Especially, Chris Guillermo, Kanwar, Sohaib, and Thom, ensured fun was always at hand. Also, a big thank you to all of my friends that have stuck with me for even longer. Martin, Cosimo, Lotte, Kaja, and Trenton, you have always been there for me and helped keep me sane. I am also infinitely grateful to Tina and Uwe, who have kept me alive over the years, even on my most daring adventures.

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PREFACE AND DISSEMINATION OF RESEARCH FINDINGS

Scientific papers published or submitted by the author in collaboration with my supervisor and other colleagues are listed in the following:

Peer-reviewed articles published in international scientific journals and included in this thesis:

Chapter 2 - **Timothy P. Jenkins**, Yasara Rathnayaka, Piyumali K. Perera, Laura E. Peachey, Matthew J. Nolan, Lutz Krause, Rupika S. Rajakaruna, and Cinzia Cantacessi. "Infections by human gastrointestinal helminths are associated with changes in faecal microbiota diversity and composition." PLoS ONE 12, no. 9 (2017): e0184719

Chapter 3 - **Timothy P. Jenkins**, Fabio Formenti, Cecilia Castro, Chiara Piubelli, Francesca Perandin, Dora Buonfrate, Domenico Otranto, Jules Griffin, Lutz Krause, Zeno Bisoffi, Cinzia Cantacessi. "A comprehensive analysis of the faecal microbiome and metabolome of *Strongyloides stercoralis* infected volunteers from a non-endemic area." Scientific Reports 8, (2018): 15651

Chapter 5 - **Timothy P. Jenkins**, Laura E. Peachey, Nadim J. Ajami, Andrew S. MacDonald, Michael H. Hsieh, Paul J. Brindley, Cinzia Cantacessi, Gabriel Rinaldi. "*Schistosoma mansoni* infection is associated with quantitative and qualitative modifications of the mammalian intestinal microbiota." Scientific Reports 8, (2018): 12072

Chapter 6 - **Timothy P. Jenkins**, Paul J. Brindley, Robin B. Gasser, Cinzia Cantacessi. "Helminth microbiomes - a hidden treasure-trove?" Trends in Parasitology, (2018): 18

Conference proceedings and seminars given:

9. Oral presentation at the Cambridge Parasitic and Neglected Tropical Diseases Network: "A

comprehensive analysis of the faecal microbiome and metabolome of *Strongyloides stercoralis* infected volunteers from a non-endemic area". Cambridge, UK (2018) – Prize for best flash talk

8. Oral presentation at the Exploring Human Host-Microbiome Interactions in Health and Disease conference: "A comprehensive analysis of the faecal microbiome and metabolome of *Strongyloides stercoralis* infected volunteers from a non-endemic area". Cambridge, UK (2018) – Prize for best short talk

7. Oral presentation at the British Society for Parasitology: "A comprehensive analysis of the faecal microbiome and metabolome of *Strongyloides stercoralis* infected volunteers from a non-endemic area". Aberystwyth, UK (2018)

6. Oral presentation at the German Society for Parasitology: "*Schistosoma mansoni* infection is associated with quantitative and qualitative modifications of the mammalian intestinal microbiota". Berlin, Germany (2018)

5. Conference organisation of the Innovation Forum – Leaders conference. Oxford, UK (2017)

4. Presentation at the annual BBSRC DTP professional internship workshop focusing on my experiences and advice. Cambridge, UK (2017)

3. Poster presentation at the research day of the Department of Veterinary Medicine: "*Schistosoma mansoni* infection is associated with quantitative and qualitative modifications of the mammalian intestinal microbiota". Cambridge, UK (2017)

2. Oral presentation at the Cambridge Parasitic and Neglected Tropical Diseases Network: "Infections by human gastrointestinal helminths are associated with changes in faecal microbiota diversity and composition". Cambridge, UK (2017)

1. Departmental seminar at the Faculty of veterinary medicine: "Helminth associated changes in composition of the faecal microbiota of a Sri Lankan community". Cambridge, UK (2017)

Prizes and awards received over the course of this PhD:

6. Best Flash Talk Prize awarded at the Cambridge Parasitic and Neglected Tropical Diseases Network conference for the best four-minute oral presentation. Cambridge, UK (2018)

5. Best Short Talk Prize awarded at the Exploring Human Host-Microbiome Interactions in Health and Disease conference for the best ten-minute presentation. Cambridge, UK (2018)

4. Wellcome Genome Campus Hackathon: Winner of the AstraZeneca Challenge - How can we map disease-drug target relationships using artificial intelligence/machine learning? Cambridge, UK (2018)

3. ABCAM Postgraduate Research Prize awarded for demonstrating notable recent research achievements or general excellence over time which is outstanding to their career level – awarded to the best PhD student within the department of Veterinary Medicine at the University of Cambridge. Cambridge, UK (2018)

2. Student Representation Award awarded by Homerton College for outstanding achievements within the Cambridge University Lacrosse Team. Cambridge, UK (2016, 2017, and 2018)

1. Best Poster Prize awarded at the Department of Veterinary Medicine Research Day for the best scientific poster Cambridge, UK (2017)

GLOSSARY

Term	Definition
Alpha diversity	In microbial ecology, the mean species diversity at the local, within-site, or within-habitat scale. It is dependent on both the number of species making up a population (richness) and the relative abundance of each species in a population (evenness).
Bacterial 16S rRNA gene	A highly conserved gene across bacteria and archaea, commonly used for taxonomic identification.
Beta diversity	The ratio between within-study group and between-study group microbial species diversity.
Core microbiome	The group of microbes consistently found within a host microbiome, which demonstrate a persistent association and provide a critical function within the habitat in which they are detected.
Dysbiosis	Microbial imbalance or maladaptation on or inside the body.
Endosymbiont	Any organism that lives within the body or cells of another organism in a symbiotic relationship with the host body or cell, often but not always to mutual benefit.
Microbial evenness	Microbial species similarity in abundance within an environment or population.
Microbial richness	The number of microbial species present in a given sample.
Microbiome	The collection of genomes from all the microorganisms found in a given environment.
Microbiome editing	Targeted manipulation of microbiota through the use of technology and/or other microbiota.
Microbiota	The collection of microorganisms detected within a given environment.

TABLE OF CONTENTS

The present thesis contains 6 chapters with a reference list at the end of each chapter. Chapters 2-5 describe original research findings and each of them contains specific sections and subsections.

CHAPTER 1

Literature review

1.1. Introduction

Globally, over two billion people are estimated to be infected by gastrointestinal (GI) soil-transmitted helminths (STHs), with a further 230 million people suffering from schistosomiasis (reviewed by [1,2]). Amongst the most medically and economically important parasitic helminths are the nematodes *Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus*, *Ancylostoma duodenale*, and *Strongyloides stercoralis*, which together are responsible for the global burden of soil-transmitted helminthiases (reviewed by [1]); the pathology these helminths cause originates from direct damage related to their attachment, migration, burrowing and feeding activities or secondary damage resulting from the host's immune response to the parasite (reviewed by [3,4]). Concurrently, the blood flukes (trematode worms) *Schistosoma mansoni* and *S. haematobium* are the main inducers of intestinal/urogenital schistosomiasis, respectively, and can cause local and systemic pathological effects through eggs becoming trapped in tissues and inducing a granulomatous response (reviewed by [2]). Together, soil-transmitted helminthiases and schistosomiasis are estimated to cause a total of over six million disability-adjusted life years (DALYs) globally, mainly affecting areas in developing countries characterised by suboptimal standards of sanitation and hygiene (reviewed by [1,2]).

In spite of global efforts to control infections by these parasites *via* mass drug administration (MDA), repeated exposures to larvae and eggs, caused by persisting environmental contamination with infective stages of the parasites, as well as high re-infection rates in at-risk populations, make interruption of the life cycles of these parasitic helminths and their elimination difficult to achieve [6,7]. These challenges, together with the existing threat of emerging drug resistance in humans (already a significant issue in veterinary species; reviewed by [8-10]) and the limited number of effective anthelmintic compounds available drive the continual search for new, integrated strategies to control these diseases. Such novel approaches, however, require a thorough understanding of the fundamental biology and epidemiology of these pathogens and their interactions with the vertebrate hosts [11].

Irrespective of our increasing knowledge of aspects of immunity, gene expression profiling and protein-protein interactions in host-parasite systems [12], major gaps still exist in our current understanding of the systems biology of parasites following invasion of, and establishment in, the vertebrate host gut. This includes the understudied area of parasite-microbiota interactions. The human gut is inhabited by a complex network of commensal microorganisms that exert a number of specialised functions beneficial to the host, such as nutrient metabolism, synthesis of essential organic compounds, protection against pathogens and development of adaptive immunity [13-16]. Studies have shown that the balance between the gut microbiota and the host body is crucial for a functional gut metabolism and overall health maintenance and that a disturbance of this balance can result in a myriad of diseases such as diabetes and obesity (reviewed by [17]). Furthermore, several studies have shown that the gut microbiota and their metabolites play a major role in protecting its host from colonisation by pathogenic organisms [19]. Indeed, they can stimulate a range of cell populations associated with maintaining the intestinal mucosal barrier and thus help provide a physical obstacle to pathogen invasion (reviewed by [17]). Therefore, given that STHs and the gut microbial flora share the same niche within the human host, it is plausible that such parasite-microbiota interactions impact substantially on the health and gut homeostasis of helminth-infected hosts [18]. Notably, the same stands true for schistosomes, since although the adults reside in the host blood, their eggs traverse the host gut epithelial layer, resulting in a direct interaction with the gut microbiota. Nevertheless, the effects that parasitic helminth infections exert on the commensal flora of the vertebrate hosts have long been neglected. The purpose of this chapter was to review relevant information of aspects of the biology of parasitic helminths of socio-economic importance (i.e. selected species of STHs, including the roundworms *A. lumbricoides* and *S. stercoralis*, the hookworms *A. duodenale* and *N. americanus*, as well as the blood fluke *S. mansoni*), the role of the mammalian microbiome and metabolome, and research approaches towards the investigation of host-helminth-microbiota-metabolite interactions. Based on the conclusions of the literature review, the aims of the thesis were formulated.

1.2. Epidemiology, life cycles and pathogenesis

1.2.1. *Ascaris lumbricoides* (Clade II, Ascaridida)

A. lumbricoides is a large (10-15 cm) roundworm, or nematode, that parasitises the human intestine and causes disease, i.e. ascariasis. Globally, ascariasis is among the most common helminthic human infections and affects approximately 804 million people, resulting in approximately one million DALYs (Fig. 1A; reviewed by [1]). *A. lumbricoides* follows a direct

life cycle and involves eggs being passed via the host's faeces (Fig. 1B; reviewed by [19]). Under favourable conditions these embryonated eggs can remain viable for up to five years (reviewed by [19]). Only upon the exposure to certain environmental cues (i.e., moist, warm, and shaded soil) the first stage larva (L1) develops within the egg and over the following two to six weeks moults to the second stage (L2), and, subsequently, to the infective third stage (L3) larva (reviewed by [19]). These infective eggs are then ingested by the host and hatch in the small intestine, where they invade the intestinal mucosa, penetrate the intestinal walls, enter the portal venous system and lymphatic channels, in which they migrate hematogenously and via lymphatics to the liver and then the lungs (reviewed by [19]). Further development occurs in the lungs over the next 10-14 days, after which the larvae penetrate the alveolar walls, ascend the bronchial tree to the throat, and are swallowed (reviewed by [19]). In the small intestine the larvae mature into adult worms and can lay up to up to 200.000 eggs per female per day (21-29 days; reviewed by [19]). Full maturity of the worms, which have a lifespan of 1-2 years, occurs 50-55 days after initial ingestions of the infective eggs and oviposition can be detected in the host faeces after 60-62 days (reviewed by [19]).

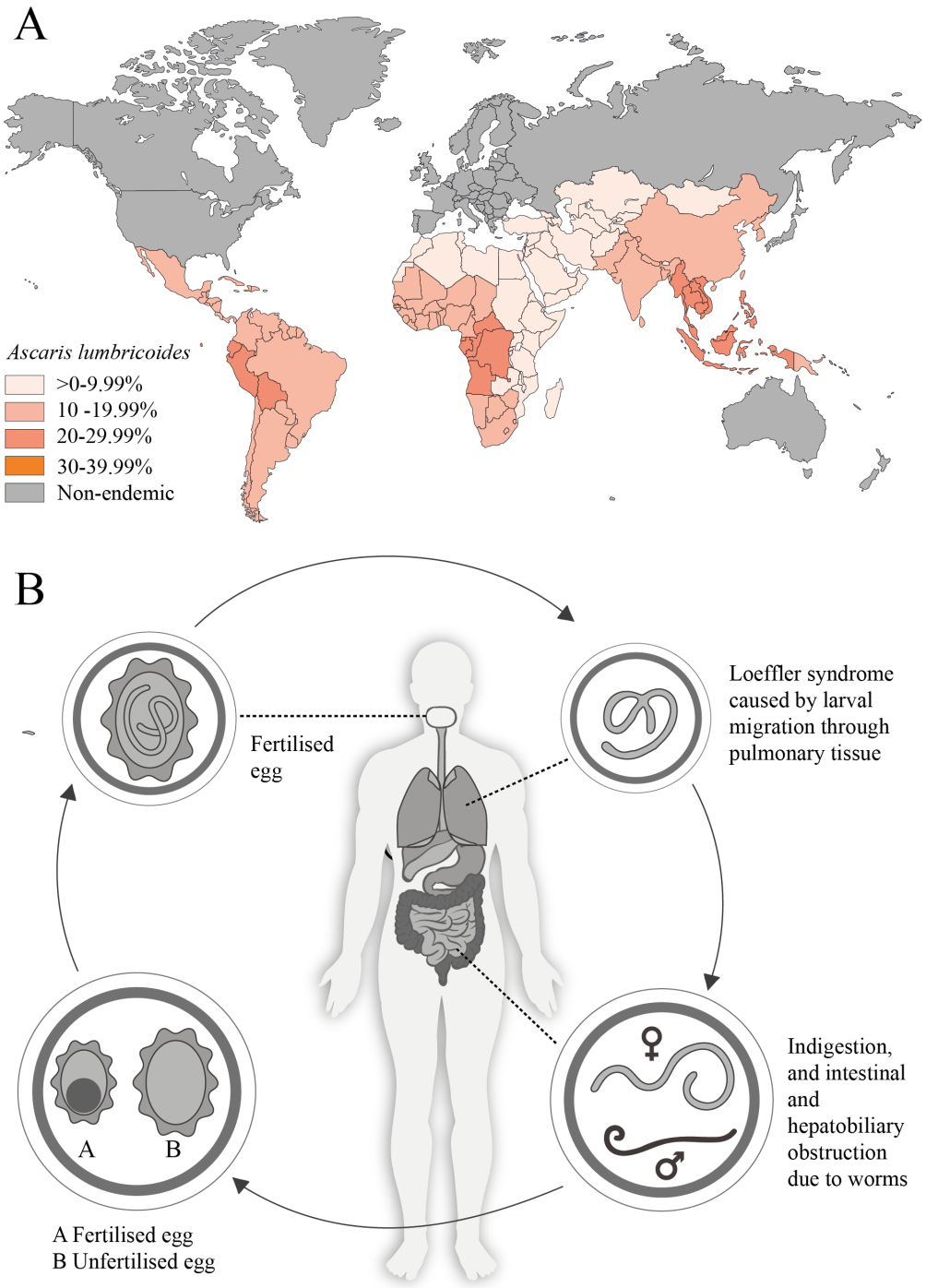

Fig. 1 Prevalence of the gastrointestinal parasitic helminth *Ascaris lumbricoides* (A) and its transmission strategies (B). Adapted from [1].

Many infections remain asymptomatic, however, heavy infections can result in severe pulmonary disease (Löeffler's syndrome) and/or partial/total obstruction of intestinal tracts, due to the propensity of the worms to interlace and form large masses (reviewed by [20]). Consequently, the classic symptoms include abdominal pain, constipation, and vomiting (reviewed by [20]). Moreover, the worms can penetrate biliary and pancreatic ducts, causing biliary colic, as well as hepatic and pancreatic dysfunction (reviewed by [20]). The diagnosis of

intestinal ascariasis is confirmed via stool microscopy for ova or via detection and examination of adult worms. The presence of significant numbers of adult worms in the small intestine of the host can also lead to malnutrition and, consequently, developmental impairment (reviewed by [20]).

1.2.2. *Strongyloides stercoralis* (Clade IV, Rhabditida)

S. stercoralis is a soil-transmitted intestinal nematode estimated to infect ~370 million people worldwide, with higher prevalence (ranging from 10-60%) recorded across tropical and subtropical regions (Fig. 2A) [21-24]. The life cycle of *S. stercoralis* is highly complex and provides the parasite with multiple routes towards survival and proliferation (Fig. 2B) [21,25]. There exist both parthenogenetic parasitic and free-living female worms. Infective filariform larvae are able to infect a putative human host both percutaneously and orally, with skin penetration on feet being the most common [1]. From there the larvae migrate to the pulmonary capillaries, penetrate alveoli, pass to the larynx, and enter the small intestine, where they develop into adults [1]. Oral infection proceeds identically following penetration of the intestinal mucosa [21,25]. Mature female worms will penetrate the epithelial layer and lodge in the duodenal and jejunal lamina propria, where they lay up to 50 eggs per day [1]. Hatching occurs within the gut mucosa and non-infective rhabditiform larvae travel to the lumen from where they are excreted within the host faeces [21,25]. At the same time some larvae might penetrate the colonic wall or perianal skin to commence a new cycle. This process is known as “autoinfection” and enables chronic long lasting (up to decades) strongyloidiasis, without repeated exposure to exogenous infective larvae [25]. Larvae or unhatched eggs that are passed in the stool are able to persist in moist soil for several weeks and can develop into infective larvae [21,25].

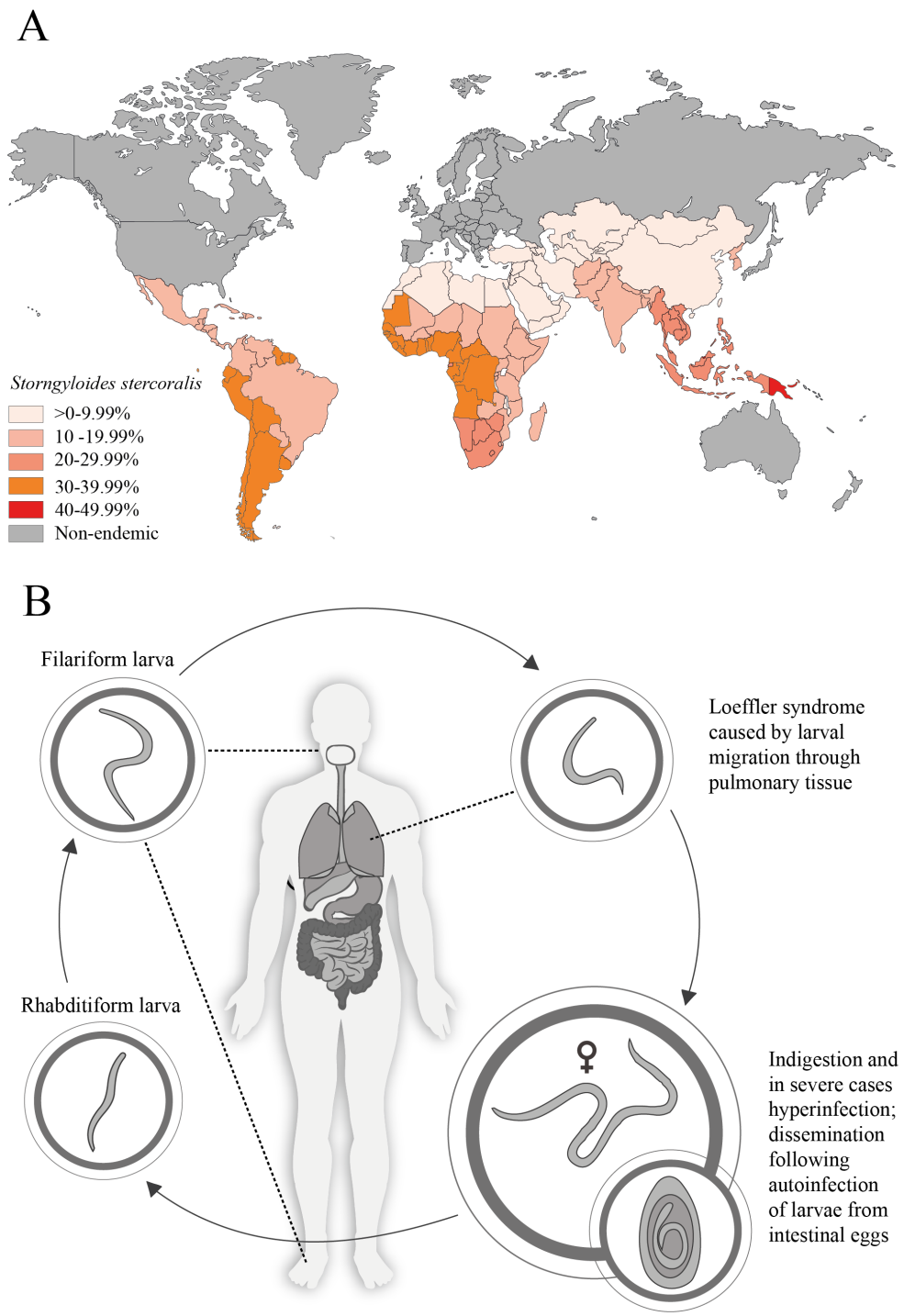

Fig. 2 Prevalence of the gastrointestinal parasitic helminth *Strongyloides stercoralis* (A) and its transmission strategies (B). Adapted from [1].

Infection with *S. stercoralis* is typically difficult to diagnose, due to patients presenting only mild or no symptoms, and stool microscopy having low sensitivity for detecting this parasite (around 50%), due to intermittent larval excretion and low infectious burden (reviewed by [26]). There are, however, reliable Real-Time PCR (rtPCR) approaches that have proven significantly more successful (up to 100%) [27]. Although most infected people remain

asymptomatic, they can experience respiratory symptoms, while the larvae migrate through the lungs; in some cases, patients present Löeffler's-like syndrome, characterised by fever, dyspnoea, and wheeze (reviewed by [26]). The most severe pathology caused by *S. stercoralis* infection is known as the hyperinfection syndrome. This occurs when patients chronically infected with this parasite become immunosuppressed and leads to over-proliferation of larvae with, often lethal, dissemination to end organs (e.g. lungs, liver, and brain; reviewed by [26]).

1.2.3. Hookworms (Clade V, Strongylida)

The hookworms *N. americanus* and *A. duodenale* are estimated to infect 477 million people world-wide and in over 58 countries, most of which lie in the tropics and sub-tropics (Fig. 3A; reviewed by [1]). The life cycle of hookworms is direct, with female worms shedding eggs through the host faeces (Fig. 3B; reviewed by [19]). Provided the right environmental conditions arise the rhabditiform L1s hatch from the eggs on the soil (reviewed by [19]). These larvae feed on soil bacteria and moult to L2s (two days), and subsequently to infective L3s (four to five days). These filariform larvae retain the L2 cuticle (i.e. sheath) and infect their host via skin penetration, following cuticular shedding (reviewed by [19]). Thereafter, the larvae travel through the blood vessels to the heart and then to the lungs, where they penetrate the pulmonary alveoli. Here they ascend the bronchial tree to the pharynx, and are swallowed and transported to the small intestine, where they reach sexual maturity within the next one to two months (reviewed by [19]). As adults, hookworms attach via their buccal capsule to the intestinal mucosa, rupture capillaries and feed on their host's blood (reviewed by [19]). Notably, although skin penetration constitutes the main route of infection, ingestion of L3s can also result in hookworm infection; in the latter case, the larvae will exsheath in the gut and develop directly to adulthood without a migration to the lungs (reviewed by [1,19]). *Ancylostoma* spp. can also undergo hypobiosis (i.e. developmental arrest) in the somatic tissue of their host and, following activation during pregnancy, undergo trans-mammary transmission to the offspring.

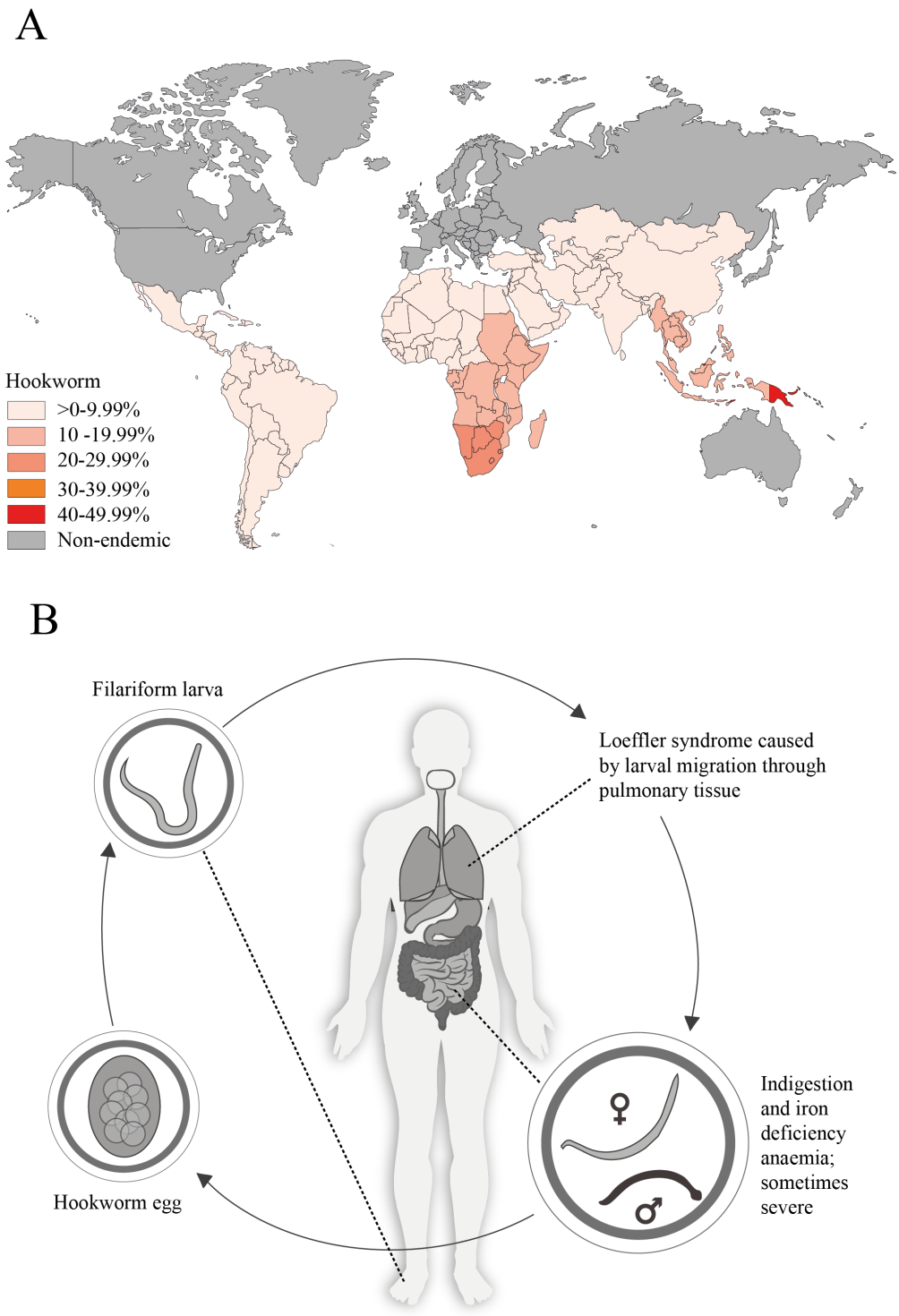

Fig. 3 Prevalence of the hookworms *Necator americanus* and *Ancylostoma duodenale* (A) and their transmission strategies (B). Adapted from [1].

Hookworm infections can cause a type-1 hypersensitivity reaction during pulmonary migration (Löeffler's syndrome), yet the main pathology stems from the blood loss the adult worms cause through direct tissue damage and consumption of red blood cells (reviewed by [1]). These focal lesions are characterised by local haemorrhage, tissue cytolysis and neutrophilic immune response (reviewed by [1]). This can clinically manifest itself in form of iron-deficiency

anaemia, which if prolonged, can result in both physical and mental impairment, as well as death in children; additionally, it can cause maternal mortality, impaired lactation, premature births, and low birth rates (reviewed by [1,28]).

1.2.4. Schistosoma mansoni (Diplostomida)

Schistosomiasis is a neglected tropical disease that affects over 230 million people worldwide and is considered the most problematic of the human helminthiases in terms of morbidity and mortality (Fig. 4A) [29]. The causative agents are not GI nematodes, like the previous helminths, but rather extra-intestinal (EI) blood flukes (trematodes) of the genus *Schistosoma* including *S. mansoni*, *S. japonicum* and *S. haematobium* amongst others. These digenetic organisms undergo four life cycle stages, two of which are completed within the human host (Fig. 4B). *S. mansoni* is one of the major schistosomiasis agents in Africa and South America. Human infections occur during the cercarial stage of the life cycle, where the freshwater-dwelling cercariae penetrate the host's skin [30,31]. Upon successful invasion, the cercariae shed their tails, develop into schistosomula, and migrate to the lungs and eventually the hepatic portal system via the bloodstream. The schistosomula mature into adults and pair, after which they migrate to the mesenteric plexus and start laying hundreds of eggs per day [30,31]. The eggs are shed into the water, hatch and infect fresh water snails as intermediate hosts, before repeating the life cycle [30,31].

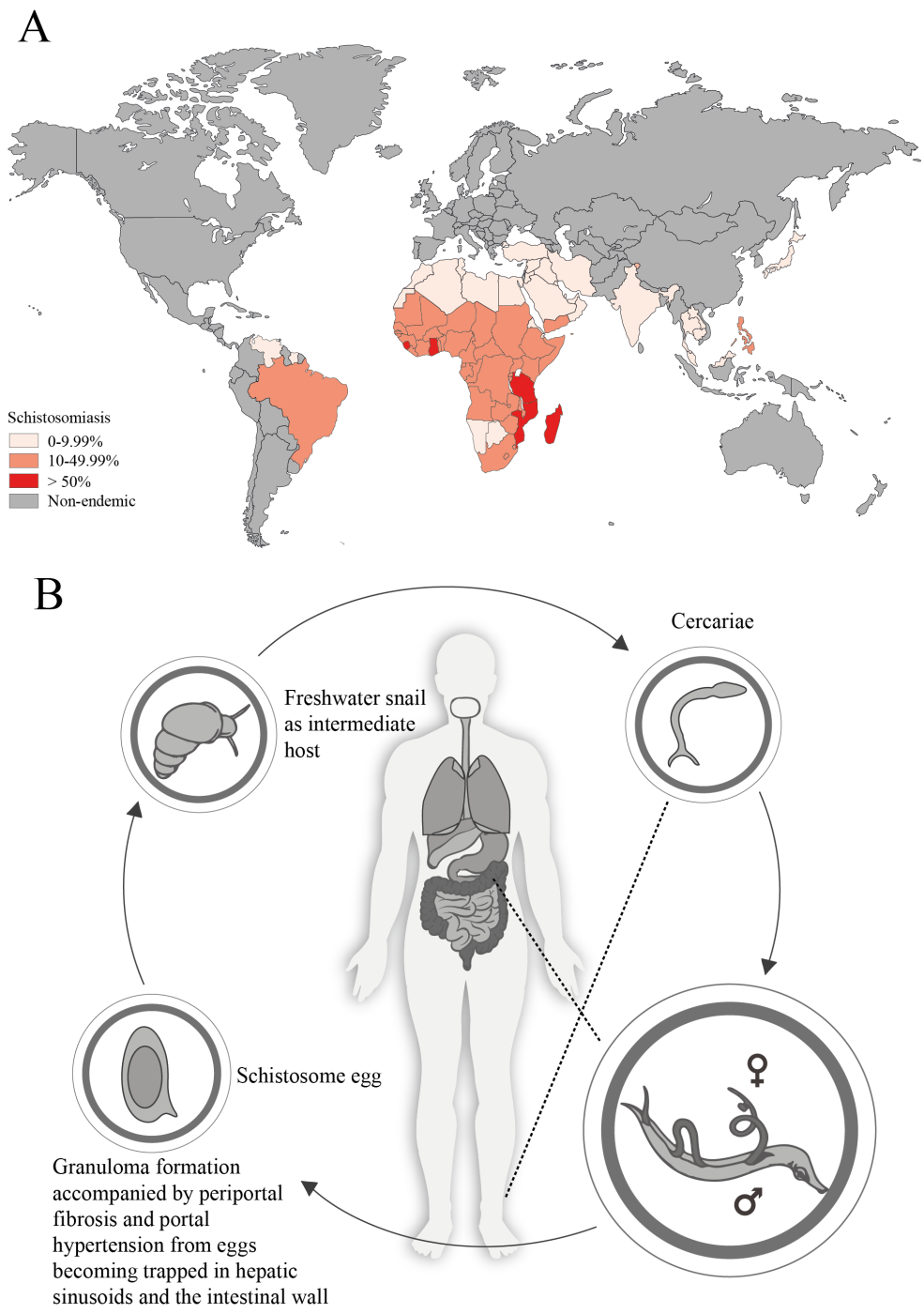

Fig. 4 Prevalence of schistosomiasis (A) and *Schistosoma mansoni* transmission strategies (B).

The main pathogenesis is caused by eggs that become trapped in host tissues and consequently trigger an immune response, while also eliciting a host granulomatous response [32]. While this granuloma formation is key in preventing more sever pathology early on, accumulation of such granulomas can result in fibroses, i.e. the primary cause of mortality associated with schistosomiasis [32].

1.3. **Aspects of immunology**

To date, a wealth of knowledge on the impact of parasitic helminths on the host immune system has been acquired in order to understand key biological questions concerning the establishment, proliferation, and potential prevention of infection. The primary incentive for parasitic helminth to actively interact with its host immune system is to avoid elimination and to instead persist and proliferate, sometimes for many years. In turn the host immune responses are faced with a compromise between immunity against the parasite and immunopathology caused by prolonged inflammatory responses. Notably, the host immune system often merely minimises helminth infection, rather than completely eradicating it, to limit immune-mediated damages (reviewed by [33]). Specifically, the host immune system typically mounts a T helper (Th) 2-type response that is generally effective in limiting infections by parasitic helminths (reviewed by [33]). However, this response may not be sufficient to entirely eliminate the infection. This can lead to the host skewing the immune response towards a regulatory phenotype to avoid potential pathogenic effects of a sustained Th2 response (reviewed by [33]). In parallel, parasitic helminths themselves have been shown to secrete immunomodulatory molecules that induce the polarization of the response towards a regulatory phenotype, thus avoiding Th2 responses that eventually may clear them out of the host (reviewed by [33]). Indeed, a murine model of *S. mansoni* infection demonstrated that wild-type mice survive and become chronically infected 56 days post-infection, whereas IL-4 (a cytokine that induces differentiation of naive helper T cells to Th2 cells) deficient mice die due to severe inflammation [34]. Indeed, the balance between regulatory T cells and a Th2 immune response is key to host health, with dysregulation often resulting in severe pathology and death, e.g. hepatic fibrosis in schistosomiasis or hyperinfections with *S. stercoralis* [35,36].

The complex interplay between helminth immune modulation and host immune response is seen across a range of phylogenetically distinct parasites, such as cestodes, trematodes, and nematodes. It is believed that the close co-evolution of these parasitic worms and their hosts has significantly shaped human immune development, as well as selecting for parasites with sufficient immune-modulatory capacity [36,37]. This is reflected in the significant overlap in the type of immune responses they cause in their human hosts; this most commonly involves a strong induction of a Th2 response alongside an overall immune dampening [5]. In turn, such Th2 responses are linked to the cytokines interleukin (IL)-3, IL-4, IL-5, IL-9, and IL-13, but also eosinophilia, goblet cell and mast cell hyperplasia, and alternative activation of macrophages [33]. Conversely, immunosuppression is predominantly mediated by regulatory T cells secreting IL-10 and transforming growth factor (TGF)-β. An immune impact on the host

requires live parasites, since parasitic helminths achieve immunomodulation through means, such as the secretion of soluble mediators that can interact with host immune cells (Fig. 5) [38]. Indeed, in murine studies it has been demonstrated that secretory proteins from adult *Nippostrongylus brasiliensis* induce strong Th2 immune responses [39], while a cysteine protease (SmCB1) from *S. mansoni* stimulates IgE production in the host [40]. Notably, the immune responses elicited by many parasitic helminths can vary across different developmental stages (i.e. eggs, larvae, and adults) across the parasites' life cycle [41-43]. For instance, people infected with *N. americanus* have been shown to have a differential pattern of proliferative immune responses to stage-specific antigens [43], while *S. mansoni* adults are understood to induce a Th1 type response, with the onset of egg deposition leading to a switch to Th2 immunity [41]. Notably, parasitic worms can also have an immunomodulatory impact via direct interactions between helminth surface molecules and host cell surface receptors [44]. The best understood system involves helminth glycans (e.g. Lacto-N-fucopentaose III and Lewis^X) that drive an activation of antigen-presenting cells that possess an anti-inflammatory phenotype [44]. Additionally, studies have shown that parasitic helminths are capable of molecular mimicry to modulate host immune responses. For instance, filarial nematodes have been shown to produce close homologues of anti-inflammatory molecules such as macrophage migration inhibitory factors [45], transforming growth factor beta [46], and suppressor of cytokine signalling 1 [47]. Lastly, there is mounting evidence that the intricate interplay between the immunomodulation by parasitic and the host immune response may be mediated, at least partially, and in-/directly via changes to the host gut microbiome [48-53].

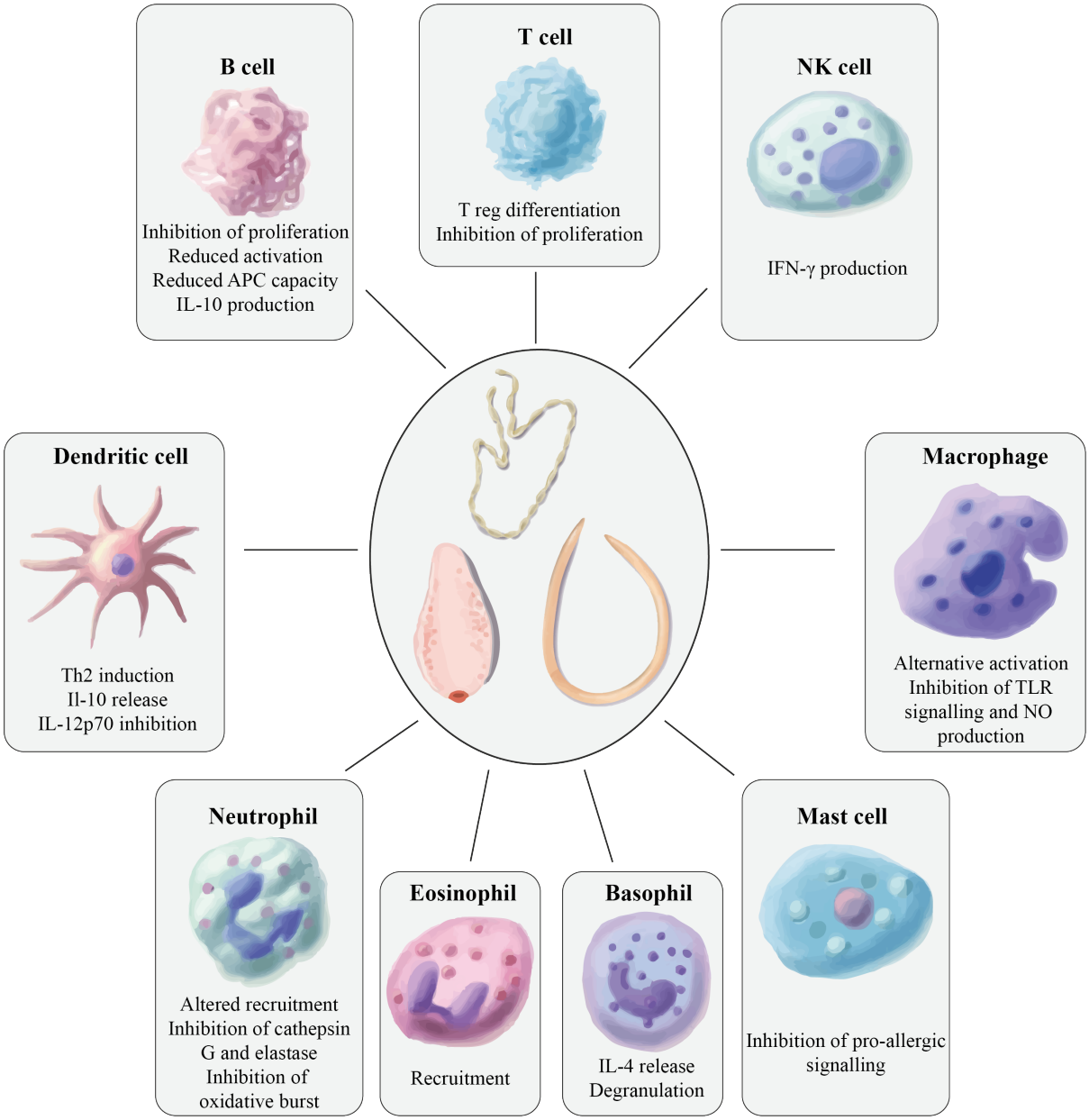

Fig. 5 Immunomodulatory strategies employed by parasitic helminths. The figure was adapted from [5].

Understanding the fundamental interactions between parasitic helminths and the host is key towards the development of novel therapies to prevent/mitigate their establishment and pathology in humans. Moreover, an improved appreciation of this complex interplay might shed light on helminth therapy, i.e. the targeted controlled infection of humans to treat immune disorders, such as asthma, allergy, inflammatory bowel disease and type 1 diabetes (reviewed by [54,55]). The idea originated from the observation that higher prevalence of parasitic infections in a population is associated with lower incidence of immune-related diseases [56]. Furthermore, the fact that these immune conditions are characterised by a boost of Th1 immunity and many parasitic helminths induce strong Th2 responses in their host added weight to the argument.

Subsequently, two murine studies were able to demonstrate that helminth infection could prevent the onset of type 1 diabetes in non-obese diabetic mice [57,58]. Furthermore, a range of human studies have investigated the therapeutic properties of parasitic nematodes in a range of immune diseases and have reported positive results in the treatment of inflammatory bowel disease, coeliac disease, as well as multiple sclerosis [59]. Yet, despite the initial success, the specific underlying mechanisms leading to these curative effects are still poorly understood and require further investigations, so that we can exploit the full therapeutic potential of parasitic helminths.

1.4. Current treatment strategies

Vaccines against parasitic GI helminths have been at the centre of research efforts within the field of parasitology for decades, with a plethora of work conducted in various host-helminth systems. This stems from the excellent prevention of helminth infection that can be achieved through this modus operandi, as well as the possibility of inducing natural immunity against these parasites, while circumventing potential concerns of resistance and adverse effects that chemotherapeutics may have on the host and its environment (reviewed by [60]). However, the strategies to achieve this goal vary and can broadly classified into three categories, (a) through the use of irradiated larvae, (b) extracted and purified native antigens, or (c) recombinant antigens [61]. Although all approaches have shown promise, recombinant antigen based vaccines are the most commercially viable, due to their comparatively higher stability and availability [61]. Nonetheless, the development of such anti-helminth vaccines is highly complex and, consequently, very costly [62]. In particular, it appears to be exceptionally difficult to achieve immune responses with recombinant vaccines, probably due to the complexity of interaction between helminth and host immune systems [62]. This has resulted in only three vaccines currently being on the market, despite the significant time and funding spent on this task. Furthermore, all of the currently available vaccines are against veterinary parasitic helminths, i.e. a native gut antigen-based vaccine for the control of *Haemonchus contortus* in Australia, an irradiated larvae-based vaccine against bovine lungworm *Dictyocaulus viviparus*, and a recombinant vaccine to control the cestode *Echinococcus granulosus* in livestock [62]. Therefore, the interventions and treatments of parasitic helminth infections, especially in humans, have been and still are reliant on the use of effective broad-spectrum anthelmintics [63,64].

Anthelmintics are a group of drugs that are designed to biochemically neutralise and remove parasitic helminths from the host. There currently are five categories of such drugs

available, i.e. benzimidazole (1-BZ), levamisole (2-LV), macrocyclic lactones (3-ML), amino-acetonitrile derivatives (4-AD) and spiroindoles (5-SI) [65]. As a result of the higher economic incentive all of the major anthelmintics currently in use were developed for the use in veterinary species, but have also shown success and proven essential for the treatment of helminth infections in humans (reviewed by [63,64]). In fact, the two most used drugs against GI helminths, such as *A. lumbricoides*, *T. trichiura* and hookworms, in humans are the benzimidazoles, mebendazole and albendazole [64] and both were originally developed for treating veterinary parasites (reviewed by [66]). Still, both drugs have been proven to be successful at significantly decreasing worm burdens in humans, as measured through egg-reduction rate (ERR; [64]). Collective data from 31 trials indicated that albendazole/mebendazole can achieve ERRs of 99.9/97.6%, 64.4/69.3%, and 92.4/76.5% for *A. lumbricoides*, *T. trichiura*, and hookworms, respectively (reviewed by [64]). Ivermectin is another successful anthelmintic and is considered the first choice of treatment against *S. stercoralis* infections [67]. The ease of administration of anthelmintic drugs, alongside their cost-effectiveness against a wide variety of GI nematodes, has led to their widespread use in humans and livestock [62-64,68]. Indeed, constant re-infections both in humans and livestock necessitate constant MDA or targeted strategic deworming programmes. However, this complete reliance on anthelmintics for the control of these parasites bears extensive risks, as indicated by the global threat of emerging anthelmintic resistance.

Anthelmintic resistance has already been observed in several GI nematodes of veterinary importance (reviewed by [8,9]). Resistant helminths have rapidly grown into a global issue and are proving to be especially problematic in South Africa, Australia, South America, and New Zealand; in these regions the extent of resistance has severely threatened the sustainability of the sheep farming [69]. Due to the significant spread and economic impact, anthelmintic resistance is expected to develop into one of the greatest challenges to the livestock industry [70]. Although anthelmintic use in humans has not matched that in veterinary species, MDA programmes in the developing world have significantly increased the frequency of administration, as well as global coverage, over the past 15 years; this drive was most recently further driven by the World Health Organization (WHO), 2012 Goals [71]. Consequently, there are growing concerns regarding the emergence of anthelmintic resistance in human STHs [72-74]. Indeed, the evidence of reduced efficacy of ivermectin in the treatment of *Onchocerca volvulus* in humans [75], and the results of efficacy evaluations linked to detection of resistance mutations in human STHs drive the demand for novel, integrated strategies to control helminth infections. However, these require a comprehensive understanding of the

fundamental biology and epidemiology of these parasites and their interactions with the mammalian host.

1.5. Human microbiome and metabolome

The microbial cells that colonize the human body and make up the human microbiome are at least as abundant as our somatic cells, while also encompassing a significantly higher genetic complexity than our own genome [76,77]. The microbiome consists of viruses, fungi, eukaryotes, archaea, and protozoans, amongst others, but bacteria are the most abundant and diverse domain found within and on our bodies; in fact, it is estimated that 500–1,000 species of bacteria exist in and on us [76]. Hence, when referring to the microbiome, the focus typically falls predominantly on the bacterial components, which also holds true for this thesis. The human microbiome is one of the most dynamically researched areas of interest of the past decades, with most work investigating the microbiota's role in the GI tract, since it harbours most of our microbes (reviewed by [78]). Humans, like many other animals, have co-evolved with their microbiome, creating complex, body-habitat-specific, adaptive ecosystems which are finely attuned to relentlessly changing host physiology [76,79]. These trillions of microbes have tremendous potential to impact host physiology, both in health and in disease [79]. Together they contribute to metabolic functions, such as nutrient catabolism and synthesis of essential organic compounds, but also play key roles in the development of adaptive immunity and protection against pathogens (Fig. 6) [13-16]. Yet, these core functions are not necessarily fulfilled by the same set of microbiota in different people [80]. In fact, some of the microbes that constitute the functional core can even vary within the same individual over the course of their life [80].

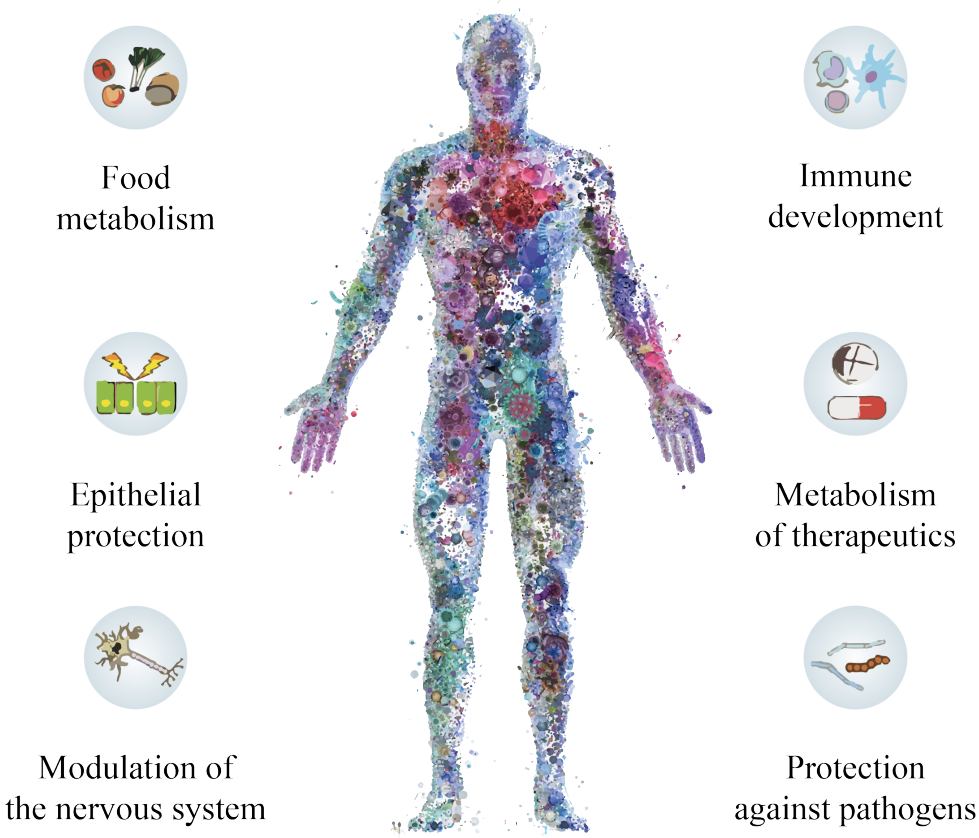

Fig. 6 Key functions of the microbiome on human health (human by Tina Hesman Saey).

Disturbances of this ecosystem and of the composition of the gut microbiota (i.e. dysbiosis) have been unequivocally linked to the onset of a range of gut and systemic diseases, such as inflammatory bowel disease, multiple sclerosis, diabetes (types 1 and 2), allergies, asthma, autism, and cancer [79]. The concept of microbial dysbiosis has proved challenging to definitively define, yet is commonly considered as a perturbation of the host microbiome that deviates from an otherwise balanced and functioning ecosystem [81] to prolong, exacerbate, or induce a detrimental health effect [79]. Consequently, the majority of research efforts have focused on identifying microbiome characteristics that broadly distinguish a healthy from an unhealthy state [79]. However, this endeavour is complicated by the naturally high level of interpersonal diversity even in the absence of disease [82,83], and which thwarts attempts to identify simple microbial elements or fluctuations that either cause disease or indicate a diseased state [79]. Nonetheless, a variety of potential features common to dysbiosis have been proposed, including shifts in gut microbial diversity and/or on a taxonomic level, through the presence and/or overabundance of opportunistic pathogens or foreign bacteria, as well as the absence or reduction of beneficial/commensal microbes [79].

The gut microbiome consists of a wide range of microbiota, which are selected from a

large meta-population of potential colonizers that are together required to ensure the coverage of a core set of functions [84,85]. This drives microbiota diversity, both in terms of richness (i.e., the number of species making up a microbial population) and microbial evenness (i.e., the relative abundance of each microbial species in a population), together known as alpha diversity [86]. This diversity within the gut microbiota provides functional redundancy, since the functional potential can be realised with a subset of the available microbiota and, if the need arises, minor functional vacancies can be filled by a different set of microbes [79]. Therefore, high levels of gut microbial diversity are generally indicative of microbiome health [87] and temporal stability [88], while the lack thereof is associated with a range of diseases such as inflammatory bowel disease [89], types 1 [90] and 2 [91] diabetes, and obesity [92] amongst others. Notably, such shifts in diversity are manifested in changes in the abundance of certain bacteria, which can act as a further biomarker of dysbiosis. Although complicated by inter-individual microbiota variability, the following bacterial families have been described to be particularly important within the human gut: *Bacteroidaceae*, *Clostridiaceae*, *Prevotellaceae*, *Eubacteriaceae*, *Rumminococcaceae*, *Bifidobacteriaceae*, *Lactobacillaceae*, *Enterobacteriaceae*, *Saccharomycetaceae*, and *Methanobacteriaceae* (reviewed by [79]). Significant alterations to the abundances of these and other taxa within a healthy ecosystem can have profound effects on the stability and functionality of the host microbiome and lead to physiological impairments such as compromised epithelial barrier function, immunity, and metabolism [87].

The gut microbiome is key in maintaining homeostasis of host metabolism through contributing to metabolism of food components, vitamin production and xenobiotic metabolism (reviewed by [93]). The resulting microbial metabolites play a significant role within the metabolism of the host, aiding in the regulation of host metabolism at different levels. Consequently, significant shifts in the gut microbiome can have a profound impact on the host metabolome and have been linked to diseases such as obesity [92] and insulin resistance [94]. Due, to this strong link between the microbiome and metabolome, parallel investigations of both systems provides a more complete picture of the overall state of the host's GI tract [95]. While analyses of the microbiome provide information on the presence/absence and abundances of microbes, it is challenging to understand their activity within the ecosystem. For example, the discovery of an expansion of certain short-chain fatty acid (SCFA) producing bacterial taxa, e.g. the mucin degrading *Akkermansia muciniphila* (produces proprionate), *Eubacterium rectale*, or *E. hallii* (both produce butyrate), could point towards an inflamed GI tract of the host, due to the role SCFAs play in inflammation (reviewed by [96,97]). Hence, metabolomics can

provide a functional readout of microbial activity, by providing information on the metabolic interplay among the host and gut microbiota [98].

1.6. Host-helminth-microbiota interactions

Thus far, the vast majority of studies that have attempted to unravel host-parasite interactions have predominantly focused on two key players, the parasite and the host immune system (e.g., [99-102]). However, over the past few years a growing body of evidence supports a key role of infections by GI helminth parasites in shaping the composition of the vertebrate gut microbiota, with significant implications for local and systemic host immunity (reviewed by [103]). It has been demonstrated that GI helminths impact on the composition of the mammalian host gut microbiota and relative abundance of individual bacterial taxa (reviewed by [104]), with downstream effects on host immunity and metabolic potential [103,105,106]. These findings have led to an improved understanding of parasite systems' biology and host-pathogen interactions, while also carrying the potential to pave the way towards novel microbiome-targeted parasite control strategies (reviewed by [104]), as well as helminth therapy approaches [49,107]. However, whilst several studies to date have been conducted in rodent models of human helminth infections [52,53,108-120], companion animals [121,122], and veterinary species [123-129], less have investigated the impact of GI helminth infection on the human microbiota [49-51,107,130-135]. Unravelling the impact of such infections on human gut microbiota is further complicated by the lack of major consistencies in findings between the existing studies. Particularly, whilst the investigation of human-helminth-microbiome interactions in real-world scenarios is key to gain insights into the actual impact such infections have in a natural setting, findings from such studies have proven challenging to untangle. The extent of inter-subject variability related to differences in diet, helminth burden, and re-infection rate, amongst others, results in a substantial amount of noise in the dataset and may contribute significantly to inconsistencies among findings from different studies (Fig. 7; reviewed by [136]).

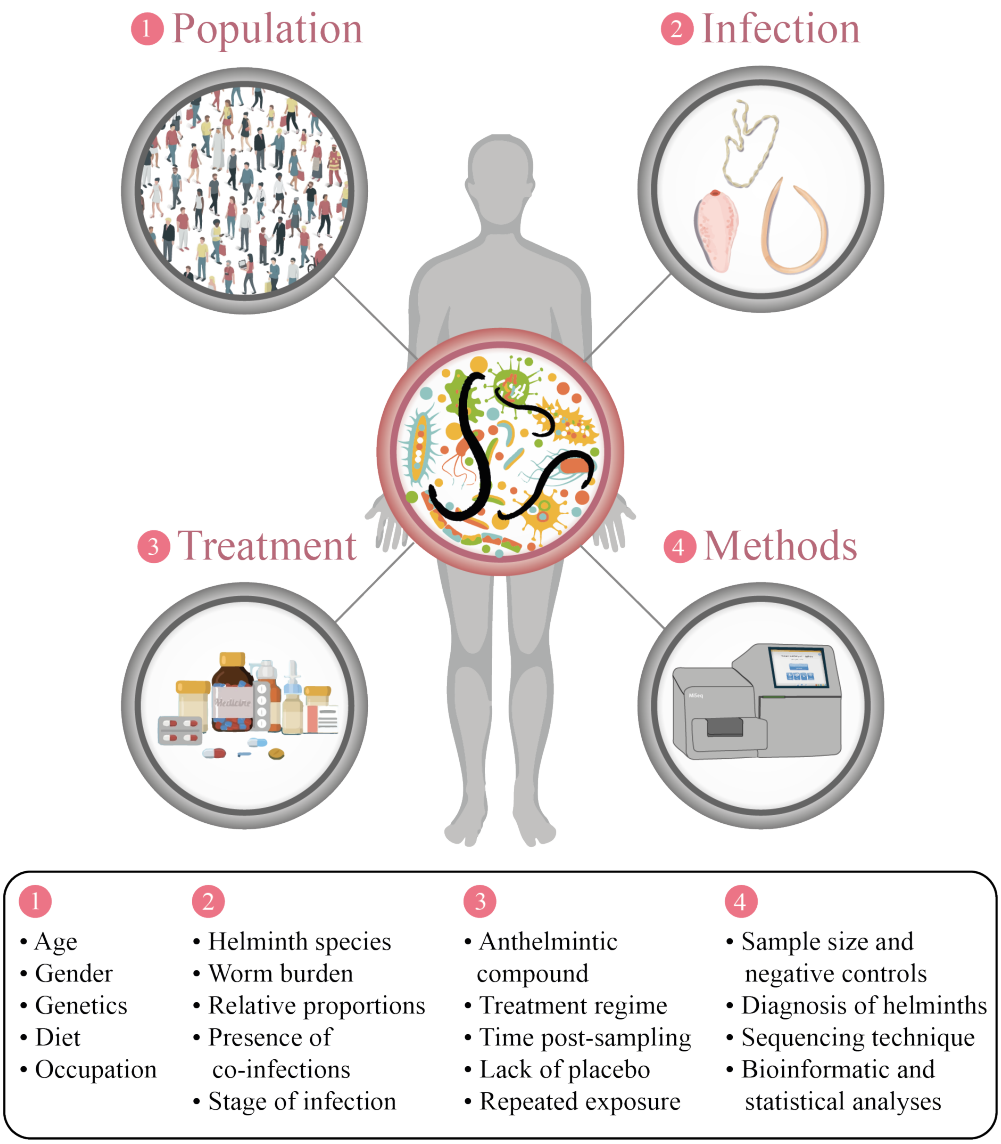

Fig. 7 Sources of variation and confounding factors potentially impacting the outcome of studies on human-helminth-gut microbiota interactions in helminth-endemic regions [135].

For example, while two studies identified increased levels of gut microbial diversity following helminth infection [50,131], the remaining studies reported no changes [130,132,134,135,137] or even decreased levels of diversity [51]. The convolutedness of the data becomes even more apparent when assessing the fluctuations of specific bacterial taxa. Indeed, two separate studies conducted in Malaysia [50,133] found that *T. trichiura* infection reduced the relative abundance of the bacterial genus *Prevotella* in the faeces of infected individuals, whereas other studies did not report significant variations in faecal populations of *Prevotella* in people either solely infected by *T. trichiura* or co-infected with other species of STHs [51,131]. Furthermore, whilst it was reported that single-species infections with *N. americanus*, *A. lumbricoides* or *T. trichiura*

were distinctly characterised in the gut microbial profiles of infected individuals, the specific features were inconsistent across two independent cohorts of helminth-infected volunteers from Liberia and Indonesia [131]. These findings further substantiate the concern that other, yet undetermined, environmental factors are likely to affect qualitative and quantitative alterations of the gut microbial profiles of infected individuals.

Nonetheless, some consistencies between the abundance of selected bacterial taxa and infections by one or more STHs exist; both *Olsenella* and *Allobaculum* were significantly more abundant in the gut microbiota of helminth-infected individuals compared to uninfected controls [131]. The study by Rosa et al. [131] was the first to report a link between infections by STHs and the abundance of these bacterial genera in the human gut. Besides 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 abundance. Indeed, specific gut microbial changes identified in cohort of Ecuadorian children co-infected with *T. trichiura* and *A. lumbricoides* could not be observed in the faecal microbiota of *Trichuris* only-infected children [51]. Analogously, certain shifts in the microbiota that were detected in studies conducted in people with mono-specific infections with, e.g. *A. lumbricoides*, could not be identified in the microbiome of human volunteers infected with the same parasite, as well as other helminth species, such as *T. trichiura* and *N. americanus* [131]. These findings suggest that a complex interplay exists between the host gut and its macro- and microbiota, which might be difficult to replicate in experimental settings (reviewed by [136]).

A further common issue with studies investigating the gut microbial impact of natural helminth infections is the availability and subsequent inclusion of 'genuine' negative controls. These would ideally consist of individuals from the same communities of parasite-infected subjects, yet who have not been previously exposed to infections by parasitic helminths. However, since such studies are typically conducted in helminth endemic areas, the presence of such control subjects is highly unlikely, and instead individuals with no evidence of patent helminth infections are recruited as control subjects [50,51,131]. Hence, interpretation of findings from such studies calls for caution, especially given that it has been demonstrated that parasite-associated alterations in the gut microbiota can persist subsequent to anthelmintic treatment [51,132].

It is worth noting that, in instances where deworming-associated changes in human gut microbial profiles were observed, these were generally moderate [130,132,133]. Accordingly, a recent study conducted on faecal samples from a rural community in Indonesia found that the

gut microbial composition of volunteers, repeatedly treated for 21 months with either albendazole or placebo, was more similar to pre-treatment samples, than to the composition of uninfected control subjects [131]. A parallel investigation focusing on the same study cohort observed reduced populations of *Prevotella* in albendazole-treated subjects in which complete deworming did not occur, compared to placebo-treated individuals with patent helminth infections [130]. Notably, failure of albendazole treatment was accompanied by a dominance of *T. trichiura* (over other helminth species) in these subjects, while placebo-treated individuals maintained infections with multiple species of helminths. Consequently, differences in the helminth species present and their relative abundances, between albendazol- and placebo-treated individuals, could explain variations in the gut microbial composition of these subjects [130]. However, while no significant associations between colonisation by *T. trichiura* and *Prevotella* abundance were detected in the Indonesian cohort [130,131], two independent studies conducted in Malaysia have previously reported a negative association between *T. trichiura* infections and *Prevotella* abundance [50,133]. In particular, Ramanan and co-authors [133] found that, subsequent to albendazole treatment, expansion of *Prevotella* populations in the human faecal microbiota was associated with reduced *T. trichiura* egg counts. Conversely, 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 albendazole and ivermectin treatment on the gut microbial profiles of a cohort of *Trichuris*-infected children from Ecuador [51]. Analogously, no qualitative or quantitative changes to faecal microbial composition were detected in two cohorts of schoolchildren from Côte d'Ivoire and Zimbabwe infected with *S. mansoni* and *S. haematobium*, respectively, following treatment with praziquantel [132,137]. Yet, successful elimination of the infection was linked to an increased abundance of *Fusobacterium* spp. pre-treatment, and expansion of the same taxa 24 hrs post-treatment [132]. Together, these findings highlight the substantial complexity in analysing and interpreting data from host-helminth-microbiota interaction studies and the considerations that need to be made in terms of study population, negative controls, and anthelmintic treatment (amongst others); however, these initial observations also have elucidated the great potential that lies within unravelling this intricate network of interactions.

Indeed, in an effort to gain a greater understanding of the specific impact that certain GI helminths have on the human microbiota under more controlled settings, investigations of experimental infections in humans have been carried out. However, while such studies carry great potential, they are rare since experimental helminth infections of humans are typically solely conducted in the context of helminth therapy. To date, three such studies exist, all

assessing the impact of experimental infections with *N. americanus* on the gut microbiota of human volunteers suffering from coeliac disease [49,107,138]. The study by Cantacessi and co-authors [49] indicated a trend towards increased microbial diversity post helminth infection, but did not suggest any significant changes in gut microbiota composition; this is likely to be associated with the limited sample size of this study (n=8) [49]. The other two studies not only assessed the effects of *N. americanus* infections on the gut microbiota of coeliac patients, via faecal [107] and duodenal [138] samples respectively, but also investigated the impact of administering increasing doses of dietary gluten over time,. Both studies reported significant increases in bacterial alpha diversity following hookworm infection and also found differences between several time points [107,138]. Notably, Giacomin and co-authors [138] reported a significant expansion of specific bacterial taxa post-infection, e.g. bacteria belonging to the phylum Bacteroidetes. However, this study lacked samples post helminth infection and pre-gluten challenge, which makes it difficult to definitively determine if the observed changes were associated with helminth infection or if they occurred due to gluten introduction [138]. Notably, a small subset of samples (n = 8), from the same helminth therapy trial of human volunteers with coeliac disease [139] as mentioned above, were subjected to metabolic analyses [140]. This study presented the first and, to date, only study investigating shifts in GI metabolites following GI helminth infection [140]. It found that short chain fatty acids (SCFAs) were increased in the study subjects experimentally infected with hookworms [140]. SCFAs have been characterised as anti-inflammatory metabolites and alongside the clinical improvement observed in the patients [139] suggested that these molecules could be at least one of the causative agents underlying the therapeutic potential of GI helminths in autoimmune conditions [140]. These results are promising and promote further investigations of the metabolic effect of GI helminth infections, yet due to the limited sample size the findings can merely be considered as a first step. The limited number of comparative studies complicates the detection of consistencies and detection of the underlying cause, but investigations of the gut microbial and metabolic impact of experimental helminth infections present an exciting opportunity to eliminate a multitude of confounding variables affected studies of natural infections.

Once we have identified certain trends in microbial or metabolic shifts, which appear to be consistent and of potential importance, via human studies of natural or experimental parasitic helminth infections, we can harness the power of murine models of human helminth infections. Indeed, one of the most consistent gut microbial responses that has been reported to date is the increase of *Lactobacillaceae* populations in mice infected with the whipworm *T. muris* [111], the hookworm *N. brasiliensis* [110], and especially the roundworm *Heligmosomoides*

polygyrus [53,114,115,141]. These bacteria belong to the phylum Firmicutes, are gram-positive, and play a key role in carbohydrate metabolism [142]. However, these bacteria gained most interest, due to their immune-modulatory functions in the host gut, primarily by promoting an expansion of T regulatory cells; this has also led to the use of these lactic acid bacteria as a probiotic supplement for GI inflammatory diseases [143]. Notably, it has been demonstrated that mice experimentally infected with *H. polygyrus* experience an expansion of *Lactobacillaceae*, while also suffering from increased worm burdens when *Lactobacillus* species were artificially administered prior to the helminth infection [115]. This led Reynolds and co-authors [115] to hypothesise that there might be a mutualistic relationship between *Lactobacillaceae* and certain parasitic helminths, whereby each promotes the activation of T regulatory mechanisms and, consequently, minimises the impact of the host immune response on the counterpart. As these findings demonstrate, the high levels of experimental control, innate to murine models, present unique opportunities towards the exploration of specific helminth-microbiota interactions observed in humans; yet, they are heavily reliant on the input of human data for translational applicability back to human settings.

Investigations of natural and experimental helminth infections, as well as murine models of such infections, all play a pivotal role in identifying common signatures across different settings and a pursuit of these diverse research avenues remains key to understanding the complexities underlying host-helminth-microbiota-metabolite interactions. However, this requires further studies that thoroughly consider inclusion/exclusion criteria for the selection of participants, include appropriate controls, and follow standardised experimental and data analysis protocols, to disentangle the potential influence of parasite-, drug- and/or population-dependent variables in natural and experimental settings [136]. Only then we will be able to draw meaningful and cross-applicable conclusions on global and system-specific gut microbial/metabolic shifts that are caused by helminths in the host.

1.7. Research approaches

Over time scientists have employed a range of different research approaches towards the exploration of the impact of parasitic helminths on the host microbiome and metabolome, ranging from bacterial culturing techniques to whole metagenome sequencing for the former and from inferred metabolomics to mass spectrometry for the latter. Until the 1990s, most studies predominantly relied on culture-based techniques for the profiling of gut microbial changes and, thus, would apply samples to growth media and record presence/absence of selected bacterial taxa [144]. However, this approach would only allow clustering and

investigation of a limited number of taxa, i.e. aerobic bacteria that would grow on existing growth media [145]. Consequently, resulting findings merely provided initial insights into this dataset and were far from a comprehensive and reliable analysis of the gut microbial composition [146]. This led to the exploration of other research approaches and resulted in the gradual shift from culture-dependent to culture-independent techniques, hence increasing the potential for discovery and novel insights [147].

One such technology is fluorescence in situ hybridization (FISH), a more complex technique that can be performed directly on bacteria. FISH makes use of the bacterial 16S ribosomal RNA (16S rRNA) gene, which is a component of 30S ribosomal small subunit and includes flanking regions that are highly conserved across bacterial taxa, as well as hypervariable regions that yield species-specific sequences and enable phylogenetic identification [148,149]. Fluorescently labelled oligonucleotide probes are hybridised to complementary target 16S rRNA sequences, allowing phylogenetic identification of known species through flow cytometry. Terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis/temperature gradient gel electrophoresis (DGGE/TGGE) are further, less targeted approaches that utilise the 16S rRNA gene in bacteria to indicate microbial diversity, rather than identify phylogeny [150,151]. In T-RFLP, fluorescently labelled primers are used to amplify the DNA followed by digestion of the 16S rRNA amplicon through restriction enzymes, and separation by gel electrophoresis. In DGGE/ TGGE the 16S amplicons are denatured by a temperature gradient or a denaturant within the gel itself, allowing for separation of bacterial taxa according to sequence differences in the 16S gene. Other techniques that involve both phylogenetic identification of bacterial taxa and a degree of quantification also came into use for microbiome investigations, e.g. Sanger sequencing or qPCR of cloned 16S amplicons and DNA microarrays [152-154]. Whilst cloning and qPCR still only target specific microbial groups, microarrays can be used for a more general gut bacteria population analysis, and can be applied to isolated DNA without introducing PCR bias. However, particularly over the past decade, innovations in next generation sequencing (NGS) and 'omics' technologies have led to more sophisticated methodologies, which are both quantitative and involve a significantly less or non-biased approach, whereby all microbial species present are sequenced and quantified. This includes sequencing 16S amplicons with either 454 Pyrosequencing® or Illumina® (still subject to minor PCR bias) [155], or the even more advanced shotgun (whole metagenome) sequencing, which prevents PCR bias by sequencing the whole genome [156]. Both of these approaches have vastly improved scientists' ability to investigate the gut microbiome and gain insights into microbial ecosystem

interactions within its host (Fig. 8) [147,157,158].

Particularly, high-throughput 16S amplicon sequencing has been employed in a vast number of studies, due to its cost-efficiency. Here, amplicon PCR primers bind to the conserved 16S region to allow the amplification of the taxa-specific gene fragments [159,160]. Upon

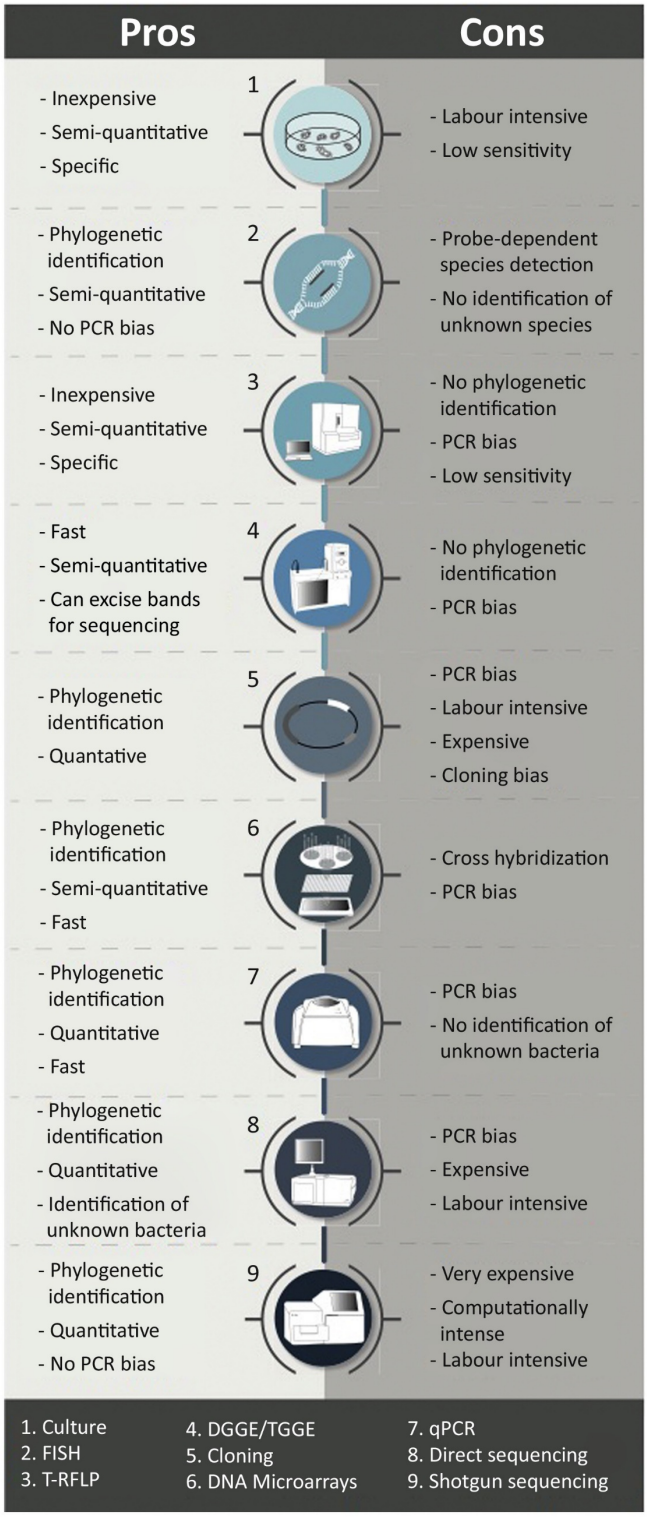

Fig. 8 Pros and cons of different microbiota profiling techniques [104].

amplification, the complete microbial community is sequenced by one of the existing NGS technologies, such as the pyrosequencing method by 454 Life Science or Illumina's sequencing methods, amongst others [158]. The resulting raw reads are stored as FASTA files, a text-based format for representing either nucleotide sequences. They are then quality filtered, based on a Phred-score that estimates probability of a base being wrong, and denoised, to decrease the potential impact of low quality reads, sequencing errors, and chimeric sequences on the sequencing results [161]. Thereafter, the remaining sequences are clustered according to sequence similarity through a 97-99% similarity threshold, to determine all operational taxonomic units (OTUs) within the dataset [162]. These OTUs are then compared to a reference database that contains known 16S rRNA sequences for taxonomic assignment, e.g. Greengenes (v13.8; http://greengenes.secondgenome.com/) or SILVA (https://www.arb-silva.de/download/archive/qiime; Silva_132) [161]. The speed of read-processing has been significantly increased by the emergence of bioinformatics platforms, such as Quantitative Insights Into Microbial Ecology (Qiime/Qiime2) [163] and Mothur [164] that facilitate this workflow through easy to use pre-built command options. The final output from these read-pipelines are OTU tables, which indicate the relative abundance of each identified taxon within every analysed sample. This then allows the thorough investigation of the dataset through biostatistical means in R or software packages specifically designed towards the analysis of microbial sequencing data, such as Calypso [165] (cgenome.net/calypso/). The OTU table can also be used to estimate metagenome content through specific software packages, e.g. PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) [166], which allows the user to predict the functional composition of a microbiota using marker gene data and a database of reference genomes, consequently enabling the investigation of inferred metabolic pathway changes without need for further experiments [166]. However, the quality of the data is limited by the reference database used for the metabolic inferences [166].

Besides 16S sequencing other innovative 'omic' technologies, such as metagenomics and metabolomics can be applied to host-parasite-microbiota interaction investigations [157,161,167]. Whole metagenome analyses involve the sequencing of the whole nucleotide content within a sample and thus include not only bacteria, but also viruses, fungi, and eukaryotes amongst others and also enable strain level identification of gut microbiota, as well as their functional potential [157]. This is achieved by fragmenting the DNA of all organisms in a given sample for random shotgun sequencing [157,167]. Similar to 16S sequencing, the resulting sequences are quality filtered and aligned to different locations for genomes present in the sample [168]. To identify taxa, sequences from any informative genetic region can be used (e.g.

16S-bacteria or 18S-fungi), while protein coding regions can provide insights into metabolic or other functional capabilities of the analysed microbiome [168]. However, all this additional information is also manifested at the significantly higher per sample costs, when compared to 16S sequencing runs. Metabolomic investigations provide a further tool to gain key insights into metabolic changes within a study cohort and can be used in conjunction with any of the above mentioned methods to correlate microbiome to metabolome changes [169]. The main technologies typically applied in such studies are either mass spectrometry (MS) based or rely on high-resolution nuclear magnetic resonance (NMR) [169]. Whilst MS is ideal for the investigations of specific metabolite groups, NMR provides an overview over the metabolic profile of a sample, as well as externally induced changes, e.g. via helminth infection [157,169].

Already, many of the above mentioned technologies are being combined to generate multi-omic datasets to offer support towards novel biological hypotheses. Notably this drive is being significantly aided by various statistical and computational methods that have been developed to integrate high-dimensional multi-omic data in the pursuit of descriptive and predictive models of gut microbial community function that can be tested in mechanistic experiments [157].

1.8. Conclusions of the literature review and research aims

Our understanding of the biology of human parasitic helminths is greater than ever before. However, so far, the research has focused on gene expression profiling, immune- and protein-protein interactions in host-parasite systems, while largely neglecting other aspects of systems biology of parasites following invasion of, and establishment in, the host gut. This includes the understudied area of parasite-microbiota interactions. It has been shown that the balance between the gut microbiota and the host is crucial for health maintenance and that a disturbance of this balance can result in a range of diseases (reviewed by [79]). Hence, given that GI nematodes and the gut microbiota share the same ecological niche within the human host, it is plausible that GI helminths and the host microbiota interact, and that this could significantly impact on the health and homeostasis of the parasite-infected hosts. Indeed, the few studies that exist to date have detected significant host microbiome shifts following GI helminth infection. However, there are few consistencies in the findings, likely due to the highly heterogeneous nature of the study designs, sample processing, data analysis, and host-parasite systems investigated. Fortunately, the availability and affordability of next generation sequencing and reliable bioinformatic/biostatistical tools now enables us to investigate such host-parasite-microbiota interactions in depth and at high throughput in a coherent and

consistent manner [157]. Furthermore, the question remains whether the impact that GI helminths have on the host microbiota are, due to their direct interactions or stem from immunological shifts upon infection and, consequently, if EI helminth infections can have a similar impact.

Therefore, the aims of this thesis were to explore the impact of such helminth infections in various systems, ranging from natural multi-species infections in a developing country to highly controlled and experimental murine models of human helminth infections, involving a single species of parasitic helminth. Meanwhile, consistent and cutting-edge protocols for sample collection, read processing, and bioinformatic/biostatistical data analysis were applied to ensure comparability and reliability of these studies. This enabled the confident identification of microbiota changes that are consistent across different settings, as well as helping to detect alterations that are specific to a certain host-helminth system. In future, these findings might help guide novel therapeutic strategies towards the control of helminth infections, as well as providing insights into potential mechanisms underlying the therapeutic potential of GI helminths in the treatment of allergic and autoimmune conditions.

The specific aims of this thesis were the following:

(1) Investigate the consequences of natural multi- or mono-species infections by helminth parasites on the composition of the human gut microbiota (Chapters 2 and 3).

(2) Elucidate the longitudinal impact of experimentally controlled mono-species helminth infections on the human gut microbiota (Chapter 4).

(3) Examine what impact an EI helminth infection has on the host microbiome in a murine model of human schistosomiasis (Chapter 5).

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CHAPTER 2

Infections by human gastrointestinal helminths are associated with changes in faecal microbiota diversity and composition

Abstract

Investigations of the impact that patent infections by soil-transmitted gastrointestinal nematode parasites exert on the composition of the host gut commensal flora are attracting growing interest by the scientific community. However, information collected to date varies across experiments, and further studies are needed to identify consistent relationships between parasites and commensal microbial species. Here, I explore the qualitative and quantitative differences between the microbial community profiles of cohorts of human volunteers from Sri Lanka with patent infection by one or more parasitic nematode species (*H+*), as well as that of uninfected subjects (*H-*) and of volunteers who had been subjected to regular prophylactic anthelmintic treatment (*Ht*). High-throughput sequencing of the bacterial 16S rRNA gene, followed by bioinformatics and biostatistical analyses of sequence data revealed no significant differences in alpha diversity (Shannon) and richness between groups (P = 0.65, P = 0.13 respectively); however, beta diversity was significantly increased in *H+* and *Ht* when individually compared to *H-* (P = 0.04). Among others, bacteria of the families *Verrucomicrobiaceae* and *Enterobacteriaceae* showed a trend towards increased abundance in *H+*, whereas the *Leuconostocaceae* and *Bacteroidaceae* showed a relative increase in *H-* and *Ht,* respectively. These findings add valuable knowledge to the vast, and yet little explored, research field of parasite – microbiota interactions and will provide a basis for the elucidation of the role such interactions play in pathogenic and immune-modulatory properties of parasitic nematodes in both human and animal hosts.

2.1 Introduction

More than one billion people worldwide are estimated to be infected by gastrointestinal (GI) soil-transmitted helminths, including the roundworm *Ascaris lumbricoides*, the whipworm *Trichuris trichiura* and the hookworms *Necator americanus* and *Ancylostoma duodenale* [1]. Infections by these nematodes alone are estimated to cause the loss of 4.98 million disability-adjusted life years (DALYs) globally [2], mainly affecting areas of developing countries characterised by suboptimal standards of sanitation and hygiene [3]. Despite global efforts to control infections by these parasites *via* mass drug administration (MDA), repeated exposures to infective larvae and high re-infection rates in at-risk populations make interruption of the life cycles of these nematodes and their elimination difficult to achieve [4,5]. These challenges, together with the realistic threat of emerging drug resistance [6] drive the continual search for new, integrated strategies to control these diseases, based on a thorough understanding of the fundamental biology and epidemiology of these pathogens and their interactions with the human hosts [7]. Recently, studies of the intimate mechanisms that regulate the relationships between GI soil-transmitted nematodes and their vertebrate hosts have involved investigations of the impact that patent parasite infections exert on the composition of the gut commensal flora and relative abundance of individual bacterial groups [8-10]. The increased attention towards detailed explorations of parasite-microbiota interactions stems from knowledge that the gut commensal flora plays several key essential roles in human health, including nutrient metabolism, protection against pathogens and regulation of both innate and adaptive immune responses [11,12]. Therefore, given that GI nematodes and the gut microbial flora share the same environment within the human host, it is plausible that parasite-microbiota interactions impact substantially on the health and homeostasis of helminth-infected hosts [10]. For instance, GI nematodes and the microbiota compete for host nutrients while, in parallel, the known immune-modulatory properties of a range of parasites may translate into dramatic modifications of the mucosal and systemic immunity to the resident bacteria [10]. The effects that GI nematode infections exert on the commensal flora of vertebrate hosts have long been neglected; however, recent studies have contributed preliminary information to this little-known field of research, mainly driven by the need to better understand the factors that determine the immune-modulatory properties of selected species of parasitic nematodes [13-17]. Our group has recently attempted to determine the impact that experimental infections by the human hookworm, *N. americanus*, exert on the composition of the gut microbiota of human volunteers [15,17,18]. While no shifts in the relative abundance of individual bacterial taxa were observed over the course of these studies, increases in microbial species richness and diversity were detected following

experimental infections [15,17,18]. However, these studies, conducted under controlled experimental settings and with a known number of infective larvae, are unlikely to represent 'real-world' infections (caused by the simultaneous presence of multiple parasite species with varying infection burdens). Thus far, to the best of our knowledge, only two studies have evaluated differences in the composition of the gut microbiota of human subjects naturally infected by GI nematodes [8,9], with contrasting results. Indeed, while in the first study, Cooper and colleagues [8] detected a reduction in faecal bacterial diversity in Ecuadorean school children naturally infected by *T. trichiura* and *A. lumbricoides* compared to uninfected children or children solely infected by the former, a study by Lee et al. [9], reported a greater richness in the faecal microbiota of a cohort of indigenous Malaysian volunteers infected by multiple GI nematodes (i.e. hookworms, whipworms and roundworms) when compared with that of a group of uninfected subjects from New York. This data highlights the need for additional explorations of the impact that natural patent infections exert on the gut microbiota of infected human subjects. In addition, in the studies by Cooper et al. [8] and Lee et al. [9], the microbial profiling of helminth-infected and uninfected subjects with that of volunteers subjected to regular anthelmintic treatment was not examined. Given the widespread use of MDA in parasite-endemic areas [5], the possible consequences of regular use of chemotherapeutics on the composition of the host gut commensal flora deserves further investigation. Amongst the developing countries in which MDA is in use, Sri Lanka provides suitable settings for such a study, given a 29% prevalence estimate for soil-transmitted helminths (including GI nematodes) in school children [19] and the implementation of mass deworming programmes since 1994 [20].

Therefore, in this chapter, I explore the qualitative and quantitative differences between the microbial community profiles of human volunteers (from diverse Sri Lankan communities) infected by one or more GI nematode species, and compare the gut microbial profiles of these subjects with those from a cohort of volunteers from the same geographical area who had been subjected to regular prophylactic anthelmintic treatment.

2.2 Materials and methods

2.2.1 Ethics statement

This study was approved and carried out in strict accordance and compliance with the guidelines of the Institutional Ethical Review Committee, Faculty of Medicine, University of

Peradeniya, Sri Lanka (Research Project No. 2015/EC/58). Written informed consent was obtained from all subjects enrolled in the study.

2.2.2 Study area and characteristics of the population

A total number of 76 subjects from nine villages in four districts of Sri Lanka were screened for the presence of patent infections by GI nematodes (Fig. 1). Participants were distributed as follows:

- 45 subjects from the Kandy district, villages of Rangala, Lolgama, Elagolla, Lunugala, Hanthana, Akurana;
- 10 from the Jaffna district, Valalai village;
- 15 from the Puttalam district, Kandakuliya village;
- 6 from the Kegalle district, Mawanella village (Fig. 1).

Subjects were both men and women, of varying ages and social background, did not report symptoms of GI disease or any other concomitant diseases, and had not been treated with antibiotics over at least 6 months prior to the study (Table 1). Participants from the villages of Rangala, Lolgama, Elagolla, Lunugala and Hanthana were workers in tea estates, while those from Kandakuliya and Valalai were fishermen (cf. Fig. 1). Subjects from Mawanella and Akurana were living in congested urban areas characterised by poor sanitary living conditions. All participants were interviewed using a standardised, pre-tested questionnaire aimed to identify means of access to water, knowledge of sanitary and hygiene standards, availability of and access to health care facilities, awareness of risks of infection by GI helminths, and frequency of anthelmintic treatments. A copy of the questionnaire is provided in Supplementary Figure S1.

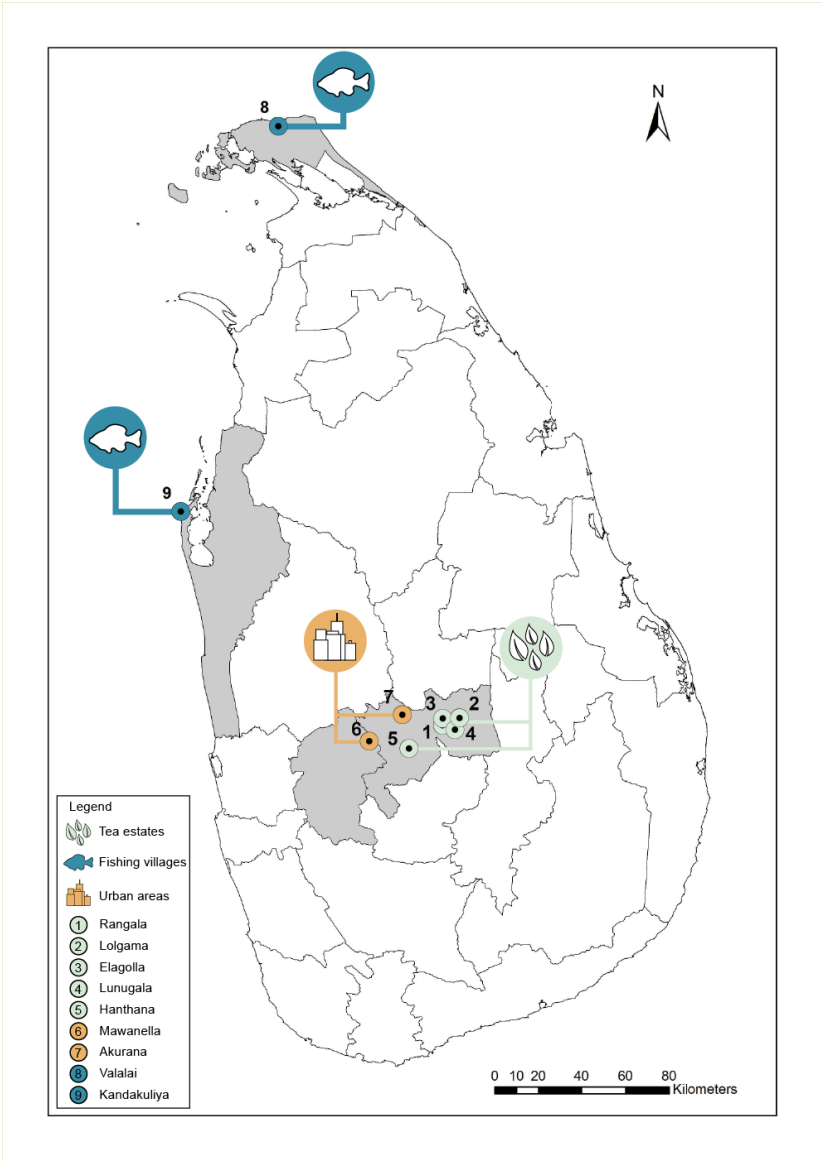

Fig. 1 Sampling locations in Sri Lanka. Numbers 1-9 represent the villages Rangala, Lolgama, Elagolla, Lunugala, Hanthana, Mawanella, Akurana (in Kandy District), Valalai (in Jaffna District) and Kandakuliya (in Puttalam District), respectively.

Table 1 Village, age (years), gender of helminth-positive (*H*+), helminth-negative (*H*-) and helminth-negative but regularly treated (*Ht*) subjects enrolled in this study. The parasite species infecting *H*+ volunteers and compound administered to *Ht* volunteers are also indicated (NA = not applicable; NP = not provided).

ID	Village	Age (years)	Gender	Parasite species / Drug
H+				
H+ 01	Hanthana	1-18	Male	*Ascaris*

H+ 02	Hanthana	1-18	Male	*Ascaris*
H+ 03	Hanthana	1-18	Female	*Ascaris*
H+ 04	Hanthana	1-18	Female	*Ascaris*
H+ 05	Hanthana	1-18	Female	*Ascaris*
H+ 06	Hanthana	1-18	Male	*Ascaris*
H+ 07	Kandakuliya	1-18	Female	*Trichuris*
H+ 08	Mawanella	1-18	Male	*Hookworm*
H+ 09	Mawanella	51+	Female	*Hookworm*
H+ 10	Rangala	19-50	Male	*Hookworm*
H+ 11	Rangala	1-18	Female	*Hookworm*
H-				
H- 01	Hanthana	1-18	Male	NA
H- 02	Hanthana	1-18	Male	NA
H- 03	Hanthana	1-18	Male	NA
H- 04	Hanthana	1-18	Female	NA
H- 05	Hanthana	1-18	Male	NA
H- 06	Hanthana	1-18	Male	NA
H- 07	Hanthana	1-18	Male	NA
H- 08	Hanthana	1-18	Female	NA
H- 09	Hanthana	NP	NP	NA
H- 10	Hanthana	1-18	Male	NA
H- 11	Hanthana	1-18	Male	NA
Ht				
Ht 01	Akurana	1-18	Female	Pyrantel pamoate
Ht 02	Akurana	51+	Female	Pyrantel pamoate
Ht 03	Kandakuliya	1-18	Female	Pyrantel pamoate
Ht 04	Kandakuliya	19-50	Male	Pyrantel pamoate
Ht 05	Kandakuliya	1-18	Female	Pyrantel pamoate
Ht 06	Kandakuliya	1-18	Female	Pyrantel pamoate
Ht 07	Mawanella	19-50	Female	Pyrantel pamoate
Ht 08	Mawanella	1-18	Female	Pyrantel pamoate
Ht 09	Mawanella	1-18	Male	Pyrantel pamoate
Ht 10	Valalai	19-50	Female	Pyrantel pamoate

Ht 11	Valalai	1-18	Male	Pyrantel pamoate

2.2.3 Sample collection and parasitological analyses

Each volunteer was asked to provide a fresh stool sample for parasitological analyses. The presence of nematode eggs/larvae in each sample was assessed using a modified sucrose floatation method described previously [21]. Stool samples were refrigerated and transported to the laboratory for processing. For each sample, approximately three grams of faeces were measured, mixed with distilled water in a capped centrifuge tube to a final volume of 15 ml. The mixtures were stirred thoroughly using a wooden applicator and centrifuged at 2045 g for 20 mins at room temperature (~27^{o}C). Following centrifugation, the supernatants were discarded and resulting pellets were re-suspended in distilled water and centrifuged (twice) until clear supernatants were obtained. The pellets were then emulsified using saturated sucrose solution, mixed thoroughly, and centrifuged for 20 min at 2045 g. Approximately 5 ml of the top meniscus of the resulting suspensions were collected in a centrifuge tube and mixed with distilled water up to a final volume of 15 ml and centrifuged for 10 min at 1370 g. This procedure was repeated and 1 ml of each suspension with the pellet was transferred to 1.5 ml eppendorf® tubes using a Pasteur pipette. Distilled water was added to a final volume of 1.5 ml and tubes were centrifuged at 1150 g for 10 min. The clear supernatants were decanted and microscope slides were prepared using the remaining 0.5 ml pellets and examined under a light microscope. Helminth eggs were identified using established morphological keys [22].

2.2.4 DNA extraction and bacterial 16S rRNA Illumina sequencing

Genomic DNA was extracted directly from each sample, as well as from two negative (no-DNA template) controls, using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), according to manufacturers' instructions, within one month from collection. High-throughput sequencing of the V3-V4 hypervariable region of the bacterial 16S rRNA gene was performed on an Illumina MiSeq platform according to the standard protocols with minor adjustments. Briefly, the V3-V4 region was PCR-amplified using universal primers [23], that contained the Illumina adapter overhang nucleotide sequences, using the NEBNext hot start high-fidelity DNA polymerase (New England Biolabs) and the following thermocycling protocol, using DNA 2ng/µl: 2 min at 98°C, 20 cycles of 15 s at 98°C – 30 s at 63°C – 30 s at 72°C, and a final elongation of 5 min at 72°C. Amplicons were purified using AMPure XP

beads (Beckman Coulter) and the NEBNext hot start high-fidelity DNA polymerase was used for the index PCR with Nextera XT index primers (Illumina) according to the following thermocycling protocol: 3 min at 95°C, 8 cycles of 30 s at 95°C – 30 s at 55°C – 30 s at 72°C, and 5 min at 72°C. The indexed samples were purified using AMPure XP beads, quantified using the Qubit dsDNA high sensitivity kit (Life Technologies), and equal quantities from each sample were pooled. The resulting pooled library was quantified using the NEBNext library quantification kit (New England Biolabs) and sequenced using the v3 chemistry (301 bp paired-end reads). Raw sequence data have been deposited in the European Nucleotide Archive database under accession number PRJEB21999.

2.2.5 Bioinformatics and statistical analyses

Raw paired-end Illumina reads were trimmed for 16S rRNA gene primer sequences using Cutadapt (https://cutadapt.readthedocs.org/en/stable/). Pre-processed sequence data were processed using the Quantitative Insights Into Microbial Ecology (QIIME) software suite [24]. Successfully joined sequences were quality filtered in QIIME using default settings. Then, sequences were clustered into OTUs on the basis of similarity to known bacterial sequences available in the Greengenes database (v13.8; http://greengenes.secondgenome.com/; 97% sequence similarity cut-off) using the UCLUST software; sequences that could not be matched to references in the Greengenes database were clustered *de novo* based on pair-wise sequence identity (97% sequence similarity cut-off). The first selected cluster seed was considered as the representative sequence of each OTU. Then, representative sequences were assigned to taxonomy using the UCLUST software. Singleton OTUs and 'contaminant' sequences (from no-DNA control samples) were removed prior to downstream analyses. Total sum normalisation (TSS) was applied followed by cumulative-sum scaling (CSS) to correct bias introduced by TSS, and log2 transformation to account for the non-normal distribution of taxonomic counts data. Statistical analyses were executed using the Calypso software [25] (cgenome.net/calypso/); samples were clustered using supervised Canonical Correspondence Analysis (CCA) (including infection status as explanatory variable). Differences in bacterial alpha diversity (Shannon diversity) and richness between groups, as well as in the abundance of individual taxa, were evaluated using paired t-test. Beta diversity was calculated using weighted UniFrac distances and differences in beta diversity were calculated using PERMDISP (Permutational Analysis of Multivariate Dispersions) through the betadisper function [26]. Differences in the composition of the faecal microbiota between groups were assessed using the LEfSe (*L*inear discriminant analysis *Ef*fect *S*i*ze*) workflow [27], by assigning

infection/treatment 'groupings' as comparison class. Metagenome functional contents were analysed using the software package PICRUSt (v1.0.0) to predict gene contents and metagenomic functional information [28]. Sequences were aligned to data available in the Greengenes database v.13.5 and OTUs were assigned using a 97% identity cut-off. The resulting OTU table was then imported into PICRUSt and used to derive relative Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway abundance [28]. Differences in KEGG pathway abundance between groups were assessed using ANOVA embedded in the software suite STAMP [29]. To minimise the risk of other variables confounding the results, binomial logistic multiple-regression models were applied to the dataset. The infection status of each study participant was used as a dependent variable and other factors including age, gender, village, education and occupation, as independent variables, including interaction terms, to identify any risk factors associated with helminth infection (cf. Supplementary Table S1).

2.3 Results

Of 76 human volunteers enrolled in this study, 11 were positive for hookworms and/or roundworms and/or whipworms (*H*+) (Table 1), while 27 were negative despite no prior anthelmintic treatment (*H*-) (Table 1 and Supplementary Table S1). A total of 38 subjects had received regular anthelmintic treatment (*H*t) with Pyrantel pamoate (Table 1 and Supplementary Table S1). Therefore, 11 samples from each of *H*- and *Ht* cohorts were selected for high-throughput sequencing of bacterial 16S rRNA and subsequent comparative analyses with samples from the *H*+ group (Table 1). Logistic multiple-regression models were applied to these samples, but none of the assessed independent variables had a significant association with infection status of the study participants. From these 33 samples, a total of 17,576,532 paired-end reads were generated (not shown) and subjected to further processing. A total of 3,694,717 high-quality sequences (per sample mean 111,960 ± 40,845) were retained after quality control. Rarefaction curves generated following *in silico* subtraction of low-quality and contaminant sequences indicated that the majority of faecal bacterial communities were represented in the remaining sequence data, thus allowing me to undertake further analyses. These sequences were assigned to 11,371 OTUs and 12 bacterial phyla.

The phyla Firmicutes (mean of 50.9 %) and Bacteroidetes (mean of 39.2 %) were predominant in all samples analysed, followed by the phyla Proteobacteria (mean of 3.6 %) and Actinobacteria (mean of 3.0%) (Supplementary Table S1). At the family level, Prevotellaceae (mean of 26.4%), Ruminococcaceae (mean of 24.7%), Lachnospiraceae (mean of 13.0%) and Bacteroidaceae (mean of 8.1%) were most abundant (Supplementary Fig. S2).

Bacteroidaceae were highly abundant in some of the *Ht* (n = 3) and *H+* (n = 2) subjects, but only in one *H-* study participant; the same samples also showed a significant reduction in Prevotellaceae (Supplementary Fig. S2). The species *Prevotella copri* was abundant in the microbiota of >75% volunteers in this study, and it made up 7-69% (mean 17.3%) of all microbe species in these samples (Supplementary Fig. S3).

Subject faecal microbial communities were ordinated by CCA, which separated samples by infection or treatment status (Fig. 2) and identified as statistically significant ($P = 0.05$). No significant differences in OTU alpha diversity (Shannon) and richness were recorded between groups ($P = 0.65$, $P = 0.13$) (Supplementary Fig. S4).

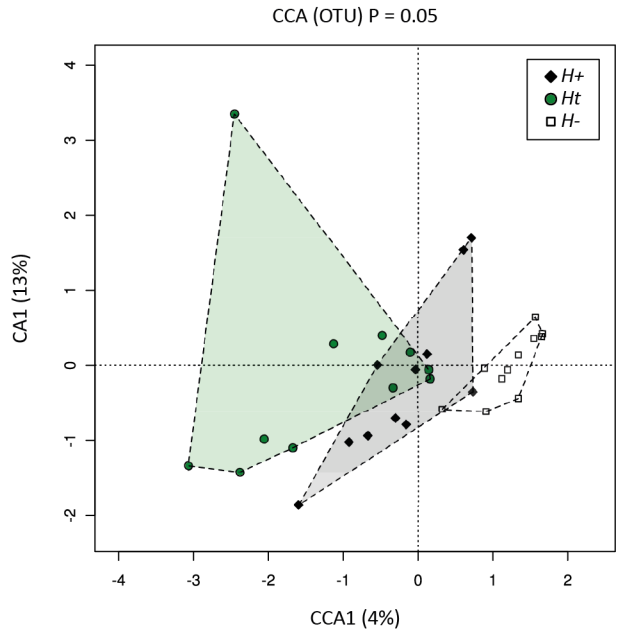

Fig. 2 Supervised Canonical Correspondence Analysis (CCA) displaying the compositional distribution of the faecal microbiota between helminth-positive (*H+*), helminth-negative (*H-*) and helminth-negative but regularly treated (*Ht*) subjects.

However, the *H+* and *Ht* microbiota was significantly more variable (i.e. characterised by increased beta diversity) compared with *H-* subjects ($P = 0.04$) (Fig. 3), which indicated differences in overall heterogeneity of species composition between sample groups, rather than in overall species composition.

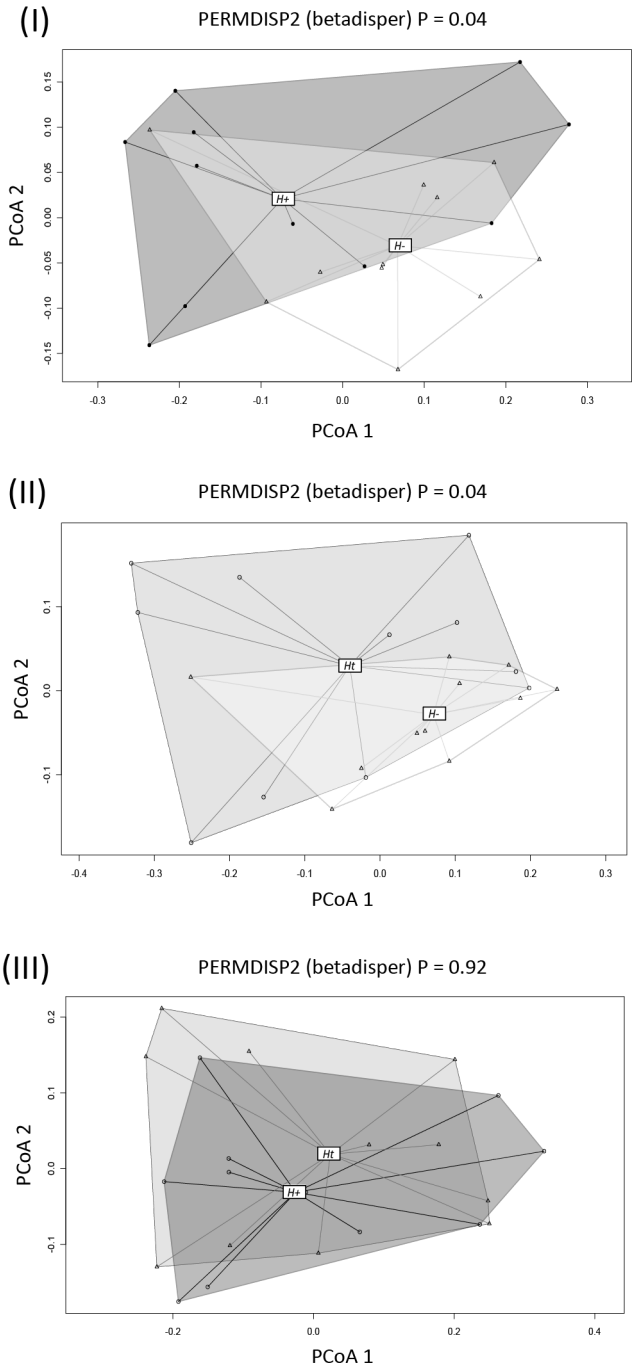

Fig. 3 Permutational Analysis of Multivariate Dispersions indicating differences in global microbial community composition of the study subjects. (I) helminth-positive (*H*+) and helminth-negative (*H*-), (II) regularly treated (*Ht*) and *H*-, and (III) *H*+ and *Ht* subjects.

Analysis by LEfSe, also supported by ANOVA, identified differences in abundance of individual taxa at the phylum, class, order, family, genus and species level between the three groups (Fig. 4). In particular, Verrucomicrobiae (Class), Verrucomicrobiales (Order), Verrucomicrobiaceae and Enterobacteriaceae (Family), *Lactococcus*, *Akkermansia* and a

genus belonging to the Enterobacteriaceae (Genus) and *Akkermansia muciniphila* (Species) showed a trend towards increased abundance in *H*+ compared to the other two groups (Fig. 4). Compared to *H*+, Leuconostocaceae and Bacteroidaceae (Family) and *Bacteroides* (Genus), were increased in *H*- and *Ht*, respectively (Fig. 4). Compared to *H*+, *Leuconostocaceae* and *Bacteroidaceae* (Family) and *Bacteroides* (Genus) were increased in *H*- and *Ht*, respectively (Fig. 4).

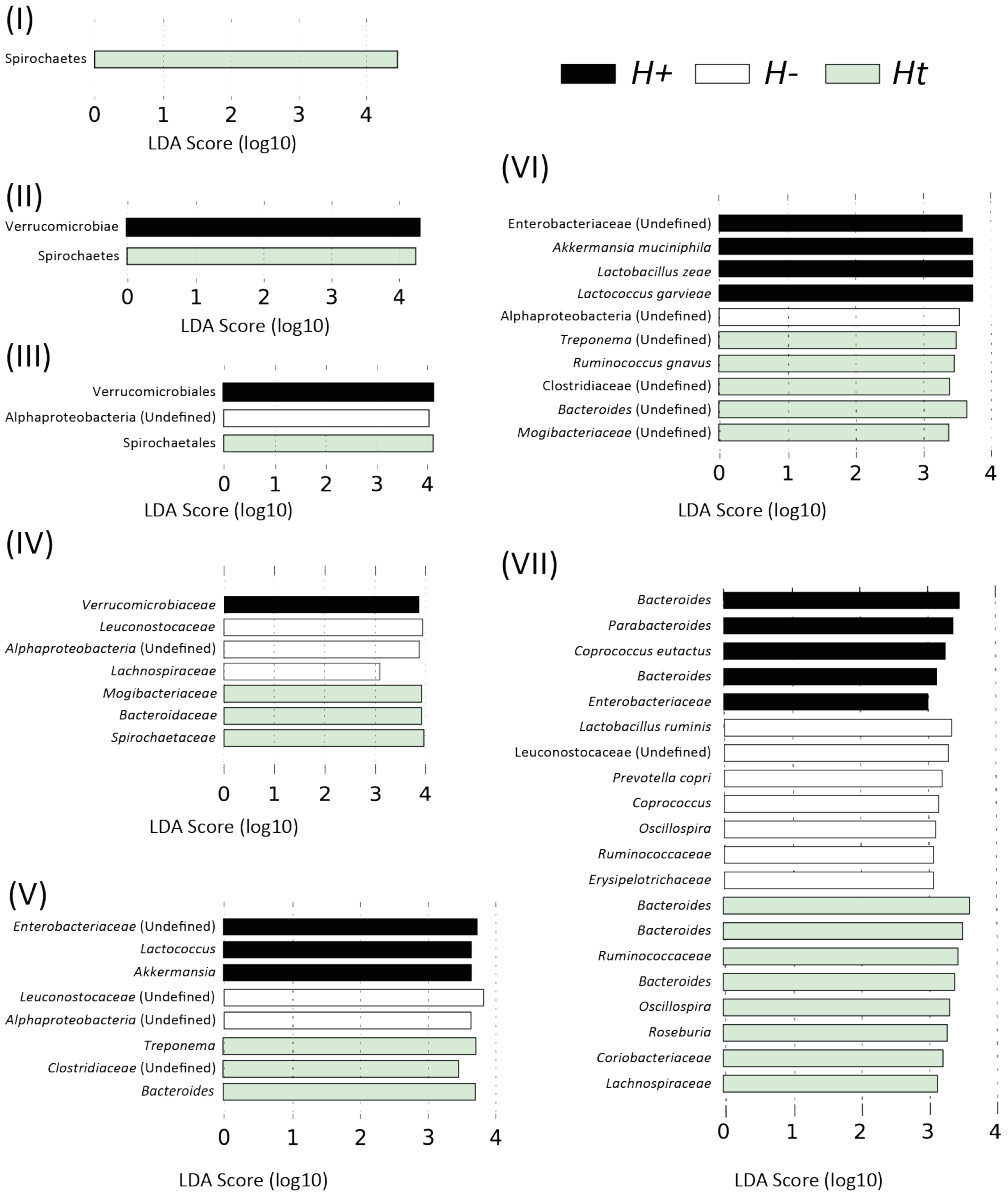

Fig. 4 Differentially abundant faecal bacteria in helminth-positive (*H*+), helminth-negative (*H*-) and helminth-negative but regularly treated (*Ht*) subjects, based on *L*DA *Eff*ect *Size* (LEfSe) analysis. Phylum (I), Class (II), Order (III), Family (IV), Genus (V), Species (VI) and OTUs (VII). Taxa highlighted in black/white/green indicate an overrepresentation in *H*+ /*H*- /*Ht*, respectively.

The same microbial metabolic and functional KEGG pathways were inferred by PICRUSt analysis in all three groups (Supplementary Fig. S5). However, 'lipid metabolism' ($P = 0.003$), 'rig-like receptor signalling pathway' ($P = 0.024$), and 'apoptosis' ($P = 0.04$), were down-regulated in *H+* compared with *H-*/*Ht* subjects, while the 'biotin pathway' was up-regulated in *H+* compared with *H-*/*Ht* ($P = 0.008$) (Fig. 5).

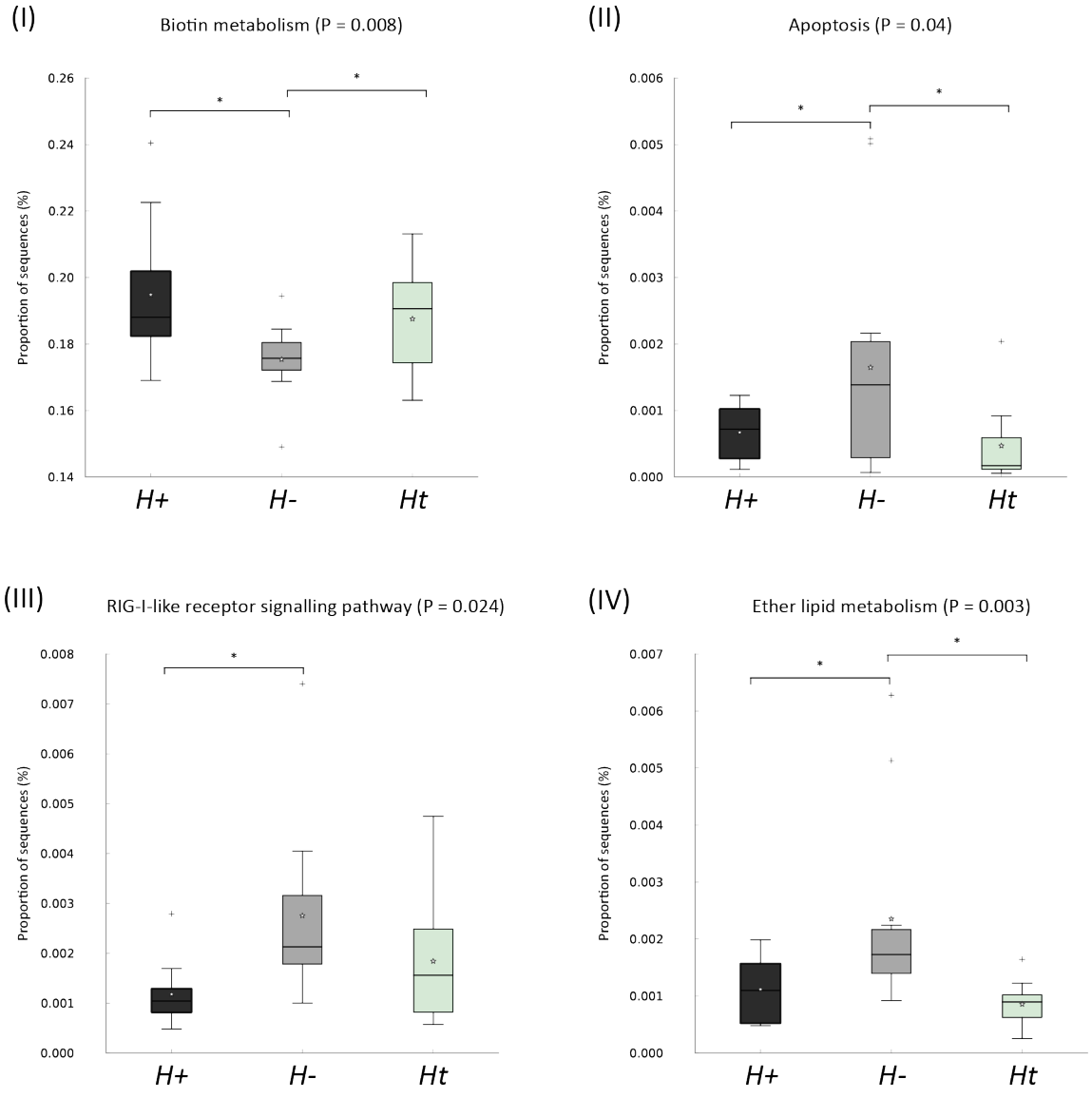

Fig. 5 Differences in relative abundance of KEGG pathways encoded in the faecal microbiota of helminth-positive (*H+*), helminth-negative (*H-*) and helminth-negative but regularly treated (*Ht*) subjects. Biotin metabolism (I), apoptosis (II), RIG-I-like receptor signalling (III) and Ether lipid metabolism (IV); significant differences (< 0.05) are indicated with asterisks (*).

2.4 Discussion

In this chapter, I analysed the effects of patent infections by parasitic helminths, as well as of repeated prophylactic administrations of anthelmintics, on the composition of the gut microbiota of human volunteers from endemic areas of Sri Lanka. Bacterial sequence data generated showed that the gut microbiota of subjects enrolled in this study was predominantly composed by species of the phylum Firmicutes, followed by those of the phyla Bacteroidetes, Proteobacteria and Actinobacteria, irrespective of infection and/or treatment status. This observation is in agreement with data from previous studies of the effects of natural or experimental helminth infections on the composition of the human gut microbiota [8,9,15,17]. In particular, at the genus level, a significant proportion of most samples analysed were represented by *Prevotella* spp., which reflects the findings from a previous study conducted in a cohort of Ecuadorean children infected by roundworms [8]. *Prevotella* spp. are known to play a key role in carbohydrate metabolism [30] and thus their expansion is likely due to a high carbohydrate/fibre diet, which is common in Sri Lanka [31]. An abundance of bacteria of the genus *Prevotella* has been linked to a concomitant decrease of *Bacteroides* spp., and vice versa, likely as a consequence of the metabolic differences between these two genera [30]. Indeed, in this study, *Bacteroides* spp. were abundant in the gut microbiota of subjects from the fishing villages Valalai and Kandakuliya, whose diet is typically high in fish-derived proteins and fats [30]. Nevertheless, *Bacteroides* were also abundant in samples from volunteers from Hanthana (tea estate). However, since the questionnaire did not include detailed questions on individual dietary habits, it was not possible to speculate whether diet-related factors may have caused the observed differences in the composition of the gut microbiota of Hanthana villagers.

In this study, the CCA analysis clustered samples according to infection or treatment status. However, analyses of OTU richness (i.e. the number of species within a sample) and alpha diversity (i.e. a measure of sample richness and evenness, the latter being defined as the relative abundance of individual species within a sample) detected no significant differences between *H+*, *H-* and *Ht* individuals. While this observation supports the findings from a previous study carried out in a cohort of human volunteers experimentally infected with *N. americanus* [18], an investigation of the impact of naturally acquired helminth infections on the composition of the gut microbiota of a cohort of Ecuadorean children [8] resulted in contrasting results. Indeed, while sole infections by *T. trichiura* could not be associated with detectable changes in microbial richness and alpha diversity, the gut microbiota of subjects with concomitant infections by *T. trichiura* and *A. lumbricoides* displayed a notable decrease of the latter [8]. Nonetheless, a recent study conducted in a cohort of naturally helminth-infected indigenous Malaysians [9], as well as other studies in experimentally infected individuals [15,17]

indicated an increase in alpha diversity associated with parasite infections. It is plausible to hypothesise that these varying observations could be linked, for example, to differences in the sample size, the 'baseline' composition of the gut microbiota of subjects enrolled, the type of species causing the infections (hookworms *vs*. whipworms *vs*. ascarids), and/or in the infection stage at which samples were collected (acute and chronic in case of experimental infections *vs*. 'undefined' in case of natural infections). Nevertheless, since a range of GI inflammatory diseases have been associated with a decrease in microbial diversity [32-34], it has been hypothesised that a helminth-mediated increase in microbial alpha diversity may represent a potential mechanism by which helminths are able to suppress inflammation [9,15,17,35]. This aspect deserves further investigation using pre-defined and standardised experimental set-ups.

Whilst I detected no differences in overall bacterial richness and alpha diversity between *H*+, *H*- and *Ht* groups, beta diversity was significantly increased in *H*+ and *Ht* subjects when each of these groups were individually compared to the uninfected *H*- cohort. Unlike alpha diversity, beta diversity provides a measure of the distance or dissimilarity between pairs or groups of samples [26]. An increased beta diversity has been previously observed in the gut microbiota of veterinary species, such as mice infected with *Trichuris muris* [36], rats infected with *Hymenolepis diminuta* [37], and goats infected with *Haemonchus contortus* [38]. In humans, the higher beta diversity observed in *H*+ individuals compared with the uninfected controls corroborates the hypothesis that helminth infections are accompanied by qualitative and quantitative changes in the composition of the host gut microbiota that are, based on data available thus far, inconsistent between individuals [15,17,18]. However, these data also suggest that anthelmintic treatment alone may be responsible for significant changes in the gut microbiota of human hosts. To date and to the best of our knowledge, no studies have shown a direct effect of anthelmintics on the composition the vertebrate gut microbiota. Nevertheless, pyrantel pamoate, the anthelmintic administered to subjects enrolled in this study, has been shown to affect, besides helminths, protozoans such as *Giardia* [39]. The microbiota profiling technique used in this chapter did not allow to investigate the effects that helminth infections or anthelmintic treatment exert on populations of commensal or pathogenic eukaryotes, and thus the hypothesis that repeated doses of pyrantel may have resulted in substantial modifications of such populations requires further testing.

Significant alterations of individual bacterial taxa were detected between *H*+, *H*- and *Ht* subjects. Amongst these, *Akkermansia muciniphila* (class Verrucomicrobiae) was significantly increased in *H*+ individuals when compared to uninfected subjects. *A. muciniphila* is an anaerobic bacterium commonly detected in the human gut microbiota, where

it primarily degrades host mucins [40]. Both vertebrate and helminth mucins have been shown to play key roles in the complex network of interactions occurring at the helminth-host interface [41]. For instance, the surface coat of the infective larval stage of the roundworm *Toxocara canis* has been shown to express high levels of a mucin-like glycoprotein (TES-120) which is shed following its binding by host antibodies or immune cells, thus suggesting that these molecules play a major role in protecting the parasite from the host immune response [42]. On the other hand, a dramatically increased production of host mucins was observed in macaques experimentally infected with the whipworm *T. trichiura* [35], likely as a consequence of the onset of Th2-type immunity stimulated by the infection [43]. Therefore, it may be possible that this observation of increased populations of *A. muciniphila* may be a direct consequence of the surge in helminth- and host-derived mucins in infected subjects. Interestingly, previous studies have shown that *A. muciniphila* populations are reduced in individuals with severe appendicitis and inflammatory bowel disease (IBD), which led the authors to hypothesise an anti-inflammatory role for this bacterial species [44-46]. Given the known anti-inflammatory properties of a range of helminth species [15,17,18,35,47,48], the role of helminth-induced expansions in populations of mucin-degrading bacteria should be tested in future studies aimed at dissecting the causality of parasite-mediated suppression of inflammation.

Amongst the Firmicutes, the family *Leuconostocaceae* (order Lactobacillales) was significantly increased in *H-* subjects. These bacteria belong to the lactic acid bacteria, a major group of autochthonous microbes that reside in the gut of humans and animals and that exert immune-modulatory functions [49]. Lactic acid bacteria are known probiotics [49]; yet, recent investigations, by our group and others, in humans experimentally or naturally infected by GI nematodes did not report significant associations between helminth infections and expanded populations of lactic acid bacteria [8,9,15,17,18]. Conversely, previous studies of murine models of nematode infections have shown a marked increase in populations of lactobacilli (belonging to the *Lactobacillaceae*, a family of lactic acid bacteria distinct from the *Leuconostocaceae* but with similar metabolic properties) in response to parasite establishment [13,48,50]. The specific groups of lactic acid bacteria shown to be associated with helminth infection are inconsistent between this and previous studies, which could be linked to host-specific responses to parasites; nevertheless, in the future, studies of parasite-microbiota interactions conducted on larger human and/or animal cohorts should particularly focus on this group of probiotics [49,51].

In order to correlate data on the composition of the human gut microbiota in response to helminth infection and anthelmintic treatment to inferred changes in bacterial metabolism, I conducted a predictive metagenomics analysis using PICRUSt. Whilst inferred KEGG

pathways were consistent across groups, as also shown in a similar investigation of the faecal microbiota of a helminth-infected community from Malaysia [9], 'biotin metabolism' was inferred to be up-regulated in *H*+ individuals compared to *H*-. Biotin is a B-vitamin with key roles in gene expression, cell signalling and chromatin structure [52]; in particular, biotin dependent signalling pathways regulate the expression of genes with key biological functions, e.g. apoptosis and cell survival [52]. Indeed, a significant down-regulation of the KEGG pathway 'apoptosis' was observed in *H*+ compared to *H*- volunteers. Overall, these findings emphasise that qualitative and quantitative compositional changes in helminth-infected individuals may be accompanied by significant alterations of the microbial metabolism which, in turn, may greatly impact host nutrition and immunity. Clearly, this data requires experimental validation using comprehensive metabolomics studies of the gut microbiota of helminth-infected hosts during acute and chronic infections.

2.5 Conclusions

Data from this chapter augments current knowledge of the effect that helminth infections and continued prophylactic treatment exert on the composition of the gut microbiota of the human host. However, inherent limitations may have impaired my ability to detect minor changes in populations of bacteria affected by parasites and/or anthelmintic treatment. Amongst these limitations, the relatively small sample size, dictated by the prevalence of helminth infections in the Sri Lankan community enrolled in this investigation, may have affected the statistical power; in addition, dietary variabilities, as well as differences in species of infecting helminths and parasite loads, while effectively representing a 'real world' scenario, may have introduced a range of confounding factors that, under the circumstances of this study, I was unable to fully evaluate. Nevertheless, I detected a significantly increased beta diversity in the microbiota of *H*+ compared with the *H*- counterpart, together with compositional changes in the gut microbiota of *H*+, *H*- and and *Ht* subjects, thus indicating a distinct effect of both helminth infection as well as of continued prophylactic treatment on the host gut microbiota. In addition, I also identified potential microbial metabolic changes associated with helminth infections, which further emphasises the need for further investigations of the role/s that helminth-induced changes in bacterial metabolism play in the complex network of host-parasite interactions. Overall, these findings add valuable knowledge to the vast, and yet little explored, research field of parasite – microbiota interactions and will provide a basis for the elucidation of the role such interactions play in pathogenic and immune-modulatory properties of parasitic nematodes in both human and animal hosts. However, to further investigate the

impact of natural helminth infections on the human microbiota in a less complex setting, I studied the impact of a single-species helminth infection in a developed country which is non-endemic for these parasites, in the following chapter.

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CHAPTER 3

A comprehensive analysis of the faecal microbiome and metabolome of *Strongyloides stercoralis* infected volunteers from a non-endemic area

Abstract

Data from recent studies support the hypothesis that infections by human gastrointestinal (GI) helminths impact, directly and/or indirectly, on the composition of the host gut microbial flora. However, to the best of my knowledge, these studies have been conducted in helminth-endemic areas with multi-helminth infections and/or in volunteers with underlying gut disorders. Therefore, in this chapter, I explored the impact of natural mono-infections by the human parasite *Strongyloides stercoralis* on the faecal gut microbiota and metabolic profiles of a cohort of human volunteers from a non-endemic area of northern Italy (*S*+), pre- and post-anthelmintic treatment, and compare the findings with data obtained from a cohort of uninfected controls from the same geographical area (*S*-). Analyses of bacterial 16S rRNA high-throughput sequencing data revealed increased microbial alpha diversity and decreased beta diversity in the faecal microbial profiles of *S*+ subjects compared to *S*-. Furthermore, significant differences in the abundance of several bacterial taxa were observed between samples from *S*+ and *S*- subjects, and between *S*+ samples collected pre- and post-anthelmintic treatment. Faecal metabolite analysis detected marked increases in the abundance of selected amino acids in *S*+ subjects, and of short chain fatty acids in *S*- subjects. Overall, this work adds valuable knowledge to current understanding of parasite-microbiota associations and will assist future mechanistic studies aimed to unravel the causality of these relationships.

3.1 Introduction

The human gastrointestinal (GI) tract is inhabited by a myriad of bacteria, viruses, archaea, fungi, and other unicellular and multicellular microorganisms, that together form the gut micro- and macrobiota [1-4]. Whilst some members of the microbiota can cause severe disease [5], most resident bacteria exert a number of specialised functions beneficial to the human host, including absorption of nutrients, synthesis of essential organic compounds, development of adaptive immunity and protection against pathogens [6-9]. Nevertheless, disturbances of the composition of the gut microbiota (i.e. dysbiosis) have been unequivocally linked to the onset of a range of gut and systemic diseases, such as chronic autoimmune and allergic disorders, obesity, diabetes and, more recently, multiple sclerosis (MS) [10-13]. On the other hand, with a few exceptions, multicellular organisms residing in the GI tract, such as parasitic worms (= helminths) are mostly considered detrimental to human health, as they can subtract nutrients, damage host tissues and release toxic waste products [14-16]. Nonetheless, in the developing world, infections by parasitic helminths have been associated with a low incidence of allergic and autoimmune diseases, as encompassed by the 'hygiene hypothesis' [17,18]; this observation has led to the 'curative' properties of a range of GI helminths being investigated in a range of clinical trials aimed to develop novel therapeutics against selected chronic inflammatory disorders, such as ulcerative colitis [19,20], Crohn's disease [21-24], coeliac disease [25,26] and MS [27-31]. Whilst preliminary results from a number of such trials are promising, a thorough understanding of the mechanisms that determine the anti-inflammatory properties of these helminths is necessary to assist the development of new effective therapeutics against these disorders. These properties are predominantly attributed to the ability of parasites and/or their excretory/secretory products to modulate host immune responses to facilitate their long-term establishment in the human gut [32-43]; nevertheless, in recent years, the ability of controlled infections by selected GI helminths to ameliorate clinical signs of chronic inflammation has been hypothesized to stem, at least in part, from direct and/or immune-mediated interactions between parasites and the resident microbial flora (reviewed by [44]). This hypothesis is supported by observations from several studies [45-50] that have reported significant associations between human infections by GI parasites (under experimental and natural settings) and shifts in the composition of the human gut microbiota towards a 'healthy' phenotype, as well as increased levels of metabolites with anti-inflammatory properties [45-50]. However, information reported to date have been derived from cohorts of human volunteers with underlying chronic gut disorders (e.g. coeliac disease [45-48]) or conditions of malnutrition and/or multi-specific helminth infections and/or exposed to multiple re-infections [50-53], with likely implications on

the ‘steady-state’ of the gut flora of these individuals. Whilst complete elimination of these confounding factors is difficult to achieve in human studies, investigations of the impact that infections by single species of GI helminths exert on the composition of the gut flora of individuals with no clinical evidence of concurring co-infections or underlying gut disorders may help disentangle the causality of parasite-microbiota relationships; in turn, this knowledge may assist the design of mechanistic experiments in available animal models of infection and disease (cf. [54-57]), aimed to achieve a better understanding of the therapeutic properties of parasites.

Strongyloides stercoralis is a soil transmitted intestinal nematode estimated to infect ~370 million people worldwide, with higher prevalence (ranging from 10% to 60%) recorded across tropical and subtropical regions [58-61]. The life cycle of *S. stercoralis* is complex, in that it involves both free-living and parasitic adult stages [58,62]. In particular, the small intestine of the vertebrate hosts (e.g. humans) harbours adult females only, which reproduce *via* parthenogenesis and lay eggs that hatch immediately, thus releasing first stage rhabditiform larvae (L1s) that are excreted with the host faeces (reviewed by [58,62]). However, L1s can also develop into invasive filariform larvae that are able to re-infect the host without being excreted (i.e. ‘autoinfection’) [62]. Once in the environment, male L1s develop through four larval stages to free-living adults; conversely, female L1s can either develop through to free-living adults (similarly to males) or reach a developmental stage infective to a new susceptible host, i.e. the infective third-stage larva (L3). Importantly, the new generation of female parasites deriving from sexual reproduction of free-living males and females is inevitably parasitic [58,62]. These infective larvae typically infect humans percutaneously and migrate to the small intestine, where the cycle recommences [58,62]. Autoinfection of a susceptible host can occur at a low level for several years, and is often subclinical or asymptomatic [58,62] although, in immunosuppressed individuals, parasites can spread to all organs and tissues causing (potentially fatal) ‘disseminated strongyloidiasis’.

Chronic infections by *S. stercoralis* provide a golden opportunity to evaluate the effect/s of long-term colonisation by parasitic nematodes on the composition of the human gut microbiota. In this chapter, I explored the impact of natural infections by *S. stercoralis* on the faecal gut microbiota and metabolic profiles of a cohort of elderly volunteers (with no clinical evidence of concurrent pathologies of infectious or non-infectious origin) from northern Italy. This area is non-endemic for parasitic nematodes, but characterised by the presence of sporadic cases of chronic infections by *S. stercoralis* in elderly individuals in which the parasite has persisted through several decades *via* autoinfection [63]. I profiled the gut microbiome pre- and post-

anthelmintic treatment with ivermectin, and compared the findings with a control cohort of uninfected individuals from the same geographical area.

3.2 Materials and methods

3.2.1 Ethics statement

This study was conducted according to the Declaration of Helsinki, and the protocol was reviewed and approved by the Institutional Ethical Review Committee for clinical experimentation for the Province of Verona (Comitato Etico per la Sperimentazione Clinica delle Province di Verona e Rovigo, protocol number 34678). Written informed consent was obtained from all subjects enrolled in the study.

3.2.2 Study area and characteristics of the population

Individual faecal samples from 20 volunteers (from four regions in northern Italy) with confirmed infections by *S. stercoralis* (*S*+) as assessed by Real-Time PCR (rtPCR; cf. [64]), performed at the Centre for Tropical Diseases of the Sacro Cuore Hospital (Negrar, Italy) during routine screening, were examined for microbiota and metabolite profiling as described below. Of these volunteers, 15 were from the Veneto region, three from Lombardia, one from Piemonte, and one from Emilia-Romagna (Supplementary Fig. S1). Subjects were both men (*n* = 12) and women (*n* = 8) of an average age of 74 (range 49-86 ± 11.5) (Supplementary Fig. S1) with no overt symptoms of GI disease and no recent history of anthelmintic treatment. Briefly, immediately following collection of individual faecal samples from each of these volunteers, aliquots (~250 mg) were examined for evidence of patent infections by GI helminths (*S. stercoralis*, *Strongyloides fuelleborni, N. americanus, Ancylostoma duodenale, Trichostrongylus* spp., *Ternidens deminutus,* and *Oesophagostomum* spp.) using the Agar Plate Copro-Culture Method (http://www.tropicalmed.eu), whilst rtPCR analyses were conducted to detect possible co-infections with *Schistosoma* spp. and *Hymenolepis nana* [64,65]. The remainders of each sample were stored at -80°C for subsequent microbiota and metabolite profiling (see below). Patent infections by *S. stercoralis* were unequivocally confirmed by DNA extractions from individual faecal samples (see below) followed by rtPCR targeting the 18S rRNA gene [64]. Upon confirmation of diagnosis, infected volunteers were treated with ivermectin (Stromectol®, Merck Sharp & Dohme BV, The Netherlands). From 13 (out of 20) *S*+ subjects (9 men and 4 women; average age of 76, range 60-84 ± 8.4, referred to as $S+_{pre\text{-}treatment}$) further individual samples were collected 6-months post-treatment (referred to

as $S+_{post\text{-}treatment}$) (Supplementary Fig. S1) and processed as described above. Samples that were negative for patent *S. stercoralis* infection at this time were progressed to microbiota and metabolite profiling (see below). In addition, individual faecal samples from 11 uninfected volunteers (*S*-) from the Veneto region (five men and six women; average age of 65, range 53-86 ± 10.7; Supplementary Fig. S1) were included for comparative analyses. These volunteers had no overt symptoms of GI disease or any other concomitant disease and had no recent history of antibiotic treatment.

3.2.3 DNA extractions and bacterial 16S rRNA gene Illumina sequencing

Genomic DNA was extracted directly from 200 mg of each faecal sample using the MagnaPure LC.2 instrument (Roche Diagnostic, Monza, Italy), following the manufacturer's instructions, and the DNA isolation kit I (Roche) and stored at -80°C until further processing. High-throughput sequencing of the V3-V4 hypervariable region of the bacterial 16S rRNA gene was performed by Eurofins Genomics on an Illumina MiSeq platform according to the standard protocols with minor adjustments. Briefly, the V3-V4 region was PCR-amplified using universal primers [66], that contained the adapter overhang nucleotide sequences for forward (TACGGGAGGCAGCAG) and reverse primers (CCAGGGTATCTAATCC). Amplicons were purified using AMPure XP beads (Beckman Coulter) and set up for the index PCR with Nextera XT index primers (Illumina). The indexed samples were purified using AMPure XP beads (Beckman Coulter) and quantified using the Fragment Analyzer Standard Sensitivity NGS Fragment Analysis Kit (Advanced Analytical) and equal quantities from each sample were pooled. The resulting pooled library was quantified using the Agilent DNA 7500 Kit (Agilent), and sequenced using the v3 chemistry (2x300 bp paired-end reads, Illumina).

3.2.4 Bioinformatics and statistical analyses

Raw paired-end Illumina reads were trimmed for 16S rRNA gene primer sequences using Cutadapt (https://cutadapt.readthedocs.org/en/stable/) and sequence data were processed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2-2018.4; https://qiime2.org) software suite [67]. Successfully joined sequences were quality filtered, dereplicated, chimeras identified, and paired-end reads merged in QIIME2 using DADA2 [68]. Sequences were clustered into OTUs on the basis of similarity to known bacterial sequences available in the Greengenes database (v13.8; http://greengenes.secondgenome.com/; 99% sequence similarity cut-off); sequences that could not be matched to references in the Greengenes database were clustered

de novo based on pair-wise sequence identity (99% sequence similarity cut-off). The first selected cluster seed was considered as the representative sequence of each OTU. The OTU table with the assigned taxonomy was exported from QIIME2 alongside a weighted UniFrac distance matrix. Singleton OTUs were removed prior to downstream analyses. Cumulative-sum scaling (CSS) was applied, followed by log2 transformation to account for the non-normal distribution of taxonomic counts data. Statistical analyses were executed using the Calypso software [69] (cgenome.net/calypso/); samples were investigated using the taxonomic visualisation tool KRONA [70] ordinated in explanatory matrices using supervised Canonical Correspondence Analysis (CCA) including infection/treatment status as explanatory variables. Differences in bacterial alpha diversity (Simpson's index) between study groups (*S*+ and *S*-, as well as $S+_{pre\text{-}treatment}$, corresponding $S+_{post\text{-}treatment}$, and *S*-) were evaluated based on rarefied data (read depth of 6063) and using analysis of variance (ANOVA); F-Tests were used to statistically assess the equality of assessed means (i.e. effect size). To take into account the paired nature of samples from $S+_{pre\text{-}treatment}$ and $S+_{post\text{-}treatment}$, differences between these sets were assessed using linear mixed effect regression. Differences in beta diversity (weighted UniFrac distances) were identified using Analysis of Similarity (ANOSIM) and effect size indicated by an R-value (between -1 and +l, with a value of 0 representing the null hypothesis [71]). Differences in the abundance of individual microbial taxa between groups were assessed using the LEfSe workflow [72], taking into account the paired nature of $S+_{pre\text{-}treatment}$ and $S+_{post\text{-}treatment}$ samples.

3.2.5 Metabolite extraction

Metabolites were extracted from 200 mg aliquots of each faecal sample using a methanol–chloroform–water (2:2:1) procedure. 600 µl of methanol–chloroform mix (2:1 v:v) were added, samples were homogenised using stainless steel beads and sonicated for 15 min at room temperature. 200 µl each of chloroform and water were added, the samples were centrifuged and the separated aqueous and lipid phases were collected. The procedure was repeated twice, and the aqueous and lipid fractions from each extraction were pooled. The aqueous layer was dried in a vacuum concentrator (Concentrator Plus, Eppendorf), while the lipid fraction was left to dry overnight at room temperature.

3.2.6 Nuclear Magnetic Resonance analysis of aqueous extracts

The dried aqueous fractions were re-dissolved in 600 µl D_2O, containing 0.2 mM sodium-3-(tri-methylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP) (Cambridge Isotope

Laboratories, MA, USA) as an internal standard and phosphate buffer (40mM NaH_2PO_4/160 mM Na_2HPO_4). The samples were analysed using an AVANCE II+ NMR spectrometer operating at 500.13 MHz for the ^{1}H frequency and 125.721 MHz for the ^{13}C frequency (Bruker, Germany) using a 5 mm TXI probe. The instrument is equipped with TopSpin 3.2. Spectra were collected using a solvent suppression pulse sequence based on a one-dimensional nuclear Overhauser effect spectroscopy (NOESY) pulse sequence to saturate the residual 1H water signal (relaxation delay = 2 s, t1 increment = 3 us, mixing time = 150 ms, solvent pre-saturation applied during the relaxation time and the mixing time). One hundred and twenty-eight transients were collected into 16 K data points over a spectral width of 12 ppm at 27 ºC. In addition, representative samples of each data set were also examined by two-dimensional Correlation Spectroscopy (COSY), using a standard pulse sequence (cosygpprqf) and 0.5 s water presaturation during relaxation delay, 8 kHz spectral width, 2048 data points, 32 scans per increment, 512 increments. Peaks were assigned using the COSY spectra in conjunction with reference to previous literature and databases and the Chenomx spectral database contained in Chenomx NMR Suite 7.7 (Chenomx, Alberta, Canada). 1D-NMR spectra were processed using TopSpin. Free induction decays were Fourier transformed following multiplication by a line broadening of 1 Hz, and referenced to TSP at 0.0 ppm. Spectra were phased and baseline corrected manually. The integrals of the different metabolites were obtained using Chenomx. Metabolites were normalised to total area and differential abundance of metabolites between *S*+ and *S*- subjects, as well as $S+_{post\text{-}treatment}$ and *S*- subjects identified using ANOVA. F-Tests were used to statistically assess the equality of assessed means, while differences between $S+_{pre\text{-}treatment}$ and $S+_{post\text{-}treatment}$ were determined through paired t-test to account for the paired nature of these samples. Associations among metabolites in the faecal metabolome of each sample group were identified by prediction of correlation networks in Calypso [69] (cgenome.net/calypso/). In particular, networks were constructed to identify clusters of co-occurring metabolites based on their association with infection status (i.e., samples from *S*+ and *S*-, as well as $S+_{pre\text{-}treatment}$, $S+_{post\text{-}treatment}$ and *S*- subjects). Metabolites and explanatory variables were represented as nodes, relative abundance as node size, and edges represented positive associations, while nodes were coloured according to infection status. Metabolite abundances were associated with infection status using Pearson's correlation. Nodes were then coloured based on the strength of the association (i.e. Spearman's rho correlation) with infection status. Networks were generated by first computing associations between taxa using Spearman's rho and the resulting pairwise correlations were converted into dissimilarities and then used to ordinate nodes in a two-dimensional plot by PCoA. Therefore, correlating nodes

were located in close proximity and anti-correlating nodes were placed at distant locations in the network.

3.2.7 Gas Chromatography–Mass Spectrometry analysis of organic extracts

100 µl of D-25 tridecanoic acid (200 µM in chloroform), 650 µl of chloroform/methanol (1:1 v/v) and 125 µl BF_3/methanol (Sigma-Aldrich) were added to 100 µl organic extract dissolved in chloroform/methanol (1:1 v/v) (half of the organic material extracted for each sample). The samples were then incubated at 80 ºC for 90 min. 500 µl H_2O and 1 ml hexane were added and each vial mixed and the two phases separated. The organic layer was evaporated to dryness before reconstitution in 200 µl hexane for analysis. Using a Trace GC Ultra coupled to a Trace DSQ II mass spectrometer (Thermo Scientific, Hemel Hempstead, UK), 2 µl of the derivatised organic metabolites were injected onto a TR-fatty acid methyl ester (FAME) stationary phase column (Thermo Electron; 30 m × 0.25 mm ID × 0.25 µm; 70% cyanopropyl polysilphenylene-siloxane) with a split ratio of 20. The injector temperature was 230 °C and the helium carrier gas flow rate was 1.2 ml/min. The column temperature was 60 °C for 2 min, increased by 15 °C/min to 150 °C, and then increased at a rate of 4 °C/min to 230 °C (transfer line = 240 °C; ion source = 250°C, EI = 70 eV). The detector was turned on after 240 s, and full-scan spectra were collected using 3 scans/s over a range of 50–650 *m*/*z*. Peaks were assigned using Food Industry FAME Mix (Restek 6098). GC–MS chromatograms were analysed using Xcalibur, version 2.0 (Thermo Fisher), integrating each peak individually, and normalised to total area. The set of metabolic profiles obtained were analysed by univariate analysis. Differential abundance of metabolites between analysis groups was identified using ANOVA, and F-Tests were used to statistically assess the equality of assessed means. Associations among metabolites identified in the faecal metabolome of each sample group were identified by prediction of correlation networks in Calypso [69] (cgenome.net/calypso/).

3.3 Results

Individual faecal samples were collected from 20 elderly [74 ± 11 years (average ± standard deviation)] volunteers with confirmed infections by *S. stercoralis* (*S*+), as well as from 11 uninfected volunteers (*S*-) of comparable age and from the same geographical areas (Supplementary Fig. S1). Additional faecal samples were collected from 13 (out of 20) *S*+ subjects six months post-anthelmintic treatment. In comparative analyses of the human faecal microbiota pre- and post-treatment, samples from the latter 13 subjects are hereafter referred to as $S+_{pre\text{-}treatment}$ and $S+_{post\text{-}treatment}$, respectively. A total of 44 faecal samples were subjected

to microbial DNA extraction and high-throughput Illumina sequencing of the bacterial 16S rRNA gene [i.e. *S*+ = 20 (including $S+_{pre\text{-}treatment}$ = 13), $S+_{post\text{-}treatment}$ = 13 and *S*- = 11] whilst a total of 31 samples [i.e. *S*+ = 14 (including $S+_{pre\text{-}treatment}$ = 8), $S+_{post\text{-}treatment}$ = 7 and *S*- = 10] were subjected to metabolite profiling *via* nuclear magnetic resonance (NMR) and gas chromatography/mass spectrometry (GC-MS) (cf. Supplementary Table S1).

High-throughput amplicon sequencing yielded a total of 5,717,403 paired-end reads (not shown), of which 1,446,150 high-quality sequences (per sample mean 26,778 ± 13,928) were retained after quality control. Rarefaction curves generated following *in silico* subtraction of low-quality sequences indicated that the majority of faecal bacterial diversity was well represented by the sequence data (Supplementary Fig. S2). These sequences were assigned to 2,630 Operational Taxonomic Units (OTUs) and 15 bacterial phyla, respectively (data available from Mendeley Data at http://dx.doi.org/10.17632/n86dtjvmbv.1). The phyla Firmicutes (64.5% average ± 14.4% standard deviation) and Proteobacteria (18.2% ± 12.8%) were most abundant in all samples analysed, followed by the phyla Actinobacteria (7.9% ± 6.4%), Verrucomicrobia (5.4% ± 7.7%) and Bacteroidetes (1% ± 1.1%) (Fig. 1). At the order level, Clostridiales were most abundant in all samples analysed (54.4% ± 15.2%), and included the two most abundant microbial families, i.e. the *Ruminococcaceae* (20.6% ± 11.6%) and the *Lachnospiraceae* (13.4% ± 8.1%) (Fig. 1). Faecal microbial community profiles were ordinated by Canonical Correspondence Analysis (CCA) (Fig. 2a), that separated samples by infection status (*S*+ and *S*-) (effect size (F) = 1.14, P = 0.03). No statistically significant differences between the gut microbial composition of $S+_{pre\text{-}treatment}$, $S+_{post\text{-}treatment}$, and *S*- subjects were detected (F = 0.97, P = 0.64; Fig. 2b). Similarly, $S+_{pre\text{-}treatment}$ and $S+_{post\text{-}treatment}$ did not show any statistically significant difference in community composition (P > 0.05, CCA).

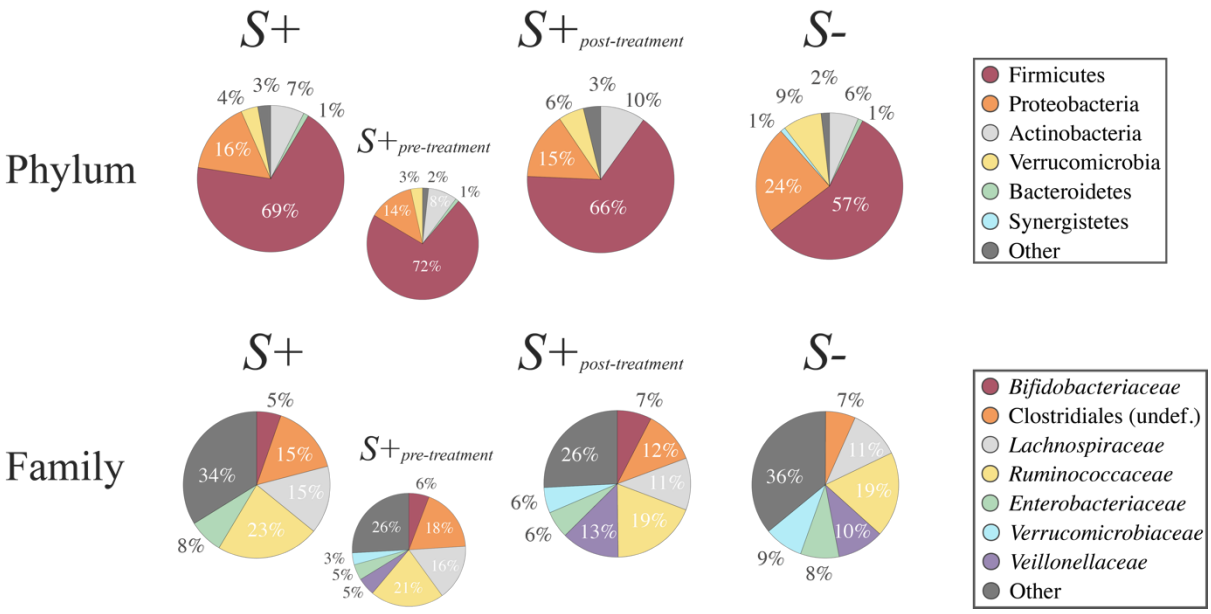

Fig. 1 Relative abundances of bacterial phyla and families detected in faecal samples from *Strongyloides stercoralis* infected and uninfected subjects (*S*+ and *S*-, respectively) and of the subset of *S*+ subjects that had received anthelmintic treatment (associated sub-circles), both prior to ($S+_{pre\text{-}treatment}$) and six months post-ivermectin administration ($S+_{post\text{-}treatment}$). Percentages in individual pie chart sections indicate the relative proportion of a given bacterial pylum or family.

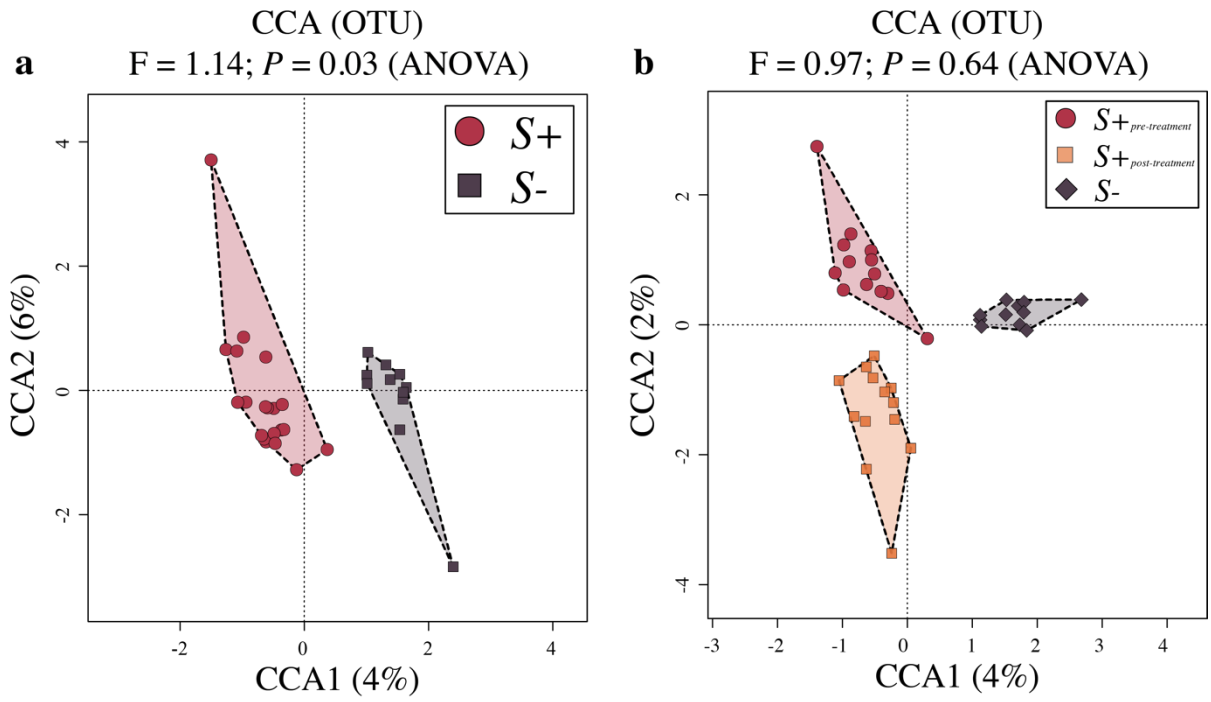

Fig. 2 Differences between the gut microbial profiles of the faecal microbiota of *S. stercoralis* infected subjects and uninfected subjects (*S*+ and *S*-, respectively) (a), and between the microbial profiles of the subset of *S*+ subjects that had received anthelmintic treatment, both prior to ($S+_{pre\text{-}treatment}$) and six

months post-ivermectin administration ($S+_{post\text{-}treatment}$), and of *S*- subjects, ordinated by supervised Canonical Correspondence Analysis (CCA; b).

Microbial alpha diversity, measured through Simpson's index, and evenness were significantly increased in the faecal microbiota of *S*+ volunteers when compared to that of *S*- (F = 5, $P = 0.03$ and F = 4.2, $P = 0.05$ respectively) (Fig. 3a). Faecal microbial richness was not significantly different between *S*+ and *S*- subjects, albeit a trend towards increased richness in samples from *S*+ subjects was observed. Simpson diversity and richness were decreased in samples from $S+_{post\text{-}treatment}$ compared to $S+_{pre\text{-}treatment}$, although only the latter was significant ($P < 0.001$; mixed effect linear regression) (Fig. 3b). Compared to *S*-, $S+_{post\text{-}treatment}$ samples showed a trend towards increased Simpson diversity and evenness, while richness was lower in samples post-treatment when compared to samples from uninfected subjects (Fig. 3b). Microbial faecal beta diversity was significantly lower in samples from *S*+ subjects when compared to *S*- subjects (effect size (R) = 0.11, $P = 0.04$; Fig. 3c), whilst beta diversity in $S+_{post\text{-}treatment}$ samples was higher than that of $S+_{pre\text{-}treatment}$ but lower than that of *S*-, albeit not significantly (Fig. 3d).

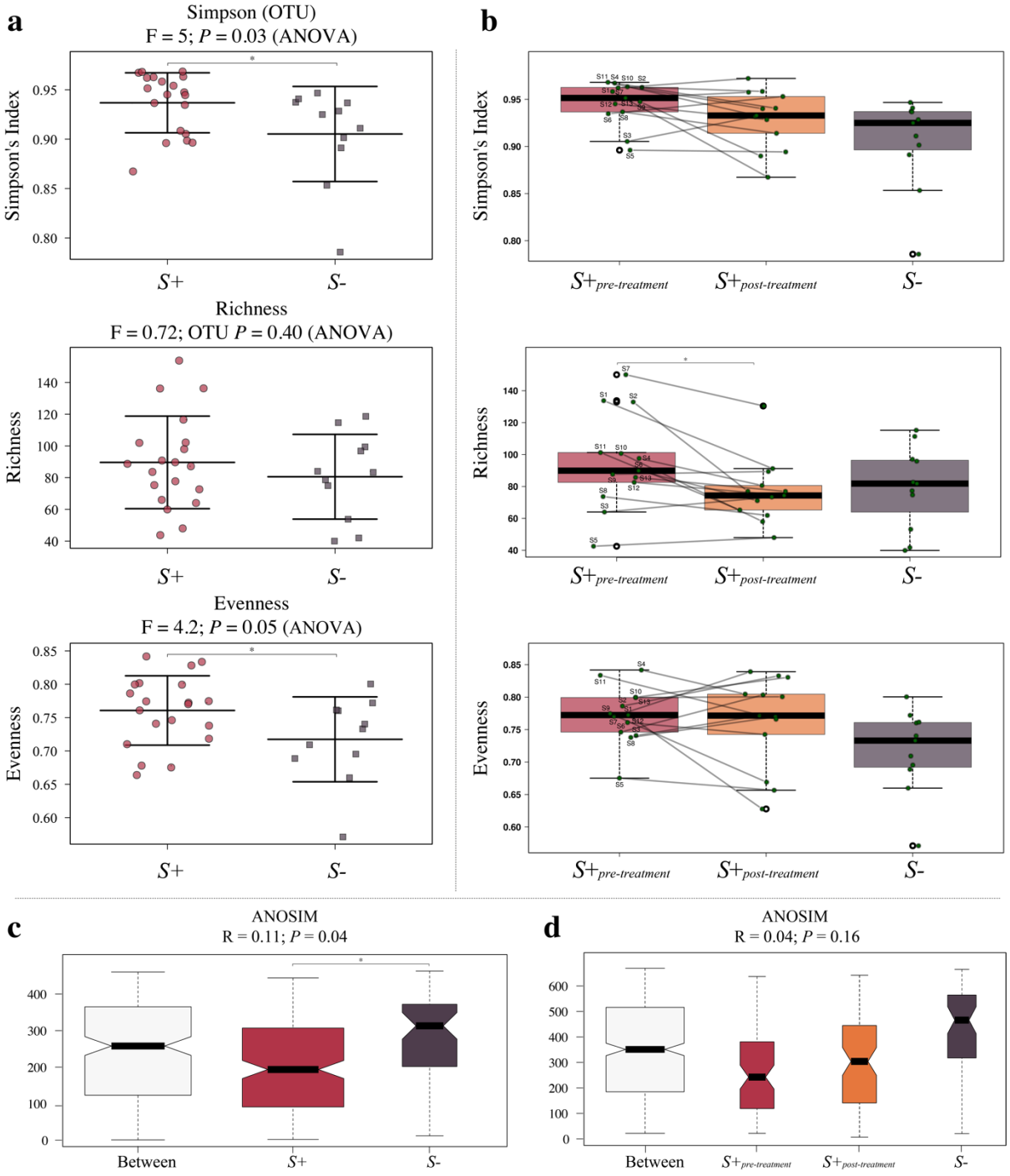

Fig. 3 (a) Differences in overall microbial Simpson (alpha) diversity, and corresponding richness and evenness, between the gut microbial profiles of *S. stercoralis*-infected and uninfected subjects (*S*+ and *S*-, respectively), and (b) between the microbial profiles of the subset of *S*+ subjects that had received anthelmintic treatment, both prior to ($S+_{pre\text{-}treatment}$) and six months post-ivermectin administration ($S+_{post\text{-}treatment}$), and of *S*- subjects. (c) Differences in microbial beta diversity between *S*+ and *S*- samples, as well as (d) between samples from $S+_{pre\text{-}treatment}$, $S+_{post\text{-}treatment}$ and *S*-. The bold and black horizontal lines in the boxplots refer to the mean of percentage abundance of metabolite associated with the cooresponding group, with top and bottom whiskers representing the standard deviation. Points

connected by lines in (b) refer to samples collected from the same study participant pre- and post-anthelmintic treatment. Significant differences between study groups are marked by an asterisk (*).

Linear discriminant analysis Effect Size (LEfSe) revealed significant differences in the abundance of individual microbial taxa (phylum to species level) between *S*+ and *S*- subjects (Fig. 4a). In particular, the faecal microbiota of *S*- subjects was significantly enriched for populations of bacteria belonging to the order Pseudomonadales (genus *Pseudomonas*) and an unidentified species belonging to the genus *Bacteroides* (Fig. 4a); conversely, bacteria belonging to the families *Leuconostocaceae*, *Ruminococcaceae*, *Paraprevotellaceae* and to the genus *Peptococcus*, amongst others, were significantly higher in the faecal microbiota of *S*+ subjects (Fig. 4a). In addition, in samples from $S+_{post\text{-}treatment}$ subjects, a significant decrease of bacteria belonging to the order Turicibacterales (genus *Turicibacter*) and an increase of Enterobacteriales (in particular associated to the genus *Shigella*) were observed compared to corresponding samples from $S+_{pre\text{-}treatment}$ (Fig. 4b and Supplementary Fig. S3). Additionally, differences were observed between the microbial profiles of $S+_{post\text{-}treatment}$ samples when compared to *S*-, with the latter displaying increased levels of bacteria belonging to the order Pseudomonadales and genus *Atopobium*, as well as *Bacteroides eggerthii*, *Clostridium celatum* and *Bifidobacterium bifidum*, and decreased levels of *Lachnobacterium*, *Roseburia faecis*, and *Eubacterium biforme*, respectively (Fig. 4c).

a

Phylum	Class	Order	Family	Genus	Species	*S+*	*S-*
Proteobacteria	Gammaproteobacteria	Pseudomonadales					
			Pseudomonadaceae				
				Pseudomonas			
					Pseudomonas (unclassified)		
Bacteroidetes	Bacteroidia	Bacteroidales	*Bacteroidaceae*	*Bacteroides*	*Bacteroides* (unclassified)		
			Paraprevotellaceae				
Firmicutes	Bacilli	Lactobacillales	*Leuconostocaceae*				
Firmicutes	Clostridia	Clostridiales	*Peptococcaceae*	*Peptococcus*			
					Peptococcus (unclassified)		
			Ruminococcaceae (OTU)				

b

Phylum	Class	Order	Family	Genus	Species	$S+_{pre\text{-}treatment}$	$S+_{post\text{-}treatment}$
Cyanobacteria							
Firmicutes	Erysipelotrichia	Turicibacterales					
			Turicibacteraceae				
				Turicibacter			
					Turicibacter (unclassified)		
Proteobacteria	Gammaproteobacteria	Enterobacteriales					
			Enterobacteriaceae				

c

Phylum	Class	Order	Family	Genus	Species	$S+_{post\text{-}treatment}$	*S-*
Proteobacteria	Gammaproteobacteria	Pseudomonadales					
			Pseudomonadaceae				
				Pseudomonas			
Bacteroidetes	Bacteroidia	Bacteroidales	*Bacteroidaceae*	*Bacteroides*	*Bacteroides eggerthii*		
Actinobacteria	Coriobacteriia	Coriobacteriales	*Coriobacteriaceae*	*Atopobium*	*Atopobium* (unclassified)		
Firmicutes	Clostridia	Clostridiales	*Clostridiaceae*	*Clostridium*	*Clostridium celatum*		
Actinobacteria	Actinobacteria	Bifidobacteriales	*Bifidobacteriaceae*	*Bifidobacterium*	*Bifidobacterium bifidum*		
Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*	*Lachnobacterium*			
					Lachnobacterium (unclassified)		
				Roseburia	*Roseburia faecis*		
			Eubacteriaceae	*Eubacterium*	*Eubacterium biforme*		

Fig. 4 Differentially abundant bacterial taxa in the faecal microbiome of *Strongyloides stercoralis* (a) infected and uninfected subjects (*S+* and *S-*, respectively), (b) infected subjects pre- and post anthelmintic treatment with ivermectin ($S+_{pre\text{-}treatment}$ and $S+_{post\text{-}treatment}$, respectively), and (c) infected subjects post-anthelmintic treatment and uninfected subjects ($S+_{post\text{-}treatment}$ and *S-* respectively), based on Linear discriminant analysis Effect Size (LEfSe) analysis. Colours correspond to Linear Discriminant Analysis (LDA) scores of 4 or higher (red) and 3.5 to 4 (yellow).

A total of 28 metabolites were identified by NMR across all samples (Fig. 5a); these were subjected to Principal Coordinates Analysis (PCoA), which unveiled no marked differences in faecal metabolic profiles between samples from *S+* and *S-* subjects, as well as from $S+_{pre\text{-}treatment}$, $S+_{post\text{-}treatment}$, and *S-* subjects (data not shown). However, association network analysis of *S+* and *S-* samples indicated clustering of several faecal metabolites

according to the infection status of the study subjects. In particular, 13 metabolites (alanine, isoleucine, glycine, phenylalanine, formate, valine, tyrosine, leucine, lysine, uracil, hypoxanthine and aspartate), were positively correlated with each other and associated with the faecal metabolic profiles of *S*+ subjects (Fig. 5b), whereas 4 (the SCFAs propionate, butyrate, and acetate, as well as succinate) were also positively correlated with each other, and associated with faecal samples from *S*- subjects (Fig. 5b). When applied to the faecal metabolic profiles of $S+_{pre\text{-}treatment}$, $S+_{post\text{-}treatment}$ and *S*- subjects, network analysis associated the 13 metabolites described above (previously associated with *S*+) with both $S+_{pre\text{-}treatment}$ and $S+_{post\text{-}treatment}$, whereas the SCFAs remained associated with the metabolic profiles of *S*- subjects (Fig. 5b). Analysis of differentially abundant metabolites between $S+_{pre\text{-}treatment}$, $S+_{post\text{-}treatment}$, and *S*- samples via ANOVA further revealed that 10 (out of 12) metabolites associated with samples from $S+_{post\text{-}treatment}$ subjects were less abundant than in $S+_{pre\text{-}treatment}$ samples, but more abundant when compared to *S*- samples, albeit not significantly (Supplementary Fig. S4). Additionally, alanine, formate, lysine, and leucine were significantly more abundant in samples from *S*+ when compared to *S*- ($F = 4.2$, $P = 0.05$; $F = 4.5$, $P = 0.03$; $F = 4.5$, $P = 0.05$; $F = 4.3$, $P = 0.05$ respectively) whilst formate was significantly more abundant in $S+_{post\text{-}treatment}$ compared to *S*- subjects ($F = 5.6$, $P = 0.01$); nevertheless, these differences were not significant following p-value correction for multiple testing (FDR > 0.05) (Fig. 5c).

Using GC-MS, a total of 13 fatty acids were detected across all analysed faecal samples, including lauric acid (C12:0), myristic acid (C14:0), pentadecanoic acid (C15:0, C15:0 iso, and C15:0 ante), palmitic acid (C16:0), margaric acid (C17:0, C17:0 iso, C17:0 ante), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and arachidic acid (C20:0) (Supplementary Fig. S5). No significant associations between any of the analysis groups were detected using PCoA and no significant differences in the relative abundance of individual metabolites were observed between samples from *S*+ and *S*- subjects, as well as $S+_{pre\text{-}treatment}$, $S+_{post\text{-}treatment}$, and *S*- subjects (data not shown).

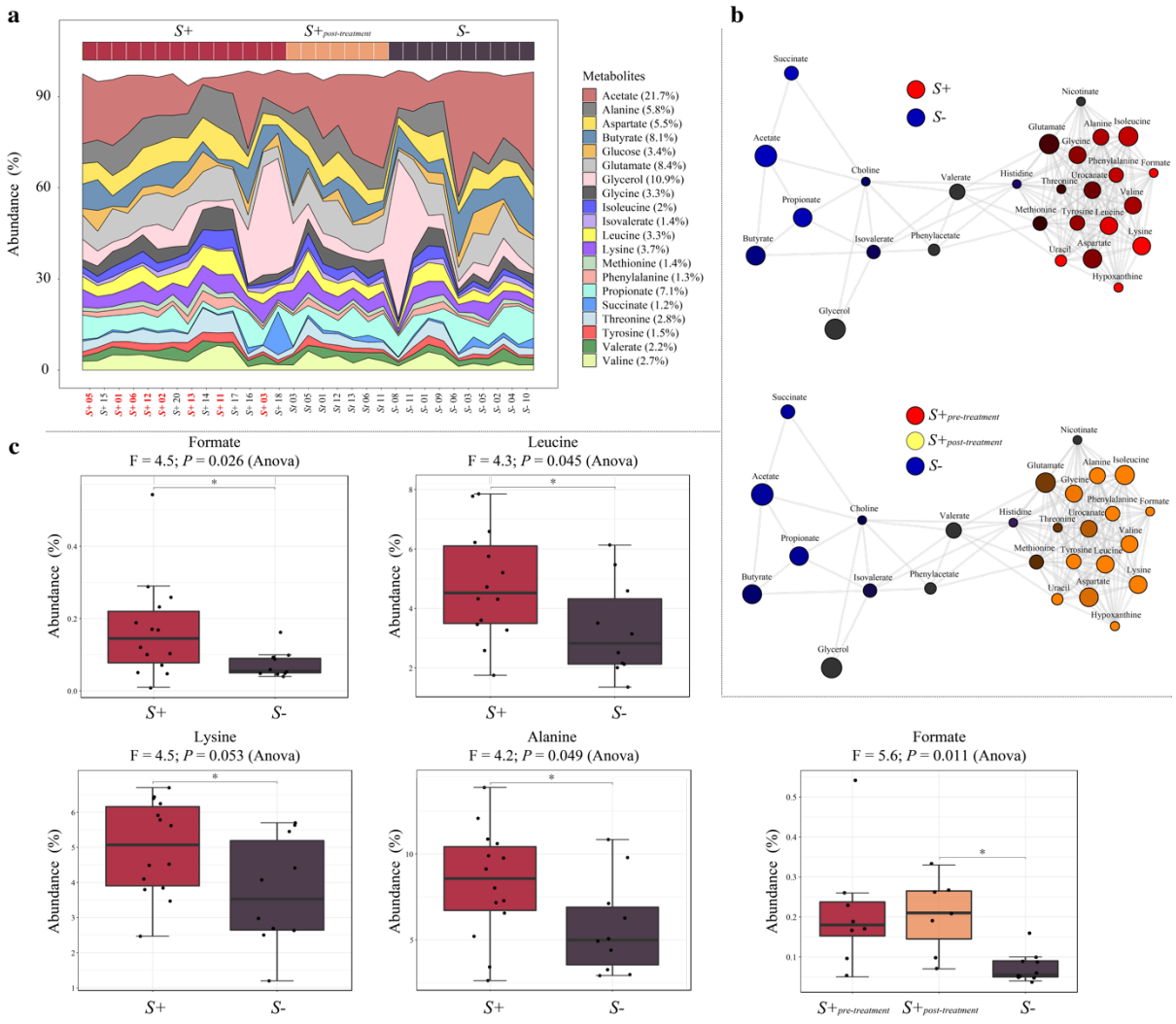

Fig. 5 (a) Area plot indicating the abundance (expressed as percentage) of metabolites detected by nuclear magnetic resonance analysis (NMR) in faecal samples from *S. stercoralis*-infected and uninfected subjects (*S*+ in red, and *S*- in purple), as well as from the subset of *S*+ subjects that had received anthelmintic treatment, both prior to ($S+_{pre\text{-}treatment}$, red sample label) and 6 months post-ivermectin administration ($S+_{post\text{-}treatment}$, in orange). (b) Network analysis displaying associations between individual or clusters of metabolites and sample groups (i.e. *S*+ in red, *S*- in blue [top network], $S+_{pre\text{-}treatment}$ in red, $S+_{post}$-treatment in yellow and *S*- in blue [bottom network]). For metabolites associated with multiple sample groups, the respective circle colours are mixed according to the strength of the association. (c) Differentially abundant metabolites detected in faecal samples from *S*+ and *S*-, as well as $S+_{pre\text{-}treatment}$, $S+_{post\text{-}treatment}$, and *S*- subjects, determined using ANOVA. The bold and black horizontal lines in the boxplots refer to the mean of percentage abundance of metabolite associated with the corresponding group, with top and bottom whiskers representing the standard deviation. Significant differences between study groups are marked by an asterisk (*).

3.4 Discussion

In this chapter, I aimed to determine the effect/s that chronic, monospecific infections by the parasitic nematode *S. stercoralis* exert on the faecal microbiome and metabolome of human volunteers from a non-endemic area of Europe, and establish whether such effects are reversed following the administration of anthelmintic treatment. Whilst the overall composition of the gut microbiota of *Strongyloides*-infected and uninfected volunteers enrolled in this study largely reflected that of human subjects harbouring GI helminths under natural or experimental conditions of infections described in previous investigations, the relatively low proportions of Bacteroidetes observed in these samples contrast findings from some previously published reports [47,48,50,51,53]. However, this discrepancy may be accounted for by differences in mean age of the cohorts enrolled in this (72 years) *versus* previous studies (i.e. 11- 51 years of age; cf. [47,48,50,51,53]); indeed, a decline in the relative abundance of Bacteroidetes in the gut microbiota of aging subjects has been documented in several studies [73-76], and is thus considered 'physiological' in the age group enrolled in this experiment.

In spite of the overall similarities in the composition of the faecal microbiome of *S*+ and *S*- subjects at phylum level, CCA analysis revealed differences in the gut microbial profiles of these two groups, thus indicating that *S. stercoralis* infection was associated with shifts in the relative abundance of individual gut bacterial species. Indeed, microbial alpha diversity was significantly higher in the faecal microbiota of *S*+ when compared to *S*-. In particular, the level of microbial alpha diversity detected in the latter group largely reflected that recorded in a cohort of healthy, elderly Italians investigated previously [77]. Conversely, microbial beta diversity was significantly decreased in the faecal microbiome of *S*+ subjects when compared to *S*-. Increased levels of microbial alpha diversity have been repeatedly observed in the gut microbiome of human subjects infected by a range of GI helminths (i.e. *Necator americanus*, *Trichuris trichiura*, and *Ascaris* sp.) [47,48,50]. Since alpha diversity measures are frequently used as proxy of microbiome 'health' (with high alpha diversity associated with a mature, homogenous, stable and healthy gut microbial environment [78,79]), it has been proposed that the direct or immune-mediated ability of GI helminths to restore gut homeostasis by promoting increases in microbial richness and evenness may represent one mechanism by which parasites exert their therapeutic properties in individuals with chronic inflammatory disorders [48,49,80].

The difference in microbial alpha and beta diversity observed between *S*+ and *S*- subjects were determined by dissimilarities in the relative abundance of selected bacterial taxa in the faecal microbial profiles of these study groups. In particular, significantly expanded populations of Clostridia and *Leuconostocaceae* could be detected in the faecal microbiome of

S+ when compared to *S*- subjects. Notably, selected strains of Clostridia strains have been identified as leading players in the maintenance of gut homeostasis, due to their roles in protecting the gut from pathogen colonisation, mediating host immune system development and modulating immunological tolerance [81]. On the other hand, members of the *Leuconostocaceae*, a family of anaerobic lactic acid-producing bacteria, have been demonstrated to stimulate the release of inflammatory Th1 type cytokines IL-12 and IFN-γ by activated antigen-presenting cells in human peripheral blood mononuclear cells, thus promoting the activation of antimicrobial immune responses [82]. However, a significant decrease in the abundance of *Leuconostocaceae* was recorded in a recent study examining the composition of the faecal microbiome of humans infected by hookworms, whipworms and ascarids [51], contrasting findings from this chapter and highlighting the need for further investigations in this area. In addition, the lactobacilli, another group of lactic acid-producing bacteria that has been positively associated with parasite colonisation in rodent models of helminth infections in several recently published studies [55,83-91], was not detected amongst the bacterial populations that were expanded in samples from *S*+ subjects. It must be also pointed out that, thus far and to the best of my knowledge, investigations conducted in human volunteers have not reported significant shifts in the abudance of lactobacilli in the gut microbiota of helminth-infected subjects [45,47,48,50-53]. Whilst expanded populations of lactobacilli following helminth colonisation may represent a rodent-specific response to infection, mechanistic studies conducted, for instance, in humanised microbiota mouse models of helminth infections may assist clarifying this point.

The faecal microbiome of *S*- subjects was enriched with a number of known opportunistic and/or potentially pathogenic bacteria, including *Bacteroides eggerthii* (higher abundances of which have been linked to increased risk for and severity of colitis in mice [92]) and species within the genus *Pseudomonas*. Expanded populations of opportunistic and potentially pathogenic microbes, coupled with an overall increase in bacterial beta diversity, have been described in the microbiome of aging humans [93,94] and may therefore underpin my observations.

Notably, whilst administration of ivermectin (a macrocyclic lactone) to a sub-group of *S*+ subjects did not result in significant differences in the levels of microbial alpha- and beta-diversity post-anthelmintic treatment (likely due to the limited number of volunteers who agreed to provide further faecal samples 6-months post-treatment), a tendency towards decreased alpha diversity and increased beta diversity, respectively, was observed in $S+_{post\text{-}treatment}$ samples when compared to $S+_{pre\text{-}treatment}$. Nevertheless, an increase in pathogenic

bacteria, including *Enterobacteriaceae* (linked to the genus *Shigella*) was observed in samples from $S+_{post\text{-}treatment}$ subjects when compared to $S+_{pre\text{-}treatment}$. This change was also accompanied by an increase in the probiotic *Lachnospiraceae* in $S+_{post\text{-}treatment}$ subjects when compared to *S*- [95], thus suggesting that anthelmintic treatment may have affected the taxonomic composition of the gut microbiota of previously infected subjects. Conversely, a recent study investigating the impact of treatment with albendazole (a benzimidazole compound) on a large cohort of human volunteers from Indonesia infected by ascarids, whipworms and/or hookworms, detected no differences between the faecal microbial composition of these volunteers and that of a placebo-treated cohort [96]. This discrepancy may be attributable to fundamental differences between the two anthelmintics investigated and/or between parasite species assessed, and/or to sample size limitations; nevertheless, future experiments carried out in large cohorts of volunteers and/or in experimental models of *Strongyloides* infection (i.e. rodents infected by *Strongyloides ratti*) may provide further clarification.

In this chapter, besides determining the qualitative and quantitative composition of the gut microbiota of *Strongyloides*-infected human volunteers, I also carried out, for the first time in helminth-infected individuals, a comprehensive analysis of the faecal metabolome of the same subjects. Indeed, given that perturbations of the gut microbiota homeostasis are known to exert downstream effects on intestinal metabolism [97,98], I hypothesized that alterations in gut microbial profiles associated to colonisation by *S. stercoralis* might be accompanied by changes in the relative abundance of individual metabolites in faecal samples, with potential implications for the overall health of infected individuals. Whilst analysis via NMR and GC-MS revealed no significant differences in the relative abundance of the vast majority of metabolites identified in samples from *S*+ versus *S*-, a number of amino acids (i.e. leucine, lysine, and alanine) were significantly more abundant in the faecal metabolome of infected individuals when compared to the uninfected cohort. Notably, anthelmintic treatment appeared to only affect the metabolites associated with helminth infection, i.e. amino acids, and thus may suggest that the helminth removal affects both the microbiome and the metabolome. An increased abundance of amino acids in the predicted faecal metabolome of helminth infected-subjects had been previously reported, albeit this information had been indirectly inferred from high-throughput metagenomics sequencing data [50,99]. Amino acids play key roles in the maintenance of the gut microbiome homeostasis and metabolism, since they support the growth and survival of bacteria in the GI tract [100]. Simultaneously, the gut microbiome exerts important functions in the metabolism of alimentary and endogenous proteins that are converted into peptides and amino acids [101,102]. In particular, the most prevalent species involved in amino

acid fermentation within the human intestine are bacteria belonging to the Class Clostridia [103-105], that were more abundant in the faecal microbiome of *S*+ subjects. Of the amino acids that were significantly more abundant in the faecal metabolome of *S*+ subjects, lysine and leucine participate in biological pathways that are key to the maintenance of the gut homeostasis [106,107]. In addition, the biological functions of lysine and leucine are closely linked [108], suggesting a possible correlation with the positive association of both these amino acids with the faecal metabolome of *Strongyloides*-infected subjects.

In contrast with the increased quantities of amino acids observed in the faecal metabolome of *S*+ subjects, the SCFAs acetate, propionate, and butyrate were significantly less abundant in this group compared to *S*-. This observation contradicts findings from a previous study in which these SCFAs were increased in faecal samples from human volunteers with coeliac disease and experimentally infected with the human hookworm, *N. americanus* [49]. Given the known anti-inflammatory properties of SCFAs, Zaiss et al. [49] had hypothesized that these molecules may play a role in the therapeutic effects of GI helminths in chronic inflammatory disorders. The discrepancy observed between this study and that by Zaiss et al. [49] may be attributable to differences between the cohorts of human volunteers investigated (acutely vs. chronically infected; middle aged vs. aged), species of parasite under consideration and infection burden (known vs. unknown). In addition, both studies are characterised limited sample sizes that may have contributed to these contrasting results.

3.5 Conclusions

In summary, in this chapter, monospecific, chronic *S. stercoralis* were associated with global shifts in the composition of the human faecal microbiome, as well as subtle changes in the faecal metabolic profiles of these individuals when compared with those of uninfected control subjects. In addition, anthelmintic treatment resulted in minor alterations of the microbiome and metabolome of these volunteers six months post-administration, albeit sample size limitations prevent any speculation on the effect/s of worm removal on the gut microbiome and metabolome. Future studies with longer monitoring of qualitative and qualitative fluctuations in gut microbiota post-treatment may assist shedding light on this point. Whilst these findings add valuable knowledge to the emerging area of host-parasite-microbiota interactions, mechanistic studies in experimental models of infection and disease or controlled clinical trial are necessary to shed light on the likely contribution of parasite-associated modifications in gut microbiome and metabolism to the anti-inflammatory properties of parasitic helminths. As a first step, I explore the impact of experimental single-species helminth

infections on host microbiota in the context of a helminth therapy trial in people suffering from relapsing-remitting multiple sclerosis in the next chapter.

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CHAPTER 4

Longitudinal changes in the gut microbiome of human volunteers with remitting-relapsing multiple sclerosis following experimental infection with the hookworm, *Necator americanus*

Abstract

Numerous studies in humans and animal models have indicated a therapeutic effect of experimental infections by gastrointestinal (GI) nematode parasites on the clinical signs and pathology of a range of autoimmune and allergic inflammatory disorders, including Inflammatory Bowel and Coeliac Diseases. Whilst the immune-regulatory properties of parasite secreted/excreted products are thought to be primarily responsible for such effects, several recent studies have hypothesized that immune modulation might stem, partly, from qualitative and quantitative changes in the composition of the host gut microbiota that follows parasite infection and establishment. In the present MHRA approved study I investigated, for the first time, the changes in gut microbial profiles of human volunteers with relapsing-remitting multiple sclerosis (RRMS) prior to and following experimental infection with 25 hookworm stage 3 larvae (*Necator americanus*; *N*+), and following administration of anthelmintic treatment, then compared the findings with data obtained from a cohort of RRMS patients subjected to placebo treatment (*PBO*). Bioinformatics analyses of bacterial 16S rRNA high-throughput sequencing data revealed significantly increased microbial alpha diversity in the gut microbiota of *N*+ compared to *PBO* subjects over the course of infection, which is indicative of a healthier gut environment. Furthermore, significant differences in the abundance of several bacterial taxa were observed between samples from *N*+ and *PBO* subjects. Most notably, Tenericutes/Mollicutes, an immune-modulatory bacterial taxon hypothesised to be associated with the pathogenesis of autoimmune disorders, was significantly increased in *N*+ subjects compared to *PBO*. Overall, these data demonstrate a significant impact of *N. americanus* infection on the human gut microbiota and lend support to the hypothesis of a

contributory role of parasite-associated changes in gut microbial composition to the therapeutic properties of hookworm parasites.

This work adds valuable knowledge to current understanding of parasite-microbiota associations and will assist future mechanistic studies aimed to unravel the causality of these relationships.

Note: The clinical findings of this trial are currently being disseminated and remain confidential. They will therefore not be discussed in this chapter.

4.1 Introduction

A number of studies have reported the beneficial effects of experimental gastrointestinal (GI) helminth infections on the pathology of a range of human autoimmune and allergic inflammatory disorders of the GI tract [1-8]. These findings sparked substantial interest in the prospect of deliberate infections of humans with live helminth parasites (i.e. helminth therapy) and already resulted in over 28 human trials (reviewed by [9,10]). To date, the most widely investigated helminth therapy approach involves the pig whipworm *Trichuris suis,* which has been demonstrated to be a safe alternative to its close relative *T. trichiura* (reviewed by [11]), a highly pathogenic human parasite (reviewed by [12]). Despite being a pig parasite, *T. suis* ova (TSO) will develop transiently in the human gut, but will be expelled within six weeks of infection; therefore, reinfections are required for long-term treatment [13]. Several studies observed a TSO treatment-induced therapeutic effect on inflammatory bowel disease (IBD; both Crohn's disease and ulcerative colitis) with improvement rates of over 70% in cohorts of 30 to 50 patients [6-8]. Moreover, TSO treatment has also been successfully trialled in patients suffering from relapsing-remitting multiple sclerosis (RRMS), a disease of the central nervous system that is characterised by inflammation, demyelination, and subsequent neural damage (reviewed by [14]). Two studies, involving three to six month TSO treatment of cohorts of four to five multiple sclerosis (MS) patients, registered slight shifts in immune and clinical profiles, which indicated minor improvements in the infected subjects [1,5]. These findings were substantiated by a subsequent trial that involved 15 study subjects and demonstrated a 34% reduction in brain lesions of the infected cohort following 5 months of TSO treatment [4]. However, helminth therapy has not solely relied on TSO treatment, but has also explored the therapeutic potential of the human hookworm (*Necator americanus*). Unlike *T. suis*, *N. americanus* is a human parasite, allowing it to persist within the human host three to five years (reviewed by [15]). Despite the risk of severe pathogenesis at high exposure, experimental hookworm infection has been demonstrated to be safe at low doses and has been shown not to provoke symptoms in safety tests [16]. Efforts have, so far, predominantly focused on intestinal immunopathologies such as a study by Croese et al. [2], which observed quantitative improvements in disease indices of nine Crohn's patients infected with 25-100 *N. americanus* larvae. Furthermore, a 21-week, double-blinded, placebo-controlled study, explored the effects of *N. americanus* infection (5 to 10 larvae) in 20 study subjects, suffering from Coeliac disease, and detected subtle therapeutic benefits [3].

Notwithstanding the indications towards the potential therapeutic benefits of helminth therapy, the biological and molecular mechanisms by which parasitic helminths might suppress

autoimmune diseases remain unclear and require further investigation. However, it has been hypothesised that the immune-regulatory properties of parasite excretory/secretory products, such as mammalian homologues of C-type lectins, galectins and cytokines (reviewed by [17]), are primarily responsible for such effects (reviewed by [18]). Indeed, natural and therapeutic helminth infections have been associated with marked immune modulation, and inducing a shift from a pro-inflammatory Th1- to a Th2-response, which, in the case of autoimmune conditions that are characterised by an overexpressed Th1-response (e.g. MS), may be beneficial (reviewed by [18]). Still, it is likely that other biological and environmental factors are involved in these processes. In particular, given the strong link that has recently been established between several autoimmune diseases (e.g. MS, IBD, type 1 diabetes and rheumatoid arthritis) and the microbiome [19-21], as well as mounting evidence towards the microbiome modulating potential of GI helminths [22-37], it has been proposed that one of the mechanisms by which such parasites can support intestinal immune homeostasis in autoimmune disorders, is via the alteration of the composition of the gut microbiota [34,37-41]. In fact, significant microbiome changes have previously been observed following the administration of hookworms to coeliac patients, who subsequently underwent a gluten challenge [30,37]. Specifically, the previously mentioned therapeutic benefits observed in the infected cohort were accompanied by significant increases in the patients' gut microbial richness and diversity, trends which are typically associated with improved microbiome homogeneity, stability and overall health [42,43].

However, the impact of parasitic GI helminths on the human microbiome have, so far, only been investigated in the context of autoimmune disorders located in the GI tract, i.e. coeliac disease [30,37]. Yet, the intrinsic intestinal inflammation and resulting gut microbial dysbiosis associated with such disorders [44,45] might distort observations of helminth induced host microbiome changes. Hence, it could be of merit to investigate such changes in the context of autoimmune conditions not primarily manifested within the GI tract, such as RRMS. Indeed, the establishment of the gut-brain-axis (reviewed by [46]), the strong association between RRMS and the human microbiome (reviewed by [47]), and the promising findings from TSO treatment of MS patients [1,4,5], make this disorder an intriguing model for the exploration of host-helminth-microbiome interactions. Therefore, in this chapter I investigated, for the first time, the longitudinal changes in gut microbial profiles of human volunteers with RRMS, prior to and following experimental infection with 25 hookworm L3 (*Necator americanus*; *N*+), and subsequent administration of anthelmintic treatment. I then compared the findings with data obtained from a cohort of RRMS patients subjected to placebo treatment (*PBO*).

4.2 Methods

4.2.1 Ethics statement

This phase 2, single centre, randomised, blinded, placebo controlled study of live hookworm (*N. americanus*) L3 larvae in patients with MS was conducted at Queen's Medical Centre, University of Nottingham, UK. This study was approved and carried out in strict accordance and compliance with the National Research Ethics Service Committee East Midlands (reference 11/EM/0140). Patients were identified, approached and recruited from the MS clinic at QMC or referred from other centres in the UK. Written informed consent was obtained from all subjects enrolled in the study. This study was registered as a clinical trial at ClinicalTrials.gov as NCT00630383.

4.2.2 Trial design

A total of 73 clinically stable patients aged 18-64 (51 female and 22 male) with relapsing remitting MS (RRMS), with at least 1 relapse in the last 12 months or two in the last 24 months and without immunomodulatory treatment, were included in this study (Worms for Immune Regulation of Multiple Sclerosis trial; NCT01470521). After screening, patients were randomised and assigned to the two treatment arms, i.e. percutaneous infection with 25 *N. americanus* larvae (*N+*; n = 36), or placebo treatment with pharmacopoeial grade water (*PBO*; n = 37). Stool samples were collected one week prior to infection/placebo-treatment (T-0.25), 1 (T1), 5 (T5), and 9 (T9) months post-infection/placebo-treatment, and two months post-anthelminthic treatment and were stored at -20°C (T12; Fig. 1). These time points constituted three investigation groups, i.e. pre-infection (T-0.25), post-infection (the combined samples from T1, T5, and T9), and post-anthelmintic treatment (T12). Disease progression (i.e. MS relapses) in patients was carefully recorded throughout the study.

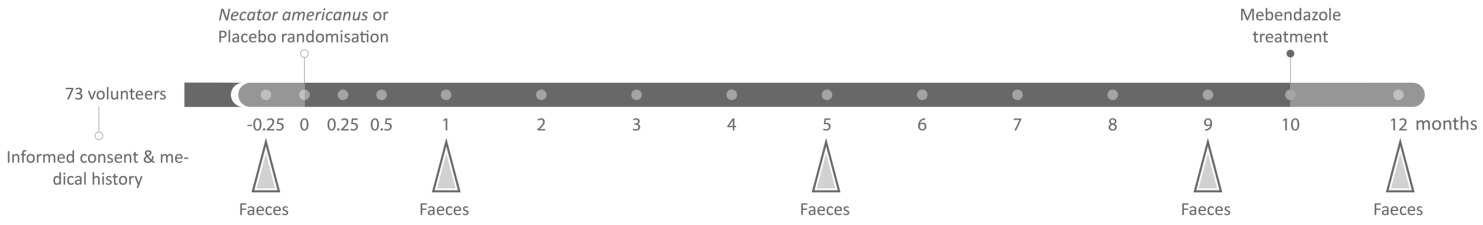


Fig. 1 Overview of the clinical study design.

4.2.3 DNA extraction and bacterial 16S rRNA Illumina sequencing

Genomic DNA was extracted directly from each faecal content sample, using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), according to manufacturers' instructions, within 1 month from sample collection. High-throughput sequencing of the V3-V4 hypervariable region of the bacterial 16S rRNA gene was performed on an Illumina MiSeq platform according to the standard protocols with minor adjustments. Briefly, the V3-V4 region was PCR-amplified using universal primers [48], that contained the Illumina adapter overhang nucleotide sequences, using the NEBNext hot start high-fidelity DNA polymerase (New England Biolabs), 2ng/µl of template DNA and the following thermocycling protocol: 2 min at 98°C, 20 cycles of 15 s at 98°C – 30 s at 63°C – 30 s at 72°C, and a final elongation step of 5 min at 72°C. Amplicons were purified using AMPure XP beads (Beckman Coulter) and the NEBNext hot start high-fidelity DNA polymerase was used for the index PCR with Nextera XT index primers (Illumina) according to the following thermocycling protocol: 3 min at 95°C, 8 cycles of 30 s at 95°C – 30 s at 55°C – 30 s at 72°C, and 5 min at 72°C. The indexed samples were purified using AMPure XP beads, quantified using the Qubit dsDNA high sensitivity kit (Life Technologies), and equal quantities from each sample were pooled. The resulting pooled library was quantified using the NEBNext library quantification kit (New England Biolabs) and sequenced using the v3 chemistry (301 bp paired-end reads). Raw sequence data will be deposited in the Mendeley database.

4.2.4 Bioinformatics and statistical analyses

Raw paired-end Illumina reads were trimmed for 16S rRNA gene primer sequences using Cutadapt (https://cutadapt.readthedocs.org/en/stable/) and sequence data were processed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2-2018.11; https://qiime2.org) software suite [49]. Successfully joined sequences were quality filtered, dereplicated, chimeras identified, and paired-end reads merged in QIIME2 using DADA2 [50]. Sequences were clustered into OTUs on the basis of similarity to known bacterial sequences available in the SILVA reference database (https://www.arb-silva.de/download/archive/qiime; Silva_132) sequences that could not be matched to references in the Silva database were clustered *de novo* based on pair-wise sequence identity (99% sequence similarity cut-off). The first selected cluster seed was considered as the representative sequence of each OTU. The OTU table with the assigned taxonomy was exported from QIIME2 alongside a weighted UniFrac distance matrix. Singleton OTUs were removed prior to downstream analyses.

Cumulative-sum scaling (CSS) was applied, followed by log2 transformation to account for the non-normal distribution of taxonomic counts data. Statistical analyses were executed using the Calypso software [51] (cgenome.net/calypso/); samples were ordinated in explanatory matrices using supervised Canonical Correspondence Analysis (CCA) including infection status as explanatory variables. Differences in bacterial alpha diversity (Shannon index) between study groups (*N+* and *PBO*) were evaluated based on rarefied data (read depth of 8712) and using analysis of variance (ANOVA); F-Tests were used to statistically assess the equality of assessed means (i.e. effect size). To take into account the paired nature of samples from *N+* and *PBO* across time points, differences between these sets were assessed using linear mixed effect regression. Differences in beta diversity (weighted UniFrac distances) were identified using Analysis of Similarity (ANOSIM) and effect size indicated by an R-value (between -1 and +l, with a value of 0 representing the null hypothesis [52]). Differences in the abundance of individual microbial taxa between groups were assessed using the LEfSe workflow [53].

4.3 Results

Out of the 73 trial subjects participating in this study 50 (36 female and 14 male) presented samples for a minimum of four out of five time points. These subjects' samples were included in the study and subsequently resulted in a cohort of 24 participants (17 female and 7 male), who were successfully infected with *N. americanus* (*N+*) and a cohort of 26 participants (19 female and 7 male), who received placebo treatment (*PBO*). Consequently, a total of 226 stool samples were selected for high-throughput sequencing of bacterial 16S rRNA and subsequent comparative analyses. From these, a total of 16,158,693 (per sample mean: 68,180 ± 70,000) paired-end reads were generated and subjected to further processing. A total of 9,100,255 high-quality sequences (per sample mean 38,397 ± 31,519) were retained after quality control. Rarefaction curves generated following *in silico* subtraction of low-quality sequences indicated that the majority of faecal bacterial communities were represented in the remaining sequence data, thus permitting me to undertake further analyses. The obtained sequences were assigned to a total of 5,611 OTUs and 14 bacterial phyla.

The phyla Bacteroidetes (*N+* = 43.8% average ± 0.4% standard deviation, and PBO = 52.2% ± 1.6%, respectively) and Firmicutes (*N+* = 51% ± 0.3%, and PBO = 43.7% ± 0.3%, respectively) were predominant in all samples analysed, followed by the phyla Proteobacteria (*N+* = 2.4% ± 1.4%, and PBO = 2.2% ± 2%, respectively), Actinobacteria (*N+* = 2.3% ± 1.3%,

and PBO = 1.8% ± 1.2%, respectively), and Tenerictures (*N*+ = 0.5% ± 3%, and PBO = 0.1% ± 3.1%, respectively; Fig. 2A). Faecal microbial community profiles were ordinated by Canonical Correspondence Analysis (CCA) (Fig. 2B) and indicated significant separation of faecal microbial profiles by infection status over the course of infection (P = 0.001). No statistically significant differences in community composition were detected pre-infection and post-anthelmintic treatment between the two study cohorts (P = 0.885, P = 0.283 respectively), or between female and male study subjects pre/post-infection and post-anthelmintic treatment (data not shown).

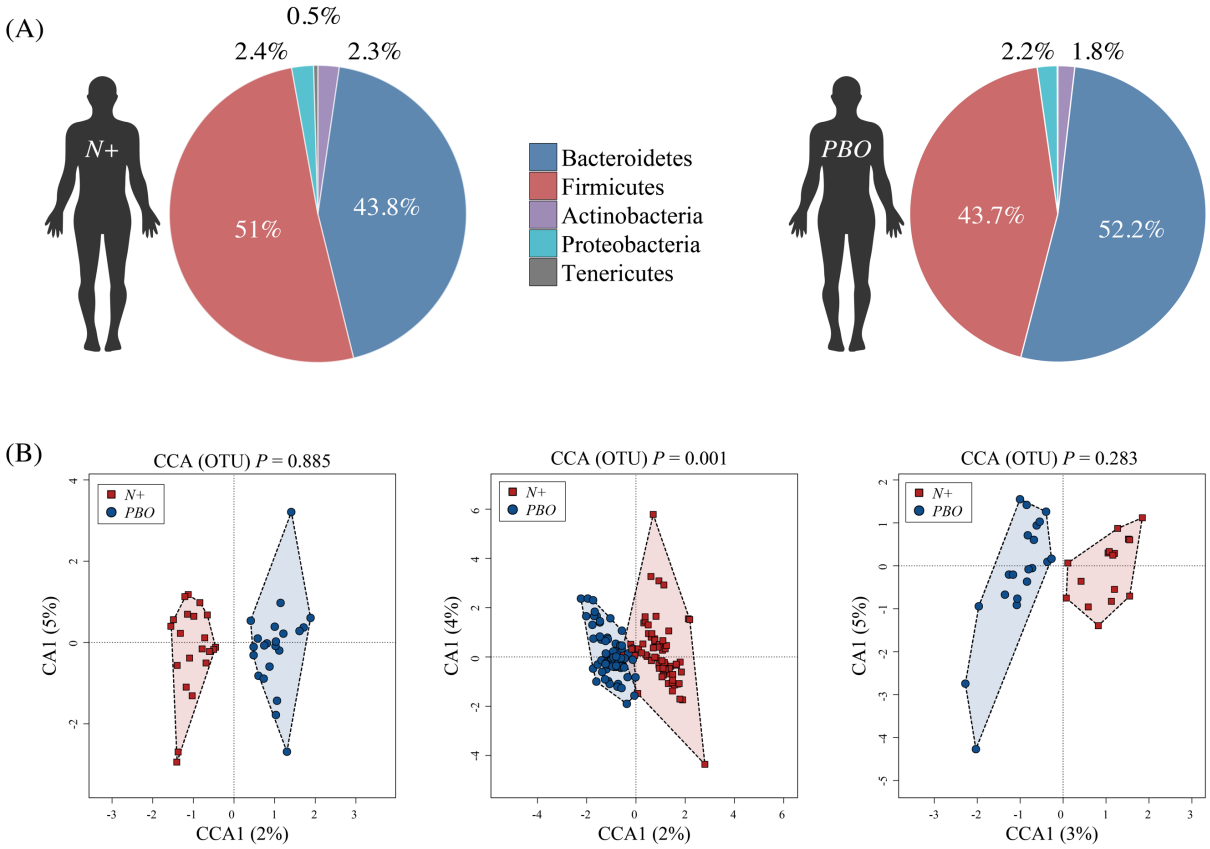


Fig. 2 Gut microbial profiles of subjects infected with *Necator americanus* (*N*+) or placebo treated (*PBO*). (A) Relative abundances of bacterial phyla detected in faecal samples from study subjects. Percentages in individual pie chart sections indicate the relative proportion of the corresponding phylum. (B) Differences between the faecal microbial profiles of *N*+ and *PBO* subjects one week prior to infection (left), one, five, and nine months post-infection (centre), and two month post-anthelmintic treatment (right) ordinated by supervised Canonical Correspondence Analysis (CCA).

Microbial alpha diversity, measured through the Shannon index was not significantly different between the faecal microbiota of *N*+ and *PBO* patients pre-infection (effect size (F)

= 0.19, *P* = 0.67; Fig. 3A). Post-infection, Shannon diversity was significantly increased in *N*+ compared to *PBO* subjects (F = 5.2, *P* = 0.025), although this significance was lost upon anthelmintic treatment (F = 2.3, *P* = 0.14; Fig. 3A). Faecal microbial richness was not significantly different between *N*+ and *PBO* patients, yet fluctuated significantly across all time points within each study group (*P* = 0.001 and *P* = 0.001 respectively; Fig. 3B; Supplementary Fig. S1). In the faecal microbiota of *N*+ subjects, richness significantly increased from T-0.25 to T1 and then progressively decreased until T12, where it increased to the highest level observed in this study. In *PBO* subjects, richness decreased at T1 compared to T-0.25, yet constantly increased from each following time point to the next, although never to the same level as observed at T-0.25. Gut microbial evenness was significantly increased in the *N*+ compared to the *PBO* cohort post-infection (Supplementary Fig. S1). No significant differences in microbial faecal beta diversity between *N*+ and *PBO* patients were observed in this study, although there appeared to be a decreased level of beta diversity in infected compared to *PBO* subjects post-infection (Supplementary Fig. S2). Beta diversity fluctuated over time in each study group, albeit not significantly (Supplementary Fig. S2).

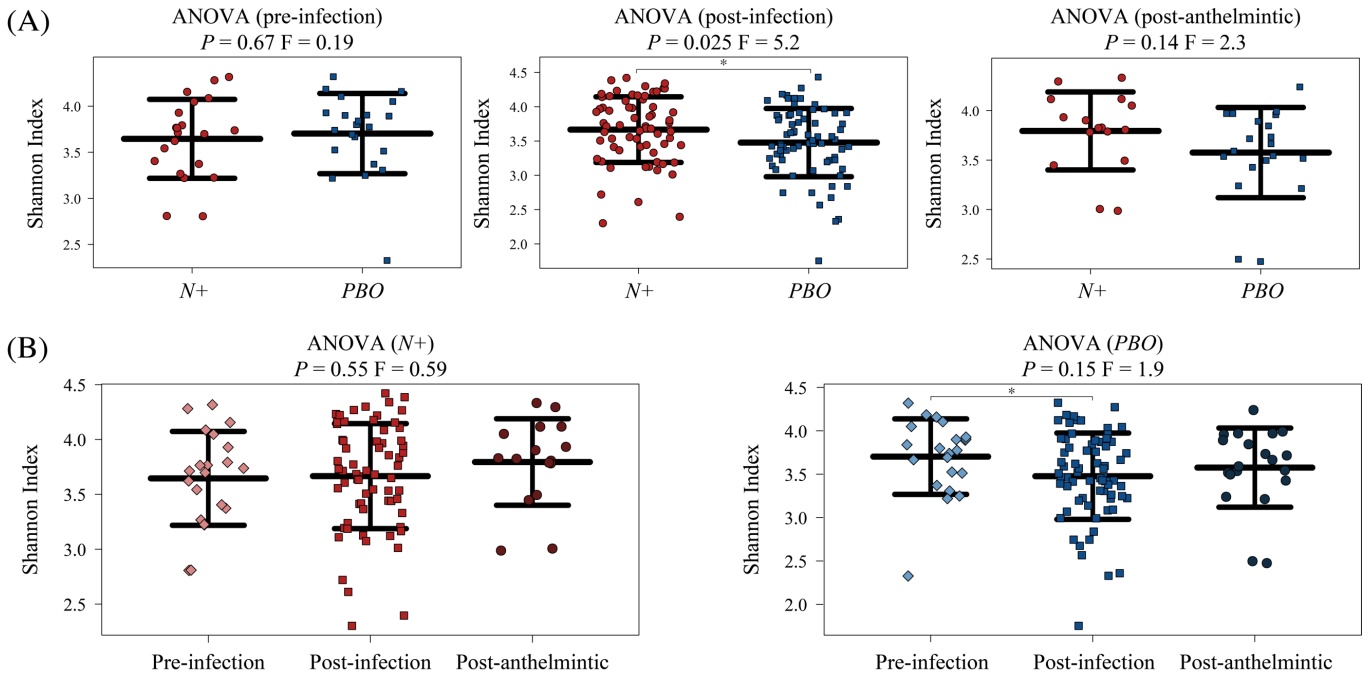
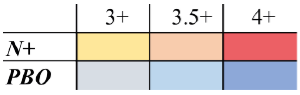


Fig. 3 Microbial alpha diversity of subjects infected with *Necator americanus* (*N*+) or placebo treated (*PBO*). (A) Differences in gut microbial Shannon diversity of *N*+ and *PBO* subjects one week prior to infection/placebo treatment (T-0.25; left), one, five, and nine months post-infection (T1, T5, and T9 combined; centre), and two months post-anthelmintic treatment (T12; right). (B) Differences in microbial richness of *N*+ (left) and *PBO* (right) subjects across all time

Linear discriminant analysis Effect Size (LEfSe) revealed significant differences in the abundance of individual microbial taxa (phylum to species level) between *N*+ and *PBO* patients at each time point (Fig. 4; Supplementary Fig. S3). The majority of differentially abundant taxa between the two study groups were detected in the later stages of the infection (i.e., T5 and T9; Supplementary Fig. S3) and post-anthelmintic treatment (T12; Fig. 4). In particular, the bacterial class Mollicutes (T5, T9, and T12; Supplementary Fig. S3), as well as the families *Ruminococcaceae* (UCG010; T1, T5, T9, and T12; Supplementary Fig. S3) and VaddinBB60 (order Clostridiales; T1, T9, and T12; Supplementary Fig. S3), were consistently more abundant in *N*+ than *PBO* subjects and remained significantly increased post-anthelmintic treatment (Fig. 4). Additionally, the genus *Haemophilus* (family *Pasteurellaceae*; T1) and the family *Flavobacteriaceae* (T12) were significantly more abundant in hookworm infected subjects (Fig. 4; Supplementary Fig. S3). Compared to faecal microbial samples from *N*+ subjects, samples from *PBO* subjects suggested significantly increased levels of the bacterial families *Peptostreptococacceae* (T5 and T9), *Streptococacceae* (T9), and Eubacteriaceae (i.e., *Eubacterium halli*; T9 and T12), as well as of several genera of the family

Lachnospiraceae, i.e. *Roseburia* (T5), *Dorea* (T9), *Tyzerella* (T9), and *Agathobacter* (T9; Fig. 4; Supplementary Fig. S3).



Phylum	Class	Order	Family	Genus	Pre-infection	Post-infection	Post-anthelmintic
Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*	UCG001			
Firmicutes	Clostridia	Clostridiales	*Peptococcaceae*				
			Ruminococcaceae	UCG009			
				Ruminiclostridium			
			Lachnospiraceae	*Anaerostipes*			
Actinobacteria							
	Coriobacteriia						
		Coriobacteriales					
			*Coriobacteriaceae**				
Bacteroidetes							
	Bacteroidia	Bacteroidales	*Bacteroidaceae*				
Firmicutes							
	Bacilli	Lactobacillales	*Carnobacteriaceae*				
	Clostridia	Clostridiales	*Peptostreptococcaceae*				
			Lachnospiraceae				
				Roseburia			
				Dorea			
				Tyzzerella			
				Fusicatenibacter			
				Agathobacter			
			Ruminococcaceae				
				UCG013			
				Anaerofilum			
Tenericutes							
	Mollicutes						
		RF39*					
		Izimaplasmatales					
		Anaeroplasmatales					
			Anaeroplasmataceae				
Proteobacteria	Gammaproteobacteria	Pasteurellales					
			Pasteurellaceae				
				Haemophilus			
Firmicutes	Clostridia	Clostridiales	VadinBB60*				
			Clostridiaceae	*Hungatella*			
			Ruminococcaceae	UCG010*			
				UCG005			
Bacteroidetes	Bacteroidia	Bacteroidales	*Sutterellaceae*	*Sutterella*			
			Bacteroidaceae	*Barnesiella*			
			Barnesiellaceae	*Coprobacter*			
	Flavobacteriia	Flavobacteriales	*Flavobacteriaceae**				
Actinobacteria	Coriobacteriia	Coriobacteriales	*Coriobacteriaceae**				
Firmicutes	Clostridia	Clostridiales	*Ruminococcaceae*	*Ruminococcus (gnavus)*			
				UCG004			
			Eubacteriaceae	*Eubacterium (hallii)*			
Actinobacteria	Actinobacteria	Bifidobacteriales	*Bifidobacteriaceae*				
				Bifidobacterium			
Tenericutes	Mollicutes	Mollicutes (RF39)*					

Fig. 4 Differentially abundant bacterial taxa in the faecal microbiota of subjects infected with *Necator americanus* (*N*+) or placebo treated (*PBO*) subjects one week prior to infection/placebo treatment (T-0.25), one (T1), five (T5), and nine (T9) months post-infection, and 2 months post-anthelmintic treatment (T12) based on Linear discriminant analysis Effect Size (LEfSe) analysis. Colours correspond to Linear Discriminant Analysis (LDA) scores of 4 or higher (*N*+ = red; *PBO* = dark blue), 3.5 to 4 (*N*+ = orange; *PBO* = light blue), and 3 to 3.5 (*N*+ = yellow; *PBO* = grey).

4.4 Discussion

RRMS is a severe pro-inflammatory demyelinating disease of the central nervous system that affects over 1.84 million people worldwide (reviewed by [14]). To date, no effective treatment exists and the search for an effective therapy without significant side-effects is ongoing (reviewed by [14]). One proposed course of action involves the use of immunomodulatory parasitic GI helminths to mediate pathology and disease progression, via induction of a Th2 response and a subsequent immune-rebalancing of the host. The recent establishment of the importance of the native microbiome in RRMS (reviewed by [47]), alongside the microbiome-modulatory properties of GI helminths [31,33,34,37,54-57], further substantiate the potential of helminth therapy in the treatment of RRMS. In the present MHRA approved study I investigated, for the first time, the changes in gut microbial profiles of human volunteers with RRMS prior to and following experimental infection with 25 hookworm L3, and following administration of anthelmintic treatment, then compared the findings with data obtained from a cohort of RRMS patients subjected to placebo treatment.

Taxonomic profiling of microbial communities using CCA revealed no substantial differences in gut microbiota composition between *N*+ and *PBO* patients prior to infection, yet indicated significant shifts post-infection; yet, the significance was lost post-anthelminthic treatment. This suggests that albeit the hookworms are modulating the host microbiota over the course of infection their gut microbial effects don't appear to persist upon the worms' removal. In particular, I identified significantly elevated alpha diversity in the *N*+ cohort compared to *PBO* patients as the likely causative agent underlying those gut microbial shifts. While alpha diversity remained stable in *N*+ patients post infection, diversity decreased significantly in *PBO* subjects over the next nine months following placebo administration. Notably, the increased levels of alpha diversity appeared to be mainly driven through increased microbial evenness, i.e. the amount of microbial species is not significantly affected (richness), but the overall distribution of all taxa is more even and balanced (evenness) [58]. Furthermore, removal of the helminths via anthelmintic treatment did not alter alpha diversity significantly in both the *PBO* and *N*+ cohorts, yet diversity levels shifted sufficiently for the significant difference between the two study cohorts to be lost. The observation of increased diversity in the *N*+ patients was of particular interest, since elevated levels of alpha diversity are typically associated with a healthier gut microbiome and overall host health (reviewed by [59]). Notably, decreases in alpha diversity have previously been reported in MS patients during relapses, which could explain the diversity drop I observed in the *PBO* cohort [60]. However, if indeed relapses were responsible for the decreased levels of microbiota diversity in *PBO* patients, it is

remarkable that *N*+ subjects appeared to be unaffected and able to maintain stable levels of alpha diversity. Furthermore, this finding is substantiated by similar observations of increased gut microbial alpha diversity in helminth treated patients with an autoimmune condition have been described in two previous studies that assessed the effects of *N. americanus* infections on the gut microbiota of coeliac patients; The authors hypothesised that this might be at least partially responsible for the therapeutic effect observed in these studies [30,37]. Therefore, it is possible that in this study the hookworms are preventing the RRMS-induced decreases in host microbiome diversity, observed in *PBO* patients, in the *N*+ study cohort.

Besides the changes in diversity that were observed in the study cohorts, I also detected shifts in specific bacterial taxa. The majority of which appeared to occur post-infection, with the number of differences between *PBO* and *N*+ patients increasing with time of infection, and reaching their peak nine months into the trial. Notably, it appears that in *PBO* patients bacterial taxa proliferated, which have been previously associated with relapsing MS patients [60]; in particular, a family of anaerobic bacteria (*Lachnospiraceae*), including the genera *Roseburia*, *Dorea*, and *Tyzzerella* (amongst others), were significantly increased in *PBO* compared to *N*+ patients post-infection. *Lachnospiraceae* constitutes one of the key taxa of the human gut microbiome that degrades complex polysaccharides to short-chain fatty acids (SCFAs), i.e. acetate, butyrate, and propionate; these SCFAs can then be used for energy by the host and can also act as anti-inflammatory agents [61]. This is of note, since *Lachnospiraceae* have been reported to be substantially increased in pathological conditions, such as inflammatory bowel diseases [62]. In particular, elevated populations of *Dorea* have been observed in irritable bowel syndrome patients [63], as well during intestinal inflammation [64]. Whilst *Dorea* are known to produce the SCFA butyrate, they also metabolise sialic acids, which are commonly found at terminal ends of mucins; release of these acids is implicated in mucin degradation, likely increasing gut permeability and compromising gut homeostasis [65]. Likely, this expansion of *Lachnospiraceae* and its respective genera in the *PBO* cohort is associated with MS disease progression and an immune shift towards a pro-inflammatory state during relapses [66]. Remarkably, although the *N*+ patients also suffer from MS, no expansion of *Lachnospiraceae* could be detected.

The most substantially and consistently increased bacterial taxon in *N*+ patients post-infection was the phylum Tenericutes. The phylum Tenericutes consists of the sole class *Mollicutes* and its taxa are known for being Gram-stain-negative, very small, and wall-less bacteria fulfilling a diverse array of roles within the mammalian microbiome (reviewed by

[67,68]). This was of note, since the relative abundance of these bacteria has been reported to differ between healthy individuals and people suffering from a wide range of autoimmune conditions, such as IBD [69,70], Diabetes Type 1 [71-73], MS [74], and experimental autoimmune encephalomyelitis (EAE; a commonly used murine model of MS)[75]. Although the trends are not always the same [72,73], the majority of these studies reported decreased levels of Tenericutes/Mollicutes in the diseased cohort, when compared to healthy controls [69-71,74,75]. Notably, although the study by Tremlett and co-authors [74] reported higher Tenericutes abundance in healthy controls, than in paediatric MS patients, it also reported that the opposite was no longer the case when the patients had been exposed to immunomodulatory drugs [74]. Indeed, these bacteria have been suggested to be easily affected by different host immune states and to particularly proliferate in Th2 environments, due to reports of increased Tenericutes populations following promoted Th2 response post helminth infection [76,77]. This hypothesis of a Tenericutes-Th2 bias was further supported through an increased relative abundance of bacteria belonging to Tenericutes/Mollicutes being reported in human cohorts naturally infected with roundworms (i.e. *Trichuris* and/or *Ascaris* and/or hookworm) [31], rats infected with *Hymenolepis diminuta* [77], and macaques with idiopathic chronic diarrhoea and which received experimental helminth treatment of *T. trichiura* [76]; all of which are Th2 promoting environments. Although the ability of parasitic helminths to modulate a taxon that has been associated with such a wide range of autoimmune conditions proves intriguing, the functional impact remains to be determined. Nevertheless, the observation of increased levels of Tenericutes in RRMS patients treated with hookworms, alongside the evidence from previous studies in different parasite/disease settings, suggests that this bacterial taxon might merit further mechanistic investigations via murine or other animal models, to help untangle its potential role and importance in the context of disease progression.

4.5 Conclusion

To conclude, this chapter identified significant differences between gut microbial profiles of RRMS patients treated with *Necator americanus* and patients who received a placebo treatment. The significant decrease of alpha diversity and the proliferation of taxa, such as *Lachnospiraceae*, observed in the *PBO* cohort was typical of trends previously reported for MS relapses. Conversely, the significantly higher levels of diversity in the *N*+ patients suggest improved gut microbial and homeostasis. This was further substantiated by the elevated relative abundance of Tenericutes, which have previously been associated with healthy, rather than immunocompromised subjects. Overall, these data lend support to the hypothesis of a

contributory role of parasite-associated changes in host gut microbiota composition to the potentially therapeutic potential of parasitic hookworm. This work adds valuable knowledge to current understanding of parasite-microbiota associations and will assist future mechanistic studies aimed to unravel the causality of these relationships. In the following chapter I investigated the impact of a murine model of human helminth infection, however the parasite in question was not GI, but rather extra intestinal. This would allow me to answer whether parasitic helminth infections can cause host microbiota shifts, without a direct interaction between the parasite and host microbiome.

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CHAPTER 5

Schistosoma mansoni infection is associated with quantitative and qualitative modifications of the mammalian intestinal microbiota

Abstract

In spite of the extensive contribution of intestinal pathology to the pathophysiology of schistosomiasis, little is known of the impact of schistosome infection on the composition of the gut commensal microbiota of its mammalian host. Here, I characterised the fluctuations in the composition of the gut microbial flora of the small and large intestine, as well as the changes in abundance of individual microbial species, of mice experimentally infected with *Schistosoma mansoni* with the goal of identifying microbial taxa with potential roles in the pathophysiology of infection and disease. Bioinformatic analyses of bacterial 16SrRNA gene data revealed an overall reduction in gut microbial alpha diversity, alongside a significant increase in microbial beta diversity characterised by expanded populations of *Akkermansia muciniphila* (phylum Verrucomicrobia) and lactobacilli, in the gut microbiota of *S. mansoni*-infected mice when compared to uninfected control animals. These data support a role of the mammalian gut microbiota in the pathogenesis of hepato-intestinal schistosomiasis and serves as a foundation for the design of mechanistic studies to unravel the complex relationships amongst parasitic helminths, gut commensal microbiota, pathophysiology of infection and host immunity.

5.1 Introduction

Schistosomiasis, a major neglected tropical disease, is considered the most problematic of the human helminthiases in terms of morbidity and mortality [1]. The causative agents are the blood flukes, trematodes of the genus *Schistosoma* including *S. mansoni*, *S. japonicum* and *S. haematobium*. Schistosomiasis in its several forms has been estimated to cause ≥3.5 million Disease Adjusted Life Years (DALYs) [2,3]. In Sub-Saharan Africa, 393 million people are estimated to be exposed to the parasite and, of these, 54 million suffer from overt schistosomiasis [4]. Humans are the definitive hosts of *S. mansoni*, and harbour the adult males and females, that live in the portal system and mesenteric veins [5,6]. The females shed eggs that traverse the lining of the mesenteric veins, proceed through the wall of the intestines, preferentially *via* the lymphoid tissue of the Peyer's patches [7-9] to the intestinal lumen and exit with the faecal stream. The eggs reach the freshwater environment and hatch a ciliated miracidium that seeks out and infects a suitable species of gastropod snail, including species of the genus *Biomphalaria*. Within the snail, the parasites multiply *via* asexual reproduction, after which the cercarial developmental stage exits the snail. The fork-tailed cercaria is the infective stage for humans, who contract the infection in water contaminated with cercariae. During the skin penetration the cercariae shed the tail and the schistosomulum stage of the blood fluke enters the circulation and is transported *via* the heart to the lungs and liver where it matures over several weeks. After about four weeks, the adult worms, which exhibit sexual dimorphism, migrate into the mesenteries of the intestines (*S. japonicum* and *S. mansoni*) or the blood vessels of the urinary bladder and other pelvic organs (*S. haematobium*), where they commence sexual reproduction releasing hundreds to thousands of eggs per day, depending on the species. These parasites can live for decades [3,6].

The pathobiology of schistosomiasis is mostly associated with tissue lesions caused by migrating parasite eggs [10]. A large proportion of schistosome eggs fail to reach the intestinal lumen, instead becoming trapped in hepatic sinusoids and the intestinal wall, where they provoke formation of collagen-rich granulomas accompanied by periportal fibrosis and portal hypertension. Granuloma formation is mediated by host immunity to egg antigens; in particular, while a strongly polarised Th2-mediated immune response is responsible for the development of large granulomas during the initial phases of parasite establishment, chronic infections are accompanied by the onset of regulatory responses that lead to the formation of smaller granulomas around newly deposited eggs [7]. In the mouse model of infection with *S. mansoni*, the immuno-regulatory environment confers protection against hepatotoxicity

(reviewed by [7]). Nevertheless, the immune-molecular mechanisms underlying these regulatory responses have yet to be fully defined. In particular, whereas *Schistosoma* egg antigens interact directly with splenic B cells in the mouse, leading to production of IL-10 and expansion of regulatory T cell (Treg) populations [11], the contribution of environmental stimuli to the initiation of these immune-regulatory mechanisms is not well understood. Notably, in a mouse model of infection by the hookworm-like nematode *Nippostrongylus brasiliensis*, parasite-induced Th2 type immune responses are accompanied by profound alterations in the composition of the commensal gut microbiota, including marked contraction of populations of segmented filamentous bacteria (SFB, Gram-positive members of the family *Clostridiaceae*) and down-regulation of pro-inflammatory IL-17 [12]. In turn, infection of SFB-deficient mice results in unaltered IL-17 gene expression [12], thereby supporting a key role for selected taxa of commensal bacteria in helminth-driven modulation of immunity. Based on these observations, it seems plausible to hypothesize that the shift between Th2-type and regulatory responses that accompany egg production and characterises chronic schistosomiasis may be triggered, at least in part and directly or indirectly, by parasite-associated modifications in the composition of the intestinal commensal microbiota.

In spite of the burgeoning interest in understanding the complex interactions occurring at the host-parasite-microbiota interface [12-25], studies investigating the impact of acute or chronic *S. mansoni* infection on the intestinal microbiota of its mammalian host are, thus far, scant. In a single study, Holzscheiter et al. [26] demonstrated that administration of broad spectrum antibiotics and antimycotics to *S. mansoni*-infected C57BL/6 mice resulted in a substantial reduction of intestinal inflammation and intestinal granuloma development, thus providing support to a direct role of the host commensal microbes in the pathogenesis of schistosomiasis. Here, I have directly addressed this issue by defining qualitative and quantitative fluctuations in intestinal microbial community profiles during infection with *S. mansoni*, and identified groups of bacteria with known roles in immune-modulation (e.g. lactobacilli), maintenance of epithelial barrier function integrity (i.e. *Akkermansia muciniphila*), and intestinal inflammation (e.g. *Dorea* and *Bacteroides acidifaciens*) that may play significant roles in the pathophysiology of acute and chronic schistosomiasis.

5.2 Materials and methods

5.2.1 Ethics statement

Swiss-Webster female mice were obtained from the NIAID Schistosomiasis Resource Center, Rockville, MD, via distribution through BEI Resources, and housed at the Animal

Research Facility of the George Washington University. The latter is accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC no. 000347) and holds an Animal Welfare Assurance by the National Institutes of Health, Office of Laboratory Animal Welfare (OLAW: A3205-01). Procedures described here were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the George Washington University, protocol number A137.

5.2.2 Infection of mice, sample collection and parasitological analyses

Ten Swiss-Webster female mice (*S*+) were percutaneously infected with 200 *S. mansoni* (NMRI strain) cercariae by tail exposure, as described [27], while 10 age- and gender-matched mice remained uninfected and were included as controls (*S*-). For each *S*+ and *S*-, five mice were euthanised 28 days after infection (D28 p.i., before the start of egg laying by sexually mature *S. mansoni* females) by an overdose of Euthasol (sodium pentobarbital and sodium phenytoin, ~40 mg per mouse) (Virbac Corporation, Fort Worth, TX) delivered by intraperitoneal injection. The remaining mice (n = 5 for *S*+ and *S*-) were euthanised, as above, at day 50 p.i. (D50 p.i., corresponding with ongoing egg laying and extensive granuloma formation). Adult *S. mansoni* were recovered from each mouse in *S*+ by portal perfusion, and livers were removed for egg isolation and counting as described previously [28]. Luminal content samples were collected from sections of the small (SI) and large intestines (LI) of individual *S*+ and *S*- mice at each D28 and D50 p.i. under sterile conditions. Briefly, after opening the abdominal cavities, the intestines were incised longitudinally with a sterile razor blade and SI and LI luminal contents were transferred directly into sterile tubes. These were snap frozen on dry ice and stored at -80°C until further use. The experiment was repeated twice for data validation, resulting in a total of 20 *S*+ and 20 *S*- mice included in the study.

5.2.3 DNA extraction and bacterial 16S rRNA gene sequencing

Genomic DNA was extracted directly from each luminal content sample, as well as two no-template negative control samples, using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), according to manufacturers' instructions, within one month from sample collection. High-throughput sequencing of the V4 hypervariable region of the prokaryotic 16S rRNA gene was performed on an Illumina MiSeq platform. The V4 region was PCR-amplified using universal primers [29], that contained the Illumina adapter overhang nucleotide sequences, using the NEBNext hot start high-fidelity DNA polymerase (New

England Biolabs), 2 ng/µl of template DNA and the following thermocycling protocol: 2 min at 98°C, 20 cycles of 15 s at 98°C – 30 s at 63°C – 30 s at 72°C, and a final elongation step of 5 min at 72°C. Amplicons were purified using AMPure XP beads (Beckman Coulter) and the NEBNext hot start high-fidelity DNA polymerase was used for the index PCR with Nextera XT index primers (Illumina), with thermocycling as follows: 3 min at 95°C, 8 cycles of 30 s at 95°C – 30 s at 55°C – 30 s at 72°C, and 5 min at 72°C. The indexed samples were purified using AMPure XP beads, quantified using the Qubit dsDNA high sensitivity kit (Life Technologies), and equal quantities from each sample pooled. The pooled library was quantified using the NEBNext library quantification kit (New England Biolabs) and sequenced using the v3 chemistry (301 bp paired-end reads). The raw sequences are available at Mendeley: doi:10.17632/y8c7vpc8zp.1.

5.2.4 Bioinformatics and statistical analyses

Paired-end Illumina reads were trimmed for 16S rRNA gene primer sequences using Cutadapt (https://cutadapt.readthedocs.org/en/stable/) and sequence data were processed using the Quantitative Insights Into Microbial Ecology (QIIME-1.9.1) software suite [30]. Successfully merged reads were quality filtered in QIIME using default settings. Thereafter, sequences were clustered into Operational Taxonomic Units (OTUs) on the basis of similarity to annotated bacterial sequences available in the Greengenes database (v13.8; http://greengenes.secondgenome.com/; 97% sequence similarity cut-off) using the UCLUST software within QIIME. Sequences that could not be matched to references in the Greengenes database were clustered *de novo* based on pair-wise sequence identity (97% sequence similarity cut-off). The first selected cluster seed was considered as the representative sequence of each OTU. Taxonomy assignment of representative sequences was accomplished with the UCLUST software. Singleton OTUs were removed prior to downstream analyses. Cumulative-sum scaling (CSS) was applied, followed by log2 transformation to account for the non-normal distribution of taxonomic counts data. Statistical analyses were executed using the Calypso software [31] (cgenome.net/calypso/). Samples were ordinated using Principle Coordinates Analysses (PCoA) based on weighted UniFrac distances. CCA was then performed, including infection status and time point as explanatory variables. In addition, Permutational Multivariate Analyses of Variance Using Distance Matrices (Adonis) [32] was used to calculate the portion of variability in the data explained by the explanatory variables, and a biplot was generated in R [33]. Following rarefaction of raw data, differences in microbial alpha diversity (Shannon

diversity) and richness between *S*+ and *S*- groups, as well as in the abundance of individual taxa, were evaluated using ANOVA. Beta diversity was calculated using weighted UniFrac distances and differences in beta diversity were calculated using Analysis of Similarity (ANOSIM) [34]. Differences in the composition of the microbiota between groups were assessed using the Linear discriminant analysis Effect Size (LEfSe) workflow [35]. Following the completion of bioinformatics analyses using QIIME (QIIME-1.9.1), QIIME2 was released (QIIME2-2018.4; https://qiime2.org). Therefore, in order to ensure that no substantial differences in findings could be detected when performing data analysis and annotation using this updated software, I undertook separate bioinformatic analyses and compared the resulting findings with those originally obtained using QIIME-1.9.1. In addition, reproducibility of findings was also ensured by replacing the Greengenes database with the SILVA reference database (https://www.arb-silva.de/download/archive/qiime; Silva_132) for sequence data annotation. Briefly, no major differences were detected between findings generated with QIIME-1.9.1 or QIIME2-2018.4 and using the Greengenes or SILVA repository as reference databases. Thus, the results from these additional analyses are not shown in the main article. Nevertheless, individual data files displaying findings from these analyses (e.g. differences in microbial alpha and beta diversity between mouse groups and infection time points, as well as differences in the relative abundances of individual OTUs) are available in the Mendeley database at doi:10.17632/y8c7vpc8zp.1.

5.3 Results

Ten Swiss-Webster female mice (*S*+) were infected with 200 *S. mansoni* cercariae, while 10 age- and gender-matched mice remained uninfected and were included as controls (*S*-). For each *S*+ and *S*- group, five mice were euthanized 28 days after infection (D28 p.i., i.e. before egg laying by sexually mature *S. mansoni* females had commenced). The remaining mice (n = 5 for each *S*+ and *S*-) were euthanized at day 50 p.i. (D50 p.i., corresponding with ongoing egg laying by sexually mature *S. mansoni* females). An average of 40 mixed-sex adult parasites were recovered from individual *S*+ mice at D28, with comparable numbers being collected at D50. Luminal content samples were collected from sections of the small (SI) and large intestines (LI) of individual *S*+ and *S*- mice at each D28 and D50. From all samples analysed in this study (n = 80), a total of 1,305,817 paired-end reads were generated and subjected to further processing (Mendeley DOI: doi:10.17632/y8c7vpc8zp.1). No amplification was obtained from two no-template negative control samples (see Materials and Methods). Following filtering of singleton Operational Taxonomic Units (OTUs), duplicate

sequences and chimeric sequences, a total of 974,491 high-quality reads (per sample mean: 12,181 ± 3,589) were retained. Rarefaction curves generated indicated that the majority of the bacterial communities were represented in the remaining sequence data (cf. Supplementary Fig. S1), which facilitated further analysis of these data. These sequences were assigned to 8,734 OTUs and seven bacterial phyla (Mendeley DOI: doi:10.17632/y8c7vpc8zp.1). Overall, the phyla Firmicutes and Bacteroidetes were predominant in all samples analysed (54.2% average ± 23.9% standard deviation, and 34.9% ± 19.4%, respectively), followed by the phylum Verrucomicrobia (8% ± 13.4%) (Fig. 1). However, differences were observed in the relative abundance of Firmicutes and Bacteroidetes between samples from the SI and LI; in particular, while Firmicutes were significantly more abundant than Bacteroidetes in the SI throughout the course of the infection (73.1% ± 18.7% *vs* 19.5% ± 11.6 for Firmicutes and Bacteroidetes, respectively), similar proportions of the two phyla were detected in LI (Firmicutes: 40.9% ± 16.6%; Bacteroidetes: 47.1% ± 15.5%) (Fig. 1). Verrucomicrobia were marginally less abundant in the microbiota from SI (5.1% ± 14.4%) than in that from LI samples (8.7% ± 12%) (Fig. 1).

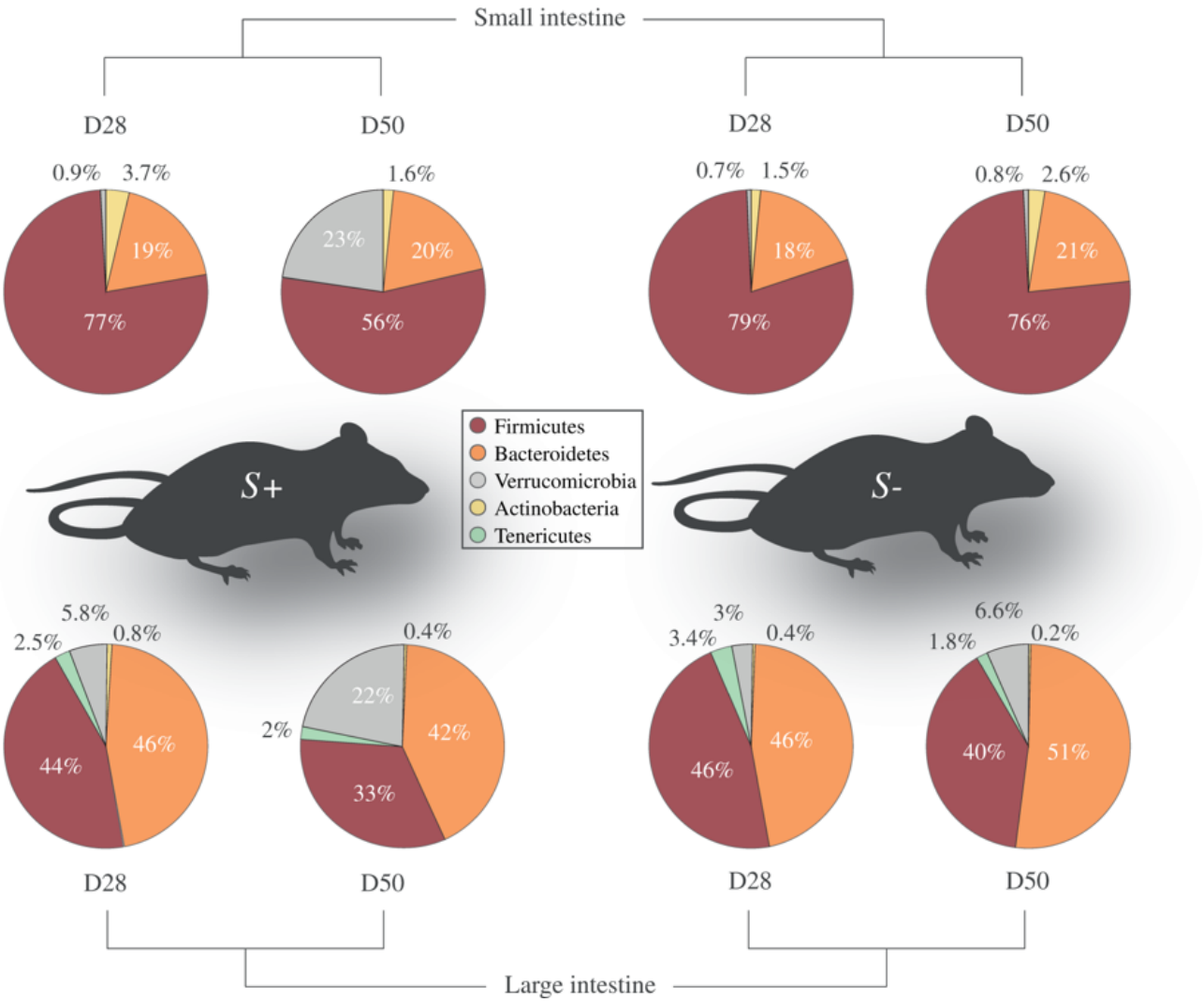

Fig. 1 Relative abundances of bacterial phyla detected in luminal content samples from the small and large intestine of mice infected by *Schistosoma mansoni* (*S*+) at 28 and 50 days post-infection (D28 and D50, respectively), as well as of uninfected controls (*S*-). Percentages in individual pie chart sections indicate the relative proportion of the corresponding phylum.

Mouse gut microbial communities were clustered by Principal Coordinates Analysis (PCoA), which clearly separated samples from *S*+ mice at D50 from all other samples (Fig. 2). Although differences between SI and LI microbial community profiles of *S*+ and *S*- mice at each time-point were statistically significant when analysed through Canonical Correspondence analyses (CCA) (SI = $P = 0.001$; LI = $P = 0.001$), the largest difference was detected between the gut microbial compositions of SI and LI of *S*+ mice at D50 and the remaining sampling groups (Fig. 2). The portion of variability explained by explanatory variables in the CCA was 0.187 and 0.218 for the SI and LI respectively (Supplementary Fig. S2). Analyses of Variance (ANOVA) of shannon diversity values obtained from the SI and LI

of *S*+ and *S*- mice at D28 and D50 revealed a significant decrease in microbial alpha diversity at D50 in both intestinal sites in *S*+ mice (SI = $P = 0.006$; LI = $P = 0.02$; Fig. 3). Beta diversity was decreased in the microbiota of *S*+ (both intestinal sites) at D28 when compared to *S*-, although these changes were not statistically significant (Fig. 4). Nevertheless, at D50, microbial beta diversity in *S*+ mice (both intestinal sites) was significantly higher than that of *S*- mice at this time point (SI: $P = 0.004$, LI: $P = 0.008$; Fig. 4).

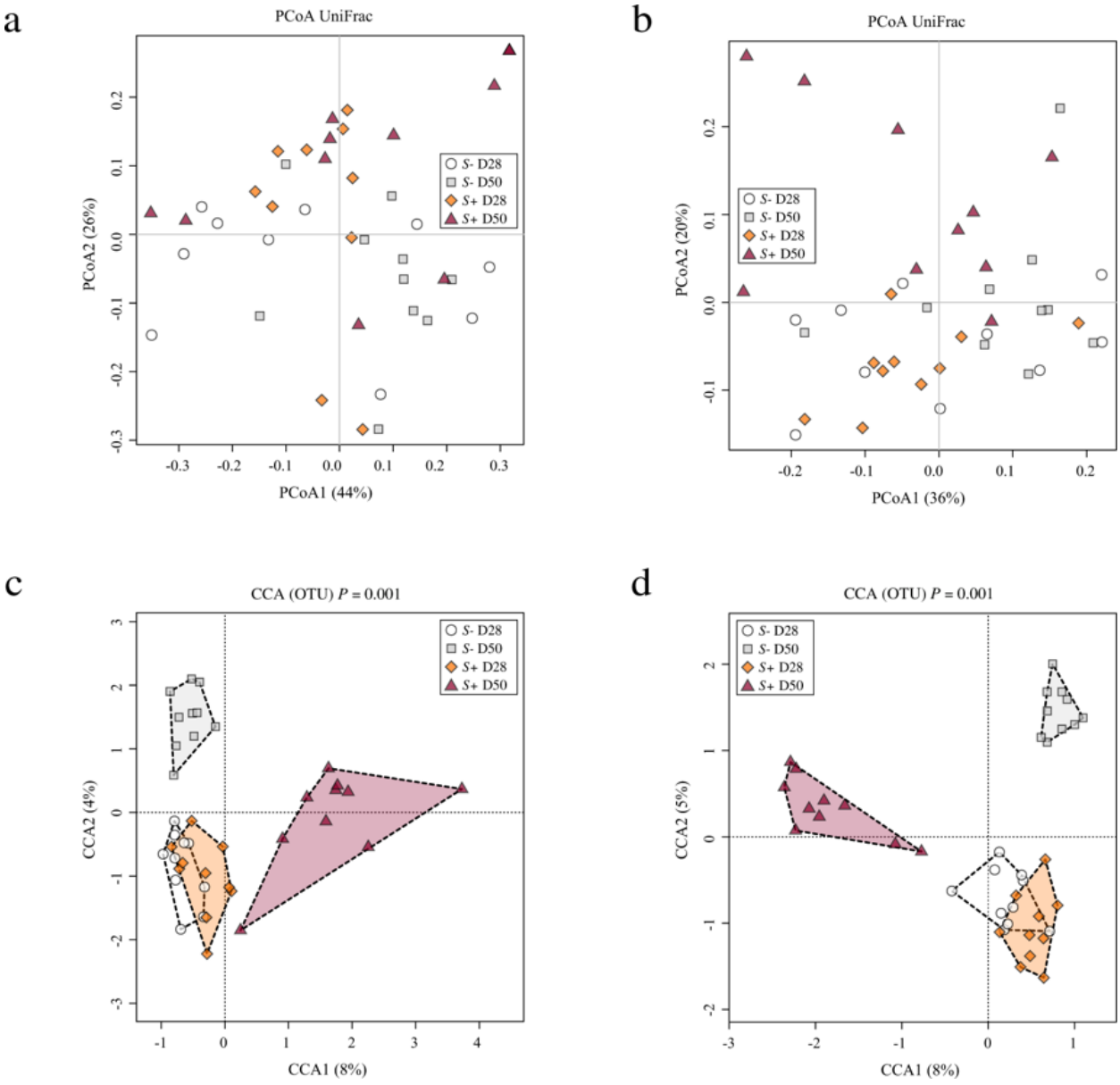

Fig. 2 The gut microbial profiles of luminal content samples from the small and large intestine of mice infected by*Schistosoma mansoni* (*S*+) at 28 and 50 days post-infection (D28 and D50, respectively), as well as of uninfected controls (*S*-), ordinated by Principal Coordinates Analysis (PCoA) (a: small

intestine; b: large intestine) and Canonical Correspondence Analysis (CCA) (c: small intestine; d: large intestine)

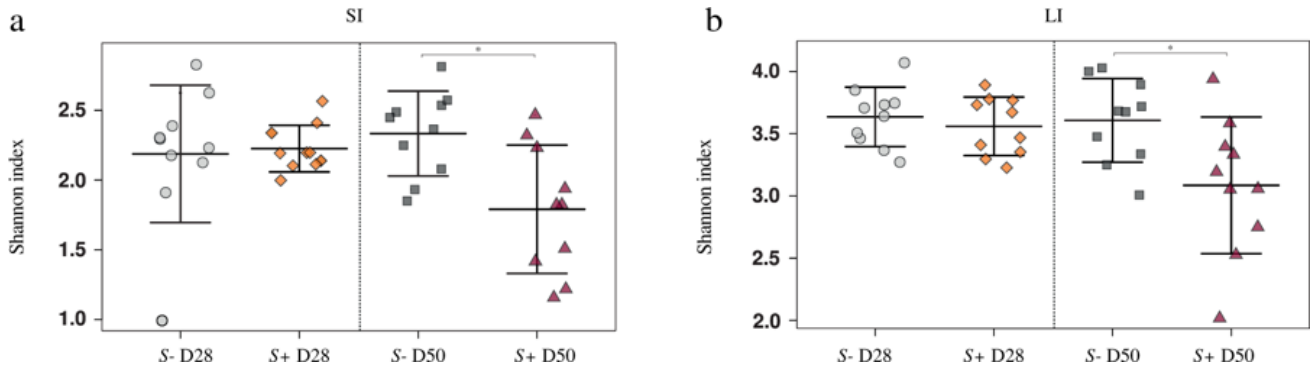

Fig. 3 Differences between microbial Shannon diversity detected in the gut microbiota of mice infected with *Schistosoma mansoni* (*S*+) at day 28 and 50 post-infection (D28 and D50, respectively) and that of uninfected control mice (*S*-), in the small (a; SI) and large intestine (b; LI). Asterisks denote significant differences ($P < 0.05$) between individual groups.

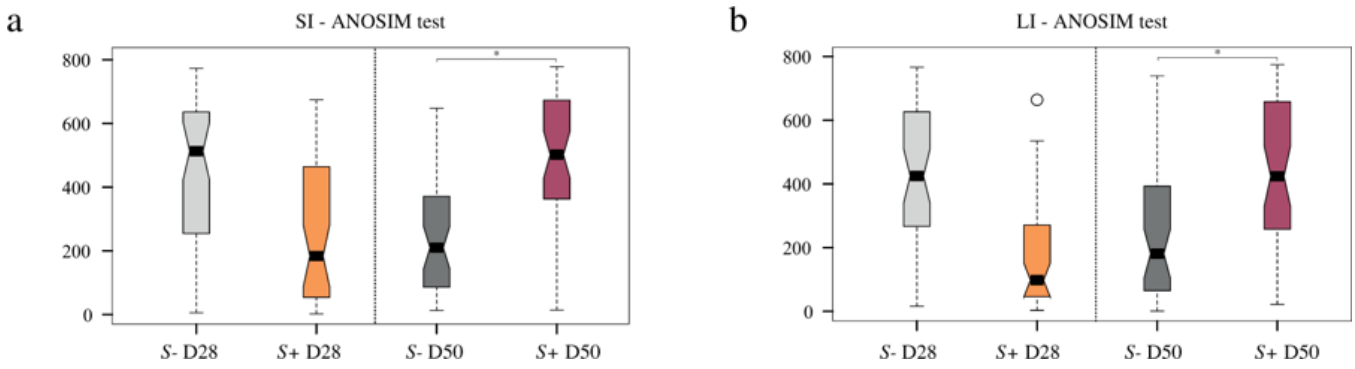

Fig. 4 Differences between microbial beta diversity detected in the gut microbiota of mice infected with *Schistosoma mansoni* (*S*+) at day 28 and 50 post-infection (D28 and D50, respectively) compared with control mice (*S*-), in the small (a; SI) and large intestine (b; LI). Asterisks denote significant differences ($P < 0.05$) between individual groups.

Differences in the abundance of individual microbial taxa were detected between datasets from *S*+ and *S*- mice, at the different intestinal sites and time-points (Table 1). In particular, a significant expansion in populations of bacteria of the Family *Verrucomicrobiaceae* (species *Akkermansia muciniphila*) was detected in both intestinal sites and time-points in *S*+ mice in comparison to *S*- (Table 1). Bacteria belonging to the *Lactobacillaceae* were also expanded in the LI of *S*+ mice at D28, while those belonging the Orders Bacteroidales, Coriobacteriales and Clostridiales were expanded in either the SI i.e.

Bacteroides acidifaciens, Lachnospiraceae and/or LI of *S*+ mice at D50, i.e. *Bacteroides acidifaciens, Parabacteroides, Adlercreutzia, Lachnospiraceae*, *Oscillospira* (Table 1). Conversely, a marked contraction of bacteria of Class Erysipelotrichia was evident in both SI and LI of *S*+ mice at D50 compared to the uninfected counterparts (Table 1).

Table 1 Bacterial taxa displaying significant differences in abundance between microbial profiles obtained from luminal content samples from mice experimentally infected with *Schistosoma mansoni* (*S*+) and uninfected controls (*S*-) based on linear discriminant analysis effect Size (LEfSe). For S+, datasets are separated for site (small and large intestine) and time point (28 and 50 days post-infection) and compared to the corresponding S- datasets. Colours correspond to Linear Discriminant Analysis (LDA) scores of 4.5 or higher (red), 4 to 4.5 (orange), and 3.5 to 4 (yellow)

						Infected (*S+*)				Uninfected (*S-*)			
						Small intestine		Large intestine		Small intestine		Large intestine	
Phylum	**Class**	**Order**	**Family**	**Genus**	**Species**	D28	D50	D28	D50	D28	D50	D28	D50
Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	*Verrucomicrobiaceae*	*Akkermansia*	*Akkermansia muciniphila*								
Bacteroidetes	Bacteroidia	Bacteroidales	*Bacteroidaceae*	*Bacteroides*	*Bacteroides acidifaciens*								
					Bacteroides (unclassified)				*			*	
			S427	*S427* (unclassified)	*S427* (unclassified)								
			Porphyromonadaceae	*Parabacteroides*									
			Rikenellaceae (unclassified)	*Rikenellaceae* (unclassified)	*Rikenellaceae* (unclassified)								
Actinobacteria	Actinobacteria	Coriobacteriales	*Coriobacteriaceae*	*Adlercreutzia*	*Adlercreutzia* (unclassified)								
Firmicutes	Bacilli	Lactobacillales	*Lactobacillaceae*	*Lactobacillus*	*Lactobacillus reuteri*								
					Lactobacillus (unclassified)								
Firmicutes	Clostridia	Clostridiales	*Lachnospiraceae*	*Lachnospiraceae* (unclassified)	*Lachnospiraceae* (unclassified)								
				Dorea	*Dorea* (unclassified)								
			Ruminococcaceae	*Oscillospira*	*Oscillospira* (unclassified)								
				Ruminococcus	*Ruminococcus gnavus*								
			Clostridiales (unclassified)	Clostridiales (unclassified)									
			Clostridiaceae	*Clostridiaceae* (unclassified)	*Clostridiaceae* (unclassified)				*				*
			Peptostreptococcaceae	*Peptostreptococcaceae* (unclassified)	*Peptostreptococcaceae* (unclassified)								
			Peptococcaceae	*Peptococcaceae* (unclassified)	*Peptococcaceae* (unclassified)								
Firmicutes	Erysipelotrichia	Turicibacterales	*Turicibacteraceae*	*Turicibacter*	*Turicibacter* (unclassified)								
Firmicutes	Erysipelotrichia	Erysipelotrichales	*Erysipelotrichaceae*	*Allobaculum*	*Allobaculum* (unclassified)								

5.4 Discussion

Increasing evidence links the immune-modulatory properties of helminth parasites to variations in the composition of the gut microbial communities of their mammalian hosts [12-15]. Attention has frequently focused on intestinal nematodes, likely because these helminths reside in contact with the microbiota of the alimentary tract [36,37]. While the adult stage of the schistosome does not reside within the lumen of the GI tract, infection with *S. mansoni* induces profound effects on gut immunity and homeostasis [26,38,39]; nevertheless, whether these effects are partly responsible for, or caused by, alterations in populations of commensal microbes remains unanswered. In this chapter, I sought to characterise the fluctuations in the composition of the microbiome of the small and large intestine of a rodent model of schistosomiasis, before and after the occurrence of intestinal damage caused by transmission of the parasite eggs, in an effort to identify populations of bacteria with active roles in the complex host-parasite interplay. Taxonomic profiling of microbial communities using PCoA and CCA revealed strong associations between gut microbiota composition and stage of infection, thus providing further evidence of a (direct or immune-mediated) microbiota-modulatory role of parasitic helminths [12-20,22-25,40-49]. In particular, a significant decrease in microbial alpha diversity, which represents mean species diversity within a population of microbes [50], was observed in both the SI and LI of *S*+ mice at D50. Accordingly, a recent study conducted in the Ivory Coast reported a lower level of alpha diversity in the faecal microbiota of *S. mansoni* infected children when compared to uninfected controls, albeit this difference did not reach statistical significance [51]. Decreases in gut microbial alpha diversity have been reported in association with GI helminth infections in several experimental systems, and to reflect a status of gut microbial dysbiosis following the onset of intestinal inflammation caused by infection [14,17,23,24]. In accordance with this hypothesis, the observation of a marked reduction in the gut microbial alpha diversity of *S*+ mice at D50 suggested a direct effect of inflammatory reactions resulting from migrating schistosome eggs. In contrast, microbial beta diversity was significantly increased in samples from *S*+ mice at D50 compared with *S*- mice, indicating that the gut microbiota of individual infected mice responded differently to the disruption of intestinal homeostasis caused by the helminth infection. A significant increase in gut microbial beta diversity had also been detected in the gut microbiota of laboratory rodents experimentally infected with the whipworm, *Trichuris suis* and the tapeworm *Hymenolepis diminuta* [17,44], as well as in human volunteers from a Sri Lankan community infected with roundworms (i.e. hookworms, ascarids and/or whipworms) [41]. These reports, coupled with the findings from this chapter, suggest increased beta diversity could represent a common feature characterising the gut microbiota of mammals

parasitized by helminths, irrespective of the phylogenetic relationships among the helminths and location in the GI tract. However, the status of infection, i.e. acute or chronic, as well as the overall parasite burden are likely to also affect helminth-induced changes in gut microbial diversity.

Significant differences in the relative abundance of a number of gut microbial taxa associated with helminth infection and intestinal inflammation were observed between *S*+ and *S*- mice, at both D28 and D50. In particular, at D28, bacterial populations from the Family *Lactobacillaceae* were significantly increased in the LI of *S*+ mice. The existence of direct and indirect relationships between lactobacilli and helminth parasites has been revealed based on data from experiments carried out in murine models of nematode infections [12,13,15-17,43,44]. In particular, in a key study, Reynolds et al. (2014) reported a marked increase in populations of *Lactobacillaceae* following infection of C57BL/6 mice with the intestinal nematode *Heligmosomoides polygyrus*. In turn, administration of *Lactobacillus* species to mice before helminth infection resulted in significantly increased worm burdens, which led the authors to hypothesize the occurrence of an immune-mediated, mutualistic relationship between selected commensal bacteria and helminths [13]. Thus, higher levels of *Lactobacillaceae* at D28 in *S*+ mice compared to *S*- mice, could represent a microbiota-modulatory effect of *S. mansoni* which, in turn, may facilitate the establishment of chronic infection. Future studies in antibiotic-treated mice recolonized with a lactobacilli-deficient gut microbiota and subjected to infection with *S. mansoni* may assist research in this area. However, it is worth noting that no significant differences in the relative proportion of populations of *Lactobacillaceae* were detected in the gut microbiota of humans naturally or experimentally infected by GI nematodes when compared to uninfected subjects or others treated with anthelmintics [20,22,24,25,40,41], and hence this mechanism might represent a rodent-specific effect of helminth infection on the host gut microbiota. Notably, the relative expansion of populations of *Lactobacillaceae* observed in the LI of *S*+ mice at D28 was no longer apparent at D50, likely suggesting that lactobacilli are negatively affected by the onset of inflammatory responses caused by the transiting schistosome eggs.

Besides lactobacilli, populations of *A. muciniphila* were significantly expanded in the microbiota from both the SI and LI of *S*+, and at both D28 and D50, when compared with the corresponding *S*- counterparts, with the largest difference recorded at the latter time point. *A. muciniphila* is a mucosal-dwelling anaerobic bacterium that degrades host mucins [52]; significantly expanded populations of *A. muciniphila* were also observed in the faecal microbiota of humans with mixed helminth infections [41]. The proliferation of *A. muciniphila*

in the GI of infected mice may be directly linked to an increased production of mucins in response to schistosome infection; this hypothesis is supported by knowledge that transcription of *Muc5ac,* encoding for a gel-forming mucin, is up-regulated in tissues of schistosome-infected mice [53]. Indeed, mammalian mucins play a key role in the complex network of interactions occurring at the helminth-host interface [54-57]. For instance, mice infected with *Echinostoma trivolvis* displayed a dramatically increased production of host mucins, which was crucial to the expulsion of the parasites [58]. In addition to an association with increased host mucin production, *A. muciniphila* adheres to the intestinal epithelium and strengthens enterocyte monolayer integrity *in vitro* [59]. Thus, it is plausible that increased levels of *A. muciniphila* play a potential protective role against the disruption of gut epithelial barrier function caused by granuloma formation.

At D50, in concomitance with schistosome egg migration and granuloma formation, bacterial taxa that have been previously associated with intestinal inflammation, e.g. of the Family *Lachnospiraceae* (i.e. *Dorea*) and genus *Bacteroides* (i.e. *Bacteroides acidifaciens*) were significantly more abundant in the gut microbiota of *S*+ compared to *S*- mice. Expanded populations of *Dorea* have been reported in irritable bowel syndrome patients [60], as well during intestinal inflammation [61], whilst *Bacteroides acidifaciens* was enriched in a mouse model of colitis [62]. Together with the increased levels of beta- and decreased levels of alpha diversity detected in *S*+ mice at D50, these findings lend credit to the hypothesis that a significant disruption of gut microbial homeostasis occurred at this time-point, and that this disruption contributed to the intestinal pathogenesis during *S. mansoni* infection. This hypothesis is supported by findings from Holzscheiter and colleagues [26], who reported a significant reduction of intestinal inflammation and intestinal granuloma development in *S. mansoni* infected mice after antibiotic treatment.

In contrast to the above-mentioned bacteria, those belonging to the Class Erysipelotrichia (Orders Turicibacterales and Erysipelotrichales) were significantly reduced in *S*+ mice at D50 when compared with uninfected mice. To my knowledge, information is not available on the relationships between this group of commensal bacteria and infections by GI helminths or schistosomes; however, in immune-deficient mice, a clear link between Turicibacterales and host immune dysfunction has been described [63,64]. For example, species of Turicibacterales are abundant in the gut microbiota of wild type mice, but completely absent from the gut of mice with defective immune responses (CD45-/- knockout) and mice lacking an adaptive immune system (RAG-/-knockout) [64]. Accordingly, I suggest that the lower abundance of Turicibacterales observed in *S*+ compared to *S*- mice could be due to

disturbances of mucosal immune functions during *S. mansoni* infection. However, it remains unclear whether and/or how a contraction in populations of Erysipelotrichia might impact the outcome of *S. mansoni* infection.

5.5 Conclusions

In conclusion, this chapter shows that infection of mice with *S. mansoni* infection is associated with profound comprehensive shifts in the global composition of the host gut microbiota, and that the changes are indicative of dysbiosis accompanying egg migration across the intestinal wall and granuloma formation. Many of the specific infection-related alterations to the microbiota involved bacterial taxa which are linked to host immune-regulation or inflammation, suggesting that the balance between immune-regulatory and pro-inflammatory bacterial taxa during schistosomiasis play a key role in determining the effective establishment of the infection, and/or severity of the disease resulting from host immune responses to infection.

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CHAPTER 6

General discussion

Understanding the impact that parasitic helminths exert on the host gut microbiota is central to a better grasp of host-parasite interactions and the fundamental molecular mechanisms that govern essential biological processes and, ultimately, could assist in identifying novel treatment approaches against parasitic helminths, as well as helminth-borne treatments of allergic and autoimmune conditions.

The present thesis (i) investigated the consequences of natural multi- or mono-species infections by helminth parasites on the composition of the human gut microbiota (Chapters 2 and 3), (ii) elucidated the longitudinal impact of experimentally controlled mono-species helminth infections on the human gut microbiota (Chapter 4), and, finally, (iii) examined what impact an extra-intestinal (EI) helminth infection has on the host microbiome in a murine model of human schistosomiasis (Chapter 5). The objectives of the present chapter were (a) to summarise the fundamental research achievements, (b) to discuss the findings and implications that can be drawn from this research in relation to host-parasite interactions, and (c) to provide an outlook on opportunities and prospects for future investigations.

Overall, this thesis has described the results from bioinformatic analyses of ~43,305,300 paired-end reads generated via 16S rRNA sequencing of human faeces (Chapters 2-5). The majority of the raw reads (Chapters 2, 3, and 5) have been deposited and made publicly available on public databases (i.e., Mendeley data and the European Nucleotide Archive) and present a substantial resource for future investigations within the field of parasitology, but also for broader investigations of the gut microbiota.

Knowledge of helminths' impact on host microbiota is pivotal to untangle the complex network of host-parasite interactions. However, until recently, such investigations had to rely on culture-based techniques for the profiling of gut microbial changes [1]. This only allowed highly limited and biased investigations into such microbiota shifts. However, recent innovations in next generation sequencing and 'omics' technologies have made quantitative and less or non-biased approaches, such as 16S rRNA sequencing, affordable and thus widely

accessible [2]. These technological advancements have vastly improved our ability to investigate gut microbiota changes and gain insights into microbial ecosystem interactions within the host gut [3-5]. At the commencement of this thesis, little data existed on the impact of parasitic helminth infections on the host microbiome. Indeed, only four studies had been published on the impact of parasitic helminths infections on the human gut microbiota at that time [6-9] and, while they established that such infections could significantly alter the host microbiota, few clear trends could be identified consistently across these studies. This lack of consistency could largely be traced back to the variability in study designs, helminth species, and data analyses techniques, amongst others, between those studies [10]. Although a complete elimination of confounding factors is improbable in investigations of human helminth infections, it is possible to minimise and carefully account for such influences [10]. Hence, in this thesis consistent DNA extraction, sequencing library preparation, and data analysis techniques have been applied to a range of human-helminth infection scenarios, as well as a murine model of human helminth infection. The aim was to apply technical consistency to draw more confident inferences from the data, while also allowing the detection of helminth induced host microbiota changes, which are consistent across different studies. Indeed, though many of the microbiota changes observed across the studies presented in this thesis appeared to be specific to the host-helminth system that was being investigated, some intriguing consistencies emerged. Firstly, low level, long term, and single species subclinical infections were associated with increased gut microbial diversity within the host and seemed to promote a stable and healthy gut microbial composition (Chapters 3 and 4). Notably, these findings are supported by data from other studies that examined the effects of experimental single species (*Necator americanus*) infections in coeliac patients and reported increased gut microbial diversity following helminth administration [7,11]. Contrarily, acute heavy burden infections, associated with pathology, appeared to have the opposite effect, i.e. reducing the overall diversity of the host's gut microbiota and associated with the presence and proliferation of potentially pro-inflammatory bacteria and/or opportunistic pathogens (Chapter 5). Indeed, heavy parasite burden infections are common practice in most studies investigating rodent models of helminth infections and have been frequently reported to lead to significant decreases in gut microbiota diversity [12-14]. Meanwhile, I was unable to detect a significant difference in alpha diversity in subjects naturally infected with different species of STHs (Chapter 1). Other studies of natural and/or mixed infections have been lacking a consistency in trends of host gut microbial diversity, with some reporting increased [8,15] and decreased [9] levels of diversity, or no change at all [16,17]. This is likely linked to the significant differences in the study cohorts and their geographic locations,

as well as the varied types of infections and species of parasite involved (reviewed by [10]). Notably, I also found that murine infections with parasitic (EI) helminths can have a significant impact on the host microbiota, even before eggs traverse through the host gut epithelial layer (Chapter 5). Though the gut microbiota perturbations significantly increased upon egg laying and consequent disruption of the gut epithelial layer, these data demonstrated that host microbiome changes can be indirectly induced by EI helminths, likely due to the parasites' strong immunomodulatory properties (reviewed by [18,19]).

Together, these data suggest that both GI and EI parasitic helminth infections have the potential to detrimentally impact the hosts they infect, besides the direct pathology they induce, but also adds further weight to the idea of a therapeutic and controlled use of helminths in the context of helminth therapy. Indeed, considering the beneficial effects parasitic helminths may have on the host gut microbiota, together with the mounting evidence towards an intrinsic link between autoimmune diseases and the gut microbiome, infection-associated changes on the microbial composition of the host gut might represent an additional route via which helminths could exert a therapeutic effect on patients suffering from such conditions [6,7,11], in addition to the release of ESPs with immunomodulatory properties (reviewed by [20]).

Besides the trends in gut microbial diversity, further patterns emerged when assessing the changes in specific bacterial taxa during helminth infections. In this thesis, I found that study cohorts characterised by increased levels of gut microbial diversity and which appeared to have an overall healthier microbiome than the cohort they were compared to, presented an increased relative abundance of the bacterial family *Leuconostocaceae* (uninfected cohort Chapter 2 and infected cohort Chapter 3) and the genus *Turicibacter* (uninfected cohort Chapter 5 and infected cohort Chapter 3), with the phylum/class Tenericutes/Mollicutes (infected Chapter 4) also proving interesting, due to the plethora of evidence from other studies indicating they could present a key taxon involved in disease progression of autoimmune conditions [21-25]. On the other hand, a decrease in gut microbial alpha diversity appeared to be associated with an increased relative abundance of the bacterial family *Enterobacteriaceae* (uninfected cohort Chapter 3 and infected cohort Chapter 2), the genus *Akkermansia* (infected cohort Chapter 2 and infected cohort Chapter 5), and the family *Lachnospiraceae*, particularly the bacterial genus *Dorea* (uninfected cohort Chapter 4, infected cohort Chapter 5). Regrettably, within this document the relevance of these bacteria could merely be inferred from evidence present in the literature and the investigation of the functional importance of these taxa fell outside the scope of this thesis. However, the bacteria identified here present excellent targets for future investigations aiming at untangling the gut microbial nuances underlying

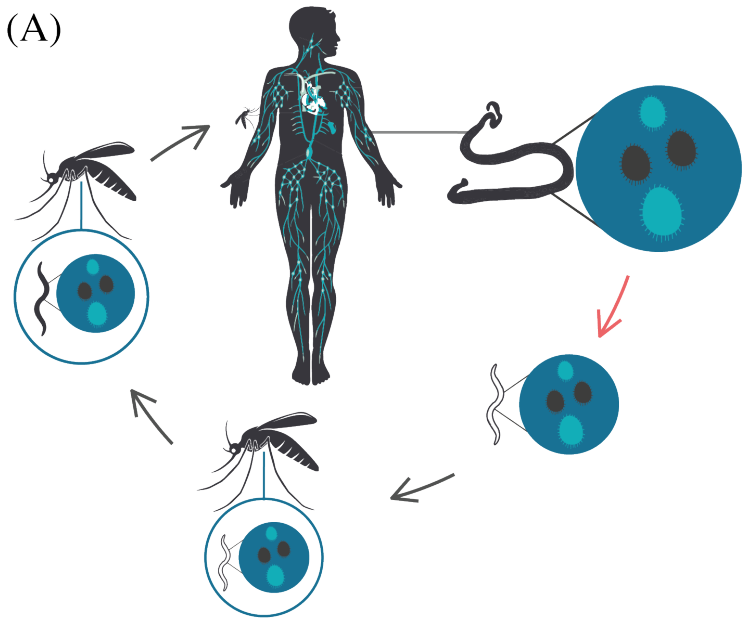
host-helminth interactions. Furthermore, these bacteria are likely not just important in the context of parasitic helminth infections, but also fall within the broader scope of human microbiome health [26] and, thus, could be of interest to studies focusing on specific bacteria involved in gut microbiome stability and gut epithelial health, as well as investigating gut inflammation.

However, despite our increasing understanding of host-parasite-microbiota interactions, we have only begun to unravel the intricate processes involved. In fact, besides the need for targeted mechanistic investigations of the trends in microbiome shifts that have emerged throughout this thesis, the question arises as to what role not only the host, but also the helminth microbiomes play in this complex system. Indeed, although in a few select helminth species the presence of a native microbiome has been established (reviewed by [27]), little is known about the occurrence, structure and function of microbial populations residing within parasite organs and tissues.

Similar to their vertebrate hosts, parasitic nematodes have complete, tubular digestive systems responsible for nutrient uptake, processing and absorption; yet, for most species of socio-economically important parasites, and GI nematodes in particular, little is known about the occurrence, structure and function of populations of resident gut microbes [28]. Nevertheless, critical evidence of the existence of essential symbiotic relationships between parasites and bacteria is provided by filarial nematodes, e.g., *Onchocerca volvulus* (causing river blindness) and *Wuchereria bancrofti* and *Brugia malayi* (causing lymphatic filariasis) (reviewed by [29]), whose propagation and survival are dependent on a genus of bacteria, i.e. *Wolbachia*, which has become the target of intense investigations aimed to develop novel filaricidal compounds [30-34]. This evidence supports the hypothesis, strongly corroborated by recent experimental findings [35,36], that the digestive system as well as other organs and tissues of GI nematodes may also harbour resident microbes with essential roles in parasite physiology and survival. Fully characterizing and understanding the structure and function of helminth microbiomes, and determining the role/s they play in key aspects of parasite biology and host-parasite interactions, could not only have broad implications for future studies of the origin of parasitism itself, but might also lead to the discovery of radically new interventions against these worms.

Investigations of the interactions between parasites and their resident bacteria rely on a thorough understanding of the dynamics of microbiome acquisition. Whilst for GI nematodes knowledge in this area is relatively limited, several investigations have documented the fine

strategy *via* which bacteria of the genus *Wolbachia* are propagated through successive generations of filarial parasites (reviewed by [27]) (cf. Fig. 1). In the filarial nematode *B. malayi*, these bacteria inhabit the lateral chords of both adult male and female worms, and the reproductive system of the latter sex, where they colonise the ovaries, oocytes and early embryos within the uteri [37]. Upon egg fertilisation, populations of *Wolbachia* segregate asymmetrically in the developing embryo, which results in an uneven distribution of these bacteria in the tissues of the resultant microfilariae [37]. In particular, the numbers of vertically transmitted *Wolbachia* remain stable (~70 per embryo [33]) throughout development of the new generation of filarial parasites into infective third-stage larvae (L3s) in the mosquito vector (reviewed by [27]). Upon L3-invasion of a new, susceptible vertebrate host, the number of *Wolbachia* bacteria rapidly increases in the hypodermal cord of developing worms, with a further expansion occurring in the reproductive tissues of sexually mature females (reviewed by [27]). Crucially, embryonic development is entirely dependent on *Wolbachia*, as treatment with tetracycline antibiotics results in a marked reduction of viable microfilariae (reviewed by [37]). To date, the obligate relationship between filarial nematodes and *Wolbachia* represents the only known example of a mutualistic association between parasitic nematodes and bacteria.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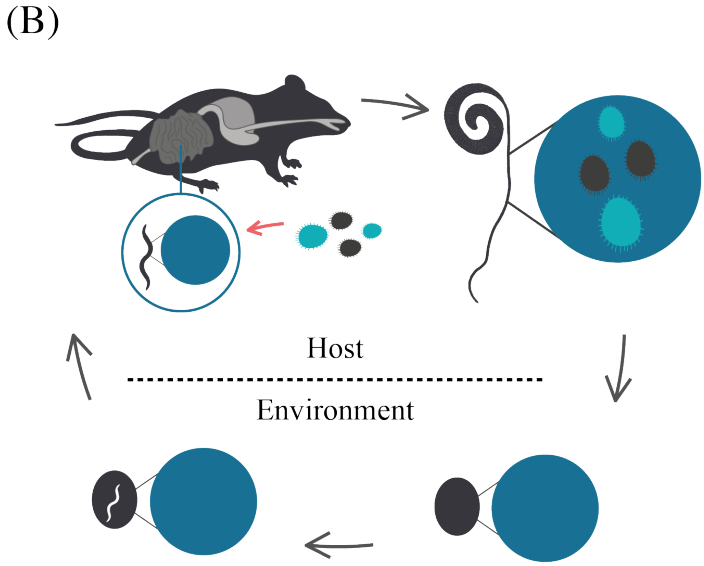
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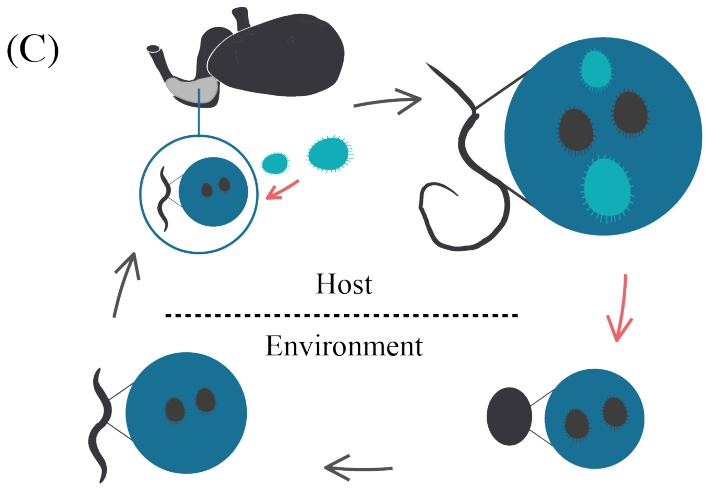
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Fig. 1 Proposed Helminth Microbiome Acquisition Strategies for *Brugia malayi, Trichuris muris*, and *Haemonchus contortus*. (A) *B. malayi* microfilariae acquire *Wolbachia*microorganisms via the female germ line, and populations of resident microbes expand throughout larval development through to infective third-stage larvae in the mosquito intermediate host. In adult male and female *B. malayi*, the bacteria localise to the lateral chords of both sexes and the female reproductive system, where they colonise the ovaries, oocytes, and early embryos within the uteri. (B) Unembryonated *T. muris* eggs are passed through murine faeces, and embryos develop inside the eggs. Upon ingestion by a susceptible host, the eggs hatch in the small intestine and release larvae that acquire selected populations of bacteria from the gut of their rodent hosts. Thereafter, the larvae mature and establish themselves as adult males and females in the colon. (C) Female *H. contortus* transfer selected populations of bacteria to the offspring via the germline. Eggs shed in the environment with the faeces of the ruminant host hatch and release first-stage larvae. Upon ingestion of the latter by a new ruminant host, the developing worms acquire further populations of bacteria from the rumen of the latter. Red arrows indicate helminth microbiome acquisition events. Empty blue circles indicate current lack of clear evidence of microbiome transfer [38].

Members of another group of helminth parasites, the digenean trematodes, are known to harbour populations of *Neorickettsia* endosymbionts which share numerous genetic similarities with *Wolbachia* (reviewed by [39]). *Neorickettsia* inhabit a range of environments suitable for the development of the infective stages of digenean parasites and their intermediate hosts (e.g., aquatic molluscs), thus lending credit to the hypothesis that a proportion of these bacteria are horizontally transmitted (reviewed by [39,40]). Nevertheless, in *Plagiorchis elegans*, a common GI helminth of a range of fishes, birds and mammals (including humans) [41], *Neorickettsia* is predominantly transmitted vertically across generations of parasites [42]. However, unlike *Wolbachia* in filarial nematodes, transmission of *Neorickettsia* in *P. elegans* occurs also through the asexual stages of this parasite [43]. Furthermore, since transmission rates of *Neorickettsia* from adult *P. elegans* to the offspring vary from 11% to 91% [43], it has been suggested that the life cycle of this flatworm is not dependent on their neorickettsial endosymbionts, but rather that *Neorickettsia* utilises *P. elegans* as a vehicle for transmission to vertebrate hosts [44].

For GI nematodes, experimental evidence of microbiome acquisition strategies is available for two species, namely *Haemonchus contortus*, an abomasal roundworm of small

ruminants [35] and *Trichuris muris*, a large intestinal whipworm of rodents [36] (cf. Fig. 1). For the former, a recent study [35] localised selected genera of bacteria (i.e. *Weissella* and *Leuconostoc*) to the gut of adult worms and to the uterus of sexually mature females by fluorescence *in situ* hybridization (FISH) and transmission electron microscopy; using DNA fingerprinting, the same genera could be identified in eggs laid by these females and, following larval culture, their L3 offspring [35]. Notably, these microorganisms could not be identified in the faecal matter on which larval culture was performed, thus providing evidence of maternal transmission of these bacteria [35]. Other bacterial genera (i.e. *Lactococcus* and *Streptococcus*) could be identified in the distal uterus of sexually mature females of *H. contortus* [35]. Nevertheless, these bacteria were not detected in newly deposited eggs and developed L3s. Since these bacteria occur in the rumen of the host [45], the authors hypothesized that female worms acquired them by ingesting ruminal fluid [35]. Whilst the roles that species of *Lactococcus* and *Streptococcus* might play in the fundamental biology of *H. contortus* is presently unknown, these data suggest that this parasite might employ a 'hybrid' microbiome acquisition strategy, with some 'core' endosymbionts (i.e. *Weissella* and *Leuconostoc*), which may play essential roles in parasite fitness and survival, being vertically transmitted, and others (i.e. *Lactococcus* and *Streptococcus*) being acquired from the host to underpin a certain level of microbiome plasticity and capacity for environmental adaptation.

A clear strategy of microbiome acquisition from the mammalian host has been recently demonstrated for *T. muris* [36] (cf. Fig. 1). The essential role that the host microbiome plays in the development and propagation of this parasite had already been demonstrated in a seminal study [46], which showed that parasite egg hatching in the large intestine of the mouse was dependent on the microbial flora within the host gut. Recently, a study by the same research group [36] demonstrated that, following egg hatching, *T. muris* acquires populations of bacteria that, together, form a 'core' nematode microbiome, which is markedly distinct from the microbiome inhabiting the environment in which the worms reside [36]. Although the *Trichuris* microbiome described predominantly comprised Firmicutes and Bacteroidetes, similar to the host microbiome, it was also rich in Proteobacteria. Proteobacteria constituted 9% of the entire *T. muris* microbiome – a 31-fold and 13-fold increase in relative proportions of this bacterial group compared with the microbiome of uninfected mice and *T. muris*-infected mice, respectively [36]. This process of selective microbiome acquisition was demonstrated to be independent from the initial host microbiome composition, and the administration of broad-spectrum antibiotics to adult *T. muris ex vivo* resulted in a marked decrease in parasite fitness

and survival rates, thus providing cogent evidence for an essential role of the host-acquired microbiome for the successful completion of the whipworm life cycle. Whether *T. muris* acquires its own microbiome passively, or actively selects populations of bacteria with functional properties which are able to facilitate its survival in the vertebrate host, remains to be established (cf. [36]). Nevertheless, the observation that mono-colonisation of germ-free mice with a single species of bacterium, i.e. *Bacteroides thetaiotaomicron*, resulted in successful egg hatching and establishment of chronic *T. muris* infection [36] provides a unique opportunity to design targeted experiments that can shed light on the precise mechanisms of acquisition of the *T. muris* microbiome. In turn, this knowledge will form the necessary basis to answer fundamental questions regarding helminth microbiome structure and function.

Studies of the structure and function of helminth microbiomes are in their infancy. Nevertheless, over the past decades, evidence has emerged about the functional association between the free-living nematode *Caenorhabditis elegans* and the bacteria inhabiting it [47,48]. Indeed, *C. elegans* is known to host a species-rich bacterial community, dominated by Proteobacteria, such as *Enterobacteriaceae* and members of the genera *Pseudomonas*, *Stenotrophomonas*, *Ochrobactrum*, and *Sphingomonas* [47,48]. Crucially, the relative proportions of bacterial populations forming the *C. elegans* microbiome vary according to the developmental stage of this nematode [48], thus suggesting that worm development relies on a range of bacterial functions that differ over time. In support of this hypothesis, worms experimentally colonised with a subset of bacterial isolates representing the *C. elegans* 'core' microbiome displayed increased fitness and survival rates, and were maintained under stressful conditions of temperature and osmolarity, compared with worms colonised solely by *Escherichia coli* [48]. In addition, compared with *E. coli*-colonised *C. elegans*, worms fed with the soil bacterium *Comamonas* displayed accelerated development, which was attributed to the ability of this bacterial group to up-regulate the expression of genes associated with the nematode's moulting program [49]. The *C. elegans* microbiome has also been demonstrated to play important roles in worm defence against pathogens; indeed, *Pseudomonas* isolates detected amongst the worm resident populations of bacteria produce anti-mycotic compounds that prevent colonisation by fungal agents [48].

Evidence from investigations of *C. elegans*, employed as a model for nematode-microbiome interactions, points to a likely functional role of the microbiomes of parasitic helminths for worm physiology, development and survival. However, the parasite microbiome itself may benefit from the protected and nutrient-rich environment that the worm host offers

[50,51]. For instance, the Gammaproteobacteria *Photorhabdus* and *Xenorhabdus*, that inhabit the gut of the entomopathogenic nematodes *Heterorhabditis* and *Steinernema*, are released upon infection of the insect host by the infective juveniles; following their release, these bacteria actively replicate and kill the insect host, while converting the insect carcass into a source of nutrients to support nematode growth and development [52,53].

For parasitic nematodes of medical and veterinary importance, the mutualistic association between filarial nematodes and *Wolbachia* offers a key example of the fundamental functions that the helminth microbiome exerts in the biology of its worm host, and *vice versa*. Indeed, besides its known role in the development and survival of filarial embryos (reviewed by [27]), *Wolbachia* is essential for worm nutrition and metabolism. The bacterium synthesizes haem, riboflavin (vitamin B_2), and flavin adenine dinucleotide, which the parasite host is unable to synthesize and that have been inferred to play an important role in filarial reproduction and development, as well as nucleotides, which are required during oogenesis and embryogenesis [34,54,55]. In addition, members of the genus *Wolbachia* participate in pathways aimed at preventing apoptosis of filarial reproductive, embryonic and somatic cells [33,56], likely by the direct targeting of the apoptotic signalling cascade [57-59]. Finally, in the filarial parasite of cattle *Onchocerca ochengi*, *Wolbachia* has been demonstrated to play a key role in host immune evasion, specifically by attracting host neutrophils and, thus, averting a potentially lethal effector response by degranulating eosinophils [60].

Over the years, the fundamental roles that *Wolbachia* play in pathways linked to reproduction, metabolism and immune defence of filarial nematodes have been the subject of intense scrutiny, focusing on developing novel chemotherapeutics to disrupt this mutualistic relationship; some have been successful [27,31,33,61]. For instance, the administration of 4-week courses of doxycycline (belonging to the tetracycline family of antibiotics) and rifampicin have been deemed effective in reducing the transmission of *O. volvulus* microfilariae to mosquito intermediate hosts and filarial embryogenesis, respectively (reviewed by [29]). Nevertheless, the length of drug administration required to achieve significant effects, along with the severe adverse reactions that tetracyclines can cause in children and pregnant women (e.g. permanent dental staining, teratogenic effects and potentially fatal hepatotoxicity [62]), limit the use of these antibiotics in mass drug administration (MDA) programs in areas where filariases are endemic. Nevertheless, these findings raise the question as to whether a deep exploration of the microbiomes of other helminth parasites of major socio-economic significance could hold

promise for the identification of novel targets for the development of antibiotic-independent control strategies against the diseases caused by these worms.

Globally, more than two billion people are at risk of infection by GI nematodes, mainly the hookworms *Ancylostoma duodenale* and *Necator americanus*, the whipworm *T. trichiura*, the roundworm *Ascaris lumbricoides*, and the threadworm, *Strongyloides stercoralis*. Collectively, these nematodes are responsible for more than 5.5 million disability-adjusted life years (DALYs) (reviewed by [63]). Moreover, GI nematodes inflict significant production losses in livestock due to the extensive morbidity and mortality associated with a range of diseases that they cause (reviewed by [64]). Complete reliance on anthelmintics for the control of these parasites (*via* MDA or targeted strategic worming programmes in humans and livestock) bears substantial risks, linked to the global threat of emerging anthelmintic resistance, as already observed in several GI nematodes of veterinary importance (reviewed by [65,66]). Yet, the discovery of alternative strategies for parasite control should be built on a thorough understanding of the fundamental biology of these pathogens, and of key mechanisms of interactions with their vertebrate hosts. A deeper knowledge of the structure and function of the microbiomes of parasitic helminths, and of mechanisms of microbiome acquisition and transmission, could lead to unprecedented discoveries in parasite physiology, pathology and reproduction, and thus, to the development of completely novel control tools. Nevertheless, for such discoveries to be harnessed, fundamental information needs to be acquired. I propose that, in the first instance, the microbiomes of representative species of GI nematodes of considerable medical and veterinary significance (Fig. 2) could be qualitatively characterized using high-throughput sequencing of the bacterial 16S rRNA gene. The selection of specimens of a range of parasite species from different hosts and geographical locations would assist the determination of species-specific 'core' parasite microbiomes. Following the establishment of reference 16S rRNA databases for each key parasite species, shotgun metagenomic sequencing of their microbiomes would provide important information on the relative abundance of each 'core' microbial species, and clues about their functional potential. The latter, coupled to investigations of the proteomes and metabolomes of the microbial communities inhabiting these parasites, could lead to a better understanding of the possible role/s that the microbiomes of parasitic helminths play in the biology and physiology of individual worms.

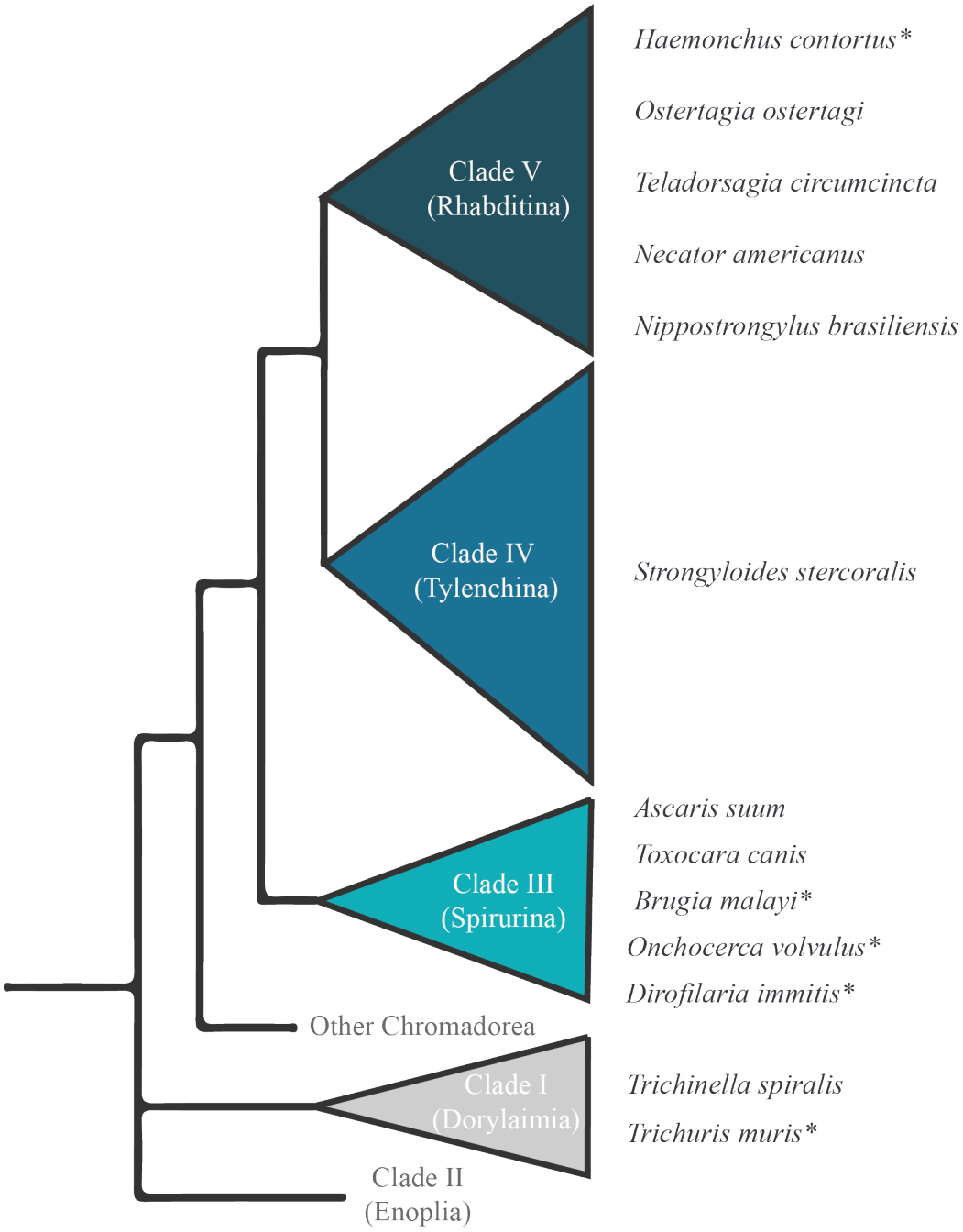

Fig. 2 Key Helminth Taxa to Be Investigated as Representatives of Their Taxonomic Clades. Asterisks (*) indicate taxa for which published data on parasite microbiome structure and/or function(s) are available [38].

Key information on the modes of transmission of helminth microbiomes could be acquired *via* experimental infections of vertebrate hosts with selected GI nematodes, followed by qualitative and quantitative comparative analyses of the host microbiomes and key parasite developmental stages. Furthermore, for selected GI nematodes (e.g. *Nippostrongylus brasiliensis*), experimental infections of germ-free or antibiotic-treated mice re-colonised with fluorescently labelled bacteria might provide clues on host-parasite microbiome transfer using *in vivo* imaging (cf. [67]). Similar techniques could be used to localise species or groups of bacteria in parasite organs and tissues, thus providing additional clues on the functions of such microorganisms in worm biology. Together, this information would form a basis for experimentation, aimed at interfering with such functions that may potentially lead to the discovery of entirely novel, antibiotic-independent strategies for parasite control (Fig. 3), for example, *via* cutting-edge microbiome editing techniques including CRISPR/Cas9, engineered probiotics, and/or bactericidal bacteriophages (reviewed by [68]).

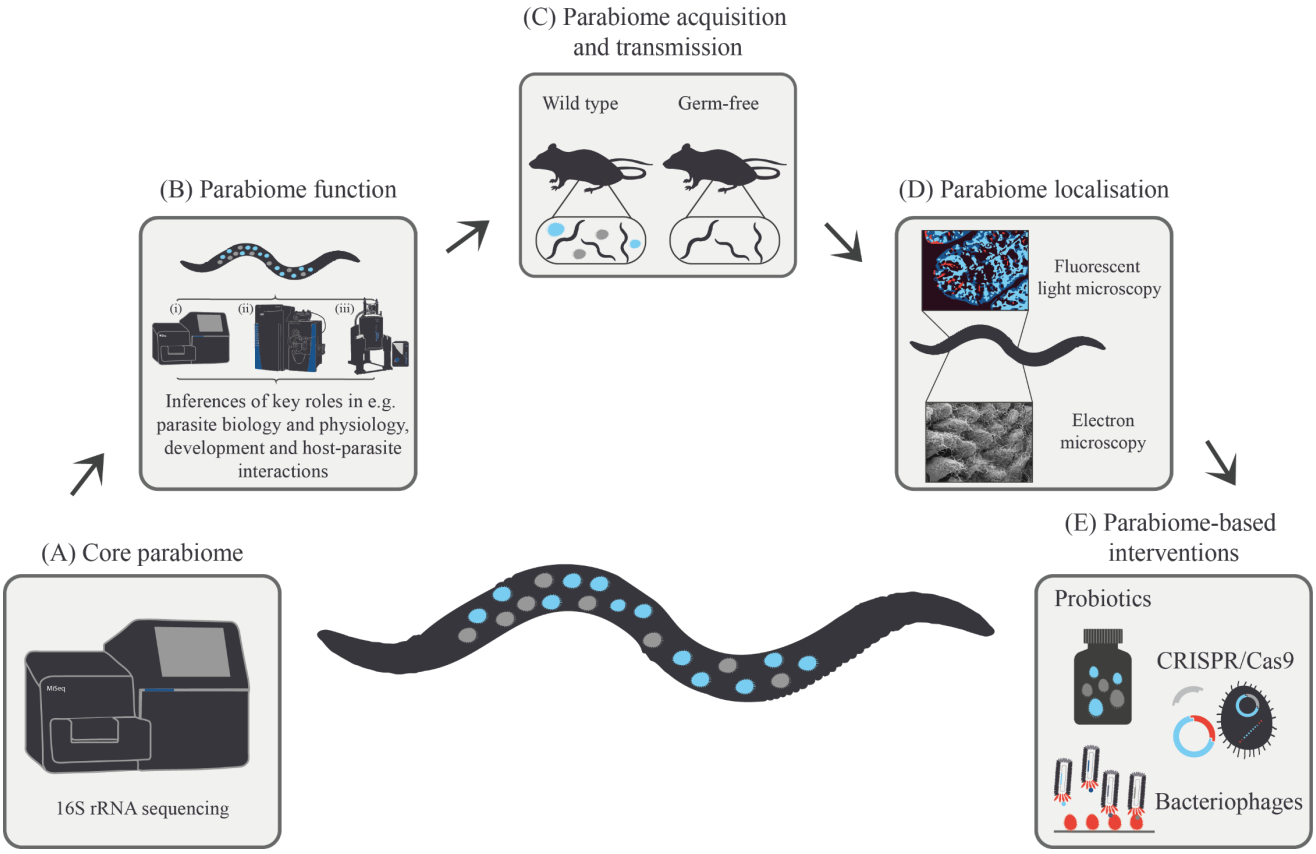

Fig. 3 Plan of Action for Efficient Investigations and Consequent Exploitation of Helminth Microbiomes [38]. (A) Bacterial 16S rRNA gene sequencing of helminth microbiomes to establish core resident microbial species. (B) Shotgun metagenomic sequencing (i), proteomic (ii), and metabolomic

(iii) analyses to establish microbial functions. (C) Experiments in wild-type and germ-free mice to determine mechanisms of acquisition and transmission of helminth microbiomes. (D) Localization of core microbial populations through fluorescent light microscopy and electron microscopy. (E) Identification and targeting of key microbial species to decrease parasite fitness (based on the previously assessed parameters) via microbiome-editing techniques [38].

Major advances have been made in describing and understanding host-parasite-microbiota interactions. Yet, as our knowledge is expanding, an ever increasing number of new questions arise, with a key one concerning the role of the parasite's own microbiome in such interactions. In spite of substantial evidence that points towards crucial role(s) of microbial species inhabiting parasitic helminths in the fundamental biology of these pathogens and host-parasite interactions, e.g. in filarial nematodes and, more recently, whipworms, current knowledge of the microbiomes of key parasitic helminths of major socio-economic significance is scarce and fragmented. Nonetheless, the relentless progress in microbiome investigation and editing technologies, and novel high-throughput bioinformatics pipelines, provides us with unprecedented opportunities to thoroughly characterize the structures and functions of such microbial populations. At the core lie questions surrounding modes of helminth microbiome acquisition and propagation to successive generations of parasites, the localisation of endosymbiont microorganisms in the organs and tissues of parasites, the functions that helminth microbiomes (including bacteria, viruses and fungi) play in parasite biology and physiology, and the effects that disrupting parasite-microbiome interactions may exert on parasite propagation and survival (see Outstanding Questions). In turn, the new knowledge can be expected to provide us with a plethora of opportunities to exploit parasite-microbiome associations to our advantage, for example, by applying cutting-edge microbiome-editing techniques as novel intervention strategies against parasitic nematodes and the diseases that they cause. Understanding both the impact of parasitic helminths on the host gut microbiota, as well as the role of the worms own microbiome will likely be pivotal for the future of host-parasite investigations and prove invaluable in the pursuit of alleviating the great burden that these helminths exert on a global scale.

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List of appendices

Chapter 2

Supplementary Table S1 Metadata associated with helminth-positive (*H+*), helminth-negative (*H-*) and helminth-negative but regularly treated (*Ht*) subjects enrolled in the study.

ID	Village	Gender	Age (years)	Education	Occupation	Infected	Parasite
H+ 01	Hanthana	Male	1 to 18	NP	NP	Yes	*Ascaris* sp.
H+ 02	Hanthana	Male	1 to 18	NP	NP	Yes	*Ascaris* sp.
H+ 03	Hanthana	Female	1 to 18	NP	NP	Yes	*Ascaris* sp.
H+ 04	Hanthana	Female	1 to 18	NP	NP	Yes	*Ascaris* sp.
H+ 05	Hanthana	Female	1 to 18	NP	NP	Yes	*Ascaris* sp.
H+ 06	Hanthana	Male	1 to 18	NP	NP	Yes	*Ascaris* sp.
H+ 07	Kandakuliya	Female	1 to 18	Grade 1 -5	NP	Yes	*Trichuris* sp.
H+ 08	Mawanella	Male	1 to 18	Grade 1 -5	NP	Yes	Nematode larvae
H+ 09	Mawanella	Female	51 and above	Grade 10	NP	Yes	Nematode larvae
H+ 10	Rangala	Male	19 to 50	Grade 1 -5	Skilled worker	Yes	Hookworms
H+ 11	Rangala	Female	1 to 18	Grade 1 -5	NP	Yes	Hookworms
H- 01	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 02	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 03	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 04	Hanthana	Female	1 to 18	NP	NP	No	NA
H- 05	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 06	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 07	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 08	Hanthana	Female	1 to 18	NP	NP	No	NA
H- 09	Hanthana	NP	NP	NP	NP	No	NA
H- 10	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 11	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 12	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 13	Hanthana	Female	1 to 18	NP	NP	No	NA
H- 14	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 15	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 16	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 17	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 18	Hanthana	Female	1 to 18	NP	NP	No	NA
H- 19	Hanthana	Female	1 to 18	NP	NP	No	NA
H- 20	Hanthana	Female	1 to 18	NP	NP	No	NA
H- 21	Hanthana	Male	1 to 18	NP	NP	No	NA

H- 22	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 23	Hanthana	Female	1 to 18	NP	NP	No	NA
H- 24	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 25	Hanthana	Male	1 to 18	NP	NP	No	NA
H- 26	Hanthana	NP	NP	NP	NP	No	NA
H- 27	Hanthana	NP	NP	NP	NP	No	NA
Ht 01	Akurana	Female	1 to 18	NP	NP	No	NA
Ht 02	Akurana	Female	51 and above	NP	NP	No	NA
Ht 03	Kandakuliya	Female	1 to 18	Grade 1 -5	NP	No	NA
Ht 04	Kandakuliya	Male	19 to 50	Grade 1 -5	Elementary occupation	No	NA
Ht 05	Kandakuliya	Female	1 to 18	Grade 1 -5	NP	No	NA
Ht 06	Kandakuliya	Female	1 to 18	Grade 1 -5	NP	No	NA
Ht 07	Mawanella	Female	19 to 50	Grade 12	NP	No	NA
Ht 08	Mawanella	Female	1 to 18	Grade 1 -5	NP	No	NA
Ht 09	Mawanella	Male	1 to 18	Grade 1 -5	NP	No	NA
Ht 10	Valalai	Female	19 to 50	Grade 1 -5	Housewife/unemployed	No	NA
Ht 11	Valalai	Male	1 to 18	NP	NP	No	NA
Ht 12	Valalai	Male	19 to 50	Grade 1 -5	Elementary occupation	No	NA
Ht 13	Valalai	Female	1 to 18	Grade 1 -5	NP	No	NA
Ht 14	Valalai	Male	51 and above	Grade 1 -5	Elementary occupation	No	NA
Ht 15	Valalai	Female	51 and above	Grade 1 -5	Housewife/unemployed	No	NA
Ht 16	Valalai	Female	51 and above	Grade 1 -5	Housewife/unemployed	No	NA
Ht 17	Valalai	Male	51 and above	Grade 1 -5	Housewife/unemployed	No	NA
Ht 18	Valalai	Male	51 and above	Grade 1 -5	Elementary occupation	No	NA
Ht 19	Valalai	Female	19 to 50	Grade 1 -5	Housewife/unemployed	No	NA
Ht 20	Kandakuliya	Female	51 and above	Grade 1 -5	Housewife/unemployed	No	NA
Ht 21	Kandakuliya	Male	51 and above	Grade 1 -5	Elementary occupation	No	NA

Ht 22	Kandakuliya	Female	19 to 50	Grade 1 -5	Elementary occupation	No	NA
Ht 23	Kandakuliya	Female	19 to 50	Grade 1 -5	Housewife/unemployed	No	NA
Ht 24	Kandakuliya	Male	1 to 18	NP	NP	No	NA
Ht 25	Kandakuliya	Male	1 to 18	NP	NP	No	NA
Ht 26	Kandakuliya	Male	1 to 18	Grade 1 -5	NP	No	NA
Ht 27	Kandakuliya	Male	19 to 50	Grade 1 -5	Elementary occupation	No	NA
Ht 28	Kandakuliya	Female	1 to 18	Grade 1 -5	NP	No	NA
Ht 29	Kandakuliya	Male	1 to 18	NP	NP	No	NA
Ht 30	Mawanella	Male	19 to 50	Grade 10	NP	No	NA
Ht 31	Akurana	Male	51 and above	NP	NP	No	NA
Ht 32	Akurana	Male	1 to 18	NP	NP	No	NA
Ht 33	Akurana	Female	1 to 18	NP	NP	No	NA
Ht 34	Akurana	Male	51 and above	NP	NP	No	NA
Ht 35	Akurana	Female	19 to 50	NP	NP	No	NA
Ht 36	Akurana	Male	19 to 50	NP	NP	No	NA
Ht 37	Akurana	Female	1 to 18	NP	NP	No	NA
Ht 38	Akurana	Male	51 and above	NP	NP	No	NA

SURVEY ON WORM INFECTIONS AND TREATMENT

District:…… Name of Village: ……..

Interviewer: …… Date: ……

1. Background Information of the head of household

I) Age …….. II) Gender M ☐ **F** ☐ III) Education …… ……..

Address: ……..

	Sex	Age	Height	Weight	Education	Occupation	Remarks
Spouse							
Child – 1							
Child – 2							
Child – 3							
Child – 4							
Child – 5							
Other							

Monthly household income: <10,000 ☐ 10,000-40,000 ☐ 40,000< ☐

Sanitary conditions:

a) Type of toilet Pit ☐ Water seal ☐ Other (specify) ……..

b) Drinking water source: Well ☐ Tap water ☐ Other ……..

c) Drinking water Boiled only ☐ filtered only ☐ not filtered or boiled ☐

d) Distance between the toilet and the well or other water source (except tap water) ……..

e) Use of detergents for cleaning: Regularly ☐ Occasionally ☐ Never ☐

f) Wear slippers when using the toilet: Yes ☐ No ☐ Sometimes ☐

g) Wash hands (with soap) after using the toilet: Yes ☐ No ☐ Sometimes ☐

Domestic Animals (Livestock/ Pets)

Type	Number	Remarks
1		
2		
3		
4		

2. Health Care Facilities

i) Does the PHI/ Health Officer visit your house? Yes ☐ No ☐

ii) If yes, how often? ……..

iii) Are there any health care activities organized by the government/ private organizations?
Yes ☐ No ☐

iv) If yes, do you take part in such activities? Yes ☐ No ☐

v) Distance to the closest government hospital? ……..

vi) Frequently visited health facility Government ☐ Private ☐

3. Specific Questions

a) Do you know about any GI worm diseases? Yes ☐ No ☐

b) What are the common diseases of children in your area? 1) ……

2) …… 3) ……..

c) Did any of your family members suffer from a worm disease during the past three months?
Yes ☐ No ☐

d) Does your family take worm treatment? Yes ☐ No ☐

e) If yes, how often? Every 6 month ☐ Yearly ☐ whenever needed ☐ Other …….

g) Are the pets/ livestock treated for worm diseases? Yes No

h) If yes, how often? Every 6 month ☐ Yearly ☐ whenever needed ☐ Other …….

4. Observation Checklist

i) Type of house: Mud ☐ Brick ☐ Other ☐

ii) Living /hygienic conditions: Good ☐ Average ☐ Poor ☐

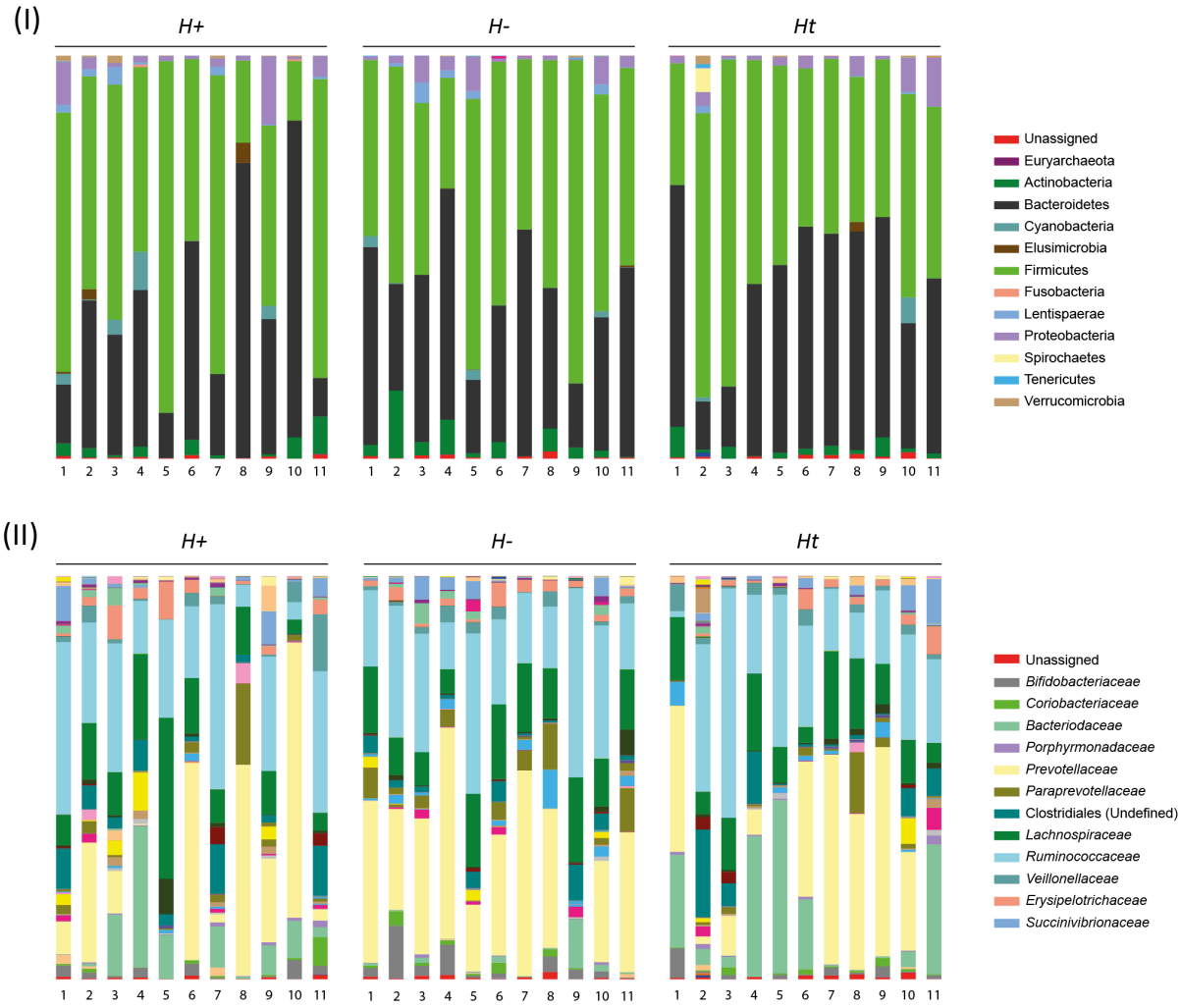
iii) Care provided for pets/ Livestock? Good ☐ Average ☐ Poor ☐

iv) Overall cleanliness: Good ☐ Average ☐ Poor ☐

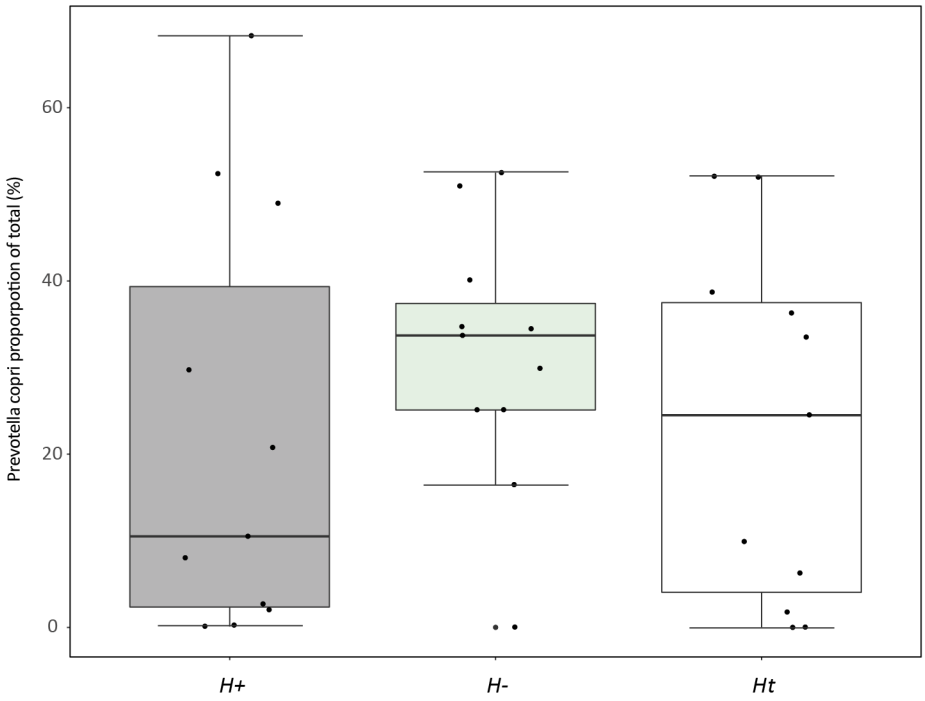
5. Other Comments ……..

……..

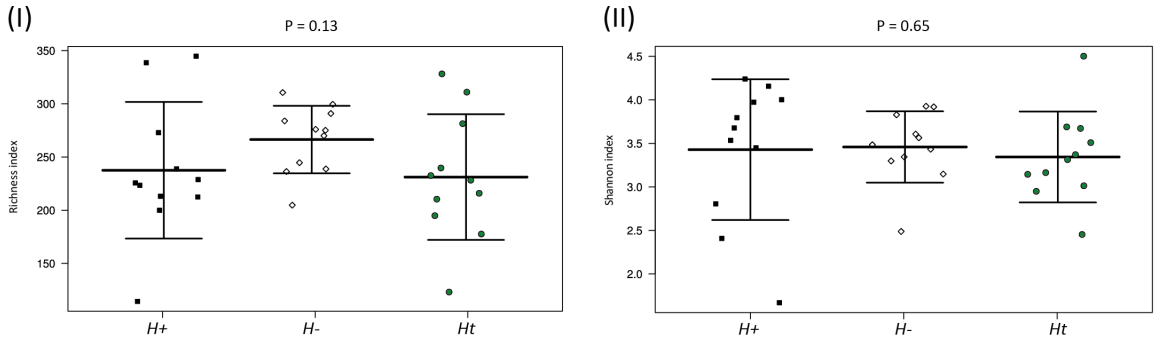
Supplementary Fig. S1 Questionnaire used for the collection of metadata from 100 human volunteers screened for the presence of patent infections by gastrointestinal nematodes.



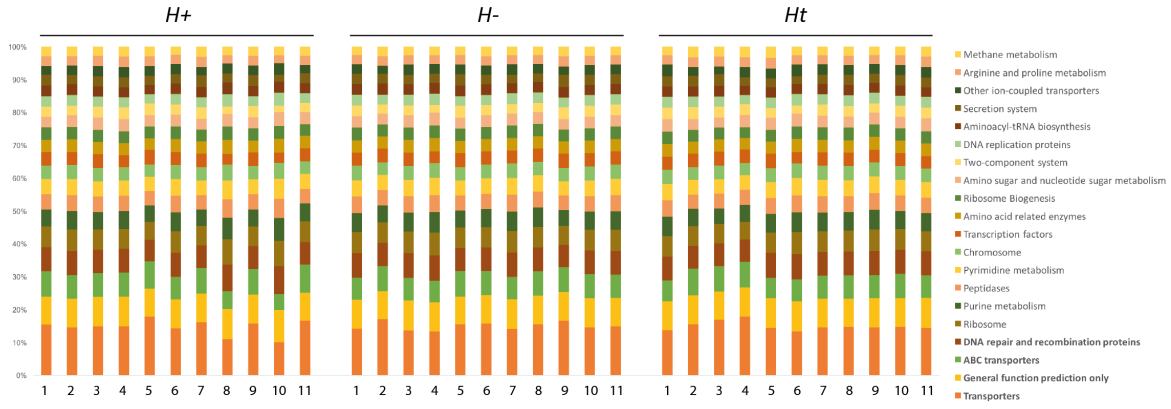
Supplementary Fig. S2 Composition of the faecal microbiota of helminth-positive (*H*+), helminth-negative (*H*-) and helminth-negative but regularly treated (*Ht*) subjects at the Phylum (I) and Family (II) level.



Supplementary Fig. S3 *Prevotella copri* abundance in helmith-positive (*H*+), helmith-negative (*H*-) and helmith-negative but regularly treated (*Ht*) subjects.



Supplementary Fig. S4 Differences in overall taxonomic species richness (I) and diversity (II) between the faecal microbiota of helmith-positive (*H*+), helmith-negative (*H*-) and helmith-negative but regularly treated (*Ht*) subjects.



Supplementary Fig. S5 Relative abundances of KEGG pathways encoded in the gut microbiota of helminth-positive (*H*+), helminth-negative (*H*-) and helminth-negative but regularly treated (*Ht*) subjects, determined by inferred metagenomic analyses with PICRUSt.

Chapter 3

Supplementary Table S1 Available metadata associated with faecal samples from *Strongyloides stercoralis*-infected and uninfected subjects (*S*+ and *S*-, respectively), as well as from the subset of *S*+ subjects that had received anthelmintic treatment, both prior to ($S+_{pre\text{-}treatment}$; in red) and 6 months post-ivermectin administration ($S+_{post\text{-}treatment}$).

Subject	Infection status	Treatment status	City	Region	Age	Sex
S*+*pre-treatment*01	Positive	Untreated	Brescia	Lombardia	82	Male
S*+*pre-treatment*02	Positive	Untreated	Verona	Veneto	81	Male
S*+*pre-treatment*03	Positive	Untreated	Novara	Piemonte	67	Male
***S*+*pre-treatment*04**	Positive	Untreated	Verona	Veneto	83	Male
S*+*pre-treatment*05	Positive	Untreated	Bologna	Emilia	60	Female
S*+*pre-treatment*06	Positive	Untreated	Brescia	Lombardia	84	Female
***S*+*pre-treatment*07**	Positive	Untreated	Verona	Veneto	75	Male
***S*+*pre-treatment*08**	Positive	Untreated	Verona	Veneto	81	Male
***S*+*pre-treatment*09**	Positive	Untreated	Verona	Veneto	69	Male
***S*+*pre-treatment*10**	Positive	Untreated	Verona	Veneto	84	Male
S*+*pre-treatment*11	Positive	Untreated	Verona	Veneto	80	Male
S*+*pre-treatment*12	Positive	Untreated	Brescia	Lombardia	80	Female
S*+*pre-treatment*13	Positive	Untreated	Padova	Veneto	60	Female
S+14*	Positive	Untreated	Verona	Veneto	86	Male
S+15*	Positive	Untreated	Verona	Veneto	69	Male
S+16*	Positive	Untreated	Verona	Veneto	81	Male
S+17*	Positive	Untreated	Verona	Veneto	59	Female
S+18*	Positive	Untreated	Verona	Veneto	49	Female
S+19	Positive	Untreated	Verona	Veneto	86	Female
S+20*	Positive	Untreated	Verona	Veneto	63	Female
S-01*	Negative	Untreated	Verona	Veneto	78	Male
S-02*	Negative	Untreated	Verona	Veneto	53	Female
S-03*	Negative	Untreated	Verona	Veneto	58	Female
S-04*	Negative	Untreated	Verona	Veneto	86	Female
S-05*	Negative	Untreated	Verona	Veneto	58	Female
S-06*	Negative	Untreated	Verona	Veneto	68	Male
S-07	Negative	Untreated	Verona	Veneto	74	Male
S-08*	Negative	Untreated	Verona	Veneto	53	Female
S-09*	Negative	Untreated	Verona	Veneto	58	Female

S-10*	Negative	Untreated	Verona	Veneto	73	Male
S-11*	Negative	Untreated	Verona	Veneto	58	Male
*S+post-treatment*01*	Negative	Treated	Brescia	Lombardia	82	Male
*S+post-treatment*02	Negative	Treated	Verona	Veneto	81	Male
*S+post-treatment*03*	Negative	Treated	Novara	Piemonte	68	Male
*S+post-treatment*04	Negative	Treated	Verona	Veneto	83	Male
*S+post-treatment*05*	Negative	Treated	Bologna	Emilia	60	Female
*S+post-treatment*06*	Negative	Treated	Brescia	Lombardia	84	Female
*S+post-treatment*07	Negative	Treated	Verona	Veneto	75	Male
*S+post-treatment*08	Negative	Treated	Verona	Veneto	81	Male
*S+post-treatment*09	Negative	Treated	Verona	Veneto	69	Male
*S+post-treatment*10	Negative	Treated	Verona	Veneto	84	Male
*S+post-treatment*11*	Negative	Treated	Verona	Veneto	80	Male
*S+post-treatment*12*	Negative	Treated	Brescia	Lombardia	80	Female
*S+post-treatment*13*	Negative	Treated	Verona	Veneto	60	Female

S+	Samples from individuals infected with *Strongyloides stercoralis*
S+pre-treatment	Samples from individuals infected with *Strongyloides stercoralis* for which samples post-anthelmitic treatment were also made available (i.e. S+*pre-treatment*)
S+post-treatment	Samples from individuals infected with *Strongyloides stercoralis* 6 months post-anthelmintic treatment
S-	Samples from uninfected individuals within the same geographical region and age group
*	Samples included in metabolomic analyses *via* nuclear magnetic resonance and gas chromatography-mass spectrometry

Age	Mean	Minimum	Maximum	Standard deviation
S+	74.0	49	86	10.8
S+pre-treatment	75.8	60	84	8.5
S+post-treatment	75.9	60	84	8.4
S-	65.2	53	86	10.6
Overall	72.3	49	86	10.9

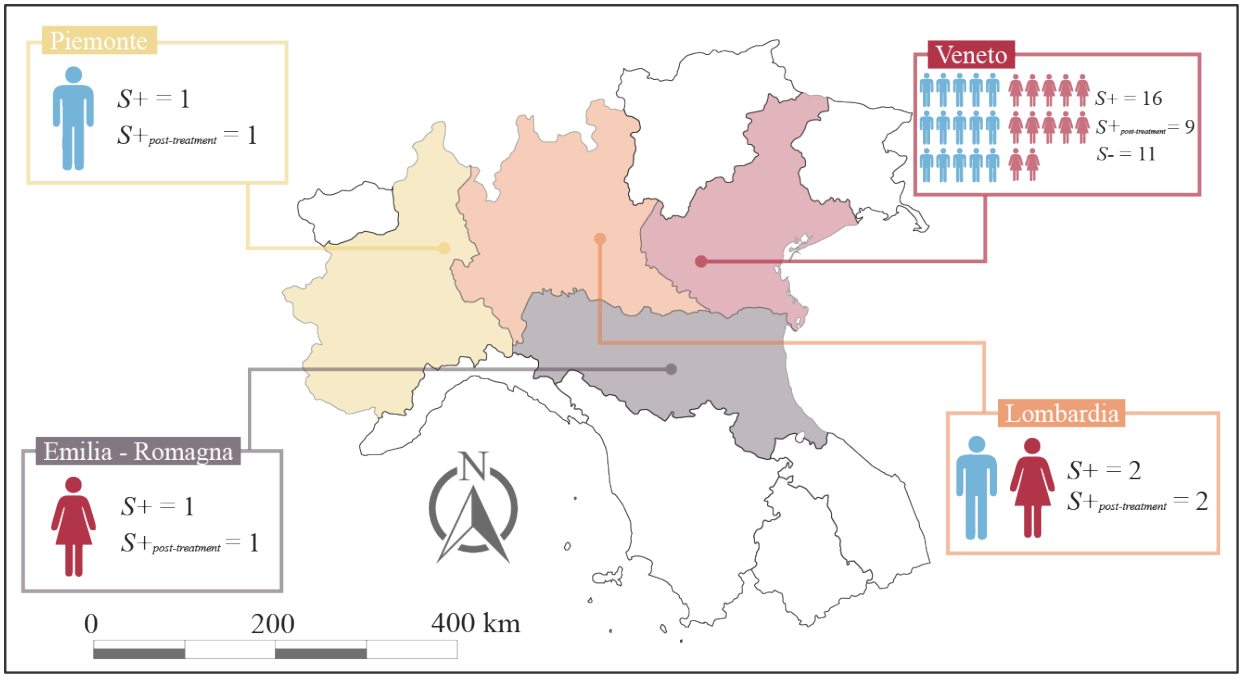
Samples subjected to high-throughput bacterial 16S rRNA sequencing					
Sex	*S+*	*S+pre-treatment*	*S+post-treatment*	*S-*	All
Male	12	9	9	5	26
Female	8	4	4	6	18
Overall	20	13	13	11	**44**

Region	Veneto	Lombardia	Piemonte	Emilia	All
S+	15	3	1	1	20
S+pre-treatment	8	3	1	1	13
S+post-treatment	8	3	1	1	13
S-	11	0	0	0	11
Overall	34	6	2	2	**44**

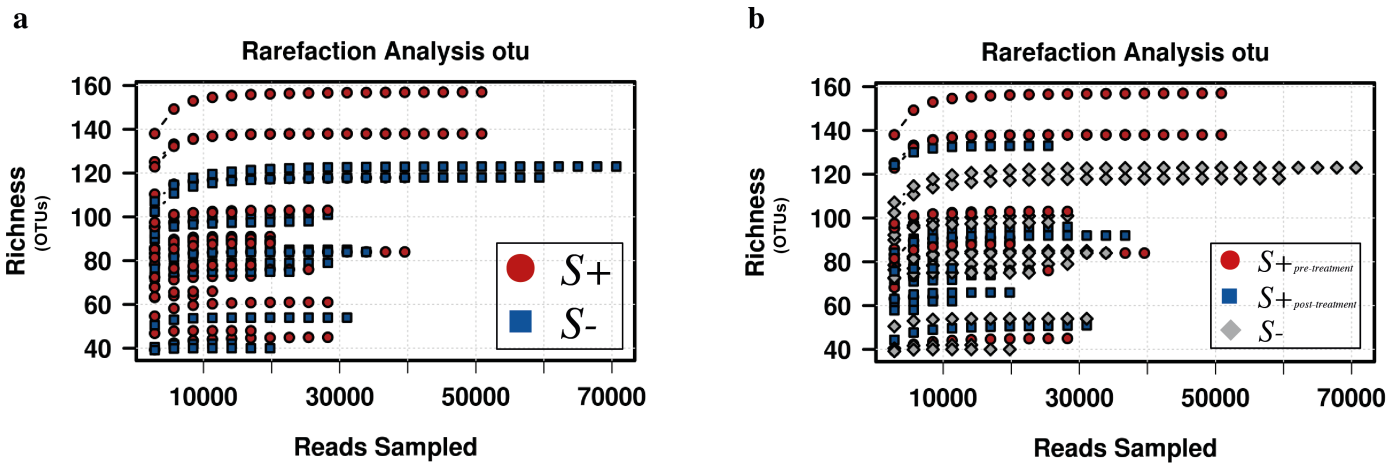
Samples subjected to metabolomic analyses

Sex	*S+*	*S+pre-treatment*	*S+post-treatment*	*S-*	All
Male	7	4	3	4	14
Female	7	4	4	6	17
Overall	14	8	7	10	**31**

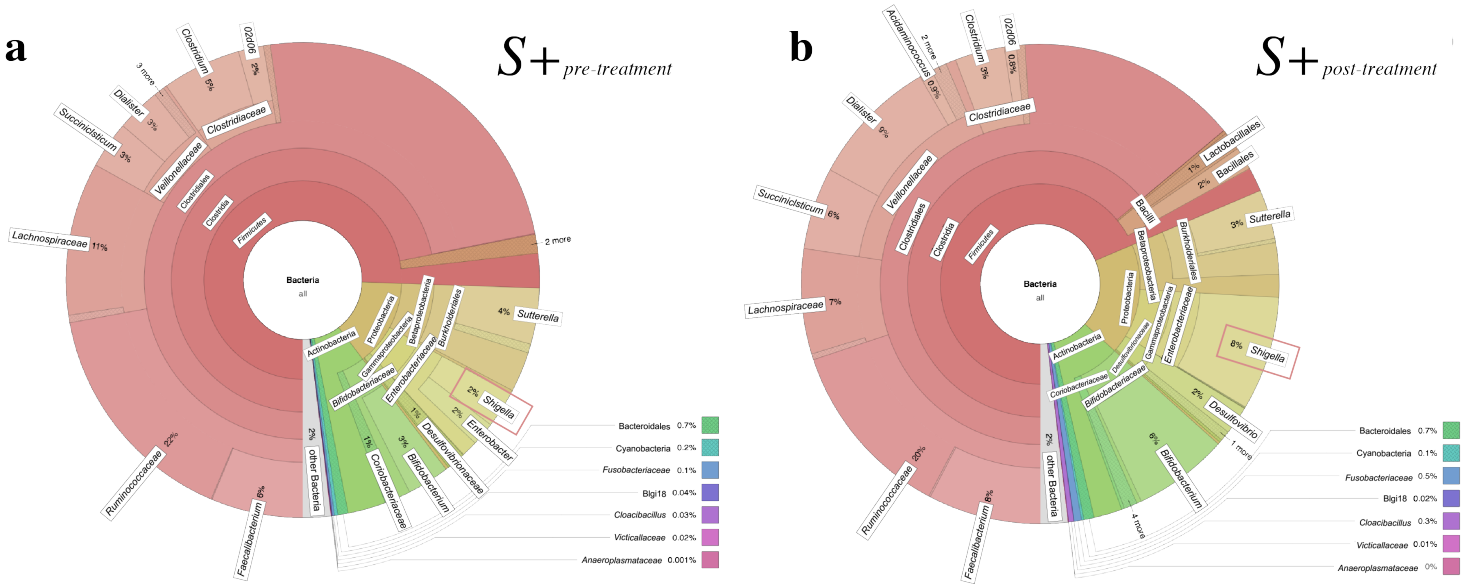
Region	Veneto	Lombardia	Piemonte	Emilia	All
S+	9	3	1	1	14
S+pre-treatment	3	3	1	1	8
S+post-treatment	2	3	1	1	7
S-	10	0	0	0	10
Overall	21	6	2	2	**31**



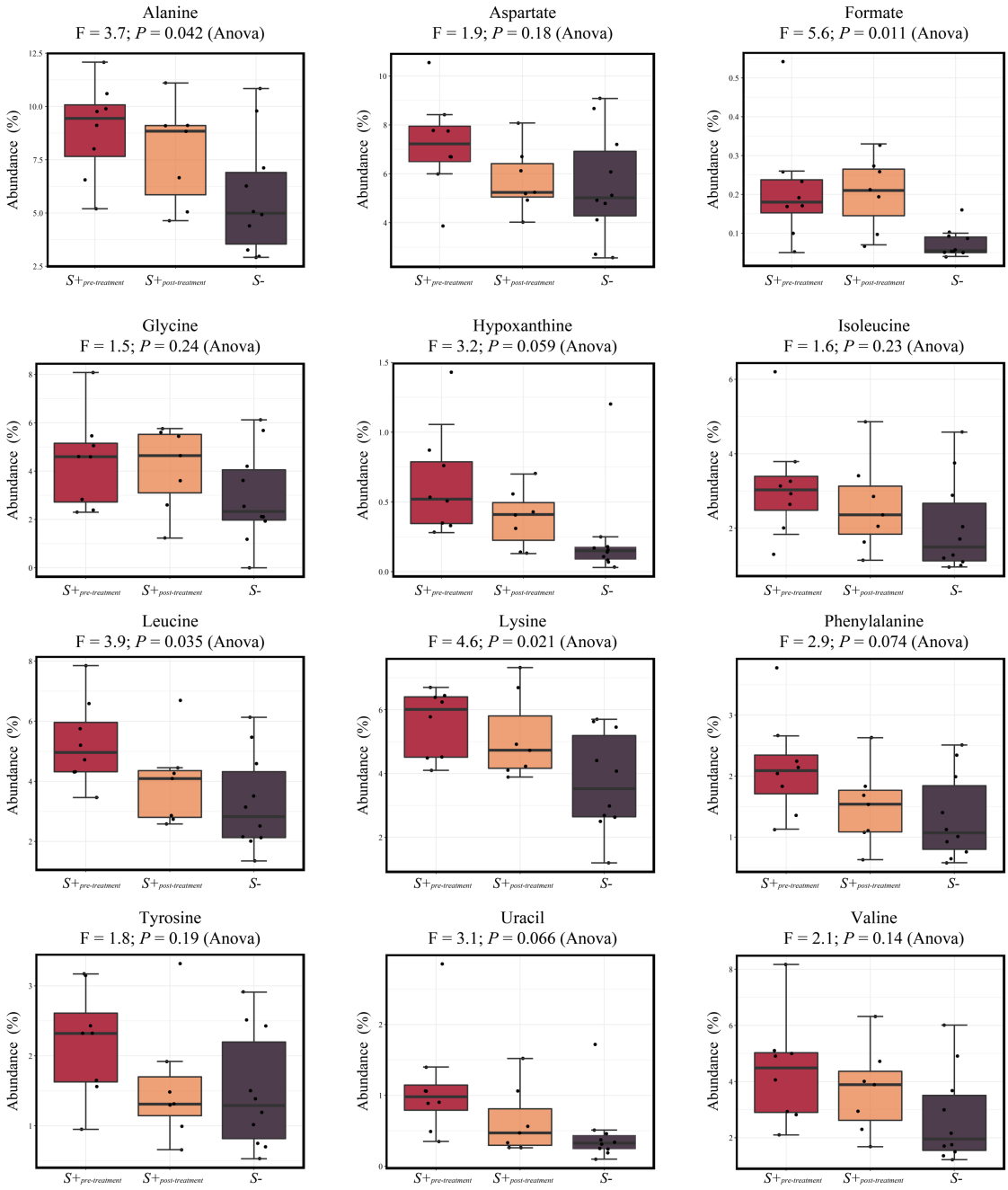
Supplementary Fig. S1 Faecal samples were collected from *Strongyloides stercoralis* infected and uninfected subjects (*S*+ and *S*-, respectively) and of the subset of *S*+ subjects that had received anthelmintic treatment and were re-sampled 6-months post-ivermectin administration ($S+_{post\text{-}treatment}$).



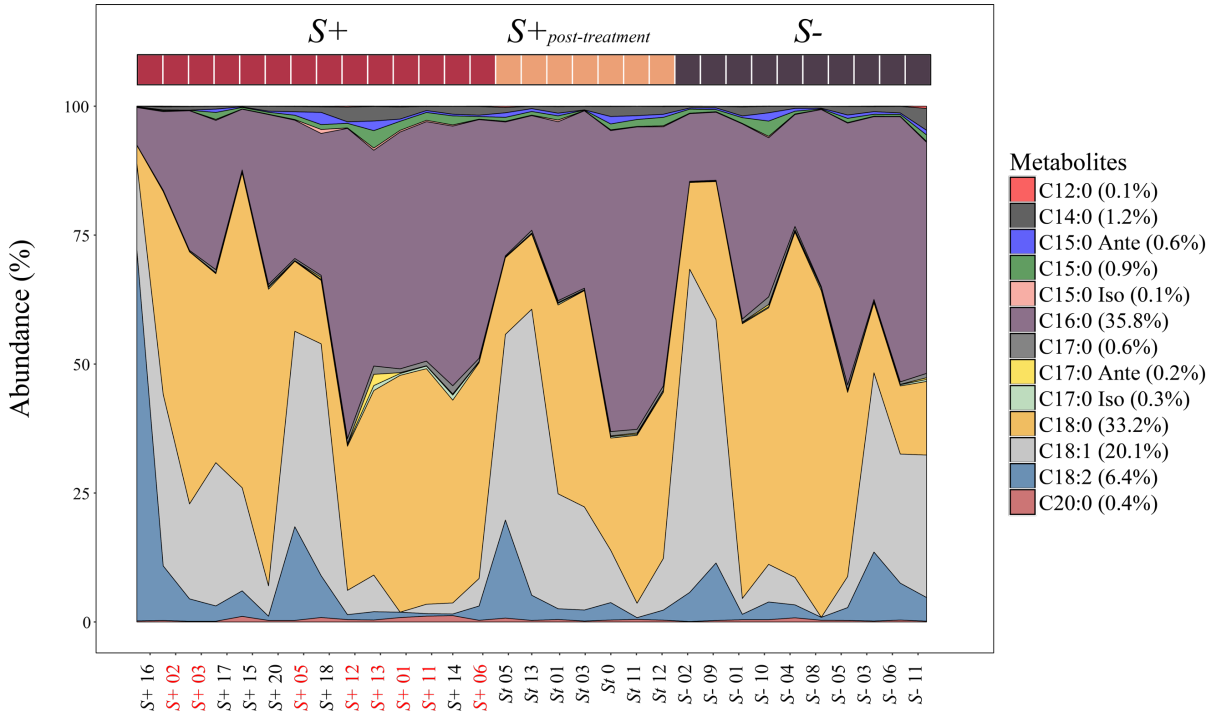
Supplementary Fig. S2 Rarefaction curves for microbial communities in faecal samples from *Strongyloides stercoralis*-infected and uninfected subjects (*S*+ and *S*-, respectively; a), as well as from the subset of *S*+ subjects that had received anthelmintic treatment, both prior to ($S+_{pre\text{-}treatment}$) and 6 months post-ivermectin administration ($S+_{post\text{-}treatment}$;b).



Supplementary Fig. S3 KRONA plot indicating taxonomic distribution of taxa associated with samples from subjects infected with *Strongyloides stercoralis* prior to ($S+_{pre\text{-}treatment}$) and 6 months post-ivermectin administration ($S+_{post\text{-}treatment}$). The pathogenic bacterial genus *Shigella* is highlighted by a red box.

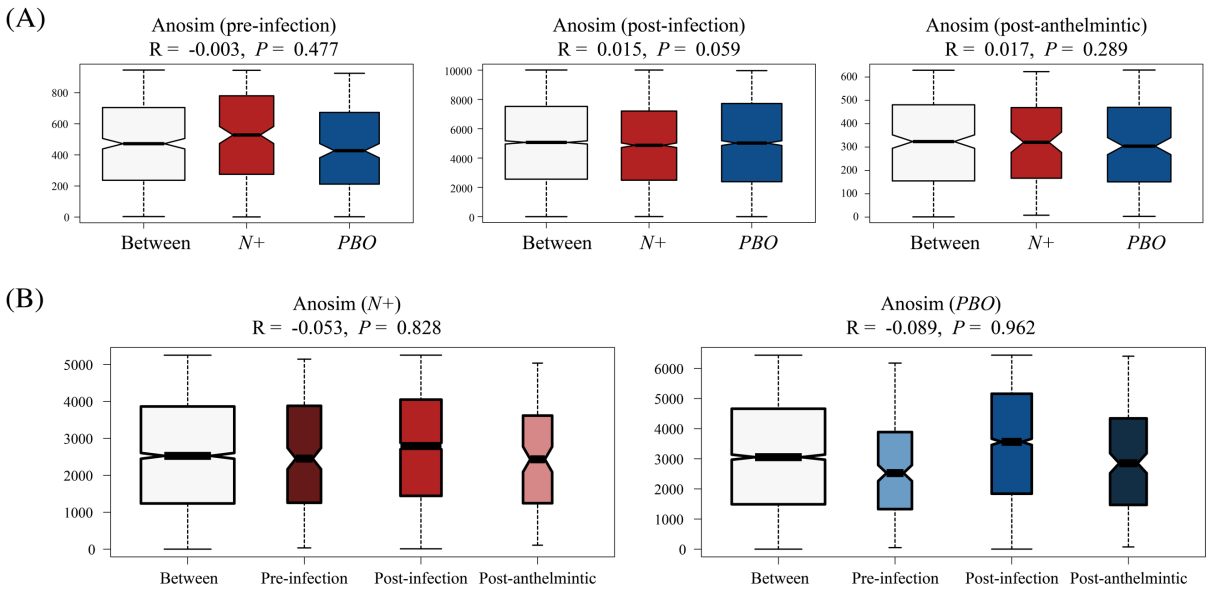


Supplementary Fig. S4 Boxplot representation of differentially abundant metabolites detected in faecal samples from subjects infected with *Strongyloides stercoralis* prior to ($S+_{pre\text{-}treatment}$) and 6 months post-ivermectin administration ($S+_{post\text{-}treatment}$), as well as uninfected controls. The bold and black horizontal lines in the boxplots refer to the mean of percentage abundance of metabolite associated with the corresponding group, with top and bottom whiskers representing the standard deviation.



Supplementary Fig. S5 Area plot indicating the abundance (expressed as percentage) of metabolites detected by nuclear magnetic resonance analysis (NMR) in faecal samples from *S. stercoralis*-infected and uninfected subjects (*S*+ in red, and *S*- in purple), as well as from the subset of *S*+ subjects that had received anthelmintic treatment, both prior to ($S+_{pre\text{-}treatment}$, sample label in red) and 6 months post-ivermectin administration ($S+_{post\text{-}treatment}$, in orange). Colours within the area plot refer to the respective metabolites, defined in the legend.

Chapter 4



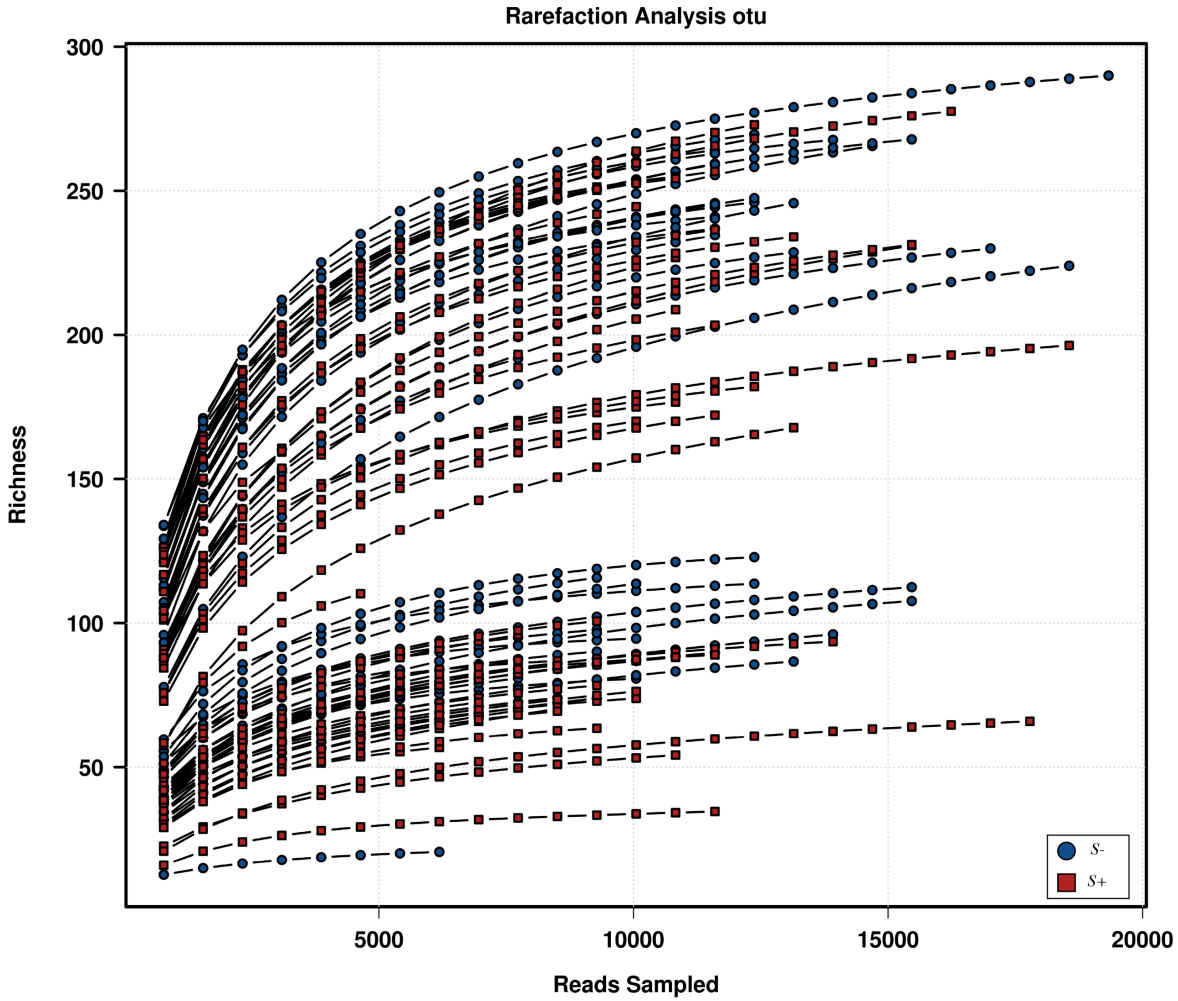
Supplementary Fig. S1 Microbial beta diversity of subjects infected with *Necator americanus* (*N*+) or placebo treated (*PBO*). **(**A) Differences in gut microbial beta diversity of *N*+ and *PBO* subjects one week prior to infection/placebo treatment (T-0.25; left), one, five, and nine months post-infection (T1, T5, and T9 combined; centre), and 2 months post-anthelmintic treatment (T12; right). (B) Differences in microbial beta diversity of *N*+ (left) and *PBO* (right) subjects across all time points.

	3+	3.5+	4+
N+			
PBO			

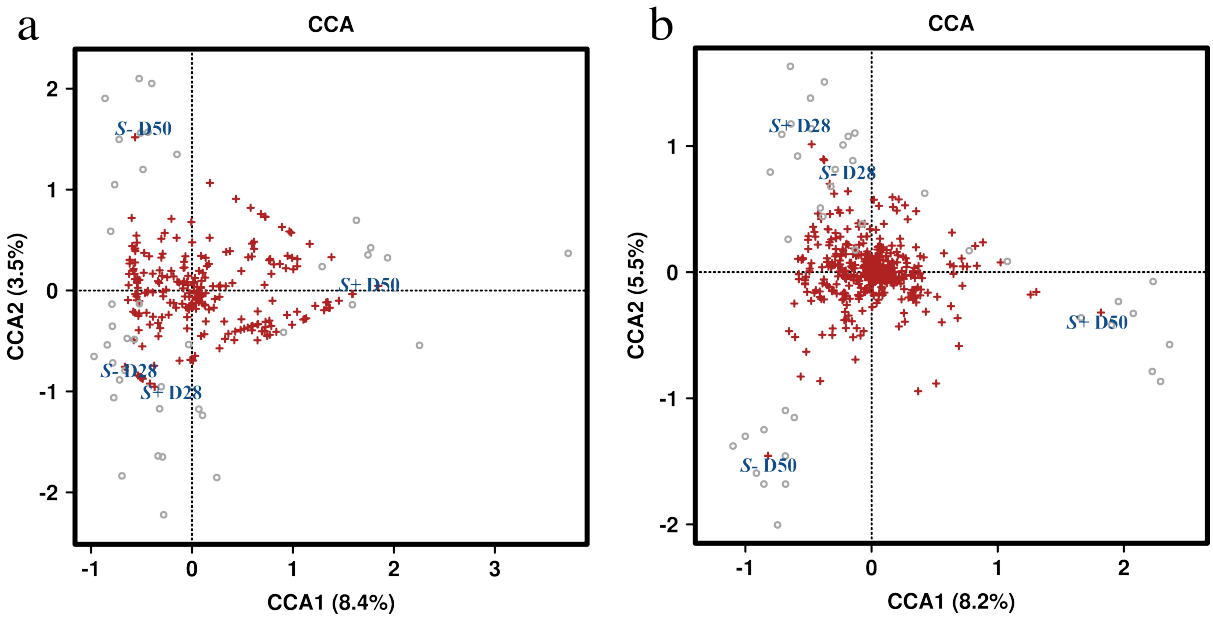
Phylum	Class	Order	Family	Genus	T1	T5	T9
Firmicutes	Clostridia	Clostridiales	VadinBB60*				
			Ruminococcaceae	UCG010*			
Proteobacteria	Gammaproteobacteria	Pasteurellales					
			Pasteurellaceae				
				Haemophilus			
Bacteroidetes	Bacteroidia	Bacteroidales	*Barnesiellaceae*	*Coprobacter*			
Firmicutes	Clostridia	Clostridiales	*Peptostreptococcaceae**				
			Lachnospiraceae	*Roseburia*			
Tenericutes*							
	Mollicutes*						
		Mollicutes (RF39)					
		Izimaplasmatales*					
Firmicutes	Clostridia	Clostridiales	*Ruminococcaceae*	UCG010			
			Lachnospiraceae	*Marvinbryantia*			
Actinobacteria							
	Coriobacteriia						
		Coriobacteriales					
	Actinobacteria	Bifidobacteriales					
Firmicutes							
	Bacilli	Lactobacillales	*Carnobacteriaceae*				
			Streptococcaceae				
	Clostridia	Clostridiales					
			*Peptostreptococcaceae**				
			Ruminococcaceae				
				UCG013			
			Eubacteriaceae	*Eubacterium (hallii)*			
				Eubacterium (ventriosum)			
			Lachnospiraceae	*Dorea*			
				Tyzzerella			
				Agathobacter			
Tenericutes							
	Mollicutes*						
		Izimaplasmatales*					
Bacteroidetes	Bacteroidia	Bacteroidales	*Sutterellaceae*	*Sutterella*			
			Barnesiellaceae	*Barnesiella*			
Firmicutes	Clostridia	Clostridiales	VadinBB60*				
			Ruminococcaceae	UCG010*			

Supplementary Fig. S2 Differentially abundant bacterial taxa in the faecal microbiota of subjects infected with *Necator americanus* (*N*+) or placebo treated (*PBO*) subjects one (T1), five (T5), and nine (T9) months post-infection, based on Linear discriminant analysis Effect Size (LEfSe) analysis. Colours correspond to Linear Discriminant Analysis (LDA) scores of 4 or higher (*N*+ = red; *PBO* = dark blue), 3.5 to 4 (*N*+ = orange; *PBO* = light blue), and 3 to 3.5 (*N*+ = yellow; *PBO* = grey).

Chapter 5



Supplementary Fig. S1 Rarefaction curves for gut microbial communities from luminal contents of mice infected with *Schistosoma mansoni* (*S*+) and that of uninfected control mice (*S*-).



Supplementary Fig. S2 Canonical Correspondence Analyses (CCA) Biplot, demonstrating the portion of variability in the data attributable to the explanatory variables. The gut microbial profiles of luminal content samples from the small and large intestine of mice infected by *Schistosoma mansoni* (*S*+) at 28 and 50 days post-infection (D28 and D50, respectively), as well as of uninfected controls (*S*-) ordinated by Canonical Correspondence Analysis (CCA) (a: small intestine; b: large intestine).