The effects of a helminth-altered gut metabolome and deworming on host immunity

by

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B.Sc., Utrecht University, The Netherlands, 2013
M.Sc., Utrecht University, The Netherlands, 2016

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We acknowledge and respect the ləkʷəŋən peoples on whose traditional territory the university stands, and the Songhees, Esquimalt and W̱SÁNEĆ peoples whose historical relationships with the land continue to this day.

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Abstract

Helminths are parasitic worms that can establish long-lived infections by modulating host immune responses. Helminth infection has been associated with a reduced prevalence of allergic disease in human populations, and impaired immunity to co-infecting pathogens. Several human and mouse studies suggest that helminths may impair host responses to concurrent bacterial infection. In this thesis, we study the extent to which helminth infection affects *Salmonella* colonization and how anthelmintic treatment (deworming) impacts immunity to *Salmonella* in a mouse model of co-infection. We find that helminth co-infection allows *Salmonella* to establish in the lumen of the small intestine. Further, we find that deworming prior to bacterial infection restores impaired immunity to *Salmonella* in the small intestine, however, deworming after *Salmonella* has established during helminth co-infection does not revert elevated bacterial burdens.

To ensure their longevity in the host, helminths release immunomodulatory molecules, and modulate immunity through changes in the gut microbiota. The microbiota is known to influence mucosal immunity through the production of metabolites, but metabolites have not received much attention in the context of helminth modification of immune responses. This thesis uncovers the impact of helminth infection on levels of short-chain fatty acids (SCFAs) and bile acids, two groups of metabolites with immunomodulatory potential. We found that helminth infection increases small intestinal levels of the branched-chain SCFA isovalerate and lowers the small intestinal bile acid concentration. We next explored the consequences of these metabolite shifts on susceptibility to bacterial infection, helminth fitness and regulatory T cells.

Collectively, these results contribute to the understanding of host-pathogen interactions in a co-infection scenario, which ultimately will help to inform strategies for disease control in helminth-endemic areas. Further, our data contributes to the characterization of the helminth-modified intestinal metabolome, which future work can build on to reveal novel immunomodulatory pathways that can be targeted to relieve symptoms in inflammatory diseases such as allergic asthma.

Acknowledgements

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My initial 'acknowledgement' was twice as long, far too long, but I will make sure all of you will hear the left-out details in person!

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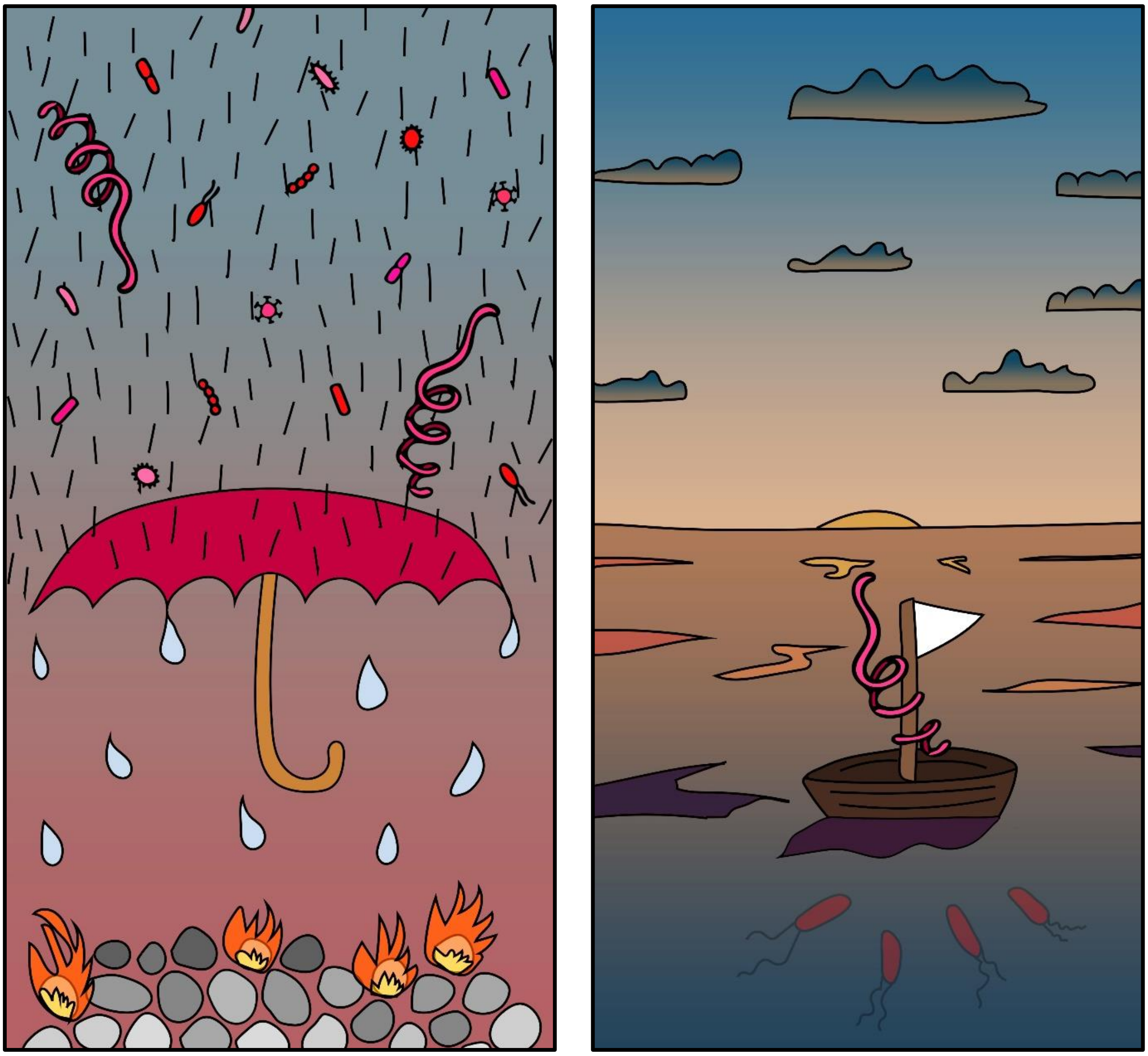
Abbreviations

A. lumbricoides	*Ascaris lumbricoides*
A. duodenale	*Ancylostoma duodenale*
A. suum	*Ascaris suum*
AMPs	Antimicrobial products
B. thetaiotaomicron	*Bacteroides thetaiotaomicron*
BMDM	Bone-marrow derived macrophage
C. rodentium	*Citrobacter rodentium*
CA	Cholic acid
CDCA	Chenodeoxycholic acid
Cfu	Colony-forming units
DC	Dendritic cell
E. coli	*Escherichia coli*
ES	Excretory-secretory
FXR	Farsenoid X receptor
H. polygyrus	*Heligmosomoides polygyrus*
HDAC	Histone deacetylases
HBSS	Hank's Balanced Salt Solution
HIV	Human immunodeficiency virus
IFN	Interferon
IBD	Inflammatory Bowel Disease
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
ILC2	Type 2 ILC
L3	Stage 3 larvae
LB	Luria-Berthani
LCA	Lithocholic acid
LPS	Lipopolysaccharide
M. tuberculosis	*Mycobacterium tuberculosis*
M2	Type 2 macrophage

MLN	Mesenteric lymph node
MCA	Muricholic acid
N. americanus	*Necator americanus*
N. brasiliensis	*Nippostrongylus brasiliensis*
NTS	Non-typhoidal *Salmonella*
PBMC	Peripherical blood mononuclear cell
PBS	Phosphate-buffered saline
PP	Pyrantel pamoate
RELM	Resistin-like molecule
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSV	Respiratory syncytial virus
***S.* Typhimurium**	*Salmonella enterica* serovar Typhimurium
S. mansoni	*Schistosoma mansoni*
SCFA	Short-chain fatty acid
SPI	*Salmonella* pathogenicity island
T. muris	*Trichuris muris*
T. trichiura	*Trichuris trichiura*
T. suis	*Trichuris suis*
T3SS	Type 3 secretion system
TB	Tuberculosis
Th2	T helper 2 (cell)
TLR	Toll-like receptor
Treg	Regulatory T (cell)
TGR5	Takeda G-coupled protein receptor 5
UDCA	Ursodeoxycholic acid
UPLC-MS	Ultra-high-performance liquid chromatography mass spectrometry
YLD	Years lived with disability

Dutch proverbs



Van de regen in de drup/na regen komt zonneschijn[*]

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[*] Dutch proverbs, literally: ‘out of the rain into the drip’ (English equivalent: out of the frying pan into the fire), and ‘after rain comes sunshine’ (every cloud has a silver lining).

Chapter 1

Literature review

Chapter Disclaimer: Parts of this chapter are published in a review article in Mucosal Immunology, on February 16th, 2018, co-authored by myself and Dr. Lisa Reynolds (attached in **Appendix**). Some sections of the review have been copied word-for-word into this chapter, and others have been rephrased, complemented with new literature, or completely omitted for the sake of the scope of this thesis. All illustrations are original and crafted by myself, and not based on existing illustrations by others but rather based on my assessment of the literature, with one exception (**Figure 1.1**, credits given in figure legend).

1.1 Introduction

Helminths are multicellular parasitic worms that remain a global health concern; around 1.5 billion people are currently infected worldwide [1]. These parasites can mount long-lasting infections, often spanning years, which has been attributed to their ability to manipulate mammalian immune responses and thereby avoid immune-mediated expulsion [2].

Modulation of mammalian immune responses by helminths not only promotes their own persistence but can additionally affect host immunity to concurrent infections and responses to bystander antigens. For example, helminth infection has been associated with impaired immunity to coinfection with various microbial parasites, bacteria, and viruses, both in human populations [3–5], and in mouse infection models [6–19]. Given that chronic helminth infections are predominantly restricted to regions of poor sanitation where people have a high exposure to microbial pathogens [20], understanding how helminth infection modulates host responses to microbial coinfection is critical for disease treatment in these populations. This thesis focuses on co-infection with helminth and bacterial pathogens and aims to further our understanding of the role of helminth co-infection in susceptibility to bacterial infection in the intestine. This work includes a deeper understanding of *Salmonella* infection in mice - a model of bacterial enteritidis- during co-infection with a natural murine helminth and uncovering of the effects of drug-induced clearance of helminth infection on bacterial burdens in the intestine.

As well as being implicated in modifying responses to bacterial co-infection, live helminth infection has been associated with a reduced prevalence of allergic disease in human populations [21], and suppression of allergic airway inflammation in mouse disease models [22–25]. Understanding how helminths modulate host immunity is therefore of global importance, as triggering the immune pathways that helminths target has the potential to be exploited for the treatment of allergic conditions; a growing burden on the healthcare system in industrialized countries [26].

Helminth infection typically elicits a type 2 immune response, characterized by the production of interleukin (IL)-4, IL-5, IL-9 and IL-13 from type 2 innate lymphoid cells (ILC2s) and T helper 2 (Th2) cells, and the recruitment of eosinophils, mast cells, and Type 2 macrophages [27]. Type 2 effector mechanisms are essential for immune-mediated helminth expulsion, yet are often ineffective, largely due to concurrent regulatory responses induced by helminth infection. For example, helminths release a variety of so-called excretory-secretory (ES) products, which include immunomodulatory proteins, glycoproteins, and miRNAs [28,29], that have been reported to modulate the activity of multiple immune cell types, and can directly induce regulatory immune cells, such as Foxp3$^+$ regulatory T cells (Tregs) [2,30]. Production of ES material by helminths likely underpins the longevity of helminths in their host, as well as affecting host susceptibility to inflammatory and infectious diseases [31].

Intestinal dwelling helminths are not alone in the intestinal tract. Mammalian intestine harbor a vast array of mutualistic and commensal microbes, collectively termed the microbiota, which includes species of bacteria, viruses, and fungi. Helminth infection not only affects the composition of microbiota species, but as a result, infection alters the array of products derived from the microbiota. In this thesis, we define the 'gut microbiota' as the microbiota species themselves, their genes and products, as well as the products in their surrounding intestinal environment. Several recent studies have investigated how shifts to the intestinal microbiota during helminth infection contribute to the immunomodulatory effects of helminth infection, which are reviewed in this chapter.

One way in which microbiota species are known to modulate host immune responses is through the production of metabolites [32]. Metabolites are intermediates or end-products of metabolism, be it microbial metabolism (such as fermentation), host metabolism or metabolism of parasites. We do not yet have a thorough understanding of metabolite production by helminths, or the contribution of host or microbiota-derived metabolites in helminth infection-driven immunomodulation. In general, metabolites have been relatively neglected in the study of potential pathways in which helminths modulate immune responses. This thesis includes pioneering work that investigates the effect of helminth infection on two classes of intestinal metabolites that have been in the spotlight for the recent years because of their impact on host immunity: short-chain fatty acids (SCFAs) [33] and bile acids [34]. Background on these classes of metabolites, and the state of knowledge of how helminths impact the intestinal metabolome (the collection of metabolites in the intestine) will be extensively discussed in this chapter.

1.2 Helminth infection

Helminth infection is a major global health issue that affects about 1 in 5 people worldwide [1]. The most common helminth infections are caused by infection with soil-transmitted helminths (e.g. roundworms, whipworms, hookworms), followed by schistosomes [35]. Broken down, the World Health Organisation's assessment of Global Burden of Disease (GBD), shows that 800 million individuals are likely infected with the roundworm *Ascaris lumbricoides*, 451 million with hookworm species (mostly *Ancylostoma duodenale* and *Necator americanus)*, 425 million with the whipworm *Trichuris trichiura*, and 190 million individuals have schistosomiasis caused by species of schistosomes [1]. Along with soil-transmitted helminths and schistosomes, a third clinically relevant group of helminths are the filarial worms. Helminths fall into either the phyla cestodes (also called ‘tapeworms’), nematodes (‘roundworms’) or trematodes (‘(river) flukes’). In this thesis, the spotlight is on those helminths that can occupy the mammalian intestine for long stretches of time, the soil-transmitted helminths, that infect over a billion people worldwide and thus form a global health burden. Helminth infections are not often a cause of death but cause significant morbidity. A measurement of morbidity is Disability-Adjusted Life Years (DALY), from which the Years Lived with Disability (YLD) can be estimated. The figures from the 2016 GDB study indicate that helminth infections contribute to 6.6 million YLD, which represent 6.5% of all YLD due to communicable, nutritional, maternal, and neonatal diseases globally [1]. Helminth infections are most common in the tropics and subtropics [36]. Climate is an important determinant for transmission as moisture and warm temperature are essential for larval development in soil [37], but also for the snails that are intermediate hosts for schistosomes, or mosquitos that are transmitting vectors for filarial parasites [38]. An equally important determining factor for helminth prevalence is poverty, which creates conditions including poor sanitation, insufficient water supply and limited access to health care, that are conducive to transmission of parasite infection [39]. Thus, helminths mainly burden the world’s economically poorest populations, and the high YLD keeps those populations from economically thriving, essentially trapping people in poverty. Because the economically poorest populations tend to be invisible for the rest of the world, helminth infections have been greatly neglected in health research. Helminth infections are thus part of the umbrella term “Neglected Tropical Diseases”. Together with the high numbers of helminth infection, this status of helminth infection indicates the dire need of more research to improve treatment strategies and understand the disease burden in helminth-endemic areas.

1.2.1 Treatment for helminth infection

Upon diagnosis of helminth infection, treatment is often targeted towards removal of worms from the gastrointestinal tract with pharmaceutical drugs. The drugs most often used for removal of worms are mebendazole and albendazole that bind specifically to nematode beta-tubulin that causes death of adult worms in a few days [40]. These are broad-spectrum drugs, but albendazole has better absorption by the gut, thus works better against tissue-migrating helminths [36]. Pyrantel pamoate is an alternative anthelmintic to treat hookworm infection and ascariasis but does not work well against Trichuris species [36]. Pyrantel pamoate works by depolarizing neuromuscular blockade and inhibition of acetylcholinesterase, following paralysis of the worms [40]. This then causes the worms to lose grip of the host tissue and further worm expulsion happens by expulsion by the host immune system. Pyrantel pamoate is very poorly absorbed by the intestine, therefore the host is unaffected by small dosages of these drugs [40]. The use of anthelmintics is not restricted to treat symptomatic helminth infection but is also used to treat populations living in helminth-endemic regions *en masse*, with the goal of greatly lowering helminth burdens in a population, to reduce transmission and ultimately to improve the health of the whole population. Morbidity that is considered asymptomatic includes impaired childhood growth and poor nutritional status. Periodic administration of anthelmintics, also known as 'deworming', has been shown to reduce morbidity within the population, from recorded improvements in health after deworming [36]. Regular treatment of school-age children reduces worm burden to under the threshold at which helminth infection causes disease. The benefits of deworming include improved iron storage, growth and physical fitness, as well as increased cognitive performance [36]. Ultimately this increases school attendance, and thus a better chance at a brighter future for the individuals in helminth-endemic areas. Similarly, reduction in morbidity in adults increases the labour force and benefits the economy of helminth-stricken nations [36]. Re-infection is common after anthelmintic treatment, therefore regular treatment is needed.

Drug resistance to anthelmintics is on the rise in nematodes that infect life-stock, because of routine treatment of animals that are kept in close proximity. If these conditions are replicated in human populations, anthelmintic resistance could pose a threat to global health [41]. However, human nematodes are exposed to less frequent drug challenge with longer intervals between treatments, and treatment is targeted at certain populations, lowering the risk of anthelmintic resistance in human helminth species. Nonetheless, the effectiveness of anthelmintic drugs in human populations is being closely monitored [42]. Development and testing of alternative anthelmintics is also on the research agenda, as well as development of vaccines against helminth infections. Vaccination is a simple, single-step interruption of infection and transmission, and is therefore the preferred method of treatment, but vaccine development for helminthiasis has substantial obstacles [43]. One major impediment is the lack of knowledge on how helminths can withstand a potent immune response and establish month- to year-long infections, and why the host immune system shows poor memory upon re-infection. Researchers have discovered that helminths are masterful manipulators of the immune system and have begun to describe the myriad of ways in which helminth can modulate immune responses. Immunomodulation by helminths will be discussed later in this chapter.

1.2.2 Animal model for chronic helminth infection

Hookworms are especially good at modulating and evading host immunity, as is demonstrated by the lack of strong immunity to hookworm species *N. americanus* and *A. duodenale* at a later age in life [44]. Whereas people overall experience less ascariasis and trichuriasis later in life, the incidence of hookworm infection increases with age until adulthood [44]. Therefore, hookworms are of special interest to examine how helminths can overcome host immune responses. To study immune responses to helminths and mechanisms of helminth-driven immunomodulation of host immunity, animal models have proven to be indispensable. Overall, helminths are well-adapted to their specific mammalian hosts, therefore infecting laboratory animals with a human helminth does not lead to chronic infections. The closest rodent helminth on the phylogenetic tree to human hookworm species *N. americanus* and *A. duodenale* is *Nippostrongylus brasiliensis*, but this helminth does not cause chronic infection in murine hosts, only an acute infection that is cleared within two weeks [45]. The next closest helminth in phylogenetic tree that chronically infects mice is *Heligmosomoides polygyrus* [46], and this is the model helminth chosen for this thesis work.

H. polygyrus is an intestinal helminth that naturally occurs in populations of wild mice. Phylogenetically it is close to the human hookworms *A. duodenale* and *N. americanus* [46] that account for the majority of hookworm cases in humans [36]. An important difference between *H. polygyrus* and human hookworm species is that human hookworm species travel to the lung before the adult worms establish in the small intestine, while *H. polygyrus* skips the lung phase and goes directly to the small intestine after the larval worms are orally ingested. Importantly, the chronic phase of infection is very similar: *H. polygyrus* establishes a long-lasting infection in the proximal small intestine of mice, which is the also the site that adult hookworms inhabit for long periods in human hosts.

The life cycle of *H. polygyrus* as occurs in wild animals, and as is mimicked in the laboratory setting, is depicted in **Figure 1.1**. Mice are infected by oral ingestion of infective *H. polygyrus* stage 3 larvae (L3) that travel to the submucosa of the small intestine within the first 24 hours. Here, the larvae undergo developmental molts, and upon maturation, around day 10 of infection, the helminth reappears in adult form in the intestinal lumen. Adult worms wrap around villi to secure their position and are thought to feed on host tissue [47]. Most adult worms segregate in the proximal part of the small intestine, mainly the duodenum, likely because the villi at this site are the longest. Coiling around villi secures their position during intestinal contractions and flow. In the lumen, female *H. polygyrus* worms produce eggs which end up being excreted into the feces. In the environment, eggs hatch in around one week and become infective larvae that can find a new host, upon which the life cycle can continue. In the lab we can mimic a soil-based environment by mixing mouse feces with deactivated charcoal and wet tissues. Larvae are harvested from these micro-environments and are kept in storage in the lab at 4 degrees until they are used to infect mice. The intensity of infection in mice can be measured by the number of eggs in the feces (non-terminal method) or enumeration of adult worms in the small intestine (terminal method). An advantage of *H. polygyrus* as a model for helminth infection is that the adult worms can be cultivated *in vitro*, and *H. polygyrus* ES products can be extracted and identified [48]. This has been done extensively, and many immunomodulatory pathways have been discovered of *H. polygyrus* ES products acting on the host immune system [49].

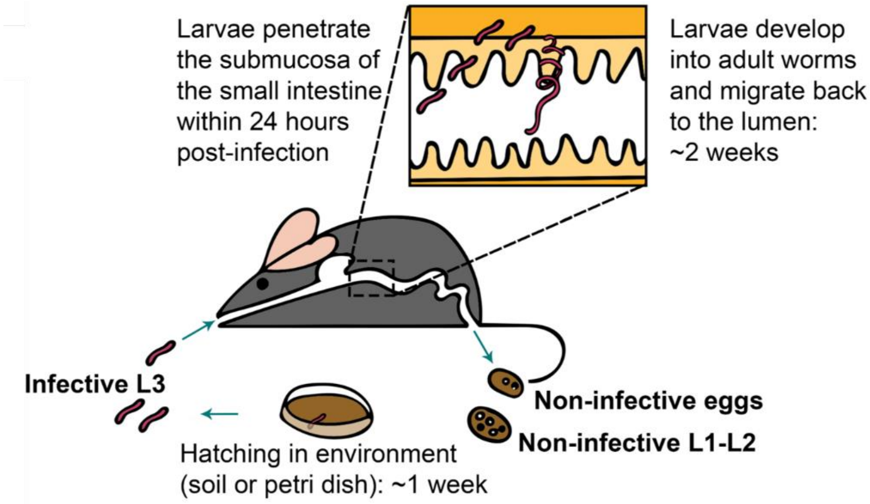

Figure 1.1. The *H. polygyrus* life cycle. Mice become infected with L3s that then undergo molts in the small intestinal tissue before re-emerging in the lumen where the adult worms wrap around the villi, mate, and lay eggs. The eggs are excreted via feces and hatch in the environment (outside of the host) and become infective larvae in about a week. Figure is re-illustrated and largely based on a previous published figure by Reynolds *et al.* [50].

1.3 Immune response to helminths

The immune response to helminth is generally dominated by the type 2 immune pathways that serve to trap, kill or expel worms, as well as regulatory immune pathways, including expansion of Tregs that serve to dampen inflammation [27,51] (**Figure 1.2**). Together, these immune pathways create an environment in which the parasite cannot fully thrive, but also limits physical damage caused by the worms and the inflammatory response to infection. The immune response against helminths with a particular focus on immunity to *H. polygyrus* will be summarized in detail below, after discussing some more general concepts regarding the immune response to helminths. It is important to appreciate that the immune response to helminths looks very different for different species, hosts, at different phases in the life cycles, at different times into the infection, and even at different infective doses. A few examples to clarify this will follow below.

1.3.1 Variety of immune responses

Different tissue tropisms of helminths naturally dictate different immune responses. For example, schistosomes release eggs in the intestinal wall, or in the liver, causing problematic levels of fibrosis in the latter tissue [52]. Helminths that dwell in the intestinal lumen generally are not directly pathogenic to the surrounding tissue [2]. The immune response is not static during an infection, in part due to the migratory nature of the worm. For example, the immune response to schistosomes evolves via 3 consecutive, immunologically distinct phases: larvae that are migrating through tissue elicit an initial type 1 response marked by an increase in interferon (IFN)-γ, which is then suppressed by a potent type 2 response prompted by eggs released by tissue-dwelling worms [53]. Over time, during the chronic phase of schistosomiasis, regulatory immune responses take over including Tregs and regulatory B cells [53]. Immune responses to *H. polygyrus* remain type 2-focused throughout the worm's lifecycle, but the type 2 cytokine production by T cells and the number of effector immune cells present in the small intestinal lamina propria differs between the larval stage in the tissue, and the time that adult worms take residence in the small intestinal lumen [54,55]. Tregs are found in larger quantities in the (intestine-draining) mesenteric lymph nodes (MLNs) and Peyer's patches (small intestinal lymphoid follicles) during larval/tissue stage, and continue to increase in number during the luminal phase [56].

Regardless of the tissue the helminth is in, the host generally shows an evolution in the immune response as it changes strategies or priorities over time [57,58]. An acute response to helminth infection is dominated by type 1, type 2 and type 3 immune responses, to abort the infection; if unsuccessful the response to subacute infection will be mainly focused on type 2 responses, which minimizes parasite load [57]. If a chronic infection is established, that can last months or years, the host response becomes immunomodulatory, mediated by regulatory cells and anti-inflammatory cytokines, that tolerates low levels of helminths and ensures immune homeostasis [57,58]. While this story of host immune evolution is oversimplified, it highlights the importance of choosing the right infection model and time points for specific research aims. Some helminths that researchers use in mouse models (e.g. *N. brasiliensis* as mentioned above) do not reach the chronic phase of infection, and are expelled rapidly, allowing for deeper understanding of an effective anti-helminth immune response, but not helminth-driven immunomodulation.

As an alternative to studying an acute model of helminth infection, another method to study immune responses required for worm expulsion is to focus on secondary infections rather than primary infections. Primary infection with helminths in mice can trigger a different immune response than a secondary infection [27]. While the primary infection with *H. polygyrus* becomes chronic, the second infection is commonly cleared by the immune system of the mouse, indicating a memory-based immune response [59]. In humans, re-infection with hookworm is common and protective memory immunity rare. The reasons for this are unclear and it is speculated that helminth infective doses in humans remain under the threshold for memory immunity to be formed [60]. As a model for human infection, secondary infections in mice are perhaps not the best representative. Nevertheless, secondary infections serve to elucidate the immune responses needed to clear helminths. An infection model that is perhaps most representative of helminth infection in humans is the so-called trickle infection, which entails administration of small dosages of infective larvae with repeated challenges [61]. In the natural setting of the host living/roaming in a helminth-endemic area, helminths are ubiquitous, and the host is constantly exposed to low numbers of parasites. Trickle infections can provoke different immune responses than a one-time bolus infection, for example with trickle infection of *H. polygyrus* authors discovered a previous underestimated, key role for granuloma formation in the innate defense to helminth infection with *H. polygyrus* [62]. In the commonly used mouse model of trichuriasis, the differences between immune responses to small infective dosages of *Trichuris muris* and larger *T. muris* doses has been clearly established: mice mount a Th2 cell response in response to high doses of *T. muris* that leads to host resistance, but lower doses of *T. muris* cause a Th1 cell response in the intestine that allows the parasite to establish a chronic infection [27].

1.3.2 *Genetic variance in mouse susceptibility to H. polygyrus*

Different mouse strains show different susceptibility to infection with *H. polygyrus*, exhibited by different response time, i.e. the time *H. polygyrus* can persist in the host [50]. The mouse strain used for most experiments in this thesis is C57BL/6 mice which have intermediate responsiveness: it takes mice 8-20 weeks to expel a bolus dose of worms, versus CBA mice that take over 20 weeks, and BALB/c (also used in this thesis, for specific aims) are faster (6-8 weeks). Several candidate resistance genes are identified, including IL-9 (role of which will be discussed later in this chapter) that correlates with worm expulsion [63]. There is also a significant sex effect on susceptibility to *H. polygyrus* with female mice of all strains clearing infections faster [50]. The reason behind the sex effect has yet to be elucidated.

1.3.3 *Epithelial cells: the first responders*

The epithelial cells that line the intestine are the first to respond to a helminth infection and consist of enterocytes as well as more specialized cells such as neuro-endocrine cells, goblet cells, Paneth cells and tuft cells. Together the epithelial cells form a barrier that protects the underlying tissue from luminal harmful agents, but also serve as a sensor for infection. The sensing capabilities come from pattern-recognition receptors such as Toll-like receptors (TLRs), that can trigger pathways that lead to immune activation. Most important in the context of helminth infection, epithelial cells can also respond to tissue damage, for example due to parasites feeding on host tissue, by releasing alarmin cytokines that stimulate ILCs and dendritic cells (DCs) to trigger further immune activation [2]. Another source of alarmins are tuft cells, a recently discovered and little-studied cell type [64,65]. Tuft cells play an important role in immunity to helminth by producing large amounts of the alarmin IL-25 [64,65]. Tuft cells are thought to be important in immune homeostasis by supporting ILC2s through constitutive production of IL-25 [64,65]. Upon sensing helminth products, such as the metabolite succinate, IL-25 production is enhanced, inducing more ILC2s that in turn cause more differentiation of tuft cells (as well as goblet cells), enhancing the IL-25 signaling loop [65]. Mice lacking tuft cells are impaired in the ability to expel helminths, unless exogenous IL-25 was administered [64]. In addition to IL-25, other alarmins are TSLP (thymic stromal lymphopoietin) and IL-33 and each of these alarmin cytokines are produced by epithelial cells upon damage and contribute to ILC2 activation. To further enhance type 2 immune signaling, alarmin-induced ILCs can stimulate Th2 differentiation in DC-primed CD4$^+$ T cells [66]. Release of alarmins and type 2 cytokines cause a so-called 'weep and sweep' reaction, by mucus production and leakage of fluids into the lumen across the epithelium as lubricant [67], and smooth muscle contraction that effectively sweeps worms out of the intestine [68–70]. In addition, the epithelial turnover rate is increased in reaction to helminth infection (in an IL-13 dependent manner) as to form a 'epithelial escalator' to expel the helminth that then fails to hold on to the intestinal villi [71]. The epithelial effector cells (such as goblet cells) and mechanisms (gut permeability and contractility) involved in the weep and sweep reaction, as well as the type 2 inflammation-induced influx of innate immune cells such as eosinophils are discussed in detail below.

1.3.4 *Goblet cells*

Goblet cells are specialized epithelial cells that secrete mucins, that make up a larger structure of mucus that covers the intestinal epithelium as well as other mucosal surfaces. Goblet cells undergo proliferation and increase mucin expression in response to external stimuli like inflammation, cytokines and microbes. Mucus is an important constituent of the physical barrier between gut lumen and intestinal tissue, and is generally impenetrable for bacteria, keeping the microbiota as well as invading pathogens at bay [72]. Secretion of the mucin Muc2 is upregulated during *H. polygyrus* infection [73], but there is no evidence yet as to whether Muc2 contributes to *H. polygyrus* expulsion, whereas Muc2 production is associated with expulsion of *T. muris* and *N. brasiliensis* [74,75]. It is thought that increased mucus production impedes helminths to physically maintain their niche, which in the case of *H. polygyrus* would be holding on to intestinal villi. It is possible that specific components in mucus other than mucins (also) contribute to immunity to helminths. Resistin-like molecule (RELM)-β is produced by goblet cells in response to IL-13 and is important for gut barrier homeostasis by controlling epithelial permeability [76,77]. RELM-β can also inhibit nematode chemotaxis, by binding to the worm's chemosensory apparatus, thus confusing the worm as to its position in the intestinal lumen [76]. The importance of RELM-β in *H. polygyrus* infection is illustrated by a delayed expulsion of worms during a secondary infection in RELM-β knock-out mice [78].

1.3.5 *Epithelial permeability*

Only modest changes in epithelial permeability are seen during primary *H. polygyrus* infection, but secondary *H. polygyrus* infection results in increased permeability of the jejunal epithelium [79]. Helminth infection can trigger increased epithelial permeability in a myriad of ways [80]. As discussed above goblet cell-derived RELM-β can increase permeability, but also direct abrasion of the epithelial layer, modulation of tight junction structures and immune activity (such as mast cells) are suggested to increase epithelial permeability during helminth infection, although the evidence for any of these pathways is not extensive [80]. Yet little is known about the way by which increased gut permeability affects helminth expulsion. It is speculated that permeability could either promote or impair worm expulsion in distinct ways [80]. Increased permeability allows more passage of antibodies, complement and even effector cells into the gut, that aid in worm expulsion [80]. However, in contrast, with increased permeability immunomodulatory products produced by helminths could cross the barrier more easily [80]. Another way in which increased epithelial permeability could impair helminth fitness is by limiting nutrient accessibility, as enhanced fluid influx in the intestinal lumen may interfere with the access to the mucosal tissue for feeding (speculated in [50]). In addition, incoming fluid could dislodge worms from villi, aiding the 'weep and sweep' method of flushing out the worm (speculated in [79]).

1.3.6 Gut contractility

Intestinal smooth muscle cell hypercontractility is observed during several helminth species including *H. polygyrus* by measuring the responsiveness of the enteric nerves to *in vitro* stimulation with acetylcholine [81,82]. Alterations in contractility was dependent on type 2 cytokine signaling in mice infected with *H. polygyrus* [81]. Naturally increased contractility would lead to decreased transit time of any given substance to travel through the intestine. Intestinal transit time was measured during *Ascaris suum* infection in pigs by tracking a radioactive dye, and the researchers found a decreased transit time was associated with worm expulsion [83]. Using a similar method, *N. brasiliensis* was shown to delay gut transit time at certain times too [84]. Recently published work from our laboratory shows that, while whole intestinal transit time is unaffected by *H. polygyrus* infection, transit time locally in the small intestine is increased by the presence of the helminth [85].

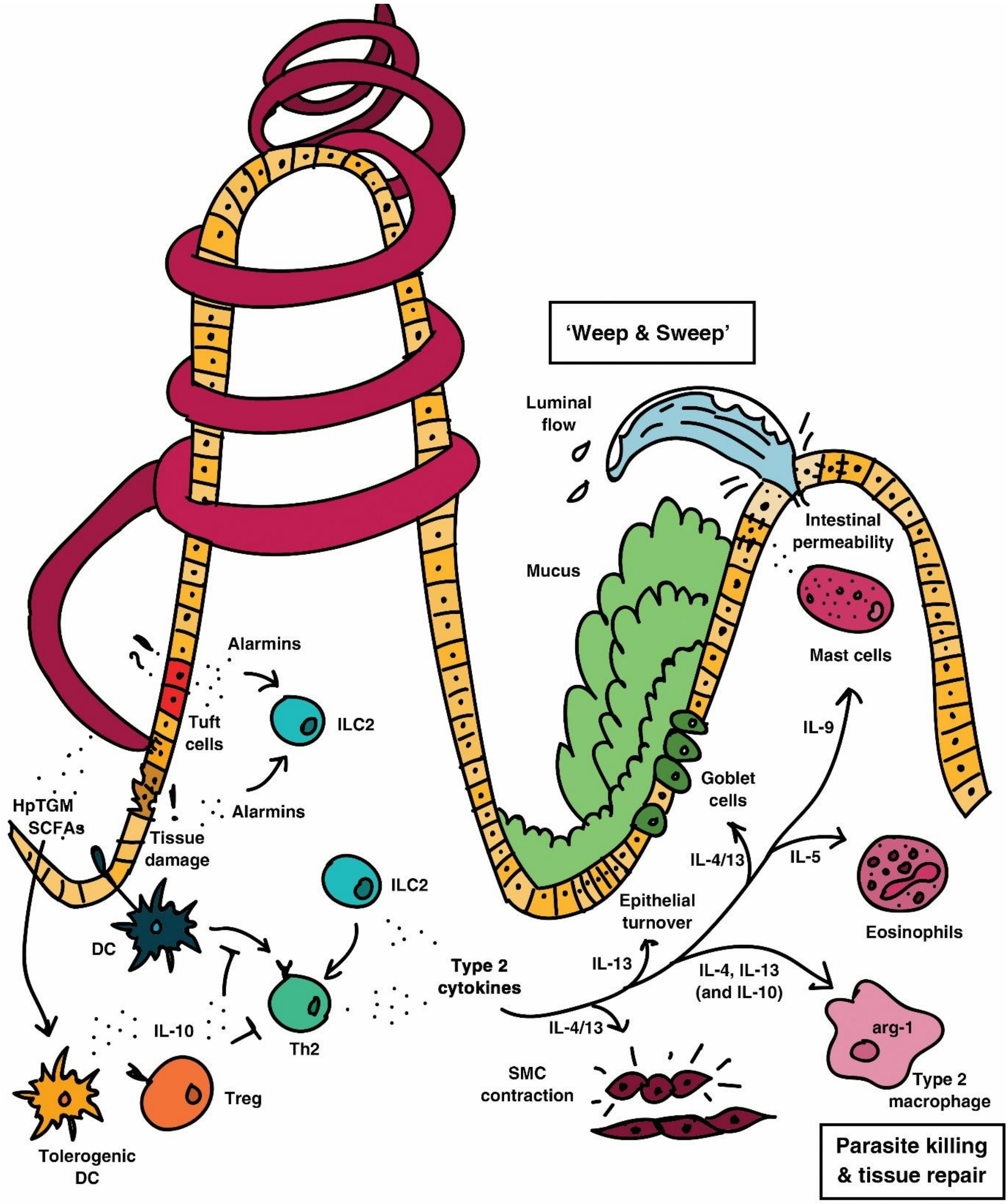

Figure 1.2. Immune responses during *H. polygyrus* infection. Tissue damage and sensing of parasite products by tuft cells results in production of alarmins from the epithelium, which activate ILC2s. Th2 differentiation is stimulated by stimulation of these ILCs, as well as by antigen-presenting cells such as DCs that present parasite antigen to T cells. Alarmin-activated ILCs and Th2 cells produce type 2 cytokines that have a range of effects on the intestinal epithelium and the submucosal tissue and trigger an influx of immune cells and polarization of resident immune cells. Signaling of IL-4 and IL-13 causes smooth muscle hypercontractility, increased epithelial turnover and mucus production.

Together with increased fluid efflux into the lumen due to increased intestinal permeability, which may be mediated through mast cells, these physiological changes are effective in dislodging worms from the villi and flushing the worms out of the intestine, in a reaction also referred to as the ‘weep and sweep’ reaction. Further, type 2 immune signaling causes recruitment of eosinophils to the small intestinal tissue, as well as polarization of macrophages to a type 2 phenotype which includes upregulation of arginine-1 (arg-1). These cellular changes are aimed to kill the parasite as well as repair the damaged tissue. To overcome the host protective immune response, helminths induce regulatory immunity, including Tregs and tolerogenic DCs. *H. polygyrus* achieves this in several ways, for example through production of a *H. polygyrus* mimic of TGF-β (HpTGM), or through harnessing the immunomodulatory properties of the resident microbiota species that produce SCFAs that can induce regulatory immunity.

1.3.7 *Type 2 immune signaling*

The effector mechanisms described above that work together in the ‘weep and sweep’ reaction are part of the type 2 immune response, which is primarily mediated through IL-4Rα- and Stat6-mediated pathways [58] that are activated by the cytokines IL-4 and IL-13. During helminth infection, the type 2 immune response is initiated at the epithelial interface, with alarmins TSLP, IL-25 and IL-33 released that induce ILCs to produce IL-13 and other cytokines. In the absence of IL-25 or IL-33 immunity to helminth infection is impaired [86], as well as with IL4-Rα or Stat6 deficiency [87]. IL-4Rα responds to both IL-13 and IL-4 and a IL-4Rα-dependent immune response includes ILC2s and Th2 cells that produce large amounts of type 2 cytokines such as IL-4, IL-5, IL-9 and IL-13 [88]. IL-4 production by Th2 cells is critical for protection against *H. polygyrus* infection, diminishing egg production and expelling worms [89]. Immunity to secondary infection can be reduced by an anti-IL-4 blocking antibody but only completely abolished when IL-4Rα is blocked [89]. IL-13 that can also signal through IL-4Rα seems to be able to compensate when IL-4 is blocked, but when both cytokines are not able to signal through their receptor, protection against *H. polygyrus* is completely lost. After primary infection, $CD4^{+}$ $IL\text{-}4^{+}$ T cells are found in distant organs such as the lungs and the liver which seem to be reservoirs for memory Th2 cells [90,91]. Antigen-specific Th2 cells that are activated during helminth infection can induce a humoral immune response, with parasite-specific immunoglobulin(Ig)G and IgE, which for immunity to *H. polygyrus* infection is important in a secondary infection only [92].

Another phenomenon that is orchestrated by type 2 immune signaling are the formation of granulomas, which are associated with resistance to helminths [93]. Within the granulomas, the host immune response focuses on the destruction of larvae that are entrapped in the submucosal layer of the intestine, and granulomas may be involved in tissue repair while the larvae grow and after adult worm escape into the gut lumen [94]. Eosinophils and macrophages are key players in the granuloma [94] and are recruited in large numbers to the small intestinal lamina propria during tissue-stage of *H. polygyrus*, and macrophage numbers are still high during the chronic stage with luminal parasites [55]. These two types of innate immune cells are discussed into further detail below.

1.3.8 *Eosinophils*

Type 2 immunity is employed in response to a parasite challenge and eosinophils are historically regarded the key cell type in this response, a cell specialized in parasite-killing. An increase in the number of eosinophils in tissue is a classic sign of helminth infection [95]. During type 2 inflammation, IL-5 production results in recruitment of eosinophils to the site of infection [95]. Eosinophils are innate immune cells equipped with granules with components of the complement system and other toxic contents, such as major basic protein, that upon release can kill parasites [95]. However, most statements about the role of eosinophils in parasite killing are based on *in vitro* evidence, the role of eosinophils in an *in vivo* helminth infection is less understood [96]. Mice that have a genetic deficiency of eosinophils showed impaired resistance to secondary infections with *N. brasiliensis* [97]. Interestingly, more larvae managed to migrate to the lung in eosinophil-deficient mice, but expulsion of adult worms in the intestine was unaffected [97]. Further, when *N. brasiliensis*-induced eosinophilia was blocked with an anti-IL-5 antibody, this had no impact on adult worm recovery [98], providing additional evidence that eosinophils are not effective at killing adult worms. Anti-IL-5 treatment during *H. polygyrus* infection had no impact on worm burden either [99]. Genetically eosinophil-deficient mice also had similar *H. polygyrus* burdens to their wild-type counterparts [100]. From *in vitro* experiments eosinophil-mediated parasite killing seems to be dependent on antibodies [101], thus eosinophils may be important in protective immunity during secondary infection with *H. polygyrus*, as humoral immunity is crucial in protection here [50]. Another role of eosinophilia in tissues is wound healing, and eosinophils express a wide variety of tissue repair regulators such as RELM-α, metalloproteases and their inhibitors, and mediators that facilitate immune cell infiltration [102]. Eosinophils can also contribute to amplification of type 2 inflammation by producing cytokines such as IL-4 [102].

1.3.9 *Type 2 macrophages*

During type 2 inflammation, tissue-resident macrophages proliferate and polarize to a type 2 phenotype (also referred to as M2). An accumulation of type 2 macrophages is found in the submucosa during secondary *H. polygyrus* infection and are associated with increased worm expulsion [103]. Type 2 macrophages are central to the anti-helminth response and tissue repair mechanisms through key molecules such as arginase-1, RELM-α and Ym1 [104,105]. Arginase-1 can directly harm parasites [103], while RELM-α and Ym1 are involved in tissue remodelling [106]. Other M2 molecules involved in tissue remodeling are TIMPs, inhibitors of metalloproteases, and IGF-1 that promotes fibroblasts [104,105]. The M2 phenotype and macrophage-mediated tissue repair is dependent on IL-4 and IL-13 signalling through IL-4Rα [107].

1.3.10 *Regulatory immune response*

To establish chronic infections, helminths must bypass or evade host immune responses devised to kill the parasites. Helminths are masterful manipulators of the host immune response. The most discussed immunomodulatory effect of helminth infection is the induction of Tregs. Tregs are induced during helminth infection and play a dual role: they protect the host from tissue damage due to excessive inflammation, while they also reduce protective immunity, and thus permit helminths to establish chronic infections [2]. Tregs and other regulatory components such as tolerogenic DCs can be induced directly by *H. polygyrus* ES products or indirectly by harnessing the immunomodulatory qualities of the gut microbiota [49]. Amongst many other well-characterized products, *H. polygyrus* ES material contains a transforming growth factor (TGF)-β mimic (HpTGM) that potently induces Tregs just like mammalian TFG-β [30]. Helminth infection causes shifts in the commensal and mutualistic microbes that are resident to the mammalian intestine, which in turn can also lead to induction of Tregs, for example via increased microbial production of SCFAs, a group of immunomodulatory metabolites [23]. The molecular pathways by which helminths modulate immunity are of special interest in the search of treatment options for inflammation-driven diseases such as allergies. Immunomodulation by helminths, and especially immunomodulatory routes via the gut microbiota and metabolome (collection of metabolites present in the gut) will form an important focus of this thesis and will be discussed in further detail later in this chapter.

1.4 Helminth co-infection with pathogenic bacteria

The first of the major research questions this thesis will address is the question as to what extent helminth infection affects immunity to concurrent infection with a bacterial pathogen. Living conditions in world areas that are endemic for helminth infection are also highly conducive to transmission of microbial pathogens. Epidemiological data shows that helminth endemic areas are also burdened with high prevalence of malaria, tuberculosis (TB) and human immunodeficiency virus (HIV) infection [108]. Poor sanitation standards and a (sub)tropical climate are key factors in why infectious disease is more widespread in certain areas in the world. The risk of co-infection with helminths and other pathogens, such as pathogenic bacteria, is very high in these areas. Bacteria can cause serious infectious disease, such as TB or life-threatening diarrhea, which contribute to morbidity and mortality in helminth-endemic areas. It is therefore worthwhile studying how helminth infection affects immunity to bacterial pathogens.

1.4.1 Human co-infection studies

Numerous studies have looked at the association of helminth infection and the incidence of malaria, TB, and HIV infection in human populations. Enough studies on these human co-infections were available for researchers to do a thorough meta-analysis in 2019 of 211 human studies through which they found that HIV and TB were positively associated with infection with one specific soil-transmitted helminth, *Strongyloides stercoralis* [109]. The odds ratios from the meta-analysis of studies looking at other helminth-TB pairs and helminth-HIV pairs were positive, but not significant [109]. Similarly, helminth-malaria co-infection studies did not produce a significant odds ratio [109]. Meta-analyses have limitations, for example, publication biases and lack of consistent testing techniques may result in over- or underestimations of effect size. On the other hand, meta-analysis such as these are useful in understanding co-infection risk in human populations across different world areas, and help informing treatment strategies that can be applied in all helminth-endemic areas. In this thesis, we are primarily interested in studying helminth co-infection with intestinal bacteria in a mouse model system, which could further our understanding of infection dynamics at the mucosal interface of the mammalian intestine and help combat bacterial-induced gastroenteritis and diarrheal disease. Human studies on these types of co-infection are much more scarce. Recently, a study was published on the association between schistosome infection and *Salmonella* burdens in human intestine [110]. High burdens of *Schistosoma mansoni* but not a light or moderate schistosome infection were associated with presence of intestinal *Salmonella* in humans living in a helminth-endemic community in a rural area of the Democratic Republic of Congo [110]. A possible interaction between *Salmonella* adhesion factors and the schistosome tegument (outer layer that covers the worm) that was found *in vitro* [111], and could be behind this association, as the tegument may provide a favorable colonization surface for *Salmonella*. The authors show that *Salmonella* can evade antibiotic killing by attaching to the tegument of the worm *in vitro* [111]. In addition to complications for antibiotic treatment of *Salmonella* in helminth infected individuals, the authors also suggest that deworming of co-infected people may lead to a release of large amounts of occult bacteria that were previously attached to the worm's surface [111]. Whether this is true has yet to be investigated, as well as whether bacterial adhesion to the helminth tegument is a mechanism of co-infection in mammalian hosts.

1.4.2 Mouse co-infection studies

Mechanistic studies looking into foundations of co-infection that require infections with harmful pathogens are not ethical to do in human subjects, thus animal models are of great importance for this research. Specifically, mouse models are very useful, due to the variety of available tools in these models, such as genetic knockouts. In mouse models, several research groups have uncovered ways in which helminths may impair immunity to concurrent bacterial infection, which are discussed below. A recurrent theme is the modulation of the anti-bacterial responses by type 2 immunity induced during helminth infection. In the bladder, schistosome eggs can impair immunity to uropathogenic *Escherichia coli* (UPEC), which Hsieh *et al.* show depends on IL4-Rα signaling that lowers CD1b expression on antigen-presenting cells, which in turn abrogates interferon gamma (IFN-γ) production by natural killer T (NKT) cells, something that is crucial to fight a bladder infection with UPEC [15]. Furthermore, Potian *et al.* showed that infection with *N. brasiliensis* impairs host resistance to *Mycobacterium tuberculosis* in the lungs through induction of type 2 macrophages [18]. In this study, IL-4R$^{-/-}$ (IL-4R knock-out) mice had a lower *M. tuberculosis* load in their lungs than wild-type mice during helminth co-infection, and when these knock-out mice received macrophages from wild-type mice, resistance to *M. tuberculosis* was once again compromised [18]. Similarly, arginine-1 expressing macrophages during *S. mansoni* infection were shown to be responsible for impairment of host defenses to *M. tuberculosis* [112]. In contrast, *H. polygyrus* infection does not seem to affect immunity to *M. tuberculosis,* despite inducing a strong Th2 response [113]. This highlights that not all helminth species affect immunity to a particular pathogen in the same manner, which could be due to many factors in which the helminth species differ. In this case the differences in co-infection effect could be dependent on the life cycle of the worm, as *N. brasiliensis* has lung phase, while *H. polygyrus* is restricted to the intestine.

In the intestine, *H. polygyrus* infection delays clearance of *Citrobacter rodentium* and exacerbates colonic inflammation that was not observed in co-infected Stat6 knock-out mice [114]. This finding suggests that type 2 inflammation impairs immunity to bacterial pathogens in the colon. Simultaneously, regulatory immunity during *H. polygyrus* infection may also promote bacterial colitis, involving helminth-primed DCs that produce IL-10 [115]. Adoptive transfer of DCs from helminth-infected mice lowered resistance to *C. rodentium* in the recipient mice that were non-helminth infected, and when these mice were subjected to anti-IL-10 antibody treatment, the deleterious effect of the DC transfer was no longer detected

[115]. Interestingly, fecal matter that was transferred from *H. polygyrus*-infected mice to naïve mice contained immunomodulatory potential and was able to replicate the effect of helminth infection on bacterial colitis [116]. Depletion of Tregs and IL-10 neutralization could revert the effects of the fecal transfer, suggesting that microbiota shifts during helminth infection may be responsible for inducing regulatory immune pathways that exacerbate colitis with *C. rodentium* [116].

1.4.3 Helminth-Salmonella co-infection

In most co-infection studies, as discussed above, inhibition of host immune responses to bacterial pathogens during helminth infection has been attributed to regulatory or type 2 immune responses. In contrast, Reynolds *et al.* demonstrated that *H. polygyrus* infection causes elevated *Salmonella* burdens in the small intestine that occur independently of Tregs or Th2 cells, or IL-4Rα signalling [7]. RAG$^{-/-}$, Stat6$^{-/-}$ and IL-4Rα$^{-/-}$ mice presented with increased *Salmonella* burdens during helminth co-infection, similar to wild-type mice [7]. The underlying mechanism in this co-infection scenario remains to be uncovered and is likely different from the mechanisms uncovered in the helminth-bacterial co-infection studies that are discussed before in this chapter. A possibility is that metabolites released by helminths or shifts in host metabolism or the gut microbiota during helminth infection create a more allowing environment for *Salmonella* to colonize the small intestine. Gross shifts in small intestinal metabolite composition can be seen when mice are infected with *H. polygyrus* [7]. Furthermore, Reynolds *et al.* show the metabolic environment in the small intestine during *H. polygyrus* infection has the capacity to increase *Salmonella* virulence [7]. *In vitro*, *Salmonella* incubated with metabolite extracts from helminth-infected mice have heightened capacity to invade host cells than bacteria incubated with metabolites form naïve animals [7]. *Salmonella* gene expression of virulence factors involved in host tissue invasion, such as *hilA*, was also upregulated when bacteria were incubated with intestinal metabolites extracted from helminth-infected mice [7]. The importance of host invasion in the ability of *Salmonella* to create a niche in the mammalian intestine is discussed further on in this chapter.

Two other studies have investigated helminth-*Salmonella* co-infection in mice and are discussed here. Su *et al.* studied large intestinal colonization with *Salmonella* during *H. polygyrus* co-infection, using streptomycin treatment prior to *Salmonella* infection. Streptomycin pre-treatment is a method that can be used by researchers to enhance *Salmonella* colonization in the luminal compartment of the large intestine by eliminating competition with the resident microbiota to colonize the intestinal niche and forging an exaggerated inflammatory environment [117]. The model is used to understand what happens with mucosal immune responses when *Salmonella* overcomes colonization resistance at large, however the method may not be very informative in terms of understanding how helminths affect bacterial infection. Antibiotic treatment does not only affect the density of the microbiota, but also has secondary effects on the intestinal epithelium [118] and mucosal immune homeostasis [119] that may obscure helminth-specific processes that facilitate bacterial infection. In their streptomycin-model of co-infection, Su et al. show that increased *Salmonella* colonization in the cecum is associated with a reduction in type 3 cytokines IL-22 and IL-17, and a reduced number of neutrophils in the cecal tissue [16]. Similar to this finding, another study showed that Schistosome eggs were able to inhibit IL-17 production in the colon and increased *Salmonella* colonization in the colon of mice that were pre-treated with streptomycin [8].

This thesis aims to further elucidate to which extent helminths affects immunity to *Salmonella* in the small intestine. The approach chosen in this thesis to further elucidate the dynamics of *H. polygyrus/Salmonella* co-infection is deworming, to investigate whether helminth-impaired immunity depends on and the presence of an ongoing helminth infection. As well as providing additional clues to what could be behind elevated *Salmonella* burdens during *H. polygyrus* infection, the outcome of deworming in our mouse model may inform whether the benefits from mass deworming programs may extend beyond protecting individuals in helminth-endemic areas from bacterial infection.

1.4.4 *Deworming in co-infection studies*

Despite deworming being the current practise to treat helminthiasis, little is known about how deworming impacts concurrent bacterial, viral or protozoan infection. The effects of deworming on concurrent infection have been tracked in human population in a handful of studies. For example, deworming was associated with improved T cell count in *Ascaris* co-infected HIV patients in two studies [120,121]. However this was only the case with *Ascaris* co-infection and not with hookworm co-infection in these studies: deworming of hookworm-HIV co-infected patients did not result in improved T cell counts [120,121]. Deworming was also not associated with improved TB outcome in a human population [122]. Whether deworming can prevent newly acquired pathogenic infection has not been tested in humans but has been studied in animals. In a study on helminth-infected African buffalos, researchers found that deworming did not affect the risk of acquiring *M. tuberculosis* infection, but did improve survival rates following onset of TB [123]. Something that comes close to challenging humans with a primary *M. tuberculosis* infection (which is obviously unethical) is testing vaccine responses to the Bacille Camette-Guérin (BCG) vaccine. In a study where researchers did this, they found that deworming of helminth-exposed humans increased mycobacterial antigen-specific vaccine responses [124]. Another possibility for mechanistic work in human populations is taking human material such as lymphocytes from blood (peripherical blood mononuclear cells; PBMCs) and studying immune responses to bacterial challenge *ex vivo*. In this way, researchers detected diminished baseline and mycobacteria-specific TLR signaling in human PBMC from patients with latent TB and concurrent filarial infection, which was restored with anthelmintic treatment [125].

Mouse studies researching effects of deworming on secondary pathogen infection are very scarce. One study showed that deworming of mice is associated with an improved Th1 response to *M. tuberculosis* and resolution of lung pathology associated with TB [112]. Prior to this thesis work it was yet unknown how deworming affects immunity to intestinal bacteria such as *Salmonella* in a mammalian host.

1.5 *Salmonella* infection

The bacterial pathogen *Salmonella enterica* can infect a large range of mammalian species, with a variety of outcomes [117]. *Salmonella* infection can cause gastroenteritis that is mostly self-limiting, but in some cases can result in life-threatening diarrhea. This is especially problematic in places where access to healthcare is poor, for example rural areas. *Salmonella* can also disseminate from the intestine and cause a rampant systemic fever that is known as typhoid fever. The type of disease is dependent on the *Salmonella* serovar and the immune status of the host. The serovars *S*. Typhi and *S*. Paratyphi are causative agents of typhoid fever in humans, while *S*. Typhimurium and *S*. Enteritidis can cause large amounts of intestinal inflammation but rarely go systemic. The latter are non-typhoidal *Salmonella* (NTS) serovars and cause a localized infection in the ileum and colon that manifests as fever, diarrhea and intestinal cramping [126]. NTS disease is a major cause of death due to diarrheal disease, and a leading cause of foodborne disease worldwide [127]. In immunocompromised individuals NTS strains have a chance to breach the gut barrier and cause life-threatening bacteremia [126]. Malnutrition, HIV infection and severe malarial anemia have been shown to be risk factors for developing NTS bacteremia [128]. Bacteremia or typhoid fever due to dissemination of *Salmonella* is not within the scope of this thesis, where the focus lies on a localized intestinal infection with helminths and *Salmonella*. Risk factors specific to intestinal infection with *Salmonella* will not be discussed at large, but as discussed earlier in this chapter, helminth co-infection may increase the risk of increased *Salmonella* in the intestine. The immune responses important in the intestinal phase of *Salmonella* infection are well-studied, and summarized below, as well as the animal models that made those discoveries possible.

1.5.1 Animal model for enteric infection with Salmonella

Mice are naturally susceptible to *S.* Typhimurium, and this subtype of *Salmonella* is often used to model human infections with NTS. A caveat is that mice show bacteremia and systemic dissemination with *S.* Typhimurium infection, whereas in humans this is rarely the case. Systemic dissemination causes typhoid fever-like symptoms in mice, making it more of a model for NTS infection in immunocompromised individuals. In the co-infection model used in this thesis, systemic dissemination of *Salmonella* is not affected by helminth infection, and thus it is not a focus in this thesis and this limitation not a big concern. Another limitation for modeling NTS gastroenteritis in mice is that *Salmonella* in mice does not cause acute mucosal inflammation as seen in human patients [126]. In contrast, infection with *S.* Typhimurium in calves mimics human NTS gastroenteritis very well in this regard, where high levels of inflammation can be observed in the infected intestine [129]. However, mouse models offer clear advantages such as the ease of animal housing and availability of knockout animals and reagents.

1.5.2 Streptomycin model and colonization resistance

A severe gastroenteritis scenario reminiscent of the human condition can be simulated by pre-treating mice with streptomycin. This antibiotic treatment can help defy the ‘colonization resistance’ of the gut microbiota. Colonization resistance is the broad term for mechanisms by which the resident microbes in the gut inhibit growth of invading pathogens, by producing metabolites or just by simply taking up space and competing for nutrients [130]. An example of metabolites produced that confer colonization resistance are SCFAs, which will be discussed in more detail later on in this introduction. Microbiota-derived SCFAs may directly inhibit *Salmonella* colonization by downregulating virulence factors needed for the bacteria to invade host tissue [131]. An example of an potent nutrient competitor of *Salmonella* is the closely related commensal *E. coli* strain Nissle 1917 which more efficiently scavenges iron, a vital nutrient for *Salmonella* colonization, in the iron-scarce environment of the inflamed intestine [132]. In similar ways, other microbiota species compete with *Salmonella* and other invading pathogens. One dosage of 20 mg streptomycin given intragastrically is sufficient to make a significant dent into colonization resistance for at least 24 hours [133], marked by a transient depletion (more than 10-fold reduction in abundance) of the microbiota [134]. Consequently, high numbers of *Salmonella* can establish in the mouse large intestine, causing large amounts of inflammation [133]. The consistency of the stool gets softer, which models the diarrhea-causing properties of *Salmonella* gastroenteritis in a subset of human patients. The streptomycin model is useful in dissecting inflammation-mediated pathways that lead to aggravated NTS gastroenteritis symptoms. However, antibiotic treatment also has unwarranted secondary effects as mentioned before in this chapter and is therefore not used to induce features of diarrhea in this thesis.

1.6 Immune response to *Salmonella* infection and bacterial immune evasion

Salmonella is a facultative intracellular pathogen, and it can also adapt to life outside the cell. The consequences for the host and type of immune response against intracellular versus extracellular colonization of *Salmonella* are discussed below (and summarized in **Figure 1.3**). It is a generally accepted concept that the immune response is beneficial to the host to limit pathogen replication and dissemination. However, under evolutionary pressures, pathogens have developed mechanisms to evade immune responses, at least temporarily, that allow establishment in the mammal host despite host defenses. Moreover, recent research shows that pathogens not only evade, but can exploit host immunity to carve out a niche in the gut and compete with the resident microbiota. *Salmonella* is a prime example of this type of pathogen. Inflammation during intestinal infection with *Salmonella* is a double-edged sword: it is equipped to efficiently kill the bacteria, while simultaneously the bacteria can exploit inflammation to overcome colonization resistance and thrive in the intestinal lumen.

1.6.1 Initiation of inflammatory responses

The key virulence trait by which *S.* Typhimurium instigates inflammation is by the ability to invade host cells in the intestinal epithelium. This ability is mediated by two type 3 secretion systems (T3SS). The first T3SS (T3SS-1) forms a needle-like structure that injects bacterial proteins into the host cell cytoplasm, that cause cytoskeleton rearrangements, resulting in internalization of the bacteria into the epithelial cells [135]. The second T3SS (T3SS-2) is then employed, that allows *Salmonella* to get trafficked in specialized cell compartments, called *Salmonella*-containing vacuoles. These vacuoles allow *Salmonella* to survive in epithelial cells and even hostile environments inside phagocytes such as macrophages [136]. Upon initial interaction with *Salmonella* with host cells, pathogen associated molecular patterns are sensed by and activate pattern-recognition receptors such as TLRs. Lipopolysaccharide (LPS), a component of the Gram-negative outer membrane of *Salmonella*, is a canonical TLR4 ligand. Signaling by *Salmonella* through TLR4 causes IL-23 production by DCs [137], which initiates type 3 inflammation, that is targeted to capture and kill extracellular bacteria and to fortify the epithelial barrier. Other bacterial components are recognized by other TLRs which causes similar downstream effects. The biofilm of *Salmonella* contains aggregated fibers called curli that are detected by TLR2 [138]. Flagella are surface structures on the bacteria that are recognized by TLR5 at the basolateral side of intestinal epithelial cells [139].

1.6.2 IL-17 signaling and neutrophils

An important part of the first line of defense to extracellular *Salmonella* is the recruitment of neutrophils. Type 3 cytokine IL-17, produced downstream of TLR activation and IL-23 signaling, contributes to neutrophil recruitment by 1) inducing epithelial production of CXC chemokines that attract neutrophils to the tissue, and 2) stimulating granulocyte production in the bone marrow [140]. Neutrophils follow the chemokine gradients and extravasate into the gut mucosa, where they encounter and kill *Salmonella* in the small window of time that the bacteria are moving from cell-to-cell. *Salmonella* in tissue is not found to replicate extracellularly, but has a brief extracellular phase when it moves to a new host cell, rendering it susceptible to neutrophil killing [126]. In addition to the benefit conferred for host defense, neutrophils can cause a lot of collateral tissue damage in the mucosa, and as a result, extravascular fluids can leak into the gut [129]. Neutrophil recruitment is therefore a double-edged sword that prevents further tissue colonization by bacteria but also contributes to diarrheal disease during *Salmonella* infection. So far, it has not been described whether *Salmonella* has evolved mechanisms to evade neutrophil killing. Moreover, it is understood that neutrophils are indispensable to limit extracellular growth of *Salmonella* in tissue [126].

1.6.3 IL-22 signalling and antimicrobial defense in the gut lumen

Another arm of the immune system, orchestrated by IL-22 signalling, offers first defense against invading microbes by fortifying the barrier function of the intestinal lining, through production of mucus and antimicrobial products (AMPs) by epithelial cells. IL-22 that is elicited during *Salmonella* infection can enhance Muc4 production [141], one of the mucins found in mucus. While mucus is impenetrable for many microbes and thus works as a natural barrier, *Salmonella* uses flagella-mediated motility, fimbriae-mediated adhesion and chemotaxis to colonize the mucus layer in the intestine [126]. In the mucus, *Salmonella* encounters AMPs excreted by the epithelium that make the mucus a hostile environment for bacteria. Thus, to reach the epithelial cells and invade host tissue, or to replicate in the mucus layer, *Salmonella* must also find protection against AMPs. In response to *Salmonella* infection, large quantities of lipocalin-2 are produced that accumulate in the lumen [141]. Lipocalin-2 prevents iron acquisition by binding to the common bacterial iron chelator, enterobactin [142]. Enterobactin is a type of siderophore, released by bacteria to scavenge iron in direct environment. Enterobactin is expressed by most members of the *Enterobacteriaceae*, including *Salmonella* [142]. However, *S.* Typhimurium also releases another siderophore named salmochelin, a derivative of enterobactin [143]. Salmochelin cannot be bound by lipocalin-2, therefore it offers *S.* Typhimurium resistance to this anti-microbial protein [144]. Salmochelin expression confers a big benefit in the inflamed intestine, whereas in absence of intestinal inflammation no fitness benefit is observed [141]. In addition, *Salmonella* expresses a high affinity zinc transporter that allows the bacteria to acquire zinc even in the presence of calprotectin, a protein complex that sequesters zinc [145,146]. Other virulence genes confer resistance to antimicrobial peptides, like defensins [126]. Overall, resistance of *Salmonella* to antimicrobial proteins and peptides represents a specific adaption to growth in the inflamed intestine. Because resident microbiota species are not well equipped against inflammatory attack, *Salmonella* faces less competition in the intestine and can cause large luminal blooms. Mice deficient in IL-22 had reduced levels of lipocalin-2 and calprotectin, leading to a loss of the competitive advantage of *Salmonella* over AMP-susceptible, closely related commensal species [146]. In fact, commensal *E. coli* outcompeted *S.* Typhimurium in the gut lumen of IL-22-deficient mice [146].

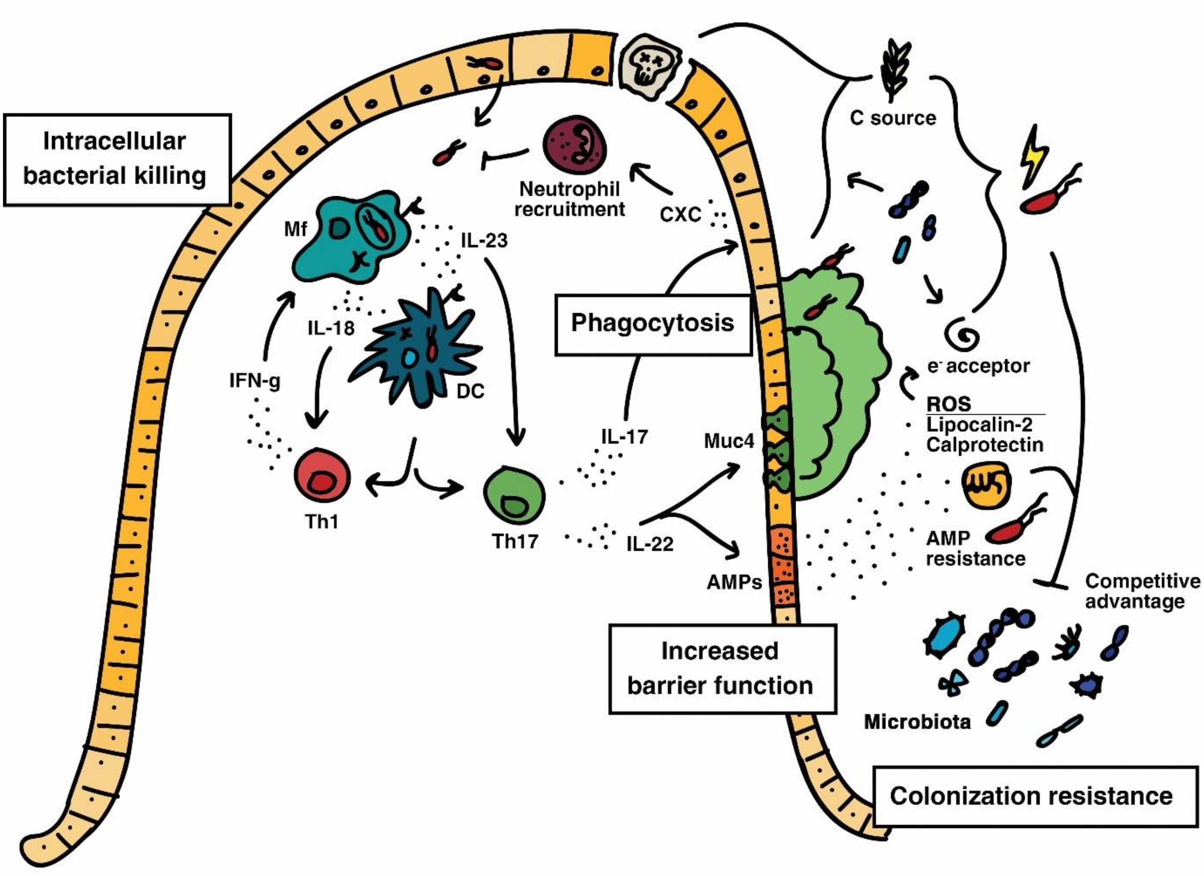

Figure 1.3. Immunity to *Salmonella* infection and bacterial immune evasion. Detection of extracellular bacteria leads to type 3 inflammation mediated by IL-23/IL-17 and IL-23/IL-22 signaling

axes, leading to phagocytosis (CXC chemokines attract neutrophils that kill bacteria by phagocytosis) and increased barrier function (more mucus and antimicrobial products). In the lumen, the resident gut microbiota confers colonization resistance, limiting pathogenic outgrowth. However, oxygenation of the intestinal lumen during *Salmonella* infection due to ROS species, and sulfate reduction by microbiota species, provide *Salmonella* with new electron acceptors that allow for utilization of additional carbon sources that are unavailable for the resident bacteria. These carbon sources include ethanolamine released from dead epithelial cells, and sialic acid released by microbiota-mediated fermentation of the mucus layer. Together with resistance to AMPs, these new energy sources give *Salmonella* a competitive edge and allow luminal blooms of *Salmonella* in the inflamed intestine. Detection of intracellular bacteria by the host immune system leads to IL-18 production and initiation of type 1 inflammation, which includes IFNγ production, which activates macrophages and increases intracellular killing within the cell (classically activated macrophages have a M1 phenotype). Without the IFNγ-mediated feedback, *Salmonella* can persist within the cell by forming and hiding in specialized vacuoles.

1.6.4 Salmonella thrives in the inflamed gut due to newly available energy sources

Another facet of the innate immune response to *Salmonella* infection and resulting inflammation, is generation of reactive oxygen species (ROS) by NAPDH oxidase, and reactive nitrogen species (RNS) by iNOS [147]. The important role of ROS in defense against *Salmonella* was demonstrated in mice deficient in NADPH oxidase, which were more susceptible to luminal blooms of *Salmonella* [148]. Similarly, RNS are important to inhibit *Salmonella* growth, as iNOS-deficient mice have increased *Salmonella* colonization in the intestinal tissue [149]. *Salmonella* can withstand low levels of oxidate stress with a ROS efflux pump that contributes to *Salmonella* replication in the intestinal lumen and inside macrophages [150]. Similarly, *Salmonella* is susceptible to high levels of NO but has evolved mechanisms to defend against moderate levels of RNS [151–153]. Although increased ROS and RNS production are of importance for host survival during *Salmonella* infection, some downstream effects of oxidative stress can in fact promote *Salmonella* colonization. The highly oxidative environment resulting from RNS and ROS release is not permissive for obligate anaerobes in the gut, that constitute more than 90% of the healthy gut microbiota, while *Salmonella* is a facultative anaerobe and can survive in oxidative environments [147]. In addition to reducing competition for *Salmonella* to colonize the gut, the oxidative environment offers electron acceptors that allows the pathogen to utilize additional carbon sources and establish high numbers. One example of those carbon sources is ethanolamine. Ethanolamine is generated from phospholipids coming from the membranes of dying intestinal cells that are sluffed off the end of the villi during epithelial turnover [154]. Because the turnover rate of enterocytes is so high, ethanolamine gets released into the intestinal lumen constantly. Ethanolamine cannot be fermented and thus is not metabolized by the microbiota [154]. However, the electron acceptors generated during inflammation enable *Salmonella* to metabolize ethanolamine and offer the pathogen a competitive advantage over the microbiota [154].

1.6.5 With a little help from my friends: some microbiota species help Salmonella thrive

The electron acceptors generated during inflammation that *Salmonella* can use include tetrathionate and nitrate [155,156]. Nitrate is a product released by the host cells during inflammatory processes, while the microbiota is responsible for the formation of tetrathionate [155,156]. Tetrathionate is formed from hydrogen sulfide that is produced in large quantities by the microbiota, and that is detoxified by the host to thiosulfate and then in the presence of ROS converted to tetrathionate [155]. In general, the microbiota assists host defenses by inhibiting pathogenic colonization, but here certain microbiota species enable pathogenic growth. There are more examples of how certain microbiota species aid *Salmonella* in establishing a niche in the intestinal lumen. Certain microbiota species help *Salmonella* get access to carbohydrate sources, for example, *Bacteroides thetaiotaomicron* that cleaves sialic acid from mucins in the larger structure of mucus [157]. Although *B. thetaiotaomicron* does not have the capacity to metabolize sialic acid, it presumably cleaves it to access underlying carbohydrate sources. *Salmonella* however, can metabolize the released sialic acid and access to it supports the growth of the pathogen [157]. Another microbiota species that can help *Salmonella* thrive is *Akkermansia municiphilia*, which disrupts the mucus layer and thereby exacerbates *Salmonella*-induced intestinal inflammation [158].

1.6.6 Macrophages: dead-end or road to further colonization?

Detection of intracellular bacteria leads to IL-18 production which can differentiate Th1 cells that produce large amounts of IFNγ, a pro-inflammatory cytokine that activates macrophages and restricts intracellular growth of bacteria [126]. Unactivated or type 2 macrophages form a preferred niche for *Salmonella* [159], while classical activation of macrophages polarizes the macrophage to a more pro-inflammatory killing machine. In the absence of a strong Th1 response *Salmonella* can quickly spread by hitchhiking with macrophages that travel to the MLNs and to systemic organs [160]. Typically, host tissue invasion is a strategy to establish systemic infection, but in the case of *Salmonella* infection it may serve another purpose too. Tissue-invading bacteria face a dead-end when adaptive immune responses kick in, but the efforts are not lost because the inflammation resulting from tissue invasion benefits the population of *Salmonella* left behind in the lumen. Such an act of self-destructive cooperation in bacterial infection can evolve through heterogeneity in the bacterial population [161]. Indeed, fimbriae are only expressed in a fraction of the bacterial population [162], allowing these bacteria to adhere to mucus, while the other fraction keeps swimming towards the epithelial tissue. The swimmers will go on to their suicidal mission, from which the mucus-dwelling *Salmonella* ultimately benefit.

1.7 Helminth immunomodulation through microbes and metabolites

Multiple mechanisms contribute to the modulation of host immunity during helminth infection. These include the products produced directly by helminths that have direct effects on host immune cells [31], as well as the indirect effects on host immunity, through an altered composition and function of the gut microbiota during helminth infection [163]. The pathways by which a helminth-modified microbiota affect host immunity have only recently begun to be explored, and it will be important to evaluate the extent to which a helminth-modified microbiota contributes to long-term host immunomodulation following clearance of live helminth infection.

Helminths, microbiota species and mammals share a long history of co-evolution, during which a complex web of intercommunication has evolved. When attempting to understand the mechanistic pathways by which helminths manipulate host disease states, it will be vital to consider the multi-directional relationships between the host, helminths, and the microbiota. These interconnected pathways have been starting to be explored in human studies [164,165], however the challenge remains to discern causal relationships, for which animal models are proving to be invaluable.

Below, I will discuss the reasons why and the ways in which helminths may shift the microbiota composition and what consequences these shifts can have on host immunity as seen in recent publications. Then, founded on this scientific knowledge of the immunomodulatory potential of the helminth-modified microbiota, I will introduce a novel avenue of research in the field of helminth immunomodulation that is less occupied with the question ‘who is there’, as in: which microbiota species are more/less abundant during helminth infection, but rather is focused on ‘what are they doing’, meaning: the metabolites the microbes produce, how helminths affect the metabolite composition in the intestine, and how the helminth-modulated metabolome may affect host immunity. Discovery of novel immunomodulatory properties of intestinal metabolites can form the basis of new therapeutics to treat inflammatory and allergic disease.

1.7.1 Helminth infection causes shifts in the microbiota

The fact that antimicrobial molecules have been described within the ES products of helminths [166–168] suggests helminths have an incentive to control the microbial species with which they share a niche. This may have evolved to protect the host from microbial exposure during helminth infection, or as a helminth-driven strategy for modulating host immune responses [169]. While some aspects of anti-helminth host immunity are dependent on the microbiota [170], it is also possible that helminths have evolved the ability to expand those microbiota species that benefit their own survival. The hatching of *T. muris* eggs in the ceca of mice is dependent on the presence of microbiota [171], and supplementation with *Lactobacillus* species renders mice more susceptible to both *T. muris* and *H. polygyrus* infection [172,173]. In addition, certain microbiota species could benefit from helminth infection, for example *Lactobacillus* species are promoted by helminth infection [172]. This indicates the existence of a mutualistic relationship between helminths and certain microbiota species, which could be a result of a shared aim: regulating mucosal immune responses in order to persist in the intestinal environment.

The bacterial microbiota provides several functions that are vital for the health of mammals. Their presence is required for the development of normal gut architecture [174], for stimulating immune development and for maintaining normal immune homeostasis [175]. Compositional differences within the microbiota have been shown to fine-tune steady-state immune differentiation, for example, specific microbiota species have been associated with the differentiation of mucosal Th17 cells [176–178] and Tregs [178–181]. The bacterial microbiota also provides protection from colonization with pathogenic microbes, either directly, by producing toxins or competing for nutrients and space, or indirectly, by promoting intestinal barrier function [182],[183]. Further, the bacterial microbiota is able to metabolize dietary components that mammals are incapable of digesting [184]. Compositional differences within the bacterial microbiota of individuals has been associated with differing levels of susceptibility to immune-driven diseases, such as allergies and inflammatory bowel disease (IBD) [185,186].

Helminth infection disrupts the composition of the microbiota within their mammalian hosts, as has been reported in numerous studies using different animal-helminth systems (recently reviewed in [187]). Species within the bacterial family Lactobacillaceae, which are capable of inducing host regulatory responses [188], have been widely reported to increase in abundance during helminth infection, irrespective of helminth or host species [172,189–195]. Aside from the commonly described upregulation of Lactobacillaceae family members, reported helminth-elicited microbiota taxonomical changes vary greatly between studies. Such variation likely arises from several factors, not least the species and infection intensity of the colonizing helminth, and their host species. The life cycle, size, and niche of helminths within their mammalian host varies drastically between helminth species and will have a profound impact on their interaction with the microbiota population of the host. Further, the site at which the microbiota is sampled (for example fecal or intestinal sections), and the sampling time-point relative to helminth exposure will impact the recorded impact on microbiota composition, since helminth-elicited taxonomical shifts vary over the course of infection in mice [172,192,196]. In addition, baseline differences in microbiota composition between individuals, that could result from a multitude of factors including host diet [197], will contribute to the impact of helminth infection on microbiota populations.

1.7.2 Putative mechanisms by which helminths impact the microbiota

Enteric helminths may affect the structure of intestinal microbial communities through multiple parallel routes (**Figure 1.4)**. Helminths may modify microbiota composition through the direct antimicrobial activity of their ES products, which has been predicted or demonstrated for components of the ES material of several helminth species [166–168]. The presence of helminths can alter both nutrient and niche availability in the intestine. For example, epithelial glucose absorption has been reported to be impaired during helminth infection [79], which likely favor microbiota species that ferment sugars. Intestinal mucus plays a major role in controlling microbiota populations [175], and changes in mucus dynamics during helminth infection could also favor growth of select species. For example, mucus-colonizing *Mucispirillum* species [198], are increased in abundance in *T. muris*-infected mice and *Trichuris suis*-infected pigs [192,196,199], and helminth-induced mucus production has been associated with a bloom of Clostridial species [200]. Finally, helminth infection may modify both innate and adaptive host immune responses that can promote clearance of intestinal microbes.

Several parameters that likely have downstream effects on microbiota composition are dependent on the type 2 immune response induced during helminth infection. For example, changes in host expression of AMPs during *T. muris* and *Nippostrongylus brasiliensis* infection are associated with the induction of Th2 cell responses in resistant mice [201,202]. During *T. muris* infection, changes in expression of the AMP Angiogenin-4, as well as altered mucin expression patterns depend on signaling through IL-4Rα [74,203]. The initiation of a type 2 response, even in the absence of live helminth infection, can be sufficient to alter intestinal microbiota populations: administering mice IL-25, an initiating factor for type 2 immunity [204], can alter AMP expression levels and alter the abundance of segmented filamentous bacteria, which replicates phenotypes seen in *N. brasiliensis*-infected mice [193].

The effects of a type 2 response on AMP expression and mucus likely reflect the need for the host to stimulate tissue repair pathways as a result of damage caused during helminth infection [205]. This is exemplified in the case of idiopathic chronic diarrhea in captive monkeys where *T. trichiura*-induced IL-4 was associated with repair of helminth-induced epithelial damage, and was accompanied with altered mucosal attachment of bacteria and microbiota composition [206]. Additionally, helminth infection has been reported to cause shifts in microbiota populations independently of IL-4 and IL-13 signaling, as microbiota taxonomical changes have been reported to occur in IL-4Rα-deficient *H. polygyrus*-infected mice [191].

Intestinal mucus forms a physical barrier against microbes but can also provide a niche and energy source for certain microbiota species, and the presence of helminths can affect host mucus in several ways: through affecting mucus-producing goblet cell proliferation [207] and through altering mucin composition, decoration, and degradation [74,208,209]. In addition, the availability of intestinal nutrients may be affected during helminth colonization, as compromised epithelial glucose absorption has been reported in the presence of helminths [79]. Furthermore, helminths and helminth-derived products can regulate host recognition of microbes through influencing host pattern-recognition receptor signaling, such as through altering the expression patterns of TLRs [210–212] and through modulating the responsiveness of immune cells to TLR ligands [213]. Helminths may also affect microbiota composition through interference with the production of microbe-specific IgA in the intestine [214]. These phenomena all form putative scenarios in which helminths leave a significant fingerprint on the gut microbiota.

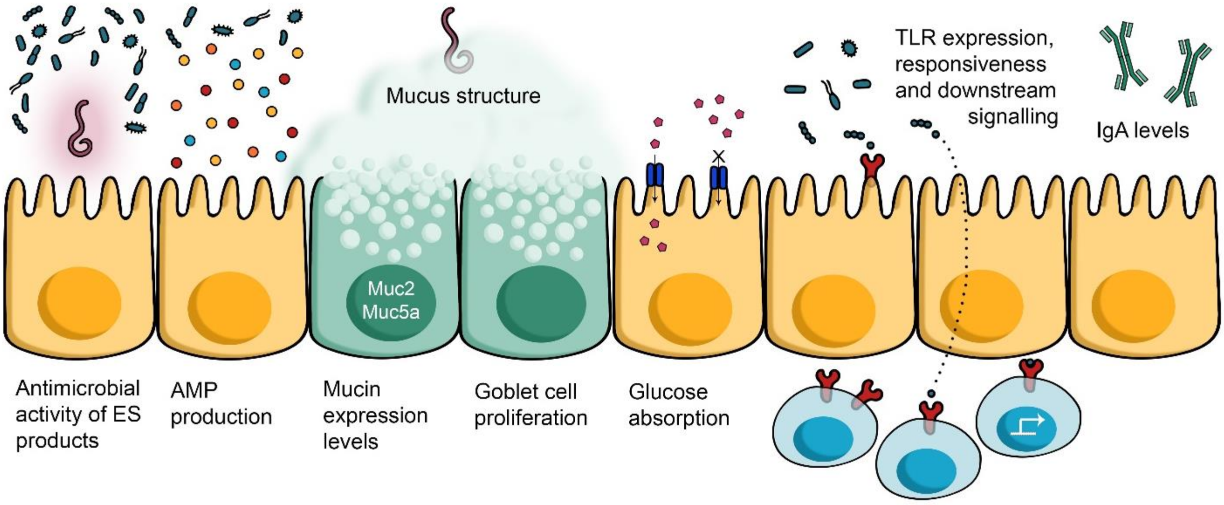

Figure 1.4. Parameters modified during helminth infection which may contribute to gut microbiota modification. Helminths may alter the microbiota composition directly, through the

antimicrobial activity of their ES products, as well as indirectly, through altering host production of AMPs. Furthermore, helminth infection causes changes in mucus structure and production, glucose availability, luminal IgA levels and TLR-mediated immune surveillance which may lead to compositional and functional changes in the microbial communities in the intestine.

1.7.3 Microbiota-dependent immunomodulatory effects of helminth infection

Since intestinal helminths and bacterial microbiota species have a shared agenda (avoiding their own expulsion from the mammalian intestinal tract), several recent studies have examined the extent to which the immunomodulatory effects of helminth infection can be attributed to changes in the gut microbiota (**Figure 1.5**). Transfer of a helminth-modified microbiota alone, in the absence of live helminth infection, has been shown to reduce airway inflammation in recipient mice in a mouse model of allergic asthma, suggesting that one of the mechanisms by which helminth can mediate allergic inflammation is through modifying the microbiota [23]. Further, while infection with *H. polygyrus* reduced the severity of allergic lung inflammation in mice, protection was lost when *H. polygyrus*-infected mice were treated with antibiotics, indicating that an intact microbiota was required for protection from airway inflammation in this experimental system [23].

A helminth-modified microbiota may also play a role in protecting from respiratory syncytial virus (RSV) infection: a pathogen which has been associated with an increased risk for allergic asthma development [215]. In a mouse model, coinfection with *H. polygyrus* ameliorated RSV-induced lung pathology and reduced viral loads, and this was dependent on the presence of the microbiota, as helminth-mediated protection from RSV was lost in germ-free mice [216]. Microbiota-dependent shifts during *H. polygyrus* infection induced type I IFN production in the lung which was required for protection from RSV infection [216]. Protection from RSV was lost in RAG$^{-/-}$ and IL-4Rα$^{-/-}$ helminth-infected mice, suggesting type 2 immunity induced by helminth infection can give rise to protective immunity in the lungs to a viral pathogen [216]. Interestingly, protection against RSV infection in the lungs also occurred after infection with irradiated *H. polygyrus* larvae, which were unable to develop into adult worms [216]. Since *H. polygyrus* larval stages were sufficient to protect against viral infection, the authors propose that the initial intestinal damage caused by larvae migrating into intestinal tissue and coincidental translocation of microbiota species into the tissue may evoke a systemic innate immune response, facilitating an antiviral response in the lungs [217].

In the intestinal tract, the bacterial microbiota can provide colonization resistance, therefore a loss of beneficial microbiota members has the potential to contribute to infectious disease [183]. Since helminths alter the microbiota composition [187], helminth colonization may protect against bacterial-driven intestinal inflammation by promoting the expansion of protective members of the microbiota that inhibit outgrowth of inflammatory bacteria. For example, a helminth-induced bloom of Clostridiales species blocked a bloom of pro-inflammatory *Bacteroides vulgatus* and ameliorated disease in a mouse model of IBD [200]. In contrast, during a mouse model of *C. rodentium*-driven colitis, a helminth-modified microbiota exacerbates, rather than protects from, intestinal inflammation [116]. Transfer of a *H. polygyrus*-modified microbiota in the absence of live helminth infection was sufficient to enhance colitis pathology initiated by *C. rodentium* infection [116]. These two studies demonstrate that the ability of a helminth-modified microbiota to protect from intestinal inflammation likely depends on the bacterial species that are driving inflammation. Together, these findings indicate that there are several mechanisms by which helminth colonization causes host immunomodulation, with the helminth-modified microbiota driving immunomodulation independently of live helminth infection in several scenarios, and that the outcomes on disease development are heavily context-dependent.

1.7.4 Metabolites derived from microbiota contribute to immunomodulation during helminth infection

The findings described above also highlight the fact that helminth infection in the gut can affect immunity at a distant site in the body, such as the lungs. How this gut-lung axis works in terms of immunomodulation is not very well understood, but the link to microbiota offers useful insights into cross-organ immumodulatory pathways. Helminth suppression of allergic airway inflammation can be mediated, at least in part, by a helminth-modified microbiota that is enriched in the capacity for SCFA production [23]. Evidencing this, *H. polygyrus*-infected mice showed a bloom in SCFA-fermenting Clostridial species and a corresponding increase in cecal SCFA levels, transfer of a *H. polygyrus*-modified microbiota was positively correlated with SCFA production, and antibiotic treatment of *H. polygyrus*-infected mice entirely abolished SCFA production and reverted the suppressive effects of helminth infection on airway inflammation [23]. Further, the effects of helminth infection on allergic inflammation were dependent on expression of GPR41, a major SCFA receptor [23,218]. A helminth-modified microbiota with a high capacity for SCFA production likely contributes to the amelioration of allergic airway inflammation through the induction of Tregs [25,219,220]. In support of this, helminth-infected mice exhibited GPR41-dependent increases in IL-10 and TGF-β in the lung during allergic airway inflammation [23]. Microbial fermentation of dietary fibers may also modulate immunity during intestinal inflammation, since Treg expression of an additional SCFA receptor, GPR43, provided protection against a T cell transfer-induced mouse model of colitis [33]. In accordance with this, transfer of a helminth-modified microbiota boosted Treg frequencies and IL-10 expression in the MLNs [116] (**Figure 1.5**). Tregs can contribute to effective immunity against *C. rodentium* through promoting Th17 induction [221], but in the context of a helminth-modified microbiota transfer, induction of Tregs has been associated with susceptibility to *C. rodentium* infection [116]. Antibodies against CD25, and IL-10 neutralization, blocked the effects of a helminth-modified microbiota transfer on *C. rodentium* infection [116], providing additional evidence that helminth can induce regulatory immunity through modifications in the microbiota. It will be important to further characterize how helminths affect the production of microbiota-derived metabolites and which consequences this has for host immunity.

1.8 Metabolite shifts during helminth infection

Modifications in the composition of the microbiota during helminth infection are difficult to pinpoint in a reproducible and consequent manner, as discussed above, and may not be as insightful as to how these microbiota shifts contribute to immune function as looking at the functional output of these compositional shifts: the microbial products including metabolites. Helminth infection has been widely reported to alter the metabolic capacity of their mammalian hosts (**Table 1.1**). Helminth-elicited metabolic shifts are likely a combined result of altered host-, bacterial-, and helminth-production of metabolites, as well as altered patterns of metabolite utilization, excretion and absorption during helminth infection. Type 2 immune responses, such as those induced during helminth infection [27], can alter host metabolic function [222], as well as modifying bacterial microbiota populations, as discussed above.

The bacterial members of the gut microbiota are major producers and utilizers of intestinal metabolites [184]. Microbiota-derived metabolites have been shown to influence immune system development systemically and at a tissue level, and can fine-tune the immune response after the system has matured [32]. While mammals lack the enzymes required to break down dietary fibers such as cellulose, certain microbiota species in the intestine can digest these carbohydrates through anaerobic fermentation. As a result, SCFAs are released, a group of abundant metabolites (including acetate, butyrate and propionate) that have been shown to influence multiple facets of immunity. Helminths themselves also both use and produce metabolites [223], and several helminth species are able to produce acetate [23,224].

Helminth infection has repeatedly been reported to affect host carbohydrate metabolism (**Table 1.1**), in studies which either described predicted effects based on the detection of specific microbiota species [189,199,225–227], or in studies that directly measured end-products of carbohydrate metabolism [23,192,199,228–230]. While carbohydrate metabolism and concentrations of SCFAs have been reported to be elevated during infections of mice with

H. polygyrus [189][23] and goats with *Haemonchus contortus* [227], a reduced activity of these metabolic pathways has been reported during *Ascaris* infection in humans [226] and *T. suis* infection in pigs [199]. The differential helminth-elicited effects on carbohydrate metabolism may reflect different immune evasion strategies between helminth species. For example, dietary fermentable carbohydrates resulted in elevated cecal SCFA levels and accelerated the expulsion of *T. suis* in pigs [231], therefore *Trichuris* species may have evolved strategies to downregulate carbohydrate metabolism. In contrast, other helminth species might benefit from SCFA production through the induction of a regulatory immune response [33,219,232], which helps to prevent their own expulsion. The identification of key microbial metabolites that are affected during helminth infection (and characterization of their effect on immune responses) will likely provide a greater insight into the mechanisms by which helminth colonization affects immunity than associations between helminth infection and the presence of (potentially redundant) specific microbial taxa.

Shifts in the intestinal metabolome during helminth infection may also which may directly affect the growth and/or virulence of enteric pathogenic bacteria [233]. While metabolites extracted from the intestine of naïve mice have been shown to inhibit the invasive capacity of *S.* Typhimurium [234], metabolites from *H. polygyrus*-infected mice were unable to suppress *S.* Typhimurium invasion of epithelial cells [7] (**Figure 1.5**). A loss of metabolite-mediated inhibition of bacterial invasion may contribute to the increased intestinal colonization of *S.* Typhimurium seen during helminth infection [7].

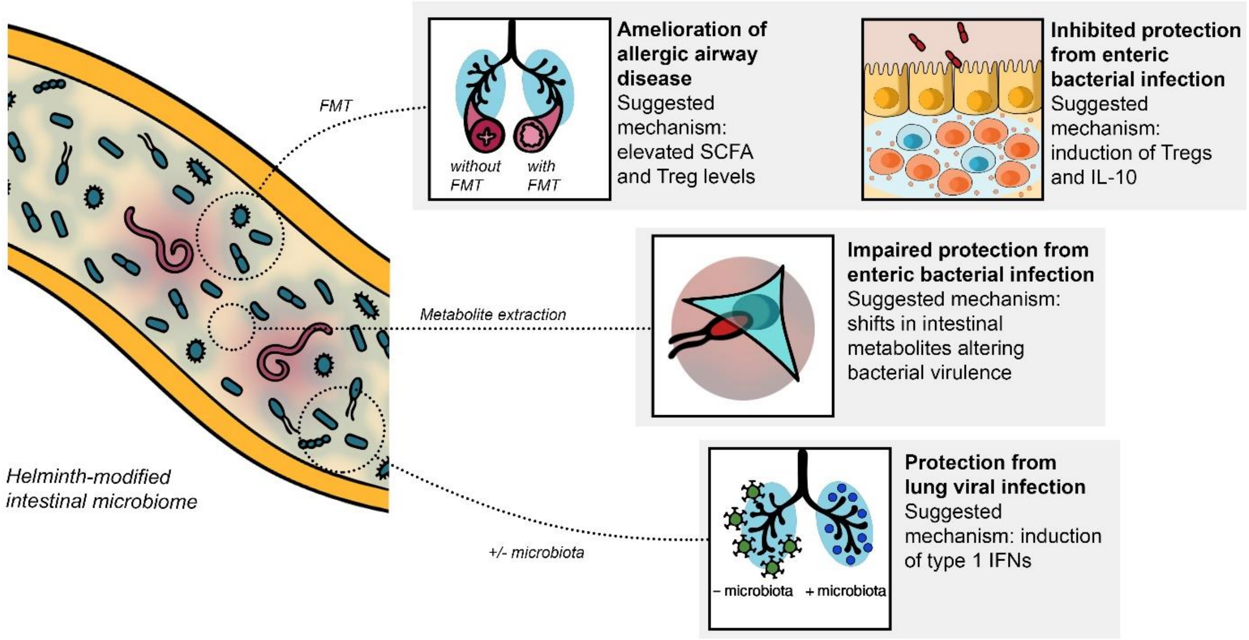

Figure 1.5. A helminth-modified microbiota directly affects host immunity. The immunomodulatory activity of a helminth-modified microbiota has been recently demonstrated in various mouse model systems. Transferring the fecal microbiota (FMT) from helminth-infected mice to non-helminth infected recipient mice can reduce levels of induced allergic airway inflammation in recipient mice, through the transfer of bacteria enriched in the production of SCFAs which likely contribute to Treg induction. A helminth-modified microbiota has been associated with impaired immunity to enteric bacterial infection, as FMT from helminth-infected mice resulted in elevated Treg frequencies and IL-10 levels in the gut-associated lymphoid tissue of recipient mice, alongside exacerbated Citrobacter-induced inflammation. In comparative studies of metabolites extracted from the small intestine of naïve or helminth infected mice, helminth-modified intestinal metabolites promoted the virulence of enteric infectious bacteria compared with metabolites from naïve mice. Finally, helminth infection can promote immunity to respiratory viral infection in mice through the induction of lung type I interferon, which was dependent on the helminth- modified microbiota, as protection from respiratory viral infection was lost in helminth-infected germ-free mice.

More research is required to pinpoint exactly which metabolites are altered in abundance during helminth infection and how these metabolic shifts impact host immunity, including regulatory immunity and anti-bacterial defense. Specifically, SCFAs are of interest, since there is some evidence that helminths impact levels of these metabolites while this has not been extensively explored, and microbially derived SCFAs may contribute to regulatory immunity as described above. Therefore, SCFAs in the context of immunomodulation and in the context of bacterial infection will be discussed in further detail now, as well as another group of metabolites, bile acids, that exist in many forms as the result of an elaborate cooperation between the host and microbiota, and that show immunomodulatory potential and interactions with pathogenic bacteria as well. Prior to this thesis, bile acids had not yet been studied in the context of helminth infection.

Table 1.1. Metabolic consequences of helminth infection. This table details studies that have either predicted (based on bacterial metagenomes) or directly measured shifts in metabolites during helminth infection. Several of the studies presented here report shifts in a vast array of directly identified or predicted metabolites that extend beyond the scope of this thesis. Abbreviations used (not previously mentioned): ^{1}H-NMR, proton nuclear magnetic resonance; ORF, open reading frame; PICRUSt, phylogenetic investigation of communities by reconstruction of unobserved states; KEGG, Kyoto Encyclopedia of Genes and Genomes; COG, Clusters of Orthologous Groups of proteins; GC-MS, gas chromatography–mass spectrometry; LC-MS, liquid chromatography-mass spectrometry; UPLC-FMTS, ultra-high-performance liquid chromatography–Fourier transform mass spectrometry.

Host Species	Helminth Species	Days Post Infection	Site sampled	Analytical Method	Metabolic Shifts Predicted/Measured During Helminth Infection	Ref.
Human (n=447) *rural Uganda*	*S. mansoni*	Unknown	Urine	^{1}H-NMR Spectroscopy and metabolite identities determined using in-house reference data	Several reported shifts, including: acetate ↑ in children, acetate ↓ in adults	[230]
Human (n=51) *rural Malaysia*	various (*Ascaris* and *Trichuris* species, hookworm)	Unknown	Feces	16S rRNA sequencing to identify OTUs, metagenomes inferred by PICRUSt, functional annotation using KEGG database	Amino acid metabolism ↑, carbohydrate metabolism ↓ (associated with *Ascaris*)	[226]
Pig	*T. suis*	21	Colon	1) Metagenomic DNA extraction and sequencing, ORF predicted using FragGeneScan, functional annotation using Pfam, COG and KEGG databases 2) volatile organic compounds analyzed by GS-MS, metabolite identities determined using NIST software and mass spectral library	1) Carbohydrate metabolism ↓, amino acid metabolism ↓ 2) Oleic acid ↑, 2,6-dimethyl-4-heptanone (butyrate metabolite) ↓, 2-methyl-2-butenal (carbohydrate metabolite) ↓	[199]
Pig	*T. suis*	53	Colon	Metagenomic DNA extraction and sequencing,	Amino acid metabolism ↑	[235]

				ORF predicted using FragGeneScan, functional annotation using Pfam and KEGG databases		
Pig	*A. suum*	56	Colon	Targeted metabolomics (GC-MS), metabolite identities confirmed by comparison with SCFA standards	SCFA (propionate, butyrate) ↑	[23]
Mouse *strain: NMRI*	*S. mansoni*	49	Urine	^{1}H-NMR Spectroscopy	Several reported shifts, including: SCFAs (acetate, propionate, butyrate) ↓	[229]
Mouse, *strain: C57BL/6*	*T. muris*	41	Feces	Untargeted metabolomics (GC-MS and LC-MS), putative metabolite identification using PUTMEDID_LCMS workflows	Various, including: various amino acids ↑, Vitamin D2/D3 derivatives ↓, fatty acids (and related metabolites) ↓, dietary plant carbohydrate breakdown products ↓	[196]
Mouse *wild:* Yellow-necked mouse	Various (including *H. polygyrus*)	Unknown	Multiple stomach and intestinal sites	16S rRNA sequencing to identify OTUs, metagenomes inferred by PICRUSt, functional annotation using COG database	Specifically associated with *H. polygyrus* colonization: carbohydrate transport and metabolism ↑ in the cecum	[189]

Mouse *strain: C57BL/6*	*H. polygyrus*	28	Cecum	Targeted metabolomics (GC-MS), metabolite identities confirmed by comparison with SCFA standards	SCFAs ↑ (acetate, propionate, butyrate)	[23]
Mouse, *strain: C57BL/6*	*H. polygyrus*	14	Duodenum and Jejunum	Untargeted metabolomics (UPLC-FMTS), putative identities assigned using Metlin database	Shifts in several metabolite features (putative identities)	[7]
Hamster *strain: SLAC*	*N. americanus*	35	Urine and Serum	^{1}H-NMR Spectroscopy	Various reported shifts, including ↓ acetate in serum	[228]
Goat	*Haemonchus contortus*	50	Abomasum	16S rRNA sequencing to identify OTUs, metagenomes inferred by PICRUSt, functional annotation using KEGG database	Carbohydrate metabolism ↑, amino acid metabolism ↑, lipid metabolism ↑	[227]

1.9 Short-chain fatty acids (SCFAs)

Levels of SCFAs are high in the intestine as a result of bacterial fermentation of complex carbohydrates from dietary fiber. Intestinal SCFAs can serve as energy building blocks for enterocytes, and can have effects on cell proliferation and function (gene expression, differentiation, chemotaxis, etc.) [236]. Through SCFA production the microbiota can modulate different immune cells to create a tolerogenic environment in the gut that allows a certain degree of microbial growth. Microbiota can also use SCFAs to enforce colonization resistance: SCFAs can have deleterious effects on pathogenic bacteria including *Salmonella*. On the other hand, pathogenic bacteria may use differing SCFA concentrations along the gastrointestinal tract as a chemical cue: subtle changes in SCFA ratios and levels are thought to be cues for *Salmonella* to determine its position in the intestine and switch on invasion genes [131,233].

Helminths have a common goal with the microbiota: to modulate immunity to ensure an intestinal niche. Several research groups have measured the levels of the three most abundant SCFAs (acetate, butyrate, propionate) during helminth infection which show that shifts in SCFAs can be different depending on the helminth species, the host and the location and perhaps even the method of quantification (**Table 1.1**). Helminths may produce SCFAs or favor the growth of certain microbiota species that allow for a tolerogenic environment and therefore an appropriate niche for both parasite and microbiota. Below, I will give a short overview of the growing body of literature that describes immunomodulatory effects of SCFAs, and subsequently I will focus on the different effects of SCFAs on *Salmonella* infection that have been described so far.

1.9.1 Receptor-mediated and epigenetic regulation of cell function by SCFAs

Epithelial cells in the intestinal lining express G-protein coupled receptors (GPCRs) that respond to luminal SCFAs. GPR41, GPR43 and GPR109a are the receptors for SCFAs that have been described so far [236]. Along with epithelial cells, many immune cells express these GPCRs too, and engagement with SCFAs can lead to modulation of several immune pathways [236]. Alternatively, SCFAs can enter the mammalian cell and act as regulators of gene expression in an epigenetic fashion [236]. Epigenetic regulation by SCFAs can also lead to immunomodulation. In the large intestine, levels of butyrate and propionate (with butyrate being the most potent) are high enough to inhibit an important class of epigenetic regulators: histone deacetylases (HDACs) [237]. HDACs remove acetyl groups from histones, resulting in tighter wrapping of DNA around the histone (chromatin condensation), repressing transcription [238]. Only high concentrations of SCFAs are sufficient to perturb HDAC function, although local fluctuations could result in transient HDAC inhibition [237]. Furthermore, to inhibit HDACs, SCFAs needs to get into cells which may require active transport. Only 60% of SCFA uptake happens by diffusion, the rest needs to be actively transported into the cell [237]. In an acidic environment, such as the intestinal lumen, there is a higher proportion of protonated SCFAs (e.g. acetic acid, butyric acid, propionic acid) versus SCFAs in conjugated base form (acetate, butyrate, propionate), and this increases the likelihood that SCFA diffuse over cellular membranes [239].

1.9.2 SCFAs promote Treg differentiation and suppressive capacity

SCFAs can promote a regulatory immune response through stimulating the differentiation and suppressive capacity of Foxp3$^+$ Tregs in the intestine [33,219,232]. SCFAs that enter undifferentiated T cells can inhibit histone deacetylation which leads to expression of Foxp3, a canonical Treg transcription factor, thus driving a regulatory phenotype and additionally stimulating production of the anti-inflammatory cytokines TGF-β and IL-10 [33,219,220,240]. In line with Treg expansion are observations that DCs adopt a more tolerogenic phenotype upon stimulation with SCFAs that may in turn engage with undifferentiated T cells and stimulate Treg development, which was dependent on GPR41 signaling [218]. In line with these effects on host immunity, a diet high in dietary fibers has been shown to dampen allergic airway inflammation and enhance tolerance to food antigens in mice [218,220,241]. Induction of Tregs and a tolerogenic environment in the mucosal tissue of the intestine also prevents unwanted immune responses against the resident microbiota and inflammation that accompanies such responses [232].

1.9.3 SCFAs modulate innate immune responses

Another method that bacterial microbiota employs to render the host unresponsive to their presence is to inhibit TLR signaling through SCFA production. When GPCR103a is stimulated with butyrate a decrease in LPS-induced activation of NF-κB, a pivotal regulator of pro-inflammatory gene expression, is observed in a colon cell line [242]. SCFA inhibition of HDACs leads to a similar inactivation of NF-κB, and downregulation of pro-inflammatory TNFα, as seen in LPS-stimulated PBMCs and neutrophils derived from humans and rats respectively [243,244].

Macrophages are also affected by SCFAs, independently of TLR-signaling, and likely through HDAC inhibition [245]. Analysis of bone-marrow derived macrophages (BMDMs) that were supplemented with butyrate and stimulated with LPS showed that TNFα was unaffected and IL-6 was downregulated, together with other inflammatory mediators, IL-12 and NO [245]. Overall, these changes result in a cell phenotype that may render the macrophages unresponsive to the microbes in the gut [245]. In line with this, another research group reported that butyrate promotes the less inflammatory M2 phenotype in BMDMs [246].

In addition to modulating the pro- and anti-inflammatory balance of immune cells and signaling, SCFAs can also have direct effects on epithelial cells that fortify the barrier function of the cells that line the gut. Mucus production was increased in specialized goblet cells in a cell line after supplementation with butyrate [247,248] which limits bacterial colonization in the intestine. Anti-microbial peptide production can also be affected by SCFAs *in vitro* as demonstrated by induction of cathelicidin in colonic epithelial cells by butyrate through HDAC inhibition [249].

1.9.4 SCFAs and Salmonella growth in vitro

SCFAs can affect *Salmonella* independently of immune functions, through modulating invasion gene expression and affecting bacterial growth, both potentially facilitating and impeding *Salmonella* infection in the intestine.

SCFAs can enter bacterial cells, especially at a lower pH such as in the intestine. At lower pH, protonated SCFA molecules are present that can freely pass the bacterial cell membrane and the resulting influx of protons results in acidification of the bacterial cytoplasm [250]. In turn, bacterial growth is inhibited because bacteria prioritizing to restore the internal pH and this energy cannot be used for metabolic processes that allow expansion of the bacterial colonies [251]. The effect of microbiota-derived propionate on *Salmonella* growth was recently shown, and this report shows a detailed dose-dependent effect of propionate on *in vitro* growth, including a prolonged lag time and decreased growth rate, and this correlated with an decreased intracellular pH [252].

While you would expect all SCFAs will acidify the bacterial lumen after entering the cell, SCFAs can have opposing effects on *Salmonella* growth. As discussed above, propionate can decrease growth rates, similar to butyrate, but acetate seems to be a positive signal for bacterial growth [253]. Acetate availability can determine whether *Salmonella* enters stationary phase or continue to grow as the colony ages [254]. Upon synthesis by bacteria, acetate can also be excreted at times, perhaps as a way of chemical communication to signal other bacteria in the colony to expand as well [255].

1.9.5 SCFAs and Salmonella invasion in vitro

While SCFAs can be deleterious for pathogenic bacteria, entry of certain SCFAs (i.e. formate and acetate) into the bacterial cell is thought to be necessary for *Salmonella* to switch to an invasive strategy [131,253,255,256]. In addition, growth inhibition is not necessarily responsible for a decrease in invasiveness, as researchers have found a decrease in *Salmonella* invasiveness upon SCFA exposure, whilst growth was not inhibited [253].

SCFA have differential effects on *Salmonella* invasion genes that are encoded in Salmonella pathogenicity island 1 (SPI1) that encode for the T3SS-1. Acetate and formate induce SPI1, whereas butyrate and propionate repress SPI1 genes [131,253,255,257–259]. Acetate can be metabolized in the bacterial cytoplasm into acetyl-phosphate which induces a response regulator SirA that induces expression of *hilA*, a key promotor of multiple SPI1 genes [131]. Propionate is metabolized into propionyl-coA and this inhibits *hilD*, another key promotor of the SPI1 genes [260]. As a result of this, *Salmonella* preferably infects the region where SCFA concentrations are ideal, which in mice is the terminal ileum (high acetate, low propionate/butyrate) (2).

Not only do SCFAs affect different SPI1 promoters in opposing ways, the pH may also dictate the differential effects on gene expression. Several researchers have found that the effects of SCFAs on *Salmonella* growth and invasion are dependent on the pH [131,253,256,258]. The reason behind this is likely that butyrate and propionate need a pH less than their pKa to freely diffuse over the bacterial membrane (while acetate can pass the bacterial membrane in protonated form as well as the conjugated base form and therefore will not be as affected by the luminal pH) [250].

1.9.6 SCFA and Salmonella in vivo

Due to the complexity of differential SCFA effects on SPI1 promoter genes and the fact that environmental conditions are of great impact on this axis too (pH, osmolarity and oxygen pressure may affect invasion gene expression [261]), I believe that it is more informative to study the effect of SCFA supplementation in animal models of *Salmonella* infection. One such study noted that addition of butyrate to chicken feed decreased *Salmonella* burdens in the gastrointestinal organs in butyrate-fed chickens compared to chickens on control diet [262]. This is in line with *in vitro* findings of butyrate-mediated decrease in SPI1 expression [131,253,258]. Along the same lines, others have tested butyrate in a mouse model of *Salmonella* infection, and specifically looked at the invasive capacity of *Salmonella* by recovering bacteria from Peyer's patches [259]. While butyrate supplementation to drinking water decreased bacterial burdens in tissue, this effect was even more profound when mice were infected with a *Salmonella* mutant that lacked *ydiD*, a gene that allows *Salmonella* to utilize butyrate for energy purposes [259]. As a consequence of genetic ablation of butyrate consumption in the bacteria, intestinal inflammation was attenuated, which was likely achieved through a decrease of bacterial invasion of the epithelium [259]. In contrast, the inhibitory effect of propionate on *Salmonella* infection was proposed to be independent of SPI-1 expression in another study [252]. The authors of this study demonstrated an invasion-independent phenotype in two ways. Firstly, by a minimal recovery of bacterial burdens from the cecal tissue whereas burdens were high in the cecal contents, suggesting there was limited invasion of host tissue but rather *Salmonella* expanded in the lumen [252]. And secondly, they showed that a SPI1 mutant was equally successful in expanding in the cecum than wild-type *Salmonella* [252].

1.9.7 Bacterial resistance to SCFAs

In agriculture, SCFAs such as propionate have been used due to their natural antimicrobial actions, however there are certain risks attached to this as bacteria can gain resistance to the effects of SCFAs. Bacteria have different grades of resistance to acidification by SCFAs, for example, gram-positive bacteria are mostly resistant, since they have increased potassium levels [250]. In addition, a few bacteria can increase potassium levels when SCFAs are present (e.g. certain *E. coli* strains), which allows them to tolerate more intracellular proton accumulation [250].

Another way by which bacteria can resist the adverse effects of SCFAs is by processing SCFAs into something that is not deleterious. Exogenous SCFAs can be metabolized into the carbohydrate cycle and can be used as an energy source by bacteria [256]. An example of this is that *Salmonella* can use butyrate as energy source and as a result the inhibitory effects of butyrate on invasion gene expression are diminished [259]. Similarly, researchers have found that repeated exposure to propionate, as seen in agriculture practices, makes *E. coli* more virulent since it adapts to use the SCFA for efficient growth with several virulence genes upregulated and also genes related to alternative carbon source utilization [263].

1.10 Bile acids

Bile acids are historically considered as simple emulsifying agents to aid fat digestion, however recent studies have discovered the pleiotropic nature of bile acids. Bile acids are hormone-like signaling molecules directly acting on cellular receptors and luminal bacteria, hereby regulating mucosal immune functions and gut inflammation [34]. The composition of bile acids is determined by the enterohepatic cycle and microbial metabolism of intestinal bile acids [264]. Bile acids are synthesized in the liver and transported into bile ducts, stored in the gall bladder, and deposited in the duodenum upon food intake, reabsorbed in the ileum and returned to the liver via the portal vein. Each step in the bile acid circulation is responsive to dietary patterns via hormone signaling, to maintain a functional yet non-toxic bile acid pool [265]. Primary bile acids are synthesized from cholesterol in the liver, while secondary bile acids are generated by microbial metabolism from primary bile acids in the intestine. In humans the primary bile acids are cholic acid (CA) and chenodeoxycholic acid (CDCA), and mice have two extra primary bile acids, which are alpha- and beta-muricholic acid (α/βMCA) and ursodeoxycholic acid (UDCA) [266]. UDCA has only recently recognized as primary bile acid produced in the liver of mice, whereas in humans it is characterized as a secondary bile acid, a product of bacterial metabolism [267]. The final step in bile acid synthesis in the liver is conjugation with taurine (and/or glycine in humans, this does not occur much in mice) and this has two consequences: 1) bile acids can form micelles and solubilize lipids, 2) bile acids cannot cross membranes (since they are fully ionized at physiological pH) and have to be actively transported back to the liver. Active transport occurs in the ileum, in this way 95% of the bile acids secreted in the intestine are recycled [264]. Following reabsorption into the underlying mucosa, accumulated bile acids interact with mucosal immune cells, before ultimately entering the portal recirculation to the liver [268]. The bile acid-microbiota interface seems to integrate nutritional cues to fine-tune innate immune responses to tolerance or resistance [265]. Following reabsorption and transport through ileocytes into the underlying mucosa, accumulated bile acids interact with mucosal immune cells, before ultimately enter the portal recirculation to the liver [268].

1.10.1 Bile acid receptors

The main bile acid receptor that is involved in ileum-liver feedback is a nuclear receptor called Farsenoid X receptor (FXR). Recirculating bile acids from the ileum to the liver suppress *de novo* bile acid synthesis by binding FXR [268]. This signaling suppresses Cyp7A1 expression, the rate-limiting enzyme of bile acid synthesis. Takeda G-coupled protein receptor 5 (TGR5) is a cell-surface receptor for bile acids, and also Pregnane X receptor (PXR) and vitamin D receptor (VDR) are bound and modulated by bile acids. Whereas FXR and TGR5 are ‘dedicated’ bile acid receptors, PXR and VDR are non-specific BA ‘sensors’ that serve to detoxify bile acids during periods of bile acid overload [269]. Bile acid receptors display different affinities for distinct bile acids. The preferred substrate for FXR is taurine-conjugated CDCA in humans and CA in mice, whereas secondary BAs and (in mice) conjugated βMCA are FXR antagonist [270] [267]. TGR5 displays highest affinity for secondary BAs and lower affinity for primary BAs, and recognizes both conjugated and unconjugated forms with different affinity [34] [264] [269]. FXR is expressed throughout the intestinal tract and in the liver, with highest concentrations in the ileum [271]. TGR5 may have extensive effects beyond the liver and intestinal tract, since it is widely expressed including endocrine glands, adipocytes, muscle, spinal cord, enteric nervous system [272].

1.10.2 Bile acids and T cell function

TGR5 is not expressed on T cells but TGR5 signaling can determine T cell fate nonetheless: TGR5 activation can direct differentiation of monocytes into tolerogenic DCs that secrete only low levels of TNFα and IL-12, which are cytokines needed for Th1 priming [273]. Furthermore, administration of a TGR5 agonist increased the numbers of Tregs in the colonic lamina propria during a mouse model of colitis [274]. Bile acid receptors FXR and VDR are expressed in CD4+ and CD8+ T cells [269] and allow bile acids to modulate T cell function. Administration of a synthetical FXR agonist INT-747 elevated colonic Treg numbers, and caused retention of DCs in the spleen, and increased serum levels of IL-10 in a mouse model of colitis [275]. This was associated with reduced homing of inflammatory cell types and increased mucosal homing of

Tregs [275]. Specific bile acids can also act on T cell fate independently of bile acids receptors. One receptor-independent way is by direct binding of bile acids to a major transcription factor, as seen with 3oxo-LCA, a derivative of lithocholic acid (LCA), that binds to *Rorγt* hereby inhibiting Th17 differentiation [276]. Another way in which bile acids can affect T cells is by affecting cellular metabolism, as seen with isoalloLCA, another derivative of LCA, that promotes the release of mitochondrial ROS in T cells that in turn switches on *Foxp3* expression and thus stimulates Treg differentiation [276]. Overall, bile acids can hamper T effector function and induce Tregs, thus helping to maintain a tolerogenic environment in the intestinal mucosa.

1.10.3 Bile acids and innate immune cells

Innate immune cells in the mucosal tissue of the intestine are dynamic sentinels that are responsive to environmental cues such as metabolite gradients [265]. Like adaptive immunity as discussed above, innate immunity generally sees anti-inflammatory effects of bile acid signaling. Macrophages from FXR-deficient mice have a higher pro-inflammatory response to LPS, and FXR activation with INT-747 represses TLR4-dependent pro-inflammatory gene expression in mouse-derived macrophages [277]. Likewise, INT-747 suppresses pro-inflammatory cytokine expression in cultured human monocytes and DCs [278]. In addition, bile acid-dependent TGR5 signaling suppresses a pro-inflammatory phenotype in macrophages in the intestinal mucosa as well as systemically [279,280], instead, TGR5 activation has been shown to drive a M2 phenotype *in vivo* which rescued mice from symptoms of colitis [274]. In line with this, TGR5 activation by endogenous and synthetic bile acids suppresses LPS-induced inflammatory cytokine production in macrophages [281,282]. Similarly, bile acid signaling suppresses NFκB activity [278,283] and increases TFGβ expression [284,285] in innate cells in the intestine. Not only cell function but also cell recruitment can be affected by bile acids, as shown by CDCA supplementation in diet which decreased the number of neutrophils and macrophages in ileum [286].

1.10.4 Bile acids and barrier function

While bile acid signaling has a dampening effect of inflammation in the intestine and renders responses in innate immune cells less pro-inflammatory, the innate intestinal barrier gets activated by bile acids signaling. FXR regulates expression of iNOS and IL-18 expression [271], which are important for the antibacterial defense at the intestinal epithelial surface. INT-747 treatment of mice with experimental colitis showed that FXR activation helps preserve barrier function measured as lower epithelial permeability, and supports antimicrobial defense by increasing expression of NO, angiogenin 1 and cathelicidin [278]. Also fortifying the intestinal barrier, CDCA supplementation in diet increased expression of Muc2 in ileum of mice [286]. Furthermore, a diet supplemented with CDCA increased synthesis of several AMPs in the terminal ileum [286].

1.10.5 Bile acids and Salmonella

Decreased bile flow into the gut due to liver injury or experimental bile duct ligation leads to bacterial overgrowth in the lumen of the small intestine [287–292], whereas feeding rodents bile or conjugated BAs during a state of low bile acid abundance can restore small intestinal overgrowth [293,294]. Until now, researchers have not looked at the effect of reduced bile flow on *Salmonella* directly, but bile duct ligation seems to have the biggest impact on gram-negative and facultative anaerobic bacteria [271,289], to which group *Salmonella* belongs. Therefore, it is possible that *Salmonella* can bloom in similar conditions where bile acids are present in lower quantities. It is thought that bile acids can affect bacterial infection through several ways. Firstly, bile acids can form micelles that can lead to direct lysis of bile-sensitive bacteria [34]. *Salmonella* is bile-resistant as it has a hardy outer membrane [295]. Bile acid signaling through the FXR receptor can also impact bacterial colonization by increasing AMP production [34]. Moreover, bile acids can affect bacterial gene expression, as *Salmonella* culture in murine bile showed decreased expression of major virulence factors and central metabolic genes [296,297]. It is not yet clear how fluctuations in bile acid composition or flow affect *Salmonella* in vivo.

1.11 Research objectives and main methods

1.11.1 Co-infection objectives

It is not well understood how helminths impact immunity to a wide range of secondary pathogens, including bacterial pathogens. There is some evidence in the existing literature that type 2 immunity, Th2 cells and Tregs induced by helminth infection can impair concurrent antibacterial responses, however, there is also evidence that helminths impact bacterial colonization independently of adaptive immune responses (see detailed description of these studies above). This indicates that there are multiple ways in which helminths affect immunity to bacterial pathogens that we have yet to explore in detail. In this thesis, we aim to reach a deeper understanding as to what extent helminth infection in mice affects small intestinal colonization of pathogenic bacteria.

As a model of helminth infection, we infect mice with small intestinal-dwelling *H. polygyrus*, a rodent model of human hookworm, and a natural pathogen of the mouse that has co-evolved with its mammalian immune system and as a result effectively manipulates host immunity and establishes chronic infections. We choose a primary infection model, to study chronicity rather than helminth expulsion, and in most experiments, we infect mice with a high dose of helminths. While this dose is less relevant to most helminth-infected individuals that are more likely do be infected regularly with small doses of helminths, we focus on more extreme cases.

To study bacterial colonization in the small intestine, we infect mice with *S.* Typhimurium. Previously we have seen that *H. polygyrus*-infected mice present with higher *Salmonella* burdens in the small intestine. *S.* Typhimurium can both replicate in tissue and in the lumen of the intestine, using very different strategies to establish in both regions. Which strategies *Salmonella* uses to overcome host immunity in the small intestine during helminth infection is unknown.

To test to what extent helminth infection affects bacterial colonization, we use a drug-cleared helminth infection, using a deworming regimen to expel helminths, to compare susceptibility of mice to *Salmonella* prior and after helminths are cleared. This addresses a question relevant to global health: can deworming protect individuals against bacterial infection? Results from these experiments will inform us to what underlying mechanism is behind susceptibility to elevated small intestinal burdens of *Salmonella* during helminth infection.

Overarching question:

- To what extent does helminth infection affect *Salmonella* colonization in the small intestine?

Major aims:

- Does drug-clearing helminth infection restore resistance to *Salmonella* colonization?
- Does *Salmonella* replicate in the lumen or tissue of the small intestine during helminth infection?

Minor aims:

- Do different doses of helminth infection differentially impact *Salmonella* colonization?
- Does the microbiota contribute to elevated *Salmonella* levels in the small intestine during helminth infection?
- Do eosinophils contribute to elevated *Salmonella* levels in the small intestine during helminth infection?

The investigation of these aims is presented in **Chapter 2**.

1.11.2 Metabolome objectives

Helminths modulate immune responses to establish chronic infections, and researchers have found several helminth products that have direct immunomodulatory functions. Metabolites have been relatively neglected in this search for immunomodulatory compounds derived from helminths. Metabolites can act as immune messengers, and in the intestine the majority of the metabolites are derived from the bacterial microbiota, which is likely part of a communal effort of the intestinal bacteria to create a tolerogenic environment to thrive in. Recent research shows that a helminth-modified microbiota can modulate immune responses in the intestine, but also at distant sites such as the lungs. It is possible that microbiota-derived metabolites are behind this localized and cross-organ immunomodulation. It is also possible that helminths modulate microbiota function to their own benefit as a tolerogenic environment benefits the helminth as well.

Host, microbiota and helminth are woven in an intricate web of interactions and studying those independently is not representative of the biological situation in mammalian intestine. Therefore, to study the contribution of metabolites to immunomodulation, the entire intestinal metabolome should be considered, that is a result of the combined metabolism of host, microbiota and helminth. There is very limited information about how helminth impact metabolites in the intestine, let alone the small intestine. In the small pool of existing studies on this topic, the majority use bacterial sequencing data to predict metabolite composition, which does not incorporate the contribution of host and helminth, or they use untargeted metabolomics that report only putative metabolite identities. In this thesis, we aim to elucidate the effects of helminth infection in mice on two major classes of metabolites that are highly abundant in the intestinal tract and have immunomodulatory properties. We use a targeted metabolomics approach that includes the use of internal standards to report metabolite abundance with high levels of confidence.

Overarching question:

- How does helminth infection affect the intestinal metabolome?

Major aims:

- How does *H. polygyrus* infection affect the levels of SCFAs in the small intestine?
- How does *H. polygyrus* infection affect the composition and abundance of bile acids in the small intestine?

Minor aims:

- How does *H. polygyrus* infection affect the levels of SCFAs in the colon and serum?
- How does *H. polygyrus* infection affect the composition and abundance of bile acids in the colon and serum?
- By what mechanism(s) does helminth infection impact bile acid levels?

The investigation of these aims is presented in **Chapter 3** and **Chapter 4**.

1.11.3 Consequences of metabolite shifts objectives

The final aims are directed to explore how metabolite shifts during helminth infection affect immunity and bacterial colonization. We start this exploration into immunomodulatory effects of helminth-modified metabolome with assessing whether metabolite shifts we observed in previous aims affect levels of Tregs. We also assess whether these metabolite shifts increase helminth fitness, as we hypothesize that helminths actively modulate the composition of the metabolome to promote their own survival. To test how specific metabolites, SCFAs and bile acids, affect levels of Tregs and helminth fitness, appropriate supplementation methods are developed and validated. With specialized diet- or supplemented drinking water metabolite frequencies can be manipulated and methods we develop can be used for future hypothesis testing.

Overarching question:

- What are the consequences of a helminth-modified metabolome on host immunity?

Major aims:

- Do shifts in SCFAs contribute to elevated *Salmonella* burdens during helminth infection?
- Do shifts in bile acids contribute to elevated *Salmonella* burdens during helminth infection?
- Do shifts in bile acids contribute to elevated Treg levels during helminth infection?

Minor aims:

- Can supplementing with certain SCFAs simulate the effects of helminth infection on SCFAs levels?
- Can a specialized diet simulate or reverse the effects of helminth infection on bile acids?

The investigation of these aims is presented in **Chapter 5** and **Chapter 6.**

Chapter 2

Impaired host resistance to *Salmonella* during helminth co-infection is restored by anthelmintic treatment prior to bacterial challenge

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Chapter disclaimer: The data in this chapter was published in PLoS Neglected Tropical Diseases, on January 20th 2021 [298] (see attached in **Appendix**). The chapter is a near exact reproduction of the final accepted manuscript, with a few changes and additions. Layout changes involve a reordering of sections, renumbering of figures, and integrating supplementary information into the main text (except for the raw data file that was appended to the online publication). Furthermore, unpublished **Figure 2.4 and 2.8** were added for completeness. References to these figures in the main body are a new addition to the published manuscript, as well as methods for "*Salmonella* invasion efficacy *in vitro*". Finally, references were added that reflect relevant research articles that were published after the date of the final manuscript submission.

2.1 Introduction

Helminths are parasitic worms that cause a significant global health concern [35]: it is estimated that more than one billion people are currently chronically infected with helminths. Anthelmintic treatment, or 'deworming', is the current treatment strategy for helminth infection and needs to be periodically administered to at-risk human and livestock populations. Helminth infection has been associated with impaired host resistance to co-infection with various pathogenic microbes, including bacterial pathogens, both in human populations [3–5], and in mouse models of co-infection [6–19]. However, it is not clear whether anthelmintic treatment is sufficient to restore host resistance to microbial pathogens.

We have previously reported that mice infected with the small intestinal helminth *H. polygyrus* are highly susceptible to colonization with the bacterial pathogen *S.* Typhimurium in the small intestine, compared to mice singly infected with *S.* Typhimurium [7]. In this chapter, we use the anthelmintic drug pyrantel pamoate to manipulate *H. polygyrus* infection status in mice and examine the resulting effect on host susceptibility to *S.* Typhimurium.

Here, we demonstrate that the presence of *H. polygyrus* is required by *S.* Typhimurium in order to initially establish high levels of colonization in the small intestinal tract, since anthelmintic treatment prior to bacterial challenge restored host resistance to *S.* Typhimurium colonization. We establish that when adult worms are present at the point of *S.* Typhimurium infection *Salmonella* remains largely in the lumen of the small intestine in close association with the adult worms, rather than invading host tissue. Indeed, expression of host tissue invasion genes by *Salmonella* was not required for establishment in the intestine during helminth infection. Additionally, despite anthelmintic treatment prior to bacterial infection being sufficient to restore host resistance to *S.* Typhimurium, we find that once *Salmonella* had established a population in the small intestinal lumen during helminth co-infection anthelmintic treatment does not result in complete clearance of *Salmonella* from the small intestine.

Our findings contribute to the understanding of how concomitant helminth infection affects bacterial pathogens in the intestinal tract. Furthermore, our data suggests that while anthelmintic treatment may reduce opportunities for bacterial pathogens to colonize the mammalian intestinal tract, anthelmintic treatment may not be sufficient to promote clearance of established bacterial pathogens.

2.2 Methods

2.2.1 Mice

All animal experiments were approved by the University of Victoria's Animal Care Committee and complied with the policies of the Canadian Council on Animal Care. 6-13 week old C57BL/6J mice were used for all experiments. Mice were initially obtained from The Jackson Laboratory (strain 000664; from a maximum-barrier room) and were subsequently bred and maintained under specific-pathogen free conditions at the University of Victoria with access to food and water *ad lib*. Mice born to different parents were randomized between treatment groups. Both male and female mice were used for experiments, as indicated in figure legends.

2.2.2 Helminth infection

The life cycle of *H. polygyrus bakeri* (*H. polygyrus*) was maintained in C57BL/6J mice according to an established protocol [299]. Experimental mice were infected with 200 (unless otherwise indicated) *H. polygyrus* stage 3 larvae by oral gavage. *H. polygyrus* burdens were tracked by counting parasite eggs released into feces, which were enumerated in a McMaster Counting Chamber slide under a light microscope.

2.2.3 Salmonella infection

All *S.* Typhimurium strains used were streptomycin-resistant (strain SL1344). Mice were infected with *Salmonella* by oral gavage with either 3 x 10^6 colony-forming units (cfu) of wild-type *S.* Typhimurium or host-invasion-deficient (Δ*invA*) *S.* Typhimurium [300], or with 3 x 10^8 cfu of a growth-attenuated (Δ*aroA*) strain of *S.* Typhimurium [301] as indicated. Inocula were prepared from stationary-phase overnight cultures in Luria-Bertani (LB) broth and were diluted in phosphate-buffered saline (PBS) prior to infection.

2.2.4 Eosinophil-deficient mice

Wild-type BALB/cJ and eosinophil-deficient ΔdblGATA BALB/cJ mice were purchased from The Jackson Laboratory (strain 000651, from a maximum barrier room) and subsequently bred and maintained under specific-pathogen free conditions at the University of Victoria with access to food and water *ad lib*. Both male and female mice were used for experiments. To minimize the potential effects of microbiota compositional differences between mice of different genotypes on experimental outcomes, female wild-type BALB/cJ and eosinophil-deficient ΔdblGATA BALB/cJ mice were co-housed for at least one week prior to beginning experiments and were kept co-housed throughout the duration of the experiment. Male mice born in different litters could not be co-housed due to fighting, but instead the bedding of male wild-type BALB/cJ and eosinophil-deficient ΔdblGATA BALB/cJ mice was swapped twice weekly starting the week prior to beginning experiments, and throughout the duration of the experiment.

2.2.5 Streptomycin treatment

Mice received an oral gavage of 20 mg of streptomycin sulfate (GoldBio) diluted in PBS, and 24 hours later, mice were infected with *Salmonella*.

2.2.6 Anthelmintic treatment

Mice were given 2.5 mg Strongid P (Zoetis) in Ultra-Pure Distilled Water (Invitrogen) by oral gavage on two consecutive days. Efficacy of anthelmintic treatment was monitored by tracking fecal *H. polygyrus* egg release. For each experiment we confirmed that fecal *H. polygyrus* egg burdens were equivalent between *H. polygyrus*-infected groups prior to beginning anthelmintic treatment in the dewormed group.

2.2.7 Bacterial burden determination

Serial dilutions of homogenized tissue were plated on Luria-Berthani (LB) plates containing 100 µg/mL streptomycin (Sigma-Aldrich) and incubated overnight at 37°C. *Salmonella* colonies were then counted and cfu per gram of tissue was calculated.

To determine *Salmonella* cfu in separated tissue and luminal gut fractions, intestinal sections were cut open longitudinally and luminal contents were scraped out using forceps. Tissue fractions were washed in PBS twice, incubated in RPMI 1640 media supplemented with 100 µg/mL gentamycin (GoldBio) for 45 minutes at room temperature, and then washed in PBS twice. The luminal and tissue fractions were homogenized, plated on streptomycin-containing LB plates and incubated overnight at 37°C, after which cfu per homogenate was calculated.

To determine the proportion of *S.* Typhimurium in association with adult *H. polygyrus* worms, worms were separated from the luminal contents prior to cfu determination. Intestinal sections were cut open longitudinally and luminal contents were scraped out using forceps. Luminal contents (containing worms) were placed in a muslin bag suspended in Hank's Balanced Salt Solution (HBSS; Gibco) in a Baermann apparatus and incubated at 37°C for 2 hours. Following incubation, the majority of worms had migrated through the muslin bag to the collection funnel and were subsequently homogenized and plated on streptomycin-containing LB plates (the few remaining worms were removed manually). The luminal contents (with no remaining worms) were recovered, homogenized, and plated on streptomycin-containing LB plates. The tissue fractions were washed in PBS twice, incubated in PBS supplemented with 100 µg/mL gentamycin (GoldBio) for 45 minutes at 37°C, then washed in PBS twice and incubated at 37°C for a further 1 hour 15 mins to be comparable to the incubation time of the worm and luminal content fractions, then homogenized and then plated on streptomycin-containing LB plates. After the plates were incubated overnight at 37°C, cfu per homogenate was calculated.

2.2.8 *Salmonella invasion efficacy in vitro*

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% heat-inactivated fecal bovine serum (Gibco), 1% GlutaMAX (ThermoFisher) and 1% non-essential amino acids (Gibco) (complete DMEM), at 37°C with 5% CO_2. Cultures of wild-type and *invA* mutant *S.* Typhimurium (kindly provided by Dr. Bruce Vallance, University of British Columbia) were used to infect HeLa cells at a multiplicity of infection of 100. Bacterial inoculates were plated on LB plates containing 100 µg/mL streptomycin (Goldbio) to confirm the cfu of *S.* Typhimurium present in inoculates. Twenty minutes post infection, HeLa cells were washed with HBSS, and complete DMEM containing 50 µg/mL gentamicin (Goldbio) was added. After a further 70-minute incubation, HeLa cells were lysed using HBSS containing 1% Triton X-100 (Biobasic) and 0.1% sodium dodecyl sulfate (Biobasic). Serial dilutions of lysate were plated onto LB plates containing 100 µg/mL streptomycin (Goldbio). The following day, *S.* Typhimurium colonies were counted, and invasion level was calculated as the percentage of *S.* Typhimurium cfu in inoculates that invaded HeLa cells.

2.2.9 *Statistical analyses*

Statistical analyses were performed in GraphPad Prism 7.04. Normality of the data was assessed by a D'Agostino-Pearson normality test and the appropriate statistical test was performed depending on the normality of the data set, the number of experimental groups being compared, and whether paired data sets were being compared or not, as indicated in the figure legends.

2.3 Results

2.3.1 *Deworming prior to bacterial challenge restores host resistance to Salmonella in the small intestine*

H. polygyrus is a natural parasite of mice and is able to establish a chronic infection in the proximal small intestine of C57BL/6J mice [50]. After 14 days of infection with *H. polygyrus*, adult helminths are present in the lumen of the duodenum and jejunum where they wrap around villi to secure their location. We have previously reported that when 14-day infected *H. polygyrus* mice are challenged with *S.* Typhimurium, *Salmonella* is able to colonize the small intestinal tract to higher levels than when no helminths are present [7]. We find that helminth co-infection enhances *Salmonella* colonization of the small intestine even in mice which lack IL-4, Stat6, and RAG1 [7], and also in mice lacking eosinophils **(Figure 2.1)**, and in bacterial microbiota-depleted mice **(Figure 2.2)**. Further, we find that there is a relationship between helminth burden and *S.* Typhimurium colonization levels: a higher infectious dose of helminths results in higher *Salmonella* colonization levels in the small intestine **(Figure 2.3)**. In addition, we find that adult worms must be present in the small intestinal lumen to robustly boost *Salmonella* colonization in the small intestine during *H. polygyrus* infection, and that the presence of *H. polygyrus* larvae does not significantly impact *Salmonella* colonization **(Figure 2.4)**.

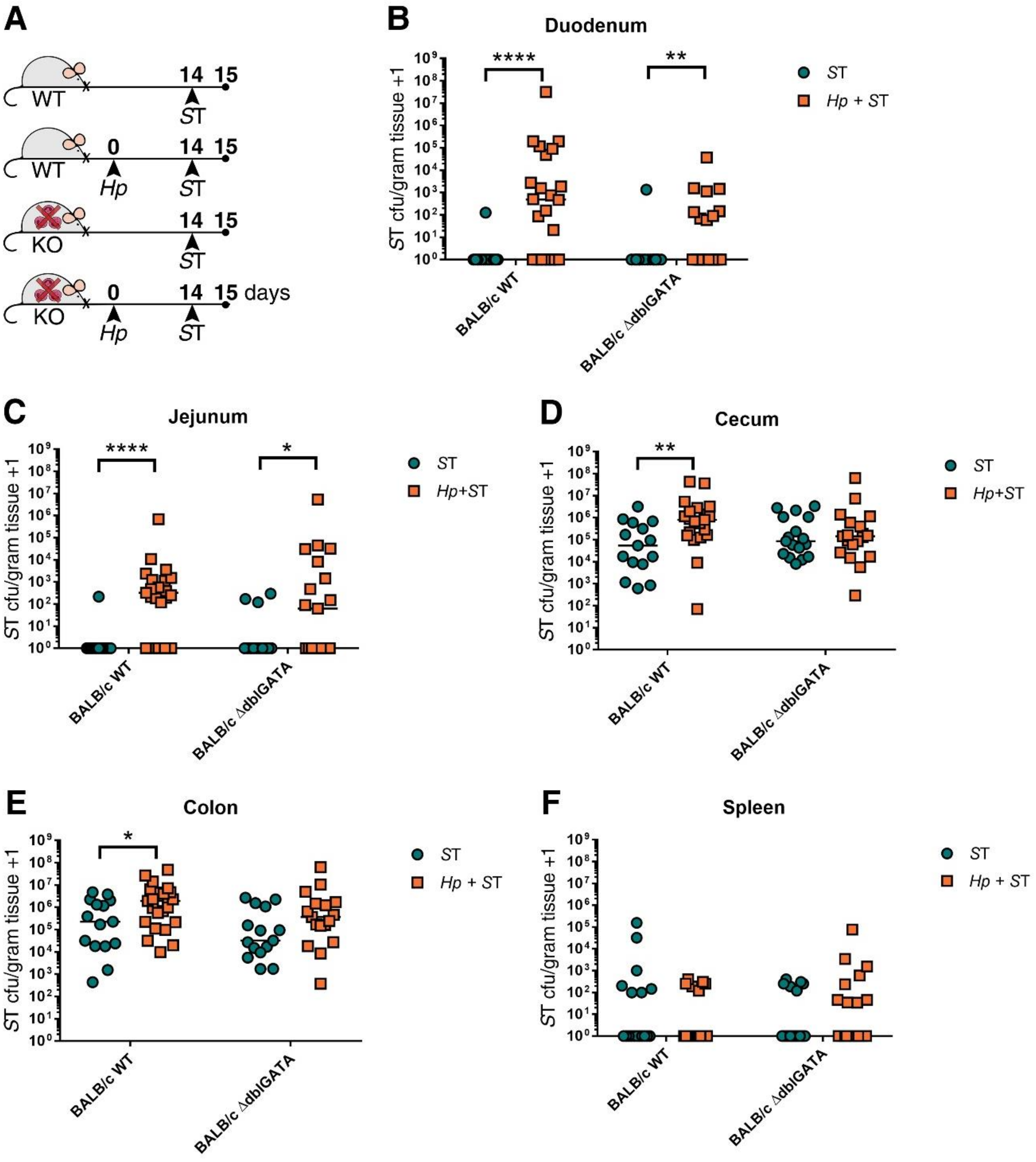

Figure 2.1. Eosinophil deficiency does not preclude the ability of helminths to promote *S.* Typhimurium (*S*T) colonization in the small intestine. (A) Experimental set-up. Naïve or *H. polygyrus* (*Hp*)-infected wild-type and eosinophil-deficient Δ*dblGATA* BALB/cJ mice were orally infected with Δ*aroA* *S*T fourteen days post *Hp*-infection. One day post-*S*T infection, *S*T colony-forming units (cfu)/gram of tissue were determined. *S*T cfu/gram of tissue in the duodenum (B), jejunum (C), cecum (D), colon (E), and spleen (F) are shown. Data shown is pooled from three independent experiments including both male and female mice. Statistical comparisons for each mouse genotype were made using a Mann-Whitney test. A line indicates the median value for each experimental group. * = $p \leq 0.05$ **= $p \leq 0.01$; **** = $p \leq 0.0001$.

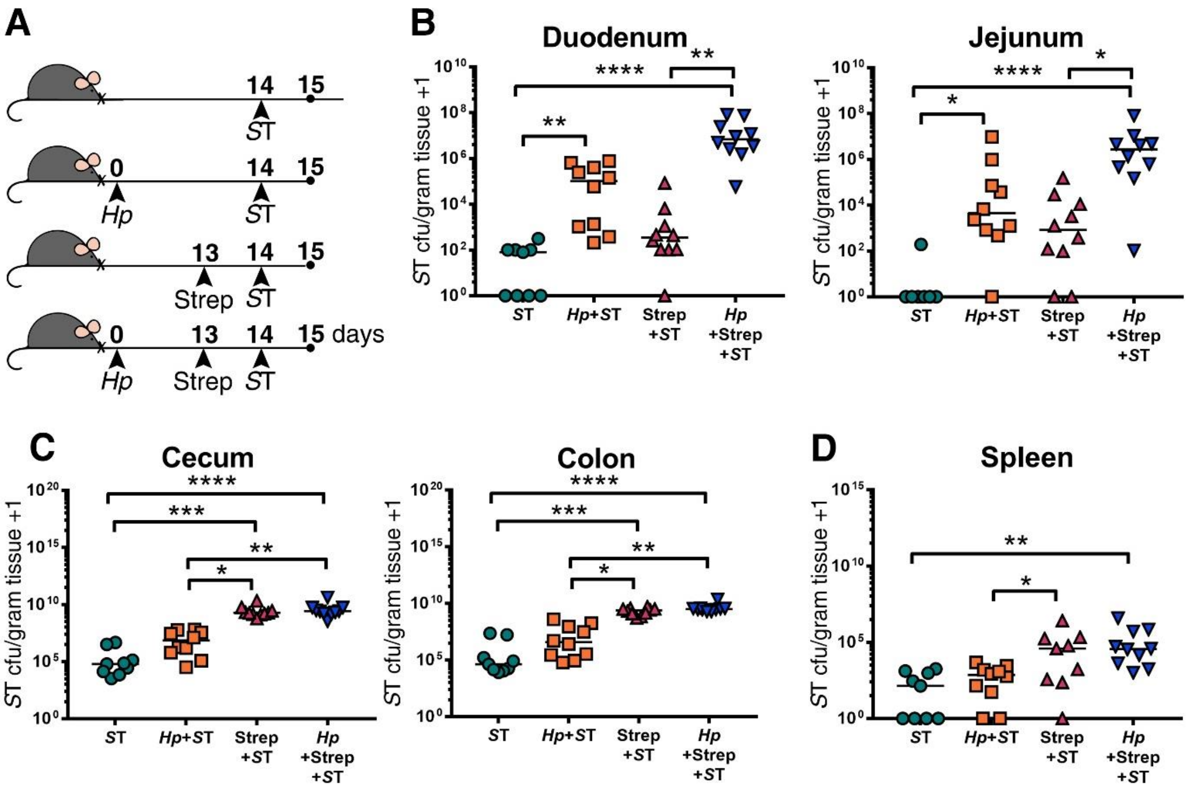

Figure 2.2. Bacterial microbiota depletion does not preclude the ability of helminths to promote *S*. Typhimurium (*S*T) colonization in the small intestine. (A) Experimental set-up. Naïve or *H. polygyrus* (*Hp*)-infected male and female C57BL/6J mice were given a 20 mg dose of streptomycin by oral gavage thirteen days post *Hp*-infection, or left untreated. One day following treatment, all mice were infected with Δ*aroA S*T. One day post-*S*T infection, *S*T cfu/gram of tissue were determined. *S*T cfu/gram of tissue in the duodenum and jejunum (B), cecum and colon (C), and spleen (D) are shown. Data shown is pooled from two independent experiments. Statistical comparisons between groups were made using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. A line indicates the median value for each experimental group. * = $p \leq 0.05$; **= $p \leq 0.01$; *** = $p \leq .0001$; **** = $p \leq 0.0001$.

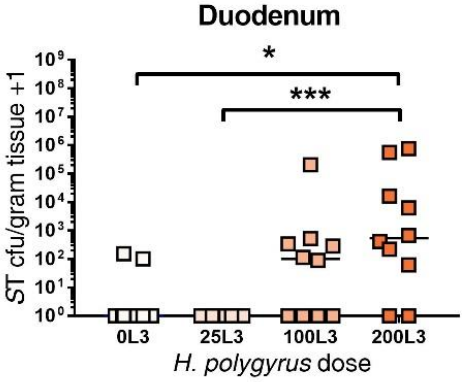

Figure 2.3. A threshold dose of *H. polygyrus* (*Hp*) is required for a loss of host resistance to *S.* Typhimurium (*S*T) in the small intestine. Female C57BL/6J mice were left naïve ('0L3') or infected with 25 ('25L3'), 100 ('100L3'), or 200 ('200L3') third stage *H. polygyrus* larvae (L3). Fourteen days later, mice were orally infected with Δ*aroA* *S*T. One day post-*S*T infection, *S*T cfu/gram of tissue were determined in the duodenum. Data shown is pooled from two independent experiments. Statistical comparisons between groups were made using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. A line indicates the median value for each experimental group. * = p ≤ 0.05; *** = p ≤ 0.001.

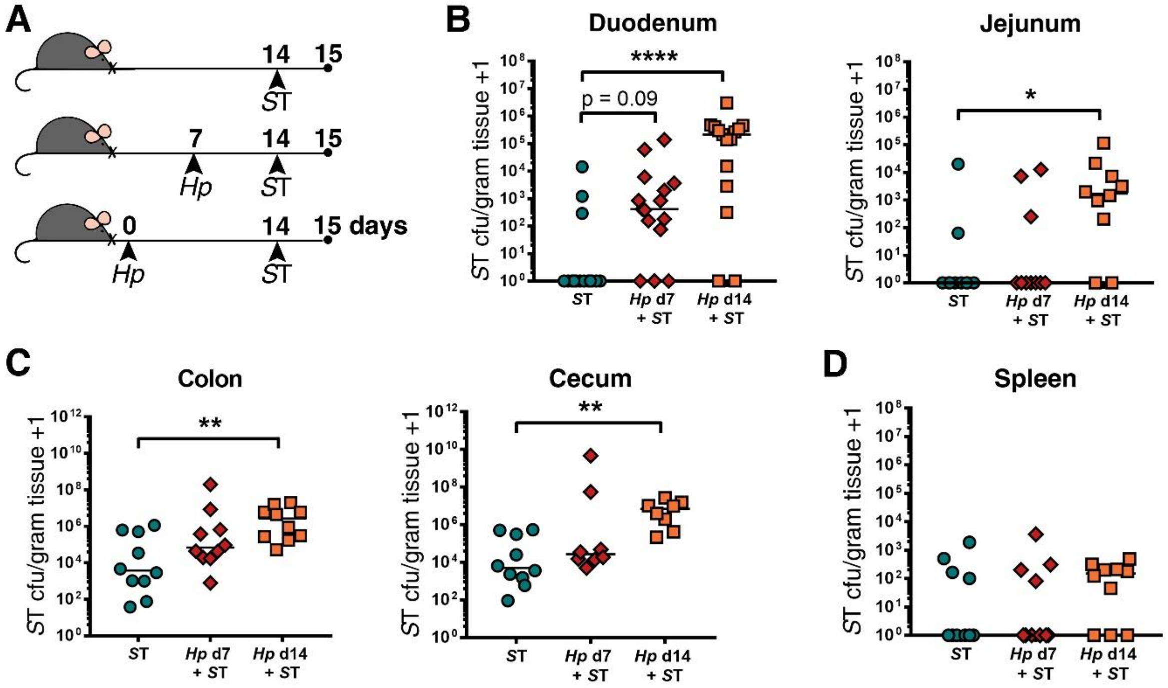

Figure 2.4. Adult worms are required to robustly boost *S.* Typhimurium (*S*T) colonization levels in the small intestine of mice during helminth infection. (A) Experimental set-up and deworming efficacy. Naïve or *H. polygyrus* (*Hp*)-infected male and female C57BL/6J mice were orally infected with Δ*aroA* *S*T, seven (d7) or fourteen days (d14) post *Hp*-infection. One day post-*S*T infection, *S*T cfu/gram of tissue were determined. *S*T cfu/gram of tissue in the

duodenum and jejunum (B), cecum and colon (C), and spleen (D) are shown. Data shown is pooled from four independent experiments. Statistical comparisons between groups were made using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. A line indicates the median value for each experimental group. ns = not significant; * = $p \leq 0.05$; **= $p \leq 0.01$; **** = $p \leq 0.0001$.

To gain deeper insight into the potential mechanisms by which helminths affect susceptibility to co-infection, we examined whether the increase in *S.* Typhimurium colonization during helminth infection requires the ongoing presence of helminths. To test this, once mice had an established *H. polygyrus* infection (day 14 of infection), we treated them with Strongid P (Zoetis). This treatment is widely used for veterinary deworming as it contains the anthelmintic compound pyrantel pamoate (PP). We found that a two-day deworming treatment was sufficient to clear *H. polygyrus* from the mouse intestine, as indicated by the absence of parasite eggs in feces **(Figure 2.5A)**. One day after successful anthelmintic treatment, mice were infected with *S.* Typhimurium, alongside mice that had an ongoing *H. polygyrus* infection and mice with no prior helminth infection **(Figure 2.5A)**. Here, we used a growth-attenuated strain of *S.* Typhimurium (ΔaroA) which allowed our subsequent experiments to explore infection dynamics several days following *Salmonella* infection without mice succumbing to the infection.

Consistent with what we have reported previously [7], those mice who had never been exposed to helminths were able to clear *Salmonella* from the small intestine one day after *Salmonella* infection, whereas *H. polygyrus*-co-infected mice presented with high bacterial burdens **(Figure 2.5B)**. Mice that received deworming treatment prior to *Salmonella* infection had significantly lower bacterial burdens in the small intestine than untreated co-infected mice, and instead, similar to mice that had never been exposed to helminths, were able to clear the majority of *Salmonella* from the small intestine **(Figure 2.5B)**. An ongoing *H. polygyrus* infection and deworming had similar, but less marked effects on *Salmonella* colonization in the large intestine **(Figure 2.5C)**, and helminth infection status did not affect *Salmonella* trafficking to the spleen **(Figure 2.5D)**. We confirmed that there was no effect of pre-treatment with anthelmintics on *Salmonella* colonization levels **(Figure 2.6)**. Together, this data shows that anthelmintic treatment restores host resistance to *Salmonella* in the small intestinal tract within one day of adult worms being cleared, suggesting that an ongoing helminth infection is required to promote *Salmonella* colonization through local and transient (only while *H. polygyrus* is present) alterations to the small intestinal environment.

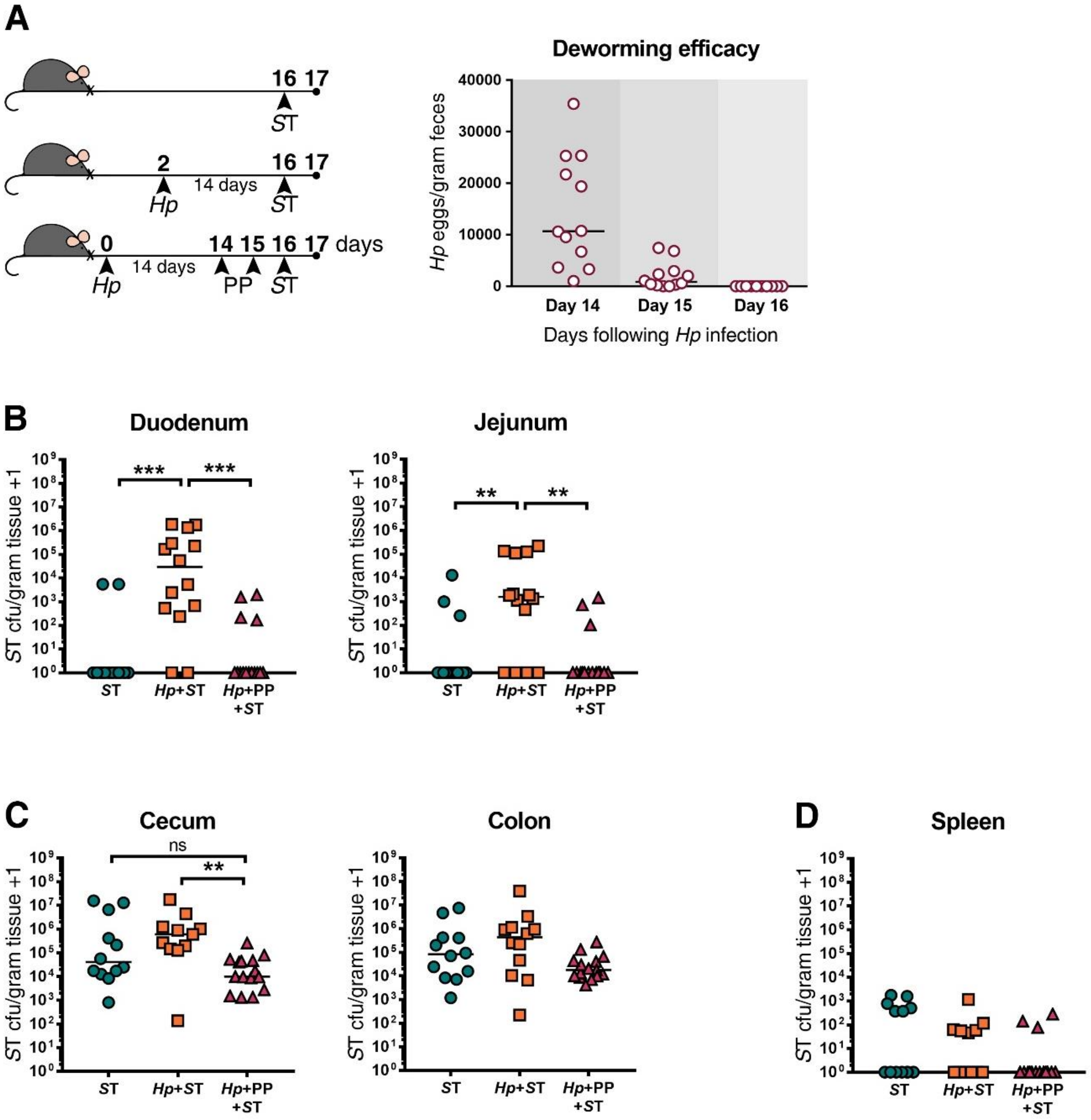

Figure 2.5. Deworming prior to bacterial challenge restores host resistance to *S.* Typhimurium (*S*T) in the small intestine of mice. (A) Experimental set-up and deworming efficacy. Naïve or *H. polygyrus* (*Hp*)-infected male and female C57BL/6J mice were orally infected with Δ*aroA S*T or given an oral dose of deworming drug (PP) for two consecutive days, fourteen days post *Hp*-infection. Mice that received PP were subsequently orally infected with *S*T. One day post-*S*T infection, *S*T cfu/gram of tissue were determined. Numbers of *Hp* eggs released in feces were quantified on days 14-16 from mice receiving deworming treatment, as a non-terminal method of assessing worm burdens, to confirm anthelmintic treatment efficacy. *S*T cfu/gram of tissue in the duodenum and jejunum (B), cecum and colon (C), and spleen (D) are shown. Data shown is pooled from three independent experiments. Statistical comparisons between groups were made using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. A line indicates the median value for each experimental group. ns = not significant; **= p ≤ 0.01; *** = p ≤ .0001.

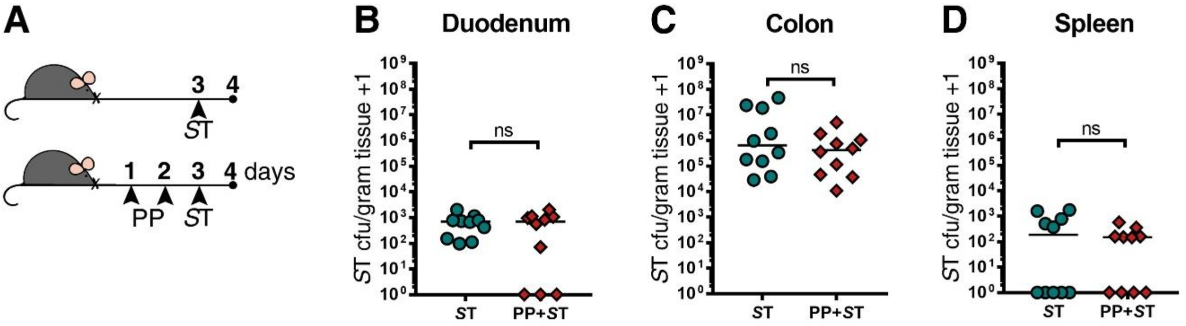

Figure 2.6. *S.* Typhimurium (*S*T) colonization is not affected by pre-treatment with a two-day oral dose of 2.5 mg dose of the anthelmintic drug pyrantel pamoate (PP) prior to *S*T inoculation. (A) Experimental set-up. Naïve male and female C57BL/6J mice were treated with 2.5 mg Strongid P (containing PP) for two consecutive days or left untreated. One day after completion of PP treatment, mice were infected with wild-type *S*T to assess *S*T cfu in the duodenum (B), or mice were infected with Δ*aroA* *S*T to assess *S*T cfu in the colon (C) and spleen (D). We used wild-type *S*T to assess the effect of PP on *S*T in the duodenum, rather than Δ*aroA* *S*T, because Δ*aroA* *S*T establishes at only low levels in the small intestine in the absence of helminths, making it impossible to detect a potentially adverse effect of PP on *S*T colonization in the small intestine. Wild-type *S*T is able to establish sufficiently in the small intestine, which allows us to determine whether PP affects colonization levels of *S*T. Data shown in both (B) and (C+D) is pooled from two independent experiments, and statistical comparisons between groups were made using a Mann-Whitney test. A line indicates the median value for each experimental group. ns = not significant.

2.3.2 The presence of helminths supports luminal expansion of Salmonella

Local changes during *H. polygyrus* infection include significant shifts in the availability of metabolites in the small intestine [7]. The composition of metabolites in the intestinal tract can alter the ability of *Salmonella* to colonize the intestinal tract through multiple mechanisms, for example, by affecting the availability of nutrients or by influencing *Salmonella* virulence gene expression [302]. We have previously shown that small intestinal metabolites from naïve mice can suppress *S.* Typhimurium genes required for host tissue invasion in *in vitro* assays, whereas small intestinal metabolites from *H. polygyrus*-infected mice lack this effect [7]. Therefore, we asked if *S.* Typhimurium was taking advantage of a helminth-altered environment that promotes *Salmonella* invasion of small intestinal tissue.

To test this, mice were co-infected with *H. polygyrus* and an invasion-deficient *S.* Typhimurium mutant (Δ*invA*) [300] **(Figure 2.7A)**. InvA expression is required to form a type 3 secretion system which allows *S.* Typhimurium to invade host cells [300]. We confirmed that a mutation in the *invA* gene indeed led to a loss of the ability of *S.* Typhimurium to invade host cells *in vitro* **(Figure 2.8)**. In our *in vivo* co-infection model, we found that colonization of the small intestine by Δ*invA S*. Typhimurium was promoted by the presence of helminths to the same extent as wild-type *S*. Typhimurium **(Figure 2.7B)**. This suggests that the helminth-modified small intestinal environment does not result in increased host tissue invasion by *Salmonella*.

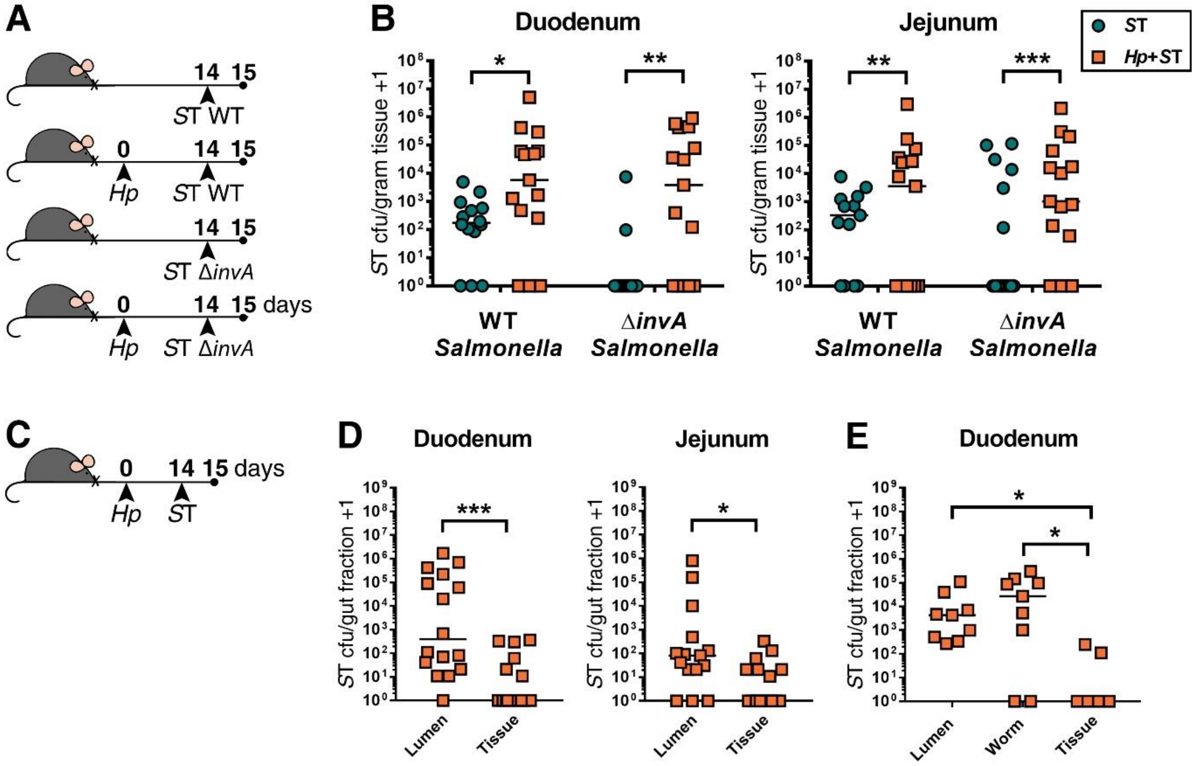

Figure 2.7. *S*. Typhimurium (*S*T) predominantly expands in the small intestinal lumen during helminth co-infection. (A) Experimental set-up. Naïve or *H. polygyrus* (*Hp*)-infected male and female C57BL6/J mice were orally infected with wild-type *S*T (WT *S*T) or invasion-deficient ST (Δ*invA S*T) fourteen days post-*Hp* infection. One day post-*S*T infection, *S*T cfu counts were determined in the duodenum and jejunum (B). Data shown is pooled from three independent experiments. Statistical comparisons between singly- and co-infected mice infected with either WT or Δ*invA S*T were calculated with a Mann-Whitney test. A line indicates the median value for each experimental group. (C) Experimental set-up. Male and female C57BL6/J mice were infected with *H. polygyrus* (*Hp*). Fourteen days post-*Hp* infection, mice were orally infected with Δ*aroA S*T. One day post-*S*T infection, small intestinal sections were dissected and *S*T cfu were determined in the duodenum and jejunum. (D) *S*T cfu in luminal and tissue small intestinal fractions. Data shown is pooled from three independent experiments. Statistical comparisons between groups were made using a Wilcoxon matched-pairs signed rank test. (E) *S*T cfu in luminal fraction with adult worms removed, in extracted *Hp* worm fraction, and in tissue fractions of the duodenum. Data shown is pooled from two independent experiments. Statistical comparisons between groups were made using a Friedman test followed by a

Dunn's multiple comparisons test. A line indicates the median value for each experimental group. * = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.001.

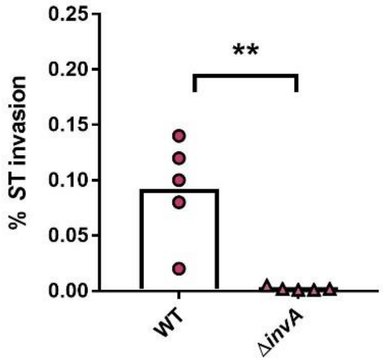

Figure 2.8. The *S.* Typhimurium (*S*T) *invA* mutant is unable to invade host cells. Wild-type and *invA* mutant ST bacteria were used to infect HeLa cells. Percentage invasion is expressed as the fraction of the *S*T inoculum that was recovered from cell lysates after incubation. Each data point represents a technical replicate of HeLa cells infected with *S*T. **P ≤ .01.

To further support our conclusion that increased bacterial host tissue invasion does not underlie increased *Salmonella* colonization during helminth infection, we tested whether *S.* Typhimurium predominantly colonizes the intestinal lumen, rather than host tissue, during helminth co-infection. Small intestinal luminal contents were separated from the intestinal tissue of helminth co-infected mice one day after bacterial infection and *Salmonella* burdens were quantified in both fractions **(Figure 2.7C)**. We found that during co-infection *Salmonella* is present in significantly higher numbers in the luminal fraction than in the small intestinal tissue **(Figure 2.7D)**. Notably, many of these bacteria were found in close association with adult *H. polygyrus* worms isolated from the luminal contents **(Figure 2.7E)**. Quantification of bacterial burdens nine days following *S.* Typhimurium infection revealed that *Salmonella* persists in the lumen of the small intestine during helminth co-infection for an extended period of time **(Figure 2.9)**. Based on our collective data, we suggest that *H. polygyrus* enables *Salmonella* to overcome host resistance to colonization and to expand primarily in the small intestinal lumen, and, to a lesser extent, in the intestinal tissue.

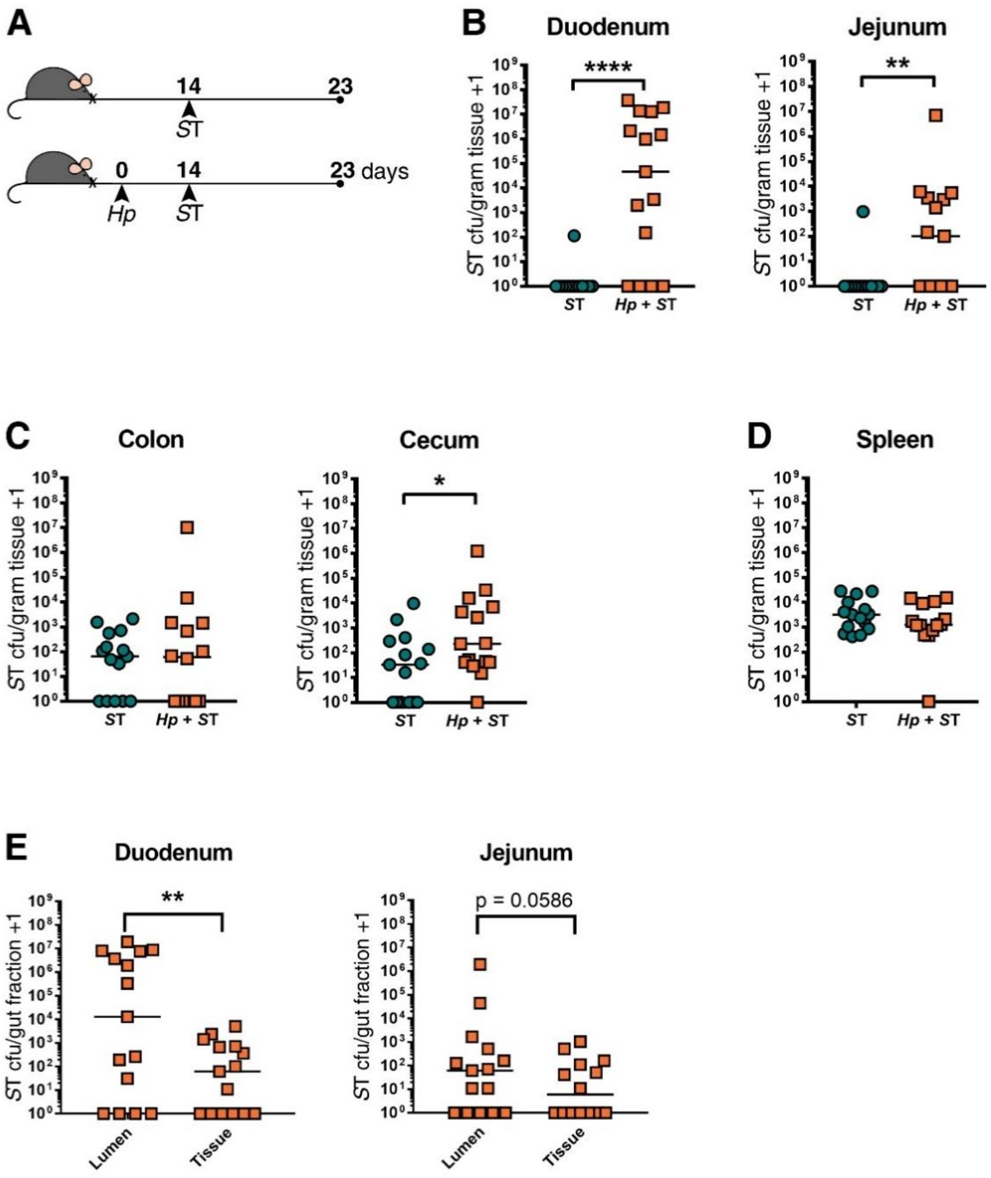

Figure 2.9. *S.* Typhimurium (*S*T) persists in the lumen of the small intestine during helminth infection for 9 days following *S*T co-infection. (A) Experimental set-up. Naïve or *H. polygyrus* (*Hp*)-infected male and female C57BL/6J mice were orally infected with Δ*aroA* *S*T fourteen days post *Hp*-infection. Nine days post-*S*T infection, *S*T cfu/gram of tissue were determined. *S*T cfu/gram of tissue in the duodenum and jejunum (B), cecum and colon (C), and the spleen (D) are shown. Data shown is pooled from three independent experiments. Statistical comparisons between groups were made using a Mann-Whitney test. In a different set of experiments following the same experimental timeline, the duodenum and jejunum were dissected to separate out tissue and luminal fractions, and *S*T cfu were determined in each fraction (E). Data shown is pooled from three independent experiments. Statistical comparisons between groups were made using a Wilcoxon matched-pairs signed rank test. A line indicates the median value for each experimental group. * = $p \leq 0.05$ **= $p \leq 0.01$; **** = $p \leq 0.0001$.

2.3.3 Salmonella does not require the ongoing presence of helminths to persist in the small intestine

It is possible that after overcoming host resistance to colonization during helminth co-infection, *Salmonella* evades or overwhelms local immune defenses which enables long-term persistence of the bacteria. Therefore, we asked whether *S*. Typhimurium required the ongoing presence of worms in order to persist in the small intestine. To test this, we assessed whether resistance to *Salmonella* could be restored by deworming once *Salmonella* had co-colonized with helminths. We first confirmed that anthelmintic treatment itself had no effect on *Salmonella* persistence, when given after *Salmonella* infection **(Figure 2.10)**. Mice were co-infected with *H. polygyrus* and ΔaroA *S*. Typhimurium and then anthelmintic-treated one day after *Salmonella* infection, alongside singly-*Salmonella* infected and co-infected mice that did not receive anthelmintic treatment **(Figure 2.11A)**. We quantified *Salmonella* burdens 48 hours after the last dose of anthelmintics **(Figure 2.11B-D)**. In mice that received deworming treatment following co-infection, a subset of *S*. Typhimurium persisted in the duodenum, resulting in bacterial burdens that did not significantly differ from levels detected in mice with an ongoing helminth infection **(Figure 2.11B).** To further resolve differences in *Salmonella* burdens between experimental groups, we followed the same experimental timeline but used the faster-growing, wild-type *S*. Typhimurium strain for infections, and confirmed that deworming following helminth-*Salmonella* co-infection did result in significantly lower small intestinal *Salmonella* burdens compared to mice with an ongoing helminth infection **(Figure 2.12)**.

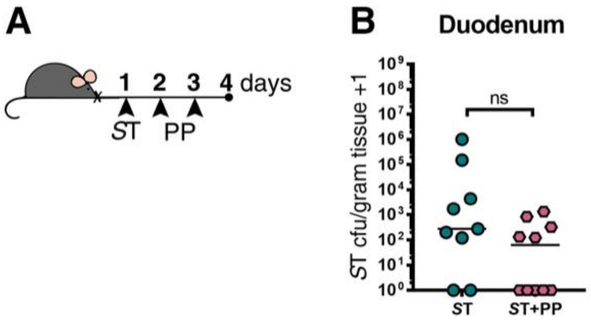

Figure 2.10. *S*. Typhimurium (*S*T) colonization levels are not affected by a two-day oral dose of 2.5 mg dose Strongid P (PP) following ST inoculation. Male C57BL/6J mice were infected with wild-type *S*T. On the first and second day after *S*T infection, mice were treated with a 2.5 mg dose of Strongid P or left untreated. One day after completion of PP treatment, *S*T cfu counts were determined in the duodenum. The purpose of this experiment was to test whether PP treatment had

any effect on *S*T colonization levels in the small intestine. Because we were looking to detect a potential reduction in *S*T burdens following PP treatment, we used wild-type *S*T rather than Δ*aroA* *S*T, since wild-type *S*T colonizes to higher levels in the small intestine which would allow us to detect a potential reduction in colonization after PP treatment. Data shown is pooled from two independent experiments. Statistical comparisons between groups were made using a Mann-Whitney test. A line indicates the median value for each experimental group. ns = not significant.

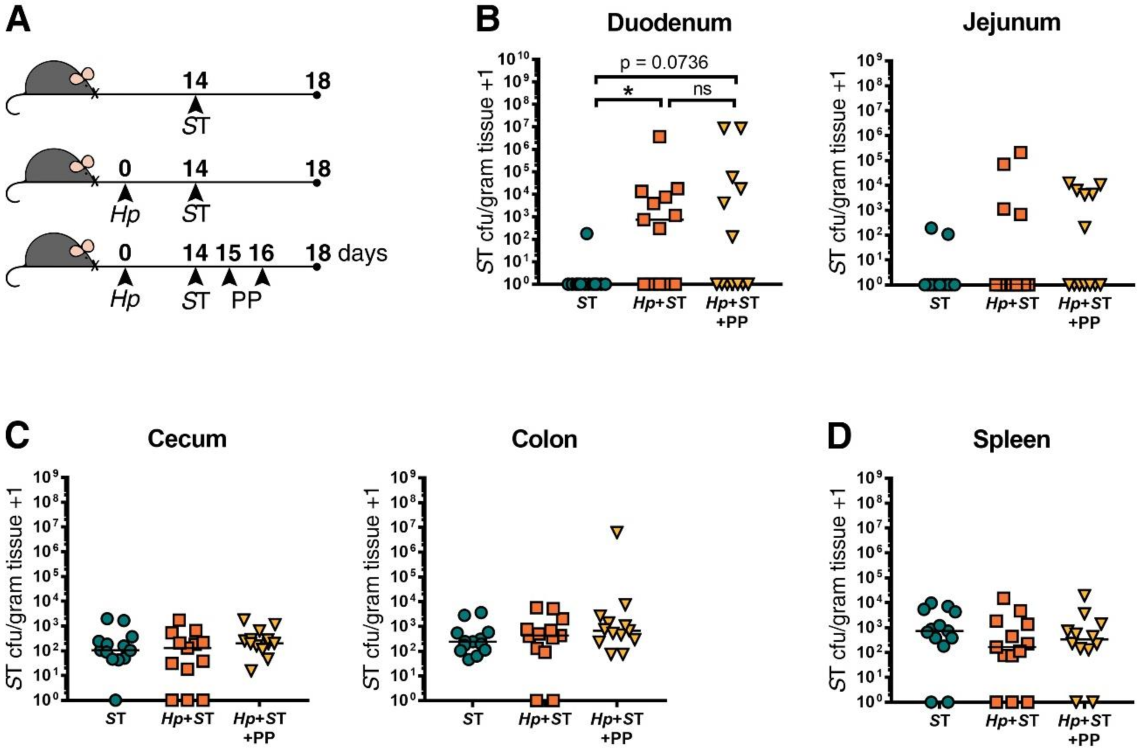

Figure 2.11. A subset of *S.* Typhimurium (ST) persists in the small intestine 24 hours after helminth clearance. (A) Experimental set-up. Naïve or *H. polygyrus* (*Hp*)-infected male and female C57BL/6J mice were orally infected with Δ*aroA* *S*T fourteen days post-*Hp* infection. One day post-*S*T infection, helminth-co-infected mice were given deworming treatment (PP) for two days or not. One day following PP treatment or no treatment, *S*T colony-forming units cfu/gram of tissue were determined. *S*T cfu/gram of tissue in the duodenum and jejunum (B), cecum and colon (C), and the spleen (D) are shown. Data shown is pooled from two independent experiments. Statistical comparisons between groups were made with a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. A line indicates the median value for each experimental group. ns = not significant; * = $p \leq 0.05$.

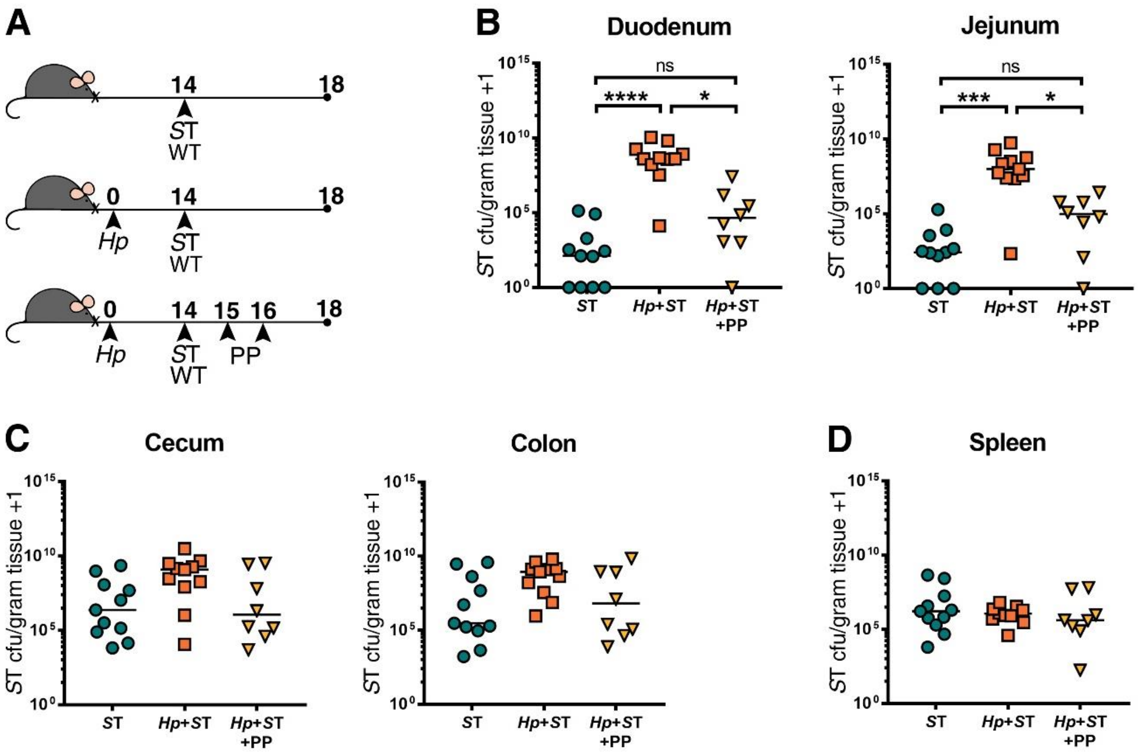

Figure 2.12. Deworming results in reduced wild-type *S.* Typhimurium (*S*T) burdens in the small intestine compared to mice with an ongoing helminth infection. (A) Experimental set-up. Naïve or *H. polygyrus* (*Hp*)-infected female C57BL/6J mice were orally infected with wild-type *S*T fourteen days post-*Hp* infection. One day post-*S*T infection, *Hp*-co-infected mice were given deworming treatment (PP) for two days or not. Two days post-treatment or no treatment, *S*T cfu/gram of tissue were determined in all groups. *S*T cfu/gram of tissue in the duodenum and jejunum (B), cecum and colon (C), and the spleen (D) are shown. Data shown is pooled from two independent experiments. Statistical comparisons between groups were made using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. A line indicates the median value for each experimental group. ns = not significant; * = $p \leq 0.05$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$.

To determine whether *Salmonella* persisting in the small intestine following helminth clearance would be ultimately cleared from this site, we followed the same experimental protocol as in **(Figure 2.11A)**, but with quantification of ΔaroA *S.* Typhimurium burdens at a later timepoint: one week after we had confirmed helminth clearance **(Figure 2.13A)**. One week following helminth clearance, we found that *Salmonella* was still detectable in the small intestine, with significantly higher *Salmonella* burdens in the small intestine than in non-helminth infected controls, and with *Salmonella* burdens that did not significantly differ from the *Salmonella* burdens of mice with an ongoing helminth infection **(Figure 2.13B)**. At this later time point, we saw higher *Salmonella* burdens in the colon of helminth co-infected mice compared to mice singly infected with *Salmonella*, which did not persist when mice received deworming treatment **(Figure 2.13C)**. This suggests that deworming reduced overall *Salmonella* burdens despite a subset of bacteria persisting in the small intestine. We found that it was specifically the luminal fraction of *Salmonella*, rather than those bacteria that had invaded the host small intestinal tissue, that was reduced after deworming **(Figure 2.14)**. We found no differences in systemic dissemination of *Salmonella* regardless of helminth infection status **(Figure 2.11D; Figure 2.14D; Figure 2.13D)**. Overall, these results show that once *Salmonella* has established in the small intestine during helminth co-infection, the presence of the worm is no longer essential for *Salmonella* to persist in the small intestine, but that anthelmintic treatment does reduce *Salmonella* colonization levels.

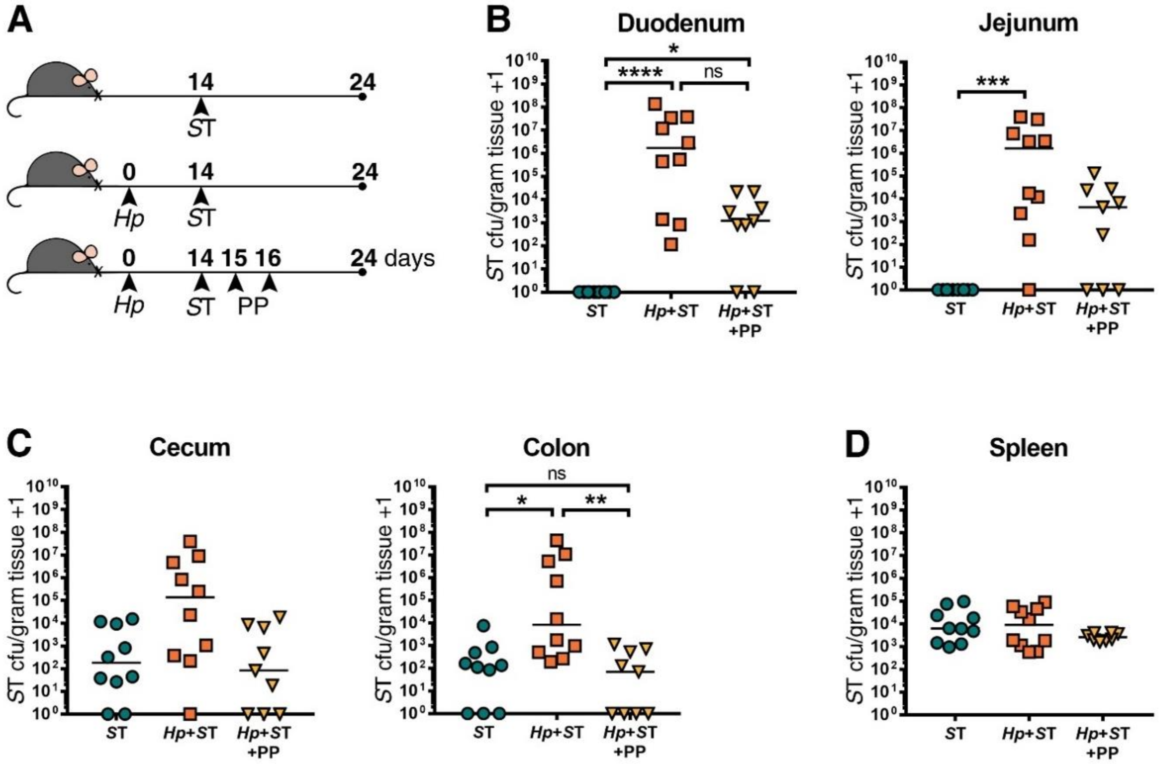

Figure 2.13. *S*. Typhimurium (ST) is able to persist in the small intestine one week after helminth clearance. (A) Experimental set-up. Naïve or *H. polygyrus* (*Hp*)-infected female C57BL/6J mice were orally infected with Δ*aroA S*T fourteen days post-*Hp* infection. One day post-*S*T infection, *Hp*-co-infected mice were given deworming treatment (PP) for two days or not. Eight days post-treatment or no treatment, *S*T cfu/gram of tissue were determined in all groups. *S*T cfu/gram of tissue in the duodenum and jejunum (B), cecum and colon (C), and the spleen (D) are shown. Data shown is pooled from two independent experiments. Statistical comparisons between groups were made using a Kruskal-Wallis test followed by a Dunn's multiple comparisons test. A line indicates the median value for each experimental group. ns = not significant; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$.

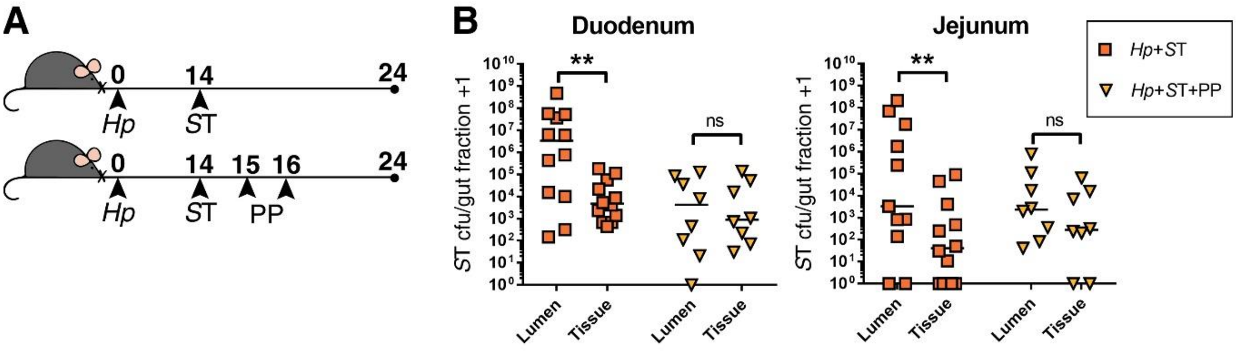

Figure 2.14. Deworming after *S.* Typhimurium (*S*T) has colonized during helminth infection leads to a reduction in luminal but not tissue-resident *S*T burdens. (A) Experimental set-up. Male and female C57BL6/J mice were infected with *H. polygyrus* (*Hp*). Fourteen days post-*Hp* infection, mice were orally infected with Δ*aroA S*T. One day post-*S*T infection, *Hp*-co-infected mice were given deworming treatment (PP) for two days or not. Eight days post-treatment or no treatment, *S*T colony-forming units cfu/gram of tissue were determined. (B) *S*T cfu in luminal and tissue small intestinal fractions. Data shown is pooled from two independent experiments. Statistical comparisons were made between the indicated groups using a Mann-Whitney test. A line indicates the median value for each experimental group. *= p ≤ 0.05.

2.4 Discussion

In this paper, we investigated the changes in host resistance to a pathogenic bacterial infection when helminth-infected hosts received anthelmintic treatment either before or after bacterial challenge. We demonstrated that helminth-infected mice that received deworming treatment prior to *S.* Typhimurium infection showed restored colonization resistance to *Salmonella* in the small intestine, within a day of helminth clearance. Based on this finding, we suggest that the benefits of mass deworming in helminth-endemic areas may extend to attenuating gastrointestinal colonization by bacterial pathogens such as *Salmonella*. The effect of anthelmintic treatment prior to challenge with microbial pathogens has been investigated in other helminth-microbial co-infection systems with similar effects. For example, in a mouse model of trematode-*Plasmodium* co-infection, exacerbation of *Plasmodium* parasitaemia due to intestinal trematode infection was reversible by deworming when mice were dewormed prior to *Plasmodium* infection [19].

Our data suggests that the effect of deworming on host susceptibility to microbial pathogens is influenced by whether deworming occurs prior to infection with microbial pathogens, or after a microbial infection has already established in the presence of helminths. In this study we found that anthelmintic treatment of helminth-*Salmonella* co-infected mice did not result in complete elimination of *Salmonella* from the small intestine one week after helminths had been cleared. This data may help to explain observations from human population studies which reported that deworming was not associated with improved TB outcome in a human population [122] and that deworming of hookworm-HIV co-infected patients was not associated with improved T cell counts [120,121]. However, when HIV patients were co-infected with the roundworm *A. lumbricoides*, deworming was associated with improved T cell counts [120,121]. A study on helminth-infected African buffalo found that anthelmintic treatment did not affect the risk of acquiring a bovine TB infection, but did find that anthelmintic improved survival rates following acquisition of TB [123]. The impact of helminth colonization and subsequent deworming on host immunity to secondary pathogens likely depends on a multitude of factors, including the particular species of helminths and microbial pathogen, the route of infection, as well as host genetic and environmental factors. Helminth species differ in their niche(s) within their host, their mechanisms of interaction with the host immune system, and their effects on the intestinal microbiota [163,187,303], and thus the impact of helminth co-infection on host susceptibility to secondary pathogens is likely highly species- and context-dependent. Our work provides an example of a helminth species that can enhance intestinal colonization of a bacterial pathogen, yet it should be noted that in other contexts helminth species have been reported to provide protection against microbial pathogens, particularly when microbial challenge occurred at non-intestinal sites [216,304–308].

We have shown that helminth infection aids in the initial colonization of *Salmonella*, but that once *S.* Typhimurium has co-colonized with helminths, *Salmonella* does not require the presence of helminths in order for a subset of bacteria to persist in the small intestine. Recently, it was shown that diet-induced microbiota perturbation enhanced the ability of *S.* Typhimurium to colonize the intestinal tract, and reverting back to the original diet after *S.* Typhimurium establishment did not result in *Salmonella* clearance [309]. This supports the hypothesis that once an opportunity arises for *S.* Typhimurium to initially establish in the intestinal tract, it may no longer require the environmental stimulus that supported colonization in order to persist.

Our current understanding of helminth-bacterial co-infections lacks information on the mechanism(s) by which certain pathogenic bacteria acquire a colonization advantage during helminth infection. Some studies have attributed this to the ability of helminths to modulate immune responses that lower host defense to other bacterial pathogens [7,8,13,15,16]. Another possible mechanism by which helminths can promote bacterial infection is through shifts in the gut metabolic environment. It is known that intestinal metabolites derived from the bacterial microbiota can contribute to colonization resistance to pathogenic bacteria [302].

For example, butyrate can inhibit *Salmonella* pathogenicity island 1 (SPI-1) expression, a gene cluster which enables *Salmonella* to invade host tissue [310]. We have previously found that *H. polygyrus* infection alters the composition of metabolites in the small intestine, and that *H. polygyrus*-modified small intestinal metabolites are unable to inhibit SPI-1 gene expression *in vitro*, in contrast to metabolites from the small intestine of naïve mice which suppress SPI-1 gene expression [7]. Despite this finding, we have demonstrated that *Salmonella* can take advantage of the helminth-modified gut environment even when *Salmonella* lacked the ability to invade host tissue, suggesting that mechanisms beyond the promotion of *Salmonella* host invasion support small intestinal colonization. In fact, we found that the primary location for *Salmonella* expansion during helminth infection was in the small intestinal lumen, and that a portion of *Salmonella* was in close proximity with adult *H. polygyrus* worms. It remains possible that shifts in metabolite availability during helminth infection support *S.* Typhimurium colonization. A previous report has described how gut inflammation can promote luminal expansion of *S.* Typhimurium due to newly available metabolic substrates, which cause a switch to bacterial aerobic respiration [302].

Eosinophilia is a hallmark of helminth infection, which we hypothesized contributed to intestinal inflammation to indirectly create a favourable environment for *Salmonella* colonization. However, using eosinophil-deficient mice, we found that *H. polygyrus* co-infection supported *Salmonella* colonization independently of the presence of eosinophils. Previously, we have shown that helminth induction of IL-4-, Stat6- and RAG1- dependent immune responses are also not essential for helminth-induced *Salmonella* colonization [7]. Additionally, we tested whether the presence of an intact intestinal bacterial microbiota was essential for *Salmonella* to benefit from helminth infection, since helminth-induced changes in the microbiota composition have been associated with changes in host immunity [163]. We found that depletion of the bacterial microbiota does not preclude the ability of helminths to promote *Salmonella* colonization in the small intestine.

Our data showing that *H. polygyrus* promotes expansion of *Salmonella* predominately in the small intestinal lumen may point to a direct interaction between helminths and *Salmonella*. Helminth infection may promote luminal *Salmonella* expansion through providing a favourable attachment surface. It has previously been reported that *S.* Typhimurium evades antibiotic lethality through intimate binding to flatworms [111]. Our data supports the hypothesis that *Salmonella* closely associates with worms to promote its initial establishment in the small intestine. We also show that *H. polygyrus* larvae, which undergo molts inside the small intestinal tissue between day 1 and ~day 10 post infection, do not promote *S.* Typhimurium colonization to the same extent as adult worms that are present in the small intestinal lumen, which is line with the hypothesis that helminths provide a surface for bacterial colonization. In contrast, based on our data obtained through anthelmintic treatment following *Salmonella* colonization, we suggest that the worm surface is no longer essential for *Salmonella* to persist in the small intestine once helminths are drug-cleared.

Together, our findings suggest that anthelmintic treatment may be beneficial in preventing new colonization events with potential enteric bacterial pathogens. Further, anthelmintic treatment given subsequent to helminth-bacterial co-infection may serve to reduce bacterial burdens. However, anthelmintic treatment may not necessarily result in complete clearance of established bacterial pathogens. These results contribute to the growing literature on the interplay between helminths and co-infecting microbial pathogens and emphasize the importance of understanding the immunomodulatory effects of particular helminth species both during an ongoing infection and following helminth clearance.

Chapter 3

Small intestinal helminth infection results in a local increase in levels of branched short-chain fatty acid isovalerate

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Chapter disclaimer: The data that is discussed in this chapter is also featured in a recent publication [85] (see attached in **Appendix**), on which I am second author). The data in this chapter formed the basis of a research project that was then headed by Mia Kennedy and results from this project are now published [85] (**Appendix**). I assisted Mia Kennedy in several experiments for this publication, and data from experiments for which Mia Kennedy was the primary investigator is mentioned in the **Discussion** of this chapter and in the introductory section of **Chapter 6**. Mass spectrometry was done by Jun Han at the UVic Genome BC Proteomics Centre.

3.1 Introduction

Helminths can establish long-lived infections by modulating host immune responses. They have evolved a wide range of immunomodulatory molecules that have started to be characterized [49]. Most of these molecules that have been discovered are proteins, while smaller molecules such as metabolites have been relatively neglected. Helminth infection causes a shift in the metabolite composition of the intestinal tract [163]. The helminth itself can produce or consume metabolites that contribute to shifts in the overall composition of metabolites and shifts in the intestinal metabolome can also be a result of altered host metabolism, or from differential activity of the microbiota. In fact, shifts in the bacterial microbiota during helminth infection can drive modulation of host immune responses [163], and perhaps these changes in host immunity are orchestrated by specific metabolites derived from the metabolism of shifting microbial communities during helminth infection. Metabolites may therefore be important players in the trifold interactions between helminths, their hosts and the host's microbiota.

One major metabolite group of interest in terms of microbiota-driven immunomodulation is the collection of SCFAs [311]. SCFAs are products of bacterial fermentation and are vital for the health of the intestine: SCFA are energy building blocks for enterocytes and also play a role in immune homeostasis in the intestine [236]. Specifically, certain SCFA were shown to support the intestinal barrier function [312] and are able to induce the upregulation of Tregs [33,179,219]. The levels of SCFA vary along the gastrointestinal tract likely due to a variety in bacterial microbiota abundance [313,314]. Prior to our work, intestinal SCFA levels during *H. polygyrus* infection have only been investigated in the large intestinal segments and not the small intestine [23,315], perhaps because of the high density of microbiota in the large intestine and the research focus of these reports on microbiota-derived metabolites. In helminth-infected mice the SCFAs acetate and propionate were found in higher abundance in fecal matter [315] and cecal contents [23]. Until our recent publication [85] (**Appendix**), it was unknown how *H. polygyrus* infection affects levels of SCFAs locally, in the small intestine.

The most abundant SCFAs, acetate, butyrate and propionate are primarily produced as a result of bacterial fermentation of dietary fibers [316]. When dietary fiber is scarce, some bacterial species switch to fermentation of amino acids that results in the release of branched SCFAs, such as isovalerate that can be produced from leucine [316]. Branched SCFAs are less abundant and often overlooked or ignored in metabolomics studies, and it is unknown how helminth infection affects levels of these metabolites.

In this chapter, we performed a comprehensive analysis of ten SCFAs (including branched SCFAs) in the small intestinal content, colonic content and serum of mice infected with *H. polygyrus* and naïve mice.

3.2 Methods

3.2.1 Mice

All experiments were approved by the University of Victoria's Animal Care Committee and were in compliance with the Canadian Council on Animal Care. Wild-type C57BL/6 mice were bought from The Jackson Laboratory (strain 000644, from a maximal barrier room) and used for experiments after a minimum acclimation period of one week, or first bred and maintained under specific-pathogen free conditions at the University of Victoria and then used for experiments. During breeding, prior and during experiments, mice had access to food and water *ad lib*. All mice were 6-13 weeks old at the beginning of experiments. Only female mice were used for experiments, as indicated in figure legends. When bred at the University of Victoria, littermates were randomized between the experimental groups.

3.2.2 Helminth infection

The life cycle of *H. polygyrus bakeri* (*H. polygyrus*) was maintained in C57BL/6J mice according to an established protocol [299]. For experiments, mice were infected with 200 *H. polygyrus* stage 3 larvae by oral gavage. Parasite burdens were tracked by counting *H. polygyrus* eggs released into feces, which were enumerated using a McMaster Counting Chamber slide under a light microscope.

3.2.3 Anthelmintic treatment

Mice were given 2.5 mg Strongid P (Zoetis) in Ultra-Pure Distilled Water (Invitrogen) by oral gavage on two consecutive days. Efficacy of anthelmintic treatment was monitored by tracking fecal *H. polygyrus* egg release. For each experiment we confirmed that fecal *H. polygyrus* egg burdens were equivalent between *H. polygyrus*-infected groups prior to beginning anthelmintic treatment in the dewormed group.

3.2.4 Quantification of SCFA levels

The proximal 6 cm of the small intestine were dissected from mice and luminal contents were collected using forceps. Contents were snap-frozen in liquid nitrogen and stored at -80°C until metabolite analysis. Blood was collected by cardiac puncture and kept on ice, and serum was prepared from blood and stored at -80°C until metabolite analysis.

An internal standard solution containing deuterium-labeled acetic, propionic and butyric acid was prepared in acetonitrile. A mixture of standard substances containing 10 SCFAs was dissolved in 50% acetonitrile at 200 μM. This solution was further diluted step by step in a same ratio of 1 to 4 (v/v) with the same solvent to have working standard solutions.

Intestinal content samples were preprocessed as follows: each sample was mixed with acetonitrile, at 3 μL per mg raw material and two 3-mm metal beads were added. The samples were homogenized on a MM 400 mill mixer at a shaking frequency of 30 Hz for 2 min, followed by sonication in an ice-water bath for 2 min. The samples were then centrifuged for clarification at 21,000 x *g* and 5 °C for 10 min. Then, 20 µL of each thawed serum sample, the supernatant of each intestinal content sample or each standard solution was mixed in turn with 20 µL of IS solution, 40 µL of 200-mM 3-nitrophenylhydrazine HCl solution and 40 µL of 150-mM EDC HCl-6%pyridine solution, both in 60% acetonitrile. The mixtures were allowed to react at 40 °C for 40 min. After reaction, 240 µL of water was added to each solution and mixed well. A 10-µL aliquot of each resultant solution was injected to run LC-MS in the multiple-reaction monitoring mode on an Agilent 1290 UHPLC system coupled to an Agilent 6495B QQQ mass spectrometer equipped with a heated electrospray ionization source and with negative-ion detection. LC separation was carried out on a C18 UPLC column (2.1*100 mm, 1.8 µm) with 0.01% formic acid in water and 0.01% formic acid in acetonitrile as the binary solvents for gradient elution (20% to 60% B in 12 min) according to the procedures previously described [317].

Concentrations of the detected compounds were calculated with internal standard calibration by interpolating the constructed linear-regression curves of individual compounds with the analyte to internal standard peak ratios measured from each sample solution.

3.2.5 Statistical analyses

Statistical analyses were performed in GraphPad Prism 7.04. First, normality of the data was assessed with D'Agostino and Pearson normality test. Next, the appropriate statistical test was performed depending on the normality of the data set and the number of experimental groups being compared as indicated in the figure legends.

3.3 Results

3.3.1 Helminth infection increases small intestinal levels of isovaleric acid which is reversible with anthelmintic treatment

To test whether helminth infection affects levels of intestinal and circulating SCFAs, we infected mice with *H. polygyrus* or left the mice naïve, and collected small intestinal and colonic content as well as serum 14 days later. Metabolite analysis revealed that helminth infection did not significantly alter the levels of the three most abundant SCFA (acetic acid, butyric acid, propionic acid) but did cause a significant increase in levels of the branched SCFA isovaleric acid in the small intestinal content (**Figure 3.1**). Six additional SCFAs (valeric, caproic, isobutyric, 2-methylbutyric, isocaproic and 3-methylvaleric acid) were analyzed, however levels of these did not show consistent trends between experimental repeats or were below the lower limit of quantification (data not shown).

We additionally tested the consequences of a 2-day administration of pyrantel pamoate aimed at expelling worms from the mice (treatment effectivity was established in **Chapter 2**) on intestinal SCFA levels. Deworming treatment allows us to assess whether changes in SCFA levels require the ongoing presence of the worm, giving clues as to what is the source of these shifts in SCFAs and whether possible impacts of the metabolite shifts extend beyond worm clearance. We found that pyrantel pamoate alone did not affect SCFA levels along the intestine, but when it was used to expel worms on day 14 of *H. polygyrus* infection, treatment reverted helminth-increased isovaleric acid levels in the small intestine back to naïve levels (**Figure 3.1**).

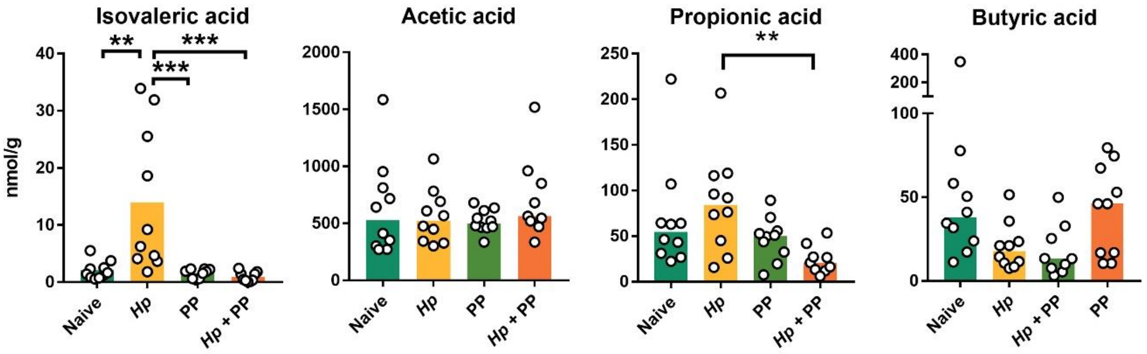

Figure 3.1. Small-intestinal levels of isovaleric acid are higher during an ongoing helminth infection and revert following helminth clearance. Female C57BL/6J mice were infected with *H. polygyrus* (*Hp*) or left naïve and were euthanized fourteen days post-*Hp* infection or received pyrantel pamoate (PP) for two consecutive days, fourteen days post-*Hp* infection, and then euthanized one day after completion of treatment. Luminal contents of the proximal small intestine were collected and analyzed for metabolites by UPLC-MS. Levels of isovaleric acid, acetic acid, propionic acid and butyric acid are shown. Data points represent individual mice and data is pooled from two independent experiments. Statistical comparisons between groups were made using a one-way ANOVA followed by a Tukey's multiple comparisons test for parametric data and a Kruskal-Wallis test followed by a Dunn's multiple comparisons test for non-parametric data. **= $p \leq 0.01$; *** = $p \leq .0001$.

In the experiments including a dewormed group of mice, isovalerate was the only SCFA that showed consistently significantly higher during helminth infection between experimental repeats. We did additional experiments comparing just naïve and *H. polygyrus*-infected mice, and with the larger sample size a significant decrease in butyric acid levels was detected in the small intestine of infected mice (**Figure 3.2**).

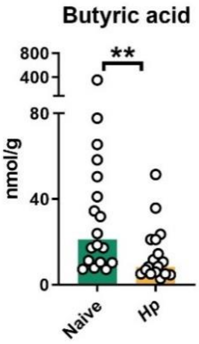

Figure 3.2. Small-intestinal levels of butyric acid are reduced during helminth infection. Female C57BL/6J mice were infected with *H. polygyrus* (*Hp*) or left naïve and were euthanized fourteen days post-*Hp* infection. Luminal contents of the proximal small intestine were collected and analyzed for metabolites by UPLC-MS. (**A-C**) Levels of butyric acid are shown. Data points represent individual mice and data is pooled from four independent experiments. Statistical comparison between groups was made using a Mann-Whitney test. **= $p \leq 0.01$

We next tested whether altered SCFA levels during helminth infection were restricted to the small intestine, or if SCFA perturbations extended along the entire length of the intestinal tract. We found that in the colonic contents SCFAs levels including isovaleric acid were unaltered by *H. polygyrus* infection (**Figure 3.3**). Pyrantel pamoate treatment of naïve or infected mice did not affect SCFA levels at this site either (**Figure 3.3**).

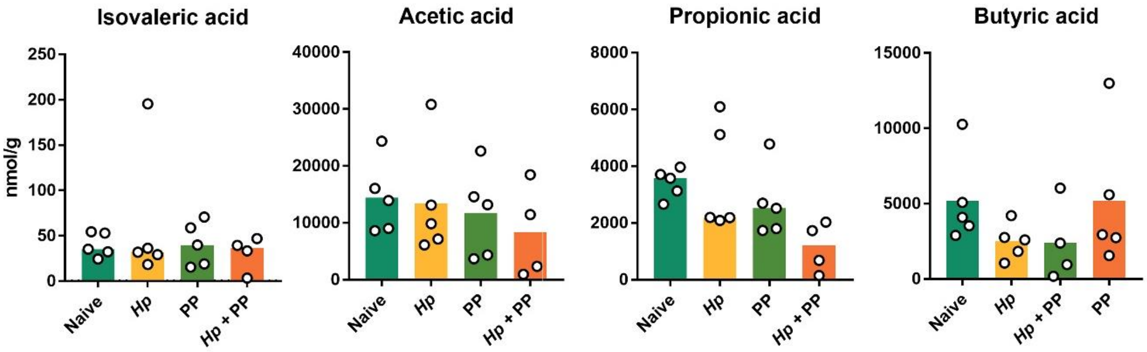

Figure 3.3. Colonic levels of SCFAs are unaffected by helminth infection and deworming. Female C57BL/6J mice were infected with *H. polygyrus* (*Hp*) or left naïve and were euthanized fourteen days post-*Hp* infection or received pyrantel pamoate (PP) for two consecutive days, fourteen days post-*Hp* infection, and then euthanized one day after completion of treatment. Luminal contents of the colon were collected and analyzed for metabolites by UPLC-MS. Levels of isovaleric acid, acetic acid, propionic acid and butyric acid are shown. Data points represent individual mice and data is from one experiment. Statistical comparisons between groups were made using a one-way ANOVA followed by a

Tukey's multiple comparisons test for parametric data and a Kruskal-Wallis test followed by a Dunn's multiple comparisons test for non-parametric data.

Finally, we tested whether helminth infection has a systemic effect on SCFA levels in the circulation. Metabolomic analysis of the serum did not reveal any differences between circulating levels of SCFAs of naïve and infected mice (**Figure 3.4**).

In conclusion, we found that an ongoing helminth infection increased levels of isovaleric acid, which was observed only locally in the small intestine and was reversible with drug clearance of the infection.

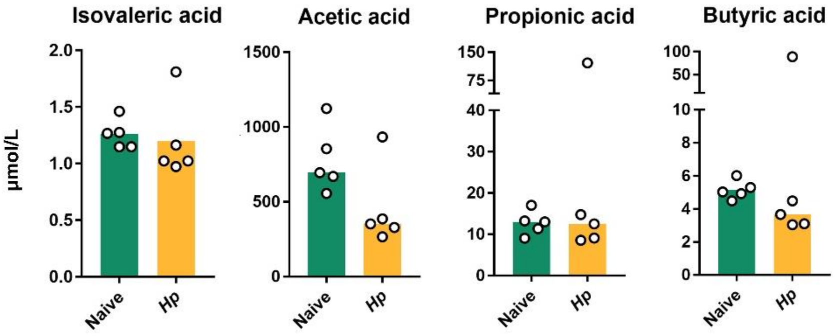

Figure 3.4. Circulating levels of SCFAs are unaltered during helminth infection. Female C57BL/6J mice were infected with *H. polygyrus* (*Hp*) or left naïve and were euthanized fourteen days post-*Hp* infection. Sera were collected and analyzed for metabolites by UPLC-MS. Levels of isovaleric acid, acetic acid, propionic acid and butyric acid are shown. Data points represent individual mice. Data is from one experiment and representative of two independent experiments. Statistical comparisons between groups were made using an unpaired t test for parametric data or Mann-Whitney test for non-parametric data.

3.4 Discussion

In this chapter (and in the publication I am second author on; [85], **Appendix**), we showed for the first time how helminth infection affects SCFA levels in the small intestine. We included branched SCFAs in our analyses and found that isovaleric acid levels were elevated in the small intestine during helminth infection. This data contributes to our understanding of how helminths impact the intestinal metabolome, which ultimately may lead to discovery of novel immunomodulatory molecules.

We did not see an increase in the levels of other SCFAs in either the small intestinal contents, colonic contents or in the serum during helminth infection. The only SCFA we found to be reduced in concentration during helminth infection was butyrate, and notably we needed to increase the sample size to detect significant differences in butyrate levels. Previous reports have shown an increase in SCFAs acetate and propionate in the large intestine during *H. polygyrus* infection [23,315]; an observation that we did not reproduce in our experiments. The experimental difference that could explain this discrepancy is that we looked at a relatively early time point: 14 days post infection, whereas the previous reports looked at four and six weeks into helminth infection. It is possible that at a more chronic phase of infection, SCFA levels are differently impacted during helminth infection. Whether isovalerate is upregulated in the small intestine during helminth infection beyond a two-week time point remains to be elucidated.

Furthermore, we included a group of mice that received a deworming treatment, which revealed that an increase in isovalerate levels requires the ongoing presence of *H. polygyrus*, as worm clearance results in reduced levels of isovaleric acid compared to mice with an ongoing helminth infection. This suggest that the helminth may be directly involved in the production of this SCFA or that helminth infection induces short-lived changes in the microbiota that results in elevated levels of isovalerate. Propionic acid levels were significantly lower in drug-cleared, previously helminth-infected mice compared to helminth-infected mice, while helminth infection alone or deworming treatment alone did not significantly impact levels of this SCFA, indicating that a combined action of infection and treatment can impact SCFA levels.

In experiments performed in parallel to those discussed in this chapter, we did not see elevated levels of isovalerate when *H. polygyrus*-infected mice were treated with antibiotics, suggesting that the bacterial microbiota plays an important role in increasing isovaleric acid levels during helminth infection [85] (**Appendix**). It is possible that in the small intestine, in addition to contributions from the bacterial microbiota, also the helminth itself contributes to SCFA production, as we found that *H. polygyrus* was able to directly produce isovaleric acid *in vitro*, especially in the presence of leucine [85] (**Appendix**). Bio-informatic analysis revealed that the *H. polygyrus* genome contains genes that encode for enzymes required for the breakdown of leucine into isovaleric acid [85] (**Appendix**). In line with our observations, SCFAs were detected in the ES material of hookworm *Ancylostoma caninum* [318,319], and *H. polygyrus* [23] and other helminth species have been previously shown to have the ability to produce acetate [23,224]. Together these results suggest that SCFA production may be a common trait amongst helminth species that could impact parasite-host interactions. It also indicates that it is possible that high isovalerate levels in helminth-infected mice as discussed in this chapter are at least in part due to *H. polygyrus*-production of isovalerate. Combined, these results inform us about the possible sources of isovalerate during helminth infection. Although *H. polygyrus* can produce isovaleric acid, our antibiotic-treatment experiments suggest that it cannot be solely responsible for an increase in isovaleric acid levels in helminth-infected mice, but rather the increase in isovalerate seems to be dependent on the interplay between *H. polygyrus* and the bacterial microbiota. It is possible that the bacterial microbiota supports *H. polygyrus* in producing isovaleric acid, for example by facilitating an increased availability of leucine, or that the microbiota contributes directly to high levels of this SCFA.

A local increase in isovalerate may influence host immunity and helminths may benefit from immunomodulation by SCFAs such as isovalerate. We found that isovalerate supplementation in drinking water of *H. polygyrus*-infected mice increased the output of parasite eggs in feces without changing the number of adult worms present in the small intestine, suggesting that isovalerate promotes helminth fecundity [85] (**Appendix**). We then hypothesized that isovalerate may modulate immune responses that may impact helminth fecundity. Several reports show that SCFAs can induce differentiation of Tregs [33,179,219] and *H. polygyrus* infection is known to lead to an expansion of Tregs [50]. Furthermore, amelioration of allergic asthma by helminth infection is dependent on the expression of a SCFA receptor [23]. We supplemented drinking water with isovalerate to mimic increased isovalerate levels during helminth infection and measured the abundance of Tregs in the small intestine and saw no differences in Treg levels due to isovalerate supplementation, indicating increased isovalerate levels alone do not increase Tregs [85] (**Appendix**).

We next investigated whether gut contractility was impacted during isovalerate-supplementation in helminth-infected mice. Exposure to isovaleric acid has been previously shown to inhibit smooth muscle contraction in *ex vivo* sections of mouse colons [320]. We hypothesized that isovalerate during helminth infection may counteract intestinal hypercontractility that is part of the host ‘weep-and-sweep’ response [2], and improve helminth fitness. To test this, we investigated gut transit time, a read-out of the time it takes for substances to travel through the intestinal tract, as an indirect way to assess gut motility *in vivo*. We supplemented drinking water of mice with isovalerate, infected mice with helminths two weeks later, and on day 14 of infection, we orally administered a red dye. We then euthanized the mice and determined how far the dye had travelled in the small intestine after 15 minutes of administration. We observed that helminth-infected mice presented with a faster small intestinal transit, as expected (since it has been shown previously that helminth infection increases smooth muscle contractility [81]). However, we found no evidence that isovalerate supplementation impacted gut transit in naïve or helminth-infected mice.

It is possible that high isovalerate levels impact other facets of host immunity, such as innate immune cell function, that may impact helminth fecundity. With our supplementation method we can assess effects of high isovalerate levels on host immunity, as well as test how isovalerate affects bacterial colonization. First, we hypothesize that shifts in metabolites during helminth infection affect the bacterial communities of the intestinal microbiota, and secondly, that metabolite shifts affect colonization of bacterial pathogens and in this way facilitates bacterial co-infection during helminth infection. Isovalerate levels during helminth infection and after deworming, as demonstrated in this chapter, follow the same trend as colonization levels of *Salmonella* in the small intestine, as showed in the previous chapter (**Chapter 2**). Determining the effects of metabolites on the composition of microbiota does not fall within the scope of this thesis, but co-infection does; the role of metabolites in the co-infection model used in this thesis is discussed in **Chapter 5**.

Overall, the work discussed in this chapter reveals new interactions between helminth and the bacterial microbiota and shows that the result of such interactions can affect helminth fitness. This chapter shows a comprehensive analysis of SCFA levels on day 14 of *H. polygyrus* infection, and with further research may reveal a novel immunomodulatory compound.

Chapter 4

Small intestinal concentrations of bile acids are reduced during helminth infection

Chapter disclaimer: Jenna Lane performed experiments under my supervision that resulted in **Figure 4.4** and **Figure 4.6**. Mass spectrometry was done by Jun Han at the UVic Genome BC Proteomics Centre.

4.1 Introduction

Helminths are potent manipulators of host immunity and do so by dampening inflammation and inducing regulatory immune pathways. The ways in which helminths modulate immune responses has been explored in recent years, and efforts have concentrated mostly on the ES products of helminths. Several ES proteins with immunomodulatory functions have been characterized [2], however there has been a lesser focus on smaller molecules such as metabolites for a potential role in helminth-mediated immunomodulation. Metabolites can act as immune messengers [32], therefore the mixture of metabolites present in the intestinal environment (the intestinal metabolome) could influence mucosal immune responses. During helminth infection, altered host and microbial metabolism, as well as the worm's metabolism, can leave a distinct fingerprint on the gut metabolome. For example, significant changes have been observed in the small intestinal metabolome during *H. polygyrus* infection in mice [7]. Several groups have attempted to characterize the changes in the intestinal metabolome of helminth-infected animals, using untargeted metabolomics [163]. The tools to annotate metabolite features remain inadequate, and therefore hypothesis-based, targeted metabolomics may prove more fruitful in discovering helminth-altered metabolite signatures. One group of metabolites that are candidates for playing a role in helminth-directed immunomodulation are bile acids.

Bile acids have been historically considered only as simple emulsifying agents to aid fat digestion, however recent studies have discovered the pleiotropic nature of bile acids. Bile acids are hormone-like signaling molecules directly acting on cellular receptors and luminal bacteria, hereby regulating mucosal immune functions and gut inflammation [34]. The composition of bile acids is determined by the enterohepatic cycle and the microbial metabolism of intestinal bile acids [264]. Bile acids are synthesized in the liver, stored in the gall bladder before being deposited in the duodenum upon food intake, and then reabsorbed in the ileum and returned to the liver via the portal vein. Each step in the bile acid circulation is responsive to dietary patterns via hormone signaling, to maintain a functional yet not toxic bile acid pool [265]. Primary bile acids (in mice: CA, CDCA, α/β-MCA, UDCA) are synthesized from cholesterol in the liver, while secondary bile acids are generated by microbial metabolism from primary bile acids in the intestine [266]. The final step in BA synthesis in the liver is conjugation with taurine or glycine (in mice mostly taurine), and this has two main consequences: 1) conjugated bile acids can form micelles and solubilize lipids, and 2) conjugated bile acids cannot diffuse across membranes (since they are fully ionized at physiological pH) and must be actively transported back to the liver. Active transport occurs in the ileum, where it is said that 95% of the BAs secreted in the intestine are recycled [264].

Following reabsorption into the underlying mucosa, accumulated bile acids can interact with mucosal immune cells, before ultimately entering the portal recirculation to the liver [268]. Examples of immunomodulation by bile acids include suppressing of pro-inflammatory signaling in macrophages and dendritic cells, and promoting Treg recruitment to the intestinal mucosa [268]. Bile acids have the capacity to directly lyse and kill bacteria [268], as well as promoting antibacterial defense [271]. Bile acids can also affect antimicrobial peptide and mucus production by enterocytes [286].

So far, no research group has characterized the bile acid profile during helminth infection. We hypothesize that changes to the bile acid profile occur during helminth infection, which might contribute to the modulation of immune responses during helminth infection. Here, we performed targeted metabolomics to characterize changes in the bile acid pool during helminth infection. Further, we assess how liver pathways responsible for bile acid biosynthesis, and how expression of small intestinal bile acid transporters, are affected by helminth infection.

4.2 Methods

4.2.1 Mice

All experiments were approved by the University of Victoria's Animal Care Committee and were in compliance with the Canadian Council on Animal Care. Wild-type C57BL/6 mice were bought from Jackson laboratories (strain 000644, from a maximal barrier room) and subsequently bred and maintained under specific-pathogen free conditions at the University of Victoria with access to food and water *ad lib*. All mice were 6-8 weeks old at the beginning of experiments. Both male and female mice were used for experiments, as indicated in figure legends. Littermates were randomized between the experimental groups, and mice were housed as 5 mice in the same experimental group, or in 2 cages of 3-4 mice of the same experimental group, as indicated in the figure legends.

4.2.2 Helminth infection

The life cycle of *H. polygyrus bakeri* (*H. polygyrus*) was maintained in C57BL/6J mice according to an established protocol [299]. Mice were infected with 200 *H. polygyrus* stage 3 larvae by oral gavage. Control animals that were kept naïve received an oral gavage with the non-sterile water that larvae were kept in but with larvae removed using a 70 µm cell strainer (VWR) and larvae removal was ensured by visual examination using a dissecting microscope. Parasite burdens were tracked by counting *H. polygyrus* eggs released into feces, which were enumerated using a McMaster Counting Chamber slide under a light microscope.

4.2.3 Sample collection

Proximal small intestine, ileum and colon were collected and luminal contents were squeezed out using forceps into cryogenic tubes (Sarstedt). Intestinal tissue was then put in a homogenizing tube containing 1 mL TRIzol (Fisher), to preserve RNA. Proximal small intestine was defined as the first 6cm of small intestine and the ileum as the last 1.5 cm. The entire liver was dissected and put into TRIzol (Fisher). Blood was collected via cardiac puncture and was coagulated on ice and spun down to obtain serum. Intestinal luminal contents, tissues and blood were kept on ice throughout dissection and stored at -80°C prior to metabolite analysis or RNA extraction.

4.2.4 Metabolite analysis

Bile acid analysis was performed at the UVic Genome BC Proteomics Centre. An Agilent 1290 UHPLC system coupled to a 6495B Agilent QQQ mass spectrometer was used. The MS instrument was operated in the multiple-reaction monitoring mode with negative-ion detection. A Waters BEH 15-cm long, 2.1-mm I.D. and C18 LC column was used and the mobile phase was 0.01% formic acid in water and 0.01% formic acid in acetonitrile for binary-solvent gradient elution. Detailed LC and MS operation parameters were the same as we described in [321].

First a mixture of standard substances containing all the targeted bile acids was dissolved in 40% acetonitrile at 10 nmol/mL, as the top standard solution, which was then was further diluted step by step at a same dilution ratio of 1 to 4 (v/v). Then, 100 µL of the standard solutions were mixed with 100 µL of an internal standard solution containing 14 D-labeled bile acids. To run UPLC-MS 10 µL of these solutions was injected. Linear-regression calibration curves were constructed between analyte-to-internal standard peak area ratios (As/Ai) versus molar concentrations (nmol/mL).

Each experimental sample was added with 10 µL/mg of 70% aqueous acetonitrile and two 3-mm metal beads. The samples were then homogenized at a shaking frequency of 30 Hz for 1 min three times on a MM 400 mill mixer, followed by sonication in a water bath for 2 min. After centrifugal clarification at 21,000 x *g* and 10˚C for 10 min. The supernatant was diluted 10 times with 40% acetonitrile. Then, 100 µL of each diluted sample solution was mixed with 100 µL of the internal standard solution, and 10 µL of this was injected for UPLC-MS. Concentrations of detected bile acids were calculated from the internal standard-calibration, linear-regression calibration curves of individual bile acids prepared in duplicate.

Sample analysis for plasma samples was done as follows. First, 20 µL of plasma was mixed with 40 µL of the internal standard solution containing 14 D-labeled bile acids and 40 µL of acetonitrile. After vortex-mixing for 30 s and sonication for 2 min in a water bath, the sample tube was centrifuged at 21,000 g for 10 min at 5˚C. Then, 80 µL of the clear supernatant was taken out and mixed with 920 µL of water. The mixture was loaded onto a 60mg/1mL Waters Oasis HLB cartridge which was activated with 1 mL of methanol and subsequently reconditioned with 1 mL of water before use. Under a positive pressure, the flow-through fraction was discarded. Bile acids were eluted with 800 µL of methanol. The collected fraction was dried under nitrogen and the residue was reconstituted in 64 µL of 40% acetonitrile. 10 µL was injected for UPLC-MS. Concentrations of detected bile acids were calculated from the internal standard-calibration, linear-regression calibration curves of individual bile acids prepared in duplicate.

Moisture content in samples was determined by 100% minus the percentage difference between wet weights (weight of the sample as dissected from the mouse) and dry weight (after lyophilization of the samples).

4.2.5 RNA extraction and cDNA generation

Tissue samples in TRIzol were thawed on ice, and a 5 mm stainless steel bead was added to each tube. The tissue was homogenized, 200 µL of chloroform was added and each tube was agitated for 15 seconds. Samples were then centrifuged for 15 min at 12,000 x *g* at 4˚C. The aqueous phase containing the RNA was transferred to a new 1.5 mL Eppendorf tube to which 500 µL of isopropyl alcohol was added to precipitate the RNA. A pellet of RNA was obtained by centrifugation for 10 min at 12,000 x *g* at 4˚C, which was then washed with 75% ethanol and centrifuged once more for 5 min at 7500 x *g* at 4˚C. After removal of supernatant, the RNA pellet was air-dried for at least 1 hr, until no liquid was visible in the tube. RNA was resuspended in an appropriate volume of UltraPure H2O (Fisher) that allowed the pellet to be entirely resuspended. The resuspended RNA was then heated at 60˚C for 15 minutes. A Nanodrop was used for RNA quality assessment and quantification. RNA concentration was normalized prior to genomic DNA removal and cDNA conversions, which were performed according to the manufacturer's instructions with the Bio-Rad iScript™ gDNA Clear cDNA Synthesis Kit. Remaining RNA was stored at -80˚C and cDNA at -20˚C.

4.2.6 Quantitative PCR

Quantitative PCR (qPCR) was performed using either the SsoAdvanced Universal SYBR Green Supermix or the Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix according to the manufacturer's instructions. All qPCR primers used are listed in the **Table 4.1**. Forward and reverse primers were added at a final concentration of 300 nM each. Final concentration of cDNA used was dependent on the results of primer optimization, the cDNA dilution was chosen for which primer efficiency was sufficient.

Primer efficiency was determined by performing qPCR on a standard curve of cDNA dilutions (of pooled samples) for each gene. The slope of the linear regression between the log of cDNA concentration and average Cq values was used to calculate efficiency. Efficiency (%) = (10-1/slope – 1) x 100%. An efficiency (%) between 90 – 110% was considered acceptable.

Each sample was run in triplicate on a Roche LightCycler® 96 Instrument. Amplification of experimental target genes was always performed alongside amplification of the housekeeping gene in the corresponding sample. Control wells containing UltraPure H2O in place of cDNA were run on each plate to establish background signal. Samples that had a Cq less than 5 cycles below the background Cq were marked as below detection limit and expression levels for these samples were set at '0'.

Expression values were calculated using the ΔΔCq method. The triplicate Cq values obtained from each sample during qPCR were averaged to give a mean Cq. The mean Cq of the housekeeping gene was subtracted from that of the target gene for each sample; this was the ΔCq value. All ΔCq values were normalized for interplate variation. The ΔCq values for the naïve control group were averaged, and this value was subtracted from all ΔCq values, both naïve and infected. This was the ΔΔCq value. An expression value (E = 2-ΔΔCq) was calculated for each sample. The mean (for parametric data) or the median (for non-parametric data) of the naïve expression values was then set at 1, to visualize fold difference of the infected samples relative to the naïve average.

Table 4.1. Primers used for qPCR to examine bile acid synthesis in the liver and bile acid transport in the small intestine.

Target	**Forward (5' to 3')**	**Reverse (5' to 3')**	**References**
mGAPDH	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG	-
CYP7A1	AGCAACTAAACAACCTGCCAGTACTA	GTCCGGATATTCAAGGATGCA	[267][322]
CYP8B1	GGCTGGCTTCCTGAGCTTATT	ACTTCCTGAACAGCTCATCGG	[267][322]
CYP2C70	TGGGCTTTTGCTCCTGCTGAAG	TCAGTGTACGGCATGTGGTTCC	[323]
BAAT	GGAAACCTGTTTAGTTCTCAGGC	GTGGACCCCCATATAGTCTCC	[324]
ASBT	TGGGTTTCTTCCTGGCTAGACT	TGTTCTGCATTCCAGTTTCCAA	[325][326][327]
OSTα	TTGTGATCAACCGCATTTGT	CTCCTCAAGCCTCCAGTGTC	[327]
OSTβ	GTATTTTCGTGCAGAAGATGCG	TTTCTGTTTGCCAGGATGCTC	[325] [326] [328]

4.2.7 Statistical analyses

Statistical analyses were performed in GraphPad Prism 7.04. First, normality of the data was assessed with D'Agostino and Pearson normality test. Next, the appropriate statistical test was performed depending on the normality of the data set, the number of experimental groups being compared, and whether correction for multiple hypothesis testing was necessary, as indicated in the figure legends.

4.3 Results

4.3.1 Helminth infection causes reduction in bile acid concentrations in the small intestine

To assess how helminth infection affects bile acid levels, we compared the intestinal levels of bile acids in naïve and *H. polygyrus*-infected mice. Mice were infected with *H. polygyrus* larvae or left naïve, and euthanized 14 days later, and the luminal content of the proximal small intestine, as well as the colon, were taken for metabolite analysis.

The metabolomics data revealed that in the proximal small intestinal lumen, total concentrations of bile acid showed a trend towards being reduced in helminth-infected mice compared to naïve mice, although this did not reach statistical significance (**Figure 4.1A**). A similar downwards trend was seen when the total bile acid data is split into subcategories: primary versus secondary, and conjugated versus unconjugated bile acids (**Figure 4.1B-D**). The data showed that in the proximal small intestine, primary conjugated bile acids are the most prevalent type of bile acids. Within this subcategory, taurine-conjugated bile acids were by far more abundant than glycine-conjugated metabolites (as is known to be the case with mice [329]), of which two were significantly reduced in helminth-infected mice (T-α-MCA and T-CDCA) (**Figure 4.1E**). A smaller but substantial part of the bile acid pool in the proximal small intestine consisted of unconjugated bile acids, highlighting a role of the small intestinal microbiota in metabolizing bile acids. In the less abundant categories, including precursors of primary bile acids and gluconated- and sulfated bile acids, there was a similar trend toward reduced bile acid concentrations when mice were infected with *H. polygyrus*, compared to naïve mice (**Figure 4.1F**).

In the colon, bile acid concentrations did not significantly differ between naïve and infected mice (**Figure 4.1G-I**). At this site, overall levels were lower than in the small intestine, as one would expect, due to reabsorption of a large part of the bile acid pool in the terminal ileum [264]. Levels of conjugated bile acids were very low, likely due to their reabsorption in more proximal parts of the intestinal tract, but also likely due to exhaustive metabolizing (i.e. deconjugating) by microbiota species of this sub-class of bile acids in the distal small intestine and in the colon.

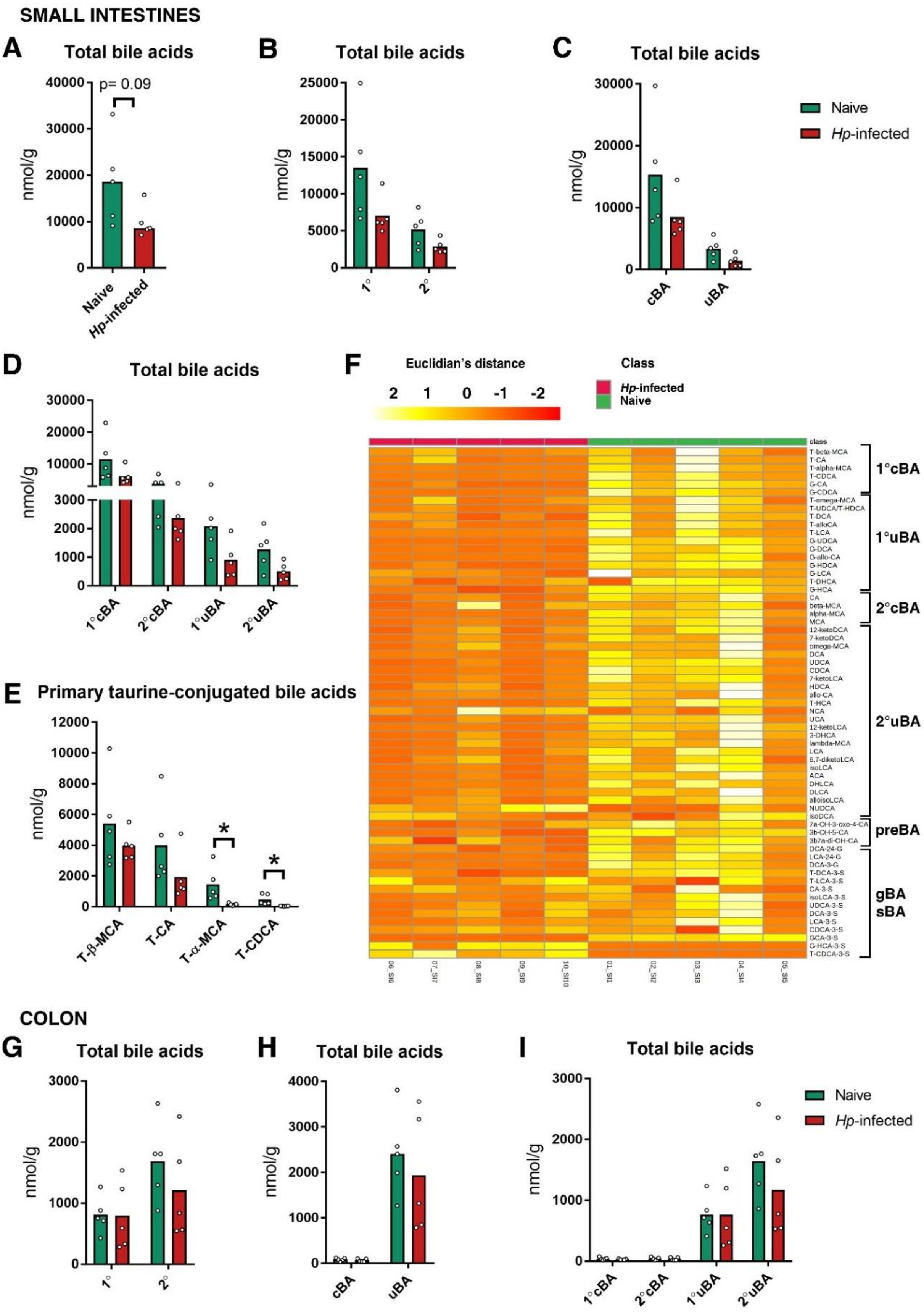

Figure 4.1. Helminth infection causes an overall reduction in bile acid concentrations in

the small intestine. Female C57BL/6J mice were infected with *H. polygyrus* (*Hp*) or left naïve and were euthanized fourteen days post-*Hp* infection. Luminal contents of the proximal small intestine and colon were collected and analyzed for metabolites by UPLC-MS. (**A-D**) Small intestinal levels of total bile acids, total primary bile acids (1°) and secondary (2°) bile acids, conjugated bile acids (cBA) and unconjugated bile acids (uBA) are shown. (**E**) Levels of taurine-conjugated 1° bile acids: T-β-MCA, T-CA, T-α-MCA and T-CDCA. UDCA is also a 1° bile acid in mice [267] however metabolite features of T-UDCA were not distinguishable from 2° bile acid taurine-conjugated hyodeoxycholic acid (T-HDCA) and thus is not included in this graph. (**F**) Heat-map of all the individual bile acids analyzed, grouped together as primary or secondary, conjugated or unconjugated subsets, and bile acid biosynthesis precursors (preBA) and gluconated or sulfated bile acids (gBA, sBA), with coloring based on Euclidian's distance as a measurement of differences between samples. (**G-I**) Colonic levels of total bile acids, total 1° and 2°, conjugated and unconjugated bile acids are shown. Data points represent individual mice and data is from one experiment. Statistical comparisons between total concentrations of bile acids were done with an unpaired t-test, and subcategories of total bile acids and the individual bile acids were made using a multiple t-test, corrected for multiple hypothesis testing with the FDR method (q value threshold = 0.05). *= q (FDR adjusted p-value) ≤ 0.05

4.3.2 Biological sex does not impact bile acid concentrations in naïve or infected mice

In an experimental repeat of our initial bile acid metabolite analysis (reported in **Figure 4.1**), we wanted to address an additional question: whether there are differences in bile acid levels between male and female mice in naïve mice and during helminth infection. For this experiment, we housed male and female mice from the same litter in separate cages per sex, and infected half of the females and males with *H. polygyrus*, while leaving half of the mice naïve. Within the experimental group (different sex, different infection status) we also divided the mice over two separate cages, to account for cage effects. Fluctuations in bile acid levels are susceptible to eating patterns [330], and the gut microbiota influences the composition of the bile acids [331], thus the results are prone to random cage effects related to divergent eating behavior and/or random introduction/expansion/elimination of certain microbes in some of the cages and not the others. In this experiment we intended to limit the potential skewing of data due to potential cage effects, by housing mice in two cages per experimental group.

Analysis by metabolomics showed that in female mice there was a significant reduction in total concentration of bile acids during helminth infection, however, the levels between male and female mice did not differ between infected or naïve mice (**Figure 4.2A**). When we divided the data into subcategories of bile acids and compared it to our previous results (**Figure 4.1**), it appeared that the patterns of the previous experiment repeat independently of sex. Moreover, statistical analysis revealed that the sex of the mice did not significantly impact bile acid concentrations during helminth infection or in naïve mice.

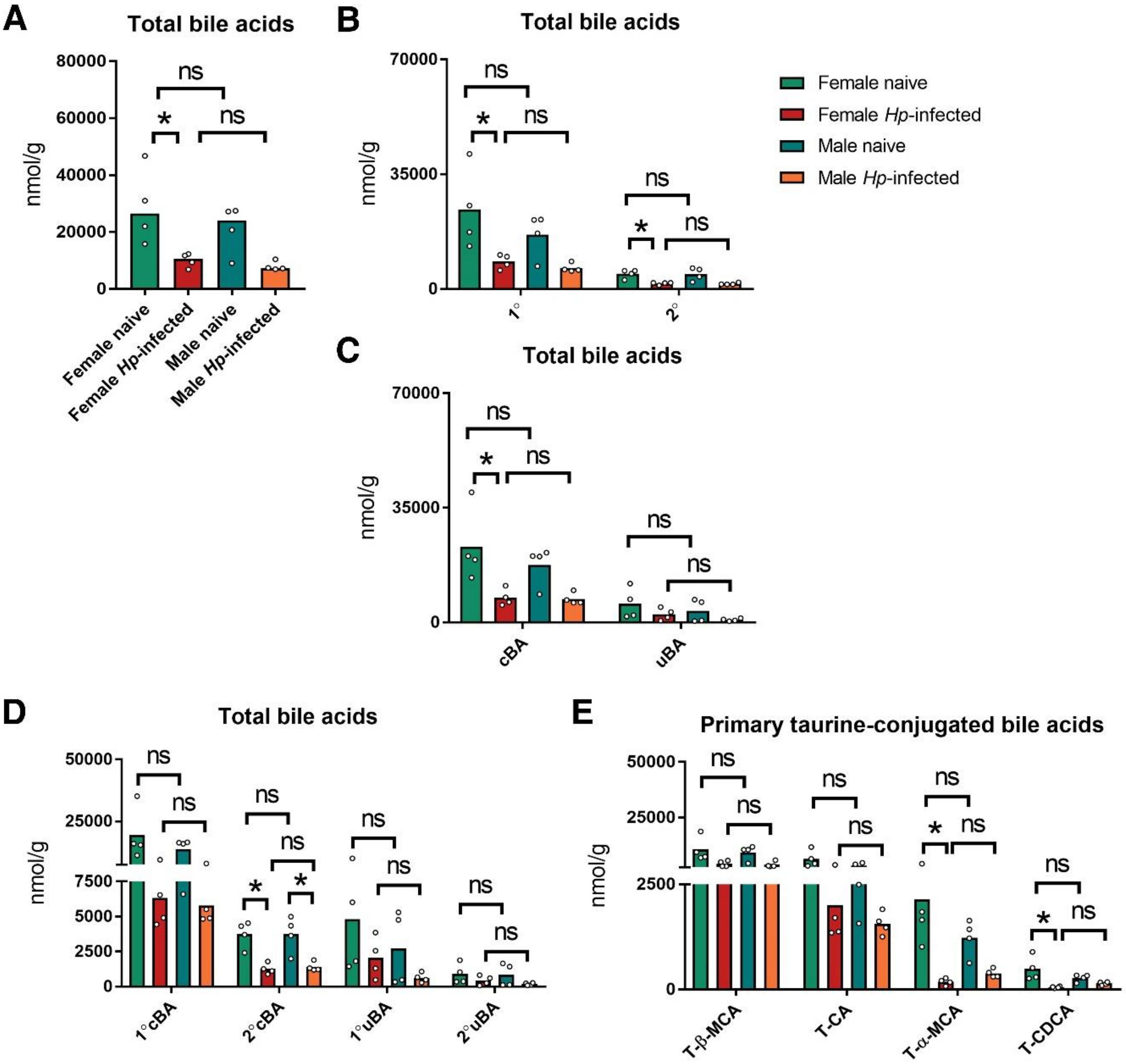

Figure 4.2. Sex does not significantly impact bile acid concentrations in naïve or helminth-infected mice. Female and male C57BL/6J mice were infected with *H. polygyrus* (*Hp*) or left naïve and were euthanized fourteen days post-*Hp* infection. Luminal contents of the proximal small intestine were collected and analyzed for metabolites by UPLC-MS. (**A-D**) Small intestinal levels of total bile acids, total primary bile acids (1°) and secondary (2°) bile acids, conjugated bile acids (cBA) and unconjugated bile acids (uBA) are shown. (**E**) Levels of taurine-conjugated primary bile acids: T-β-MCA, T-CA, T-α-MCA and T-CDCA. Data points represent individual mice and data is from one experiment. Statistical comparisons were made using a 2-way ANOVA by a Tukey's multiple comparisons test. *= $p \leq 0.05$

4.3.3 Reduction in bile acid concentrations is not due to decreased biosynthesis in the liver

Because did not detect sex differences in this data set, we decided to pool the data from female and male mice together, and with increased statistical power we were able to detect robust trends between naïve and *Hp*-infected mice (**Figure 4.3A-D**). This data confirmed our conclusion that helminth infection causes a reduction in bile acid concentration in the mouse small intestine. Here, multiple hypothesis testing of individual bile acid levels revealed that mostly conjugated bile acids contribute to a reduction in the concentration of bile acids in helminth infected mice (**Table 4.2**). We next asked whether the reduction in bile acid levels was due to decreased production of primary bile acids in the liver.

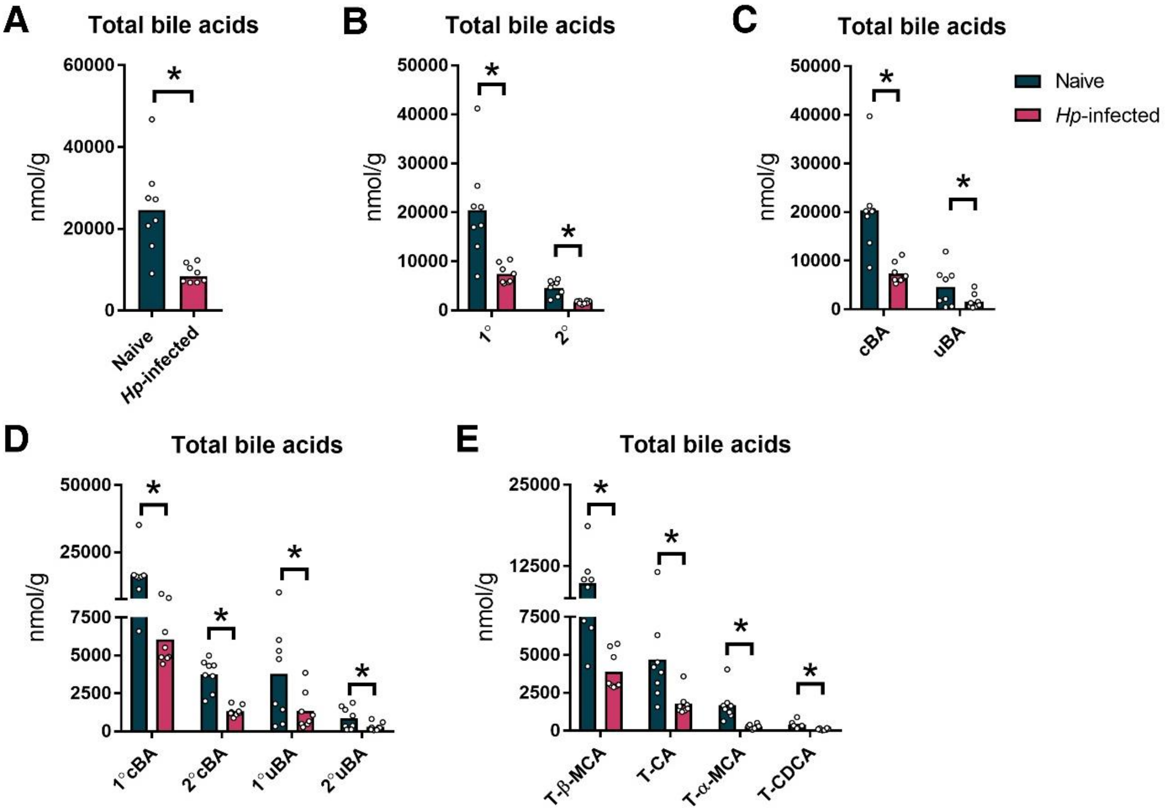

Figure 4.3. Helminth infection significantly reduces bile acid concentrations in female and male mice. Female and male C57BL/6J mice were infected with *H. polygyrus* (*Hp*) or left naïve and were euthanized fourteen days post-*Hp* infection. Luminal contents of the proximal small intestine were collected and analyzed for metabolites by UPLC-MS. (**A-D**) Small intestinal levels of total bile acids, total primary bile acids (1°) and secondary (2°) bile acids, conjugated bile acids (cBA) and unconjugated bile acids (uBA) are shown. (**E**) Levels of taurine-conjugated primary bile acids: taurine-conjugated beta-muricholic acid (T-β-MCA), taurine-conjugated cholic acid (T-CA), taurine-conjugated alpha-muricholic acid (T-α-MCA) and taurine-conjugated chenodeoxycholic acid (T-CDCA). Data points represent individual mice, and data is from one experiment. Statistical comparisons between total concentrations of bile acids were done with an unpaired t-test. Subcategories of total bile acids,

and all individual bile acids (including taurine-conjugated primary BA in (**E**), for statistics of all individual bile acids see **Table 4.1**) were made using a multiple t-test, corrected for multiple hypothesis testing with the FDR method (q value threshold = 0.05). *= p≤ 0.05 or q (FDR adjusted p-value) ≤ 0.05.

Table 4.2. Helminth infection primarily reduces levels of conjugated bile acids demonstrated by multiple hypothesis testing of levels of individual bile acids. Results from multiple hypothesis testing with multiple t-test that are corrected with the FDR method. Desired FDR was set at 0.05 and as a result q values (FDR-corrected p values) under 0.05 that were positive for discovery are marked as 'yes' and false discoveries as 'no'. The p values are from the individual t-tests, 'Mean1' corresponds to the mean value of naïve samples, and 'Mean2' refers to helminth-infected samples. #T-UDCA is a primary conjugated bile acid, but cannot be distinguished from T-HDCA, a conjugated secondary bile acid and therefore is included in the latter category.

	Discovery	**p value**	**Mean1**	**Mean2**	**q value**
Primary conjugated bile acids					
Tauro-beta-muricholic acid	Yes	0.00212	9840	3891	0.0078
Taurocholic acid	Yes	0.02167	4711	1783	0.0354
Tauro-alpha-murocholic acid	Yes	0.00193	1684	283.7	0.0078
Taurochenodeoxycholic acid	Yes	0.00431	382.6	98.83	0.0119
Glycocholic acid	Yes	0.00026	16.74	3.217	0.0023
Glyco-beta-muricholic acid	Yes	0.002	8.929	1.647	0.0078
Glyco-alpha-muricholic acid	Yes	0.00039	2.198	0.2155	0.0029
Glycochenodeoxycholic acid	Yes	0.00174	0.7718	0.059	0.0078
Secondary conjugated bile acids					
Tauro-omega muricholic acid	Yes	0.00014	2089	894.1	0.0021
Tauro(urso/hyo)deoxycholic acid#	Yes	0.00336	698.9	193.3	0.0108
Taurodeoxycholic acid	Yes	0.00341	297	67.95	0.0108
Tauroallocholic acid	Yes	0.01783	637.3	171.9	0.0343
Taurohyocholic acid	Yes	0.02463	13.36	6.984	0.0386
Taurolithocholic acid	Yes	0.00024	4.242	0.7891	0.0023
Glyco-omega-muricholic acid	Yes	0.00585	2.375	0.4152	0.0152
Glycoursodeoxycholic acid	Yes	0.00149	0.729	0.0577	0.0078
Glycoallocholic acid	No	0.04518	1.877	0.3675	0.0586
Glycodeoxycholic acid	Yes	0.00367	0.6418	0.0399	0.0108
Glycohyodeoxycholic acid	Yes	0.00084	0.2934	0.0346	0.0053
Glycohyocholic acid	Yes	0.01995	0.05204	0.0137	0.0343
Glycolithocholic acid	Yes	0.01874	0.00501	0.0003	0.0343
Primary unconjugated bile acids					
Cholic acid	No	0.05081	2326	680	0.064
beta-Muricholic acid	No	0.26401	1118	599.1	0.2156
alpha-Muricholic acid	Yes	0.02602	265.4	36.41	0.0386
Ursodeoxycholic acid	No	0.09228	90.02	22.75	0.0989
Chenodeoxycholic acid	No	0.07361	56.28	21.14	0.0877
Murocholic acid	No	0.11175	5.807	2.008	0.112

Secondary unconjugated bile acids					
7-Ketodeoxycholic acid	Yes	0.02914	243	60.34	0.0389
omega-muricholic acid	No	0.09422	172.9	73.92	0.0989
12-Ketochenodeoxycholic acid	No	0.17499	136.6	66.21	0.1582
Deoxycholic acid	No	0.05691	103.9	29.26	0.0697
Allocholic acid	No	0.12512	55.68	17.06	0.1226
hyodeoxycholic acid	No	0.29298	17.27	8.263	0.2349
7-Ketolithocholic acid	No	0.08588	16.1	6.318	0.0989
Ursocholic acid	Yes	0.01217	14.22	2.711	0.0283
Norcholic acid	No	0.17575	6.238	15.26	0.1582
3-Oxocholic acid	No	0.08896	4.114	1.151	0.0989
Apocholic acid	No	0.16753	3.687	1.444	0.1572
Lambda-muricholic acid	No	0.15719	2.307	1.114	0.1507
Lithocholic acid	No	0.19365	0.7191	0.3762	0.1642
6,7-Diketolithocholic acid	No	0.18183	0.8561	0.3937	0.1595
Dioxolithocholic acid	No	0.09263	0.3961	0.0798	0.0989
12-Ketolithocholic acid	No	0.18441	0.3823	0.153	0.1595
3b-OH-5-cholestenoic acid	Yes	<0.00001	0.398	0.0934	<0.0000
7a-OH-3-oxo-4-cholestenoic acid	No	0.39086	0.2054	0.1623	0.2972
Isodeoxycholic acid	Yes	<0.00001	0.06741	0.0007	<0.0000
Isolithocholic acid	No	0.94003	0.05004	0.048	0.6686
Nordeoxycholic acid	No	0.32408	0.02955	0.0783	0.2552
Dehydrolithocholic acid	Yes	0.02865	0.03652	0.0115	0.0389
3b,7a-diOH-5-cholestenoic acid	No	0.10083	0.01638	0.008	0.1034
Norursodeoxycholic acid	No	0.22218	0.00626	0.0148	0.1849
Alloisolithocholic acid	No	0.68978	0.02275	0.0167	0.507
Dehydrocholic acid	No	0.50304	0.02816	0.02	0.376
Gluconated/sulfated bile acids					
Deoxycholic acid-24-glucuronide	Yes	0.01802	0.1946	0.0313	0.0343
Chenodeoxycholic acid-24-glucuronide	Yes	0.01	0.1047	0.0104	0.0245
Lithocholic aicd-24-glucuronide	Yes	0.01623	0.05465	0.0089	0.0341
Ursodeoxycholic acid-24-glucuronide	Yes	0.02024	0.05132	0.0094	0.0343
Taurodeoxycholic acid-3-sulfate	Yes	0.02672	2.736	1.188	0.0386
Cholic acid-3-sulfate	Yes	0.02713	3.503	0.8614	0.0386
Deoxycholic acid-3-sulfate	Yes	0.01446	0.4052	0.1135	0.0319
Lithocholic aicd-3-sulfate	No	0.37765	0.01186	0.0036	0.2922
Chenodeoxycholic acid-3-sulfate	No	0.89585	0.01166	0.0103	0.6477

To test whether the bile acid biosynthesis was altered during helminth infection, we extracted RNA from the mouse livers, and quantified RNA expression levels by qPCR. In the liver, several cytochrome P450 enzymes play important a role in the biosynthesis primary bile acids from cholesterol (**Figure 4.4A**). The rate-limiting enzyme encoded by *Cyp7a1* manages the first step of the biosynthesis of bile acids from cholesterol, and we found no differences in expression of this enzyme between naïve and infected animals (**Figure 4.4B**). In contrast, *Cyp8b1* was significantly downregulated in helminth-infected mice compared to naïve mice (**Figure 4.4C**), and *Cyp2c70* was upregulated during infection (**Figure 4.4D**). The enzyme encoded by *Cyp8b1* is a 12α-hydroxylase and determines the production of CA (which is 12α-hydroxylated) versus CDCA (which is not) [332], but a downregulation of *Cyp8b* cannot explain a reduction in the concentration of all primary bile acids. Moreover, a 40% reduction in *Cyp8b1* expression was observed in experiments with heterozygous *Cyp8b1*$^{+/-}$ mice and the authors of reported that this did not lead to a reduction in cholic acid, and did not affect the size of the overall bile acid pool [333]. In addition, the increase we observed in the expression of *Cyp2c70* (responsible for converting CDCA in αMCA and βMCA) did not correlate with the lower MCA levels we detected in our mice. Therefore, different expression levels of these liver enzymes alone are unlikely to explain a general reduction in bile acid concentrations that we observed during helminth infection in the small intestine. Before release into the gallbladder, bile acids are conjugated to taurine or glycine by bile acid-CoA:amino acid N-acyltransferase (encoded by *Baat*) (**Figure 4.4A**). In our experiments, *Baat* expression levels were not affected by helminth infection (**Figure 4.4E**). The results discussed here are preliminary results and ongoing experimental repeats should reveal whether the trends as discussed above are robust.

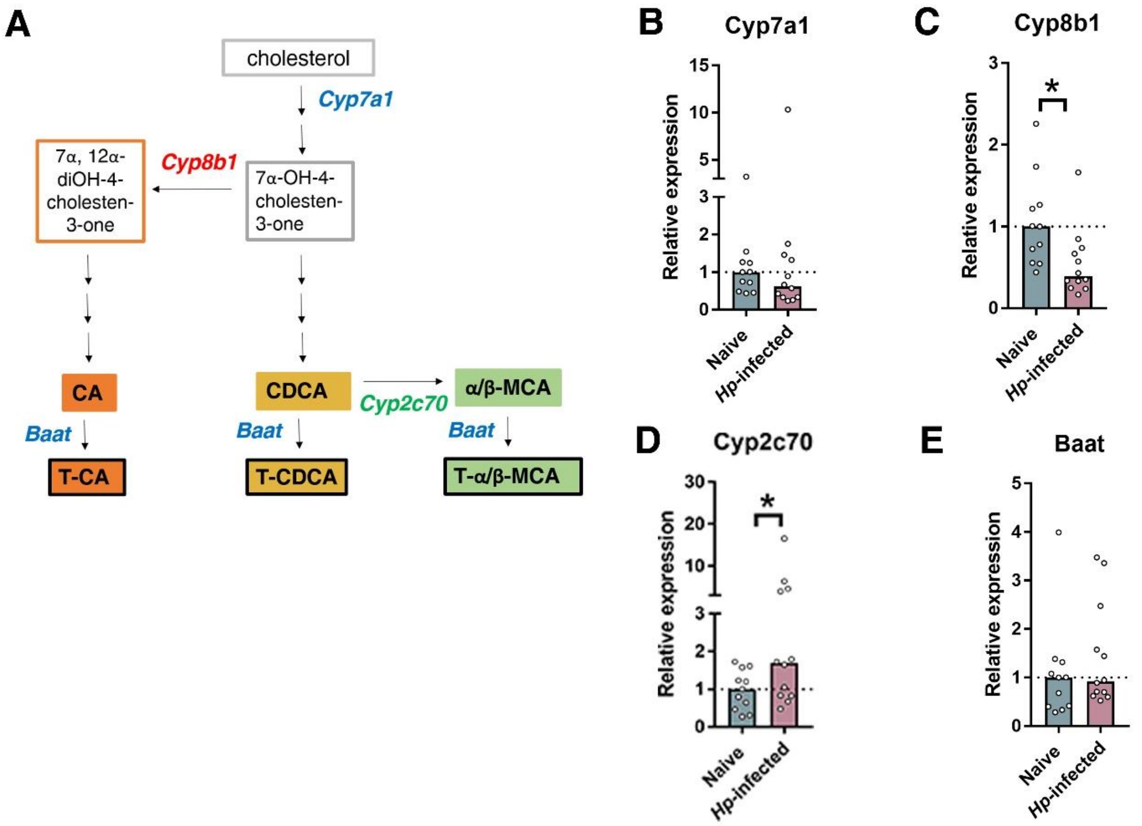

Figure 4.4. Expression levels of enzymes involved in bile acid synthesis are altered by helminth infection. Expression of genes in the hepatic pathways of BAs biosynthesis during helminth infection relative to expression in naïve mice. RNA was extracted from mouse livers, and expression levels of the genes were quantified using RT-qPCR. (**A**) Hepatic pathways of bile acid biosynthesis are shown, highlighting the genes tested, in red genes that we found were downregulated during helminth infection, in green those that were upregulated and in blue similarly expressed between naïve and infected mice. Expression levels relative to house-keeping genes are shown for Cyp genes responsible for generating primary bile acids (**B-D**) and BAAT (**E**) that mediates the final step of bile acid biosynthesis, the conjugation of bile acids to an amino acid. Each data point represents an individual mouse, and data is pooled from two independent experiments. Statistical comparisons were made using an unpaired t-test for parametric data, and a Mann-Whitney test for non-parametric data.

4.3.4 Reduction in bile acid concentrations is correlated with increased fluid content in the small intestinal lumen

In our initial analysis of bile acids in small intestinal luminal content, abundance of individual bile acids was normalized to the wet, 'actual' weights of samples after dissection. In our experiments, there was a significant difference in the moisture content between samples from naïve and helminth-infected small intestines (**Figure 4.5A**). This prompted us to consider normalizing the data to dry weights of the samples that were recorded after lyophilisation prior to mass spectrometry. While we think that this is not the best way to represent the physiological situation in the mouse intestine, it did provide us with additional information: normalized to dry weights, there were no significant differences in bile acid levels between naïve and *H. polygyrus*-infected mice (**Figure 4.5B-E**). It is possible that the increased moisture content of small intestinal contents is diluting the bile acid pool, resulting in a lower concentration in the small intestinal lumen of helminth-infected mice. It may be that the contribution of increased fluid efflux into the intestinal lumen is far greater than subtle differences in the *de novo* synthesis of bile acids, which may explain why the changes in liver pathways we measured were not reflected in the total bile acid pool.

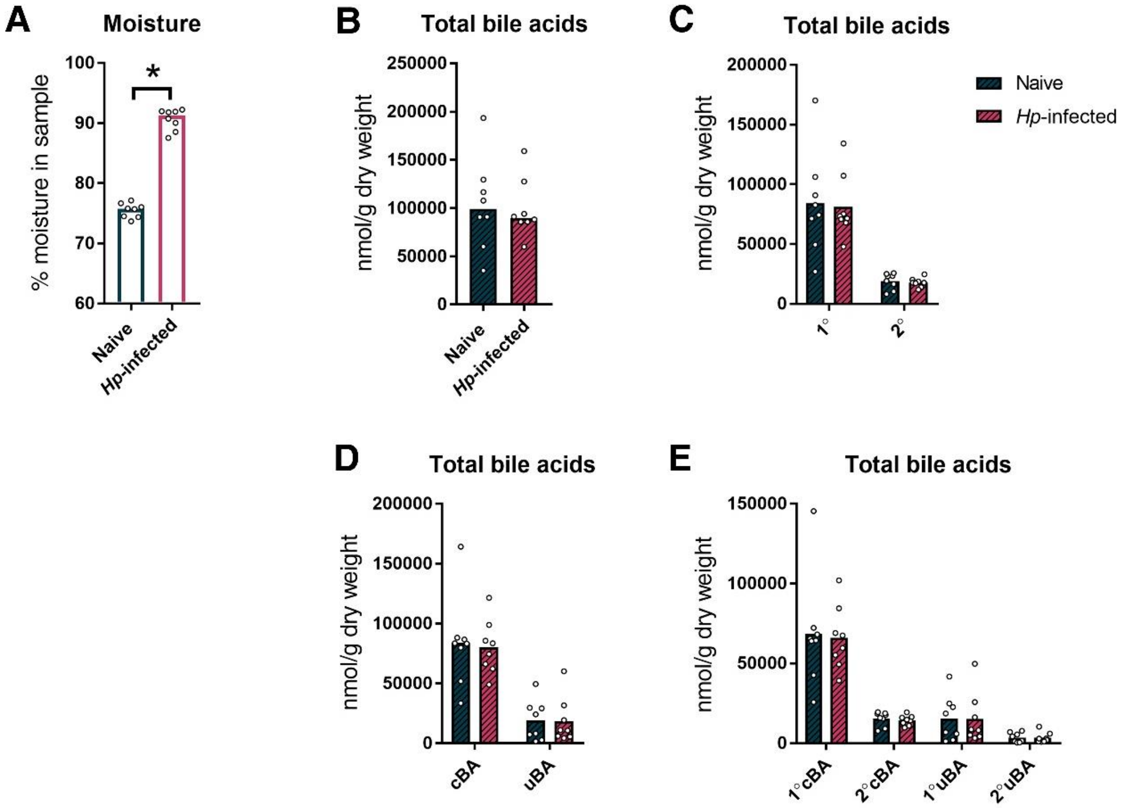

Figure 4.5. Reduction in bile acids during helminth infection is not observed when metabolite concentrations are normalized to dry sample weight (**A**) Moisture content in the 'wet' samples. (**B-D**) Small intestinal levels of total bile acids, total primary bile acids (1°) and secondary (2°) bile acids, conjugated bile acids (cBA) and unconjugated bile acids (uBA) are shown. Data is normalized to dry weights that were obtained after lyophilisation of the 'wet' samples. Data points represent individual mice and data is from one experiment. Statistical comparisons between total concentrations of bile acids, and different moisture content, was done with an unpaired t-test, and subcategories of total bile acids were made using a multiple t-test, corrected for multiple hypothesis testing with the FDR method (q value threshold = 0.05). *= p≤ 0.05 or q (FDR adjusted p-value) ≤ 0.05

4.3.5 *Circulating levels of bile acids are not altered during helminth infection*

Next, we asked whether reabsorption of bile acids in the tissue along the small intestine may be affected by a lower concentration of bile acid during helminth infection. After transport into the small intestinal tissue, bile acids can interact with the mucosal immune system [34], and once taken up into the blood, can even affect systemic immunity [334].

To test if bile acid transport from the intestine was affected by helminth infection, we quantified expression of bile acid transporter genes in enterocytes in the proximal small intestine and the ileum. We quantified gene expression levels of the apical sodium-dependent bile acid transporter (*Asbt*) [335] and organic solute transporter (OST) that assists transport over the basal membrane and consists of two subunits (encoded by *Ostα* and *Ostβ*) [336]. We observed significant downregulation of *Asbt* and *Ostβ* during helminth infection in the proximal small intestine (**Figure 4.6A-C**), whereas the expression levels of the bile acid transporters in the ileum remained the same between naïve and helminth-infected mice (**Figure 4.6D-F**). These results are preliminary as experimental repeats are ongoing as well as experiments to directly compare expression levels of the transporters between proximal small intestinal and ileal tissue.

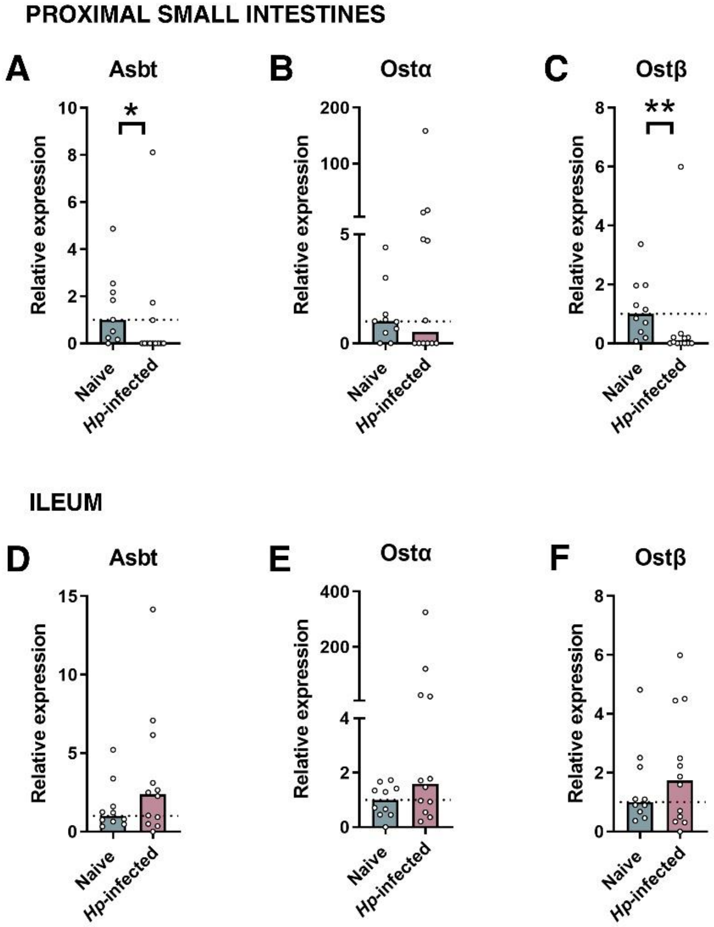

Figure 4.6. Several bile acid transporter genes are downregulated in the small intestine during helminth infection. Expression of genes involved in bile acid transportation across small intestinal and ileal epithelium. RNA was extracted from intestinal tissue, and expression levels of the genes were quantified using RT-qPCR. Naïve levels of expression are set to average the value '1' for each gene. Expression levels relative to house-keeping genes are shown for bile acid transporter genes in the proximal small intestinal tissue (**A-C**) and ileal tissue (**D-F**). Each data point represents an individual mouse, and data is pooled from two independent experiments. Statistical comparisons were made using an unpaired t-test for parametric data, and a Mann-Whitney test for non-parametric data. *= p≤ 0.05; **= p≤ 0.01

Because we found changes in expression of bile acid transporter during helminth infection, we then questioned whether the concentrations of bile acid in the serum were impacted during infection. Upon metabolite analysis, we did not detect any differences in circulating levels of bile acid between naïve and *H. polygyrus*-infected mice (**Figure 4.7**).

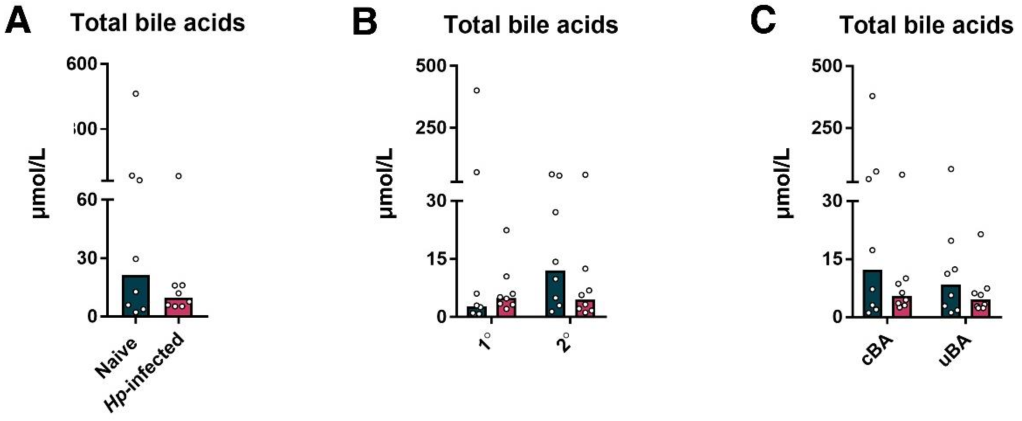

Figure 4.7 Helminth infection does not affect circulating levels of bile acids. Serum levels of total bile acids, total primary bile acids (1°) and secondary (2°) bile acids, conjugated bile acids (cBA) and unconjugated bile acids (uBA) are shown. Data points represent individual mice and data is from one experiment. Statistical comparisons between total concentrations of bile acids was done with a Kruskall-Wallis test, and subcategories of total bile acids were made using a multiple t-test, corrected for multiple hypothesis testing with the FDR method (q value threshold = 0.05). *= p≤ 0.05 or q (FDR adjusted p-value) ≤ 0.05

4.4 Discussion

This is the first report, to our knowledge, of how bile acids are affected by helminth infection. At day 14 of *H. polygyrus* infection, bile acid concentrations in the small intestine are significantly reduced. We hypothesize that the reduction in concentrations is primarily due to the efflux of fluids into the intestinal lumen at the preferred niche of *H. polygyrus* adult worms, the duodenum and jejunum. We did not detect differences in bile acid levels when luminal samples were normalized to their dry weights. Further, we find that helminth infection affects important genes in the biosynthesis of bile acids, but this likely only has a small role in the reduction of bile acid concentrations in the lumen, if at all. The expression levels of bile acid transporters are reduced during helminth infection in the proximal small intestine as well, which we hypothesize is likely as an attempt to maintain appropriate bile acid levels in the lumen. Serum levels of bile acids are not affected by helminth infection.

It is the central dogma that active transport of bile acids takes place in the ileum [336], and we could not find evidence in the literature of any reports on bile acid transport in the proximal small intestine. We are the first to show that gene expression of bile acid transporters can be measured in the proximal small intestine of mice. Reduced transport of bile acids out of the small intestinal lumen could in theory lead to increased excretion versus recycling bile acids into enterohepatic circulation, however, we observed no changes in circulating levels of bile acids during helminth infection. Our working hypothesis is that the contribution of the proximal small intestinal transporters to the recycling of bile acids is likely rendered insignificant by the contribution of ileal bile acid transport (the expression of ileal bile acid transporters was unaltered by helminth infection). It remains possible that a causal relationship in the other direction exists: a lower bile acid concentration in the intestinal lumen may cause reduced transporter expression. Modulation of ileal bile acid transporter expression has been described and includes regulation by bile acids [336], while in the proximal parts of the intestine such a feedback mechanism has not yet been explored. In the proximal small intestine, it may be particularly important to maintain high luminal levels of bile acids to solubilize fat molecules and facilitate their absorption, versus the ileum where transport ensures bile acids are recycled and not lost due to excretion. Therefore, it seems possible that the expression levels of bile transporters in the duodenum and jejunum are affected by the lower luminal concentrations of bile acids during helminth infection, as a feedback mechanism to maintain the luminal availability of bile acids. However, we have not yet provided evidence for such a causal role and further research is required.

In the literature, it is often stated that in the ileum 95% of the bile acids is recycled [34,264,266]. However, we find that levels in the colon are higher than we would expect from this statement: closer to 15% of the bile acids we find in the proximal small intestine remained in the intestinal flow up till the colon. In the colon, bile acids that are deconjugated (which is the majority of the pool at this site) can passively diffuse over membranes, and in this way be recycled. Therefore, the statement that 95% of the bile acids are recycled may be correct, however this does not only occur in the ileum. We find evidence that active transport occurs in the proximal small intestine, and passive transport in the colon may contribute as well.

The intestinal microbiota plays important roles in bile acid metabolism [266]. Moreover, the microbiota forms an essential component in increasing the diversity of the bile acid pool, by removing the conjugated amino acid from the bile acid as they are derived from the liver, and by further metabolizing bile acids to form a wide arsenal of secondary bile acids. Those variable bile acids can have distinct effects (with different effect sizes) on cell function, exemplified by a study that shows that different derivatives of LCA have differential impact on Treg and Th17 differentiation [276]. The gut microbiota does not only impact metabolism of secondary bile acids, but also synthesis of primary bile acids, through engagement of FXR in the ileum, a receptor that controls liver bile acid synthesis through negative feedback from the intestine [266]. We have not yet investigated the contribution of the microbiota to the altered bile acid concentrations in the small intestine of helminth-infected mice. We suspect that microbiota shifts do not contribute to the changes we see in bile acid levels, as we do not see changes in bile acid synthesis enzymes in the liver that could indicate altered inhibitory feedback from the intestine, neither do we see specific changes in conjugated versus unconjugated bile acids, or primary versus secondary bile acids, rather we see an overall reduction in bile acids during helminth infection which suggest effects of the gut microbiota on bile acid composition do not underlie differences between naïve and infected animals.

Lower concentrations of intestinal bile acids may impair fat metabolism and could cause gastrointestinal disease. An extreme example of low abundance of bile acids is seen with an obstruction of the bile duct, which leads to small intestinal bacterial overgrowth [337]. Bile acids can disrupt bacterial cell membranes and thus bacterial growth in the small intestine is kept in check by maintaining a steady level of bile flow [264]. It is possible that in helminth-infected mice, lower bile acid concentrations may lead to susceptibility to small intestinal overgrowth. Co-infections with helminths and bacterial pathogens are common but the cross-kingdom interactions are not well understood (as discussed in **Chapter 1** in this thesis). The role of helminth-, host- and bacterially derived metabolites should be considered in shaping immunity to concurrent bacterial challenge during helminth infection. The next chapter (**Chapter 5**) describes our attempts to characterize the contribution of SCFAs and bile acids to the breakdown of immunity against *Salmonella* in the small intestine during helminth co-infection.

In addition to direct effects on bacterial growth, a helminth-induced reduction in bile acid concentrations and decreased bile acid transportation in proximal small intestinal tissue may impact cellular parts of mucosal immunity. Innate immune cells as well as T- and B cells harbor bile acid receptors that when activated can modulate their function [34]. A disrupted bile acid homeostasis can have down-stream effects on the intricate balance of pro-inflammatory and regulatory pathways [34]. Lastly, a scarcity of bile acids will likely have impacts on the intestinal microbiota composition. During helminth infection, the gut microbiota composition is shifted, and these shifts can underlie some of the immunomodulatory properties of helminths, such as induction of Tregs [163].

In conclusion, we show that helminth infection alters the concentration of bile acids in the small intestinal lumen and affects transport of bile acids in the proximal small intestine. Future efforts should focus on dissecting whether these changes in bile acid homeostasis affect immunity, either directly by altering bacterial virulence, or modulating immune cells, or indirectly, by driving compositional changes in the intestinal microbiota.

Chapter 5

The impact of the helminth-modified metabolome on *Salmonella* colonization in the small intestine

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Chapter Disclaimer: Results in **Figure 5.2B** of this chapter are published [85] (**Appendix**). Metabolomics analysis was done by Jun Han at the UVic Genome BC Proteomics Centre.

5.1 Introduction

Metabolite shifts in the small intestine during helminth infection include an increase in the concentration of isovalerate (a branched SCFA; **Chapter 3**), and a reduction in the concentration of bile acids (**Chapter 4**), and these metabolite shifts correlate with increased susceptibility in helminth-infected mice to high *Salmonella* bacterial burdens in the small intestine (**Chapter 2**). Intestinal metabolites can impact bacterial colonization in two major opposing ways: metabolites can be released by resident microbiota species that suppress virulence of invading pathogens (colonization resistance) [302], and fluctuations in metabolite composition can promote the colonization/proliferation of pathogenic bacteria such as *Salmonella* that are excellent at adapting to different metabolic requirements [338].

SCFAs can directly affect *Salmonella* virulence *in vitro* and confer resistance to infection *in vivo* [131,252,253,256,259,262]. For example, in the intestine, propionate derived from the microbiota protects against *Salmonella* expansion [252]. However, in an inflamed gut, *Salmonella* is able to switch to aerobic respiration to utilize SCFAs, making the pathogen resistant to SCFA-mediated colonization resistance [259,339]. An aerobic milieu during inflammation contains alternative electron acceptors for bacterial respiration, and this grants *Salmonella* access to additional carbon sources through beta-oxidation [154,155,340,341]. It is possible that inflammation during helminth infection creates novel metabolic opportunities for *Salmonella* to expand, including utilization of isovalerate.

On the other hand, isovalerate may have antibacterial properties. High levels of isovalerate (making up 50% of the SCFA abundance) were found in the blubber of sea mammals [342] and blubber-associated microorganisms were found to produce isovalerate, and extracts of these marine microbes had broad-spectrum antibiotic activity [343]. In addition, isovalerate has been shown to directly stimulate antimicrobial peptide production: 0.5 mM isovalerate could elevate levels of antimicrobial cathelicidin in a human cell line [344]. While *Salmonella* is resistant to cathelicidin attack [345], broad-spectrum antibacterial activity such as cathelicidin induction could impact bacterial microbiota communities, clearing the road for *Salmonella* to establish a niche in the small intestine.

In the small intestine, bile acids are one of the most abundant molecules and beyond their primary role in fat digestion, these metabolites may aid in defense against invading pathogens as well [34]. Decreased bile flow into the gut due to liver injury or experimental bile duct ligation leads to bacterial overgrowth in the lumen of the small intestine [271,287–292], whereas feeding rodents conjugated bile acids during a state of low bile acid abundance can counteract small intestinal overgrowth [293,294]. Until now, researchers have not looked at the effect of reduced bile flow on *Salmonella* directly, but bile duct ligation does mainly seem to affect Gram-negative and facultative anaerobic bacteria, which includes *Salmonella*

[271,289]. Bile acids can affect bacterial infection through several ways. Firstly, bile acids can form micelles that can lead to direct lysis of bile-sensitive bacteria [34]. However, *Salmonella* is bile-resistant as it has a hardy outer membrane [295]. Bile acids can also signal through the FXR receptor, which can promote antibacterial programs such as secretion of AMPs [34]. Moreover, bile acids can affect bacterial gene expression, and *Salmonella* cultured in murine bile showed decreased expression of major virulence factors and central metabolic genes [296,297]. Bile acids may form an important part of the host defense against *Salmonella* colonization, therefore it is possible that low levels of bile acids are responsible for a luminal bloom in *Salmonella* during helminth infection.

In this thesis chapter, we tested the hypothesis that metabolite shifts during helminth infection impact *Salmonella* colonization. Specifically, we tested whether isovalerate availability during helminth infection or low bile acid abundance in the small intestine promoted *Salmonella* colonization.

5.2 Methods

5.2.1 Mice

All experiments were approved by the University of Victoria's Animal Care Committee and were in compliance with the Canadian Council on Animal Care. Wild-type C57BL/6 mice were bought from The Jackson Laboratory (strain 000644, from a maximal barrier room) and used for experiments after a minimum acclimation period of one week, or first bred and maintained under specific-pathogen free conditions at the University of Victoria and then used for experiments. During breeding and prior to and during experiments, mice had access to food and water *ad lib*. All mice were 6-8 weeks old at the beginning of experiments. Both male and female mice were used for experiments, as indicated in figure legends. When bred at the University of Victoria, littermates were randomized between the experimental groups.

5.2.2 H. polygyrus-conditioned media

Two mice were infected with 400 *H. polygyrus bakeri (H. polygyrus)* stage 3 larvae and euthanized two weeks later. Small intestinal contents were placed in a muslin bag suspended in HBSS (Gibco) in a Baermann apparatus and incubated at 37°C for 2 hours. Following incubation, the majority of worms had migrated through the muslin bag to the collection funnel. The collected worms were washed 6 times with HBSS and moved into a biological safety cabinet for another 6 washes with HBSS + 100 U/mL penicillin and 100 μg/mL streptomycin. Then, worms were soaked in RPMI + 1 mg/mL gentamicin for 45 min, in a shaking incubator at 37 °C, following 12 more thorough washes with HBSS to wash of the antibiotics. Worms were then transferred to minimal media (Difco Peptone Water, BD) and incubated horizontally in a shaking incubator at 37 °C for 6 hours. This media ('worm conditioned media'), and control minimal media, were then used to grow *S.* Typhimurium Δ*aroA* (strain used in the *in* vivo co-infections in Chapter 2) from a stationary-phase overnight culture.

5.2.3 Helminth infection

The life cycle of *H. polygyrus* was maintained in C57BL/6J mice according to an established protocol [299]. For experimental infections, mice were infected with 200 *H. polygyrus* stage 3 larvae by oral gavage. Control animals that were kept naïve received an oral gavage with the non-sterile water that larvae were kept in but with larvae removed using a 70 µm cell strainer (VWR) and visual examination using a dissecting microscope. Parasite burdens were tracked by counting *H. polygyrus* eggs released into feces, which were enumerated using a McMaster Counting Chamber slide under a light microscope.

5.2.4 In vivo Salmonella infection

S. Typhimurium strain used was streptomycin-resistant (strain SL1344). Mice were infected with *Salmonella* by oral gavage with 3 x 10^8 cfu of a growth-attenuated (Δ*aroA*) strain of *S.* Typhimurium [301]. Inocula were prepared from stationary-phase overnight cultures in LB broth and were diluted in PBS prior to infection.

5.2.5 Growing Salmonella in vitro with ex vivo metabolite extractions

Intestinal contents were collected from the proximal 6 cm of the small intestine from naïve or day 14 *H. polygyrus*-infected mice. For every 10 mg of intestinal content, 100 µL acetonitrile (VWR) was added, and samples were gently rocked at 4 °C overnight. Samples were then spun at 13,000 rpm at 4 °C for 15 min, and supernatants containing metabolites were collected. Supernatants were sterile-filtered and aliquoted into 125 µL (containing 12.5 mg) aliquots, and acetonitrile was evaporated using a speed vacuum concentrator. Samples were stored at -80 °C prior to functional analysis. To enrich minimal media with *ex vivo* metabolite extracts, extracts from individual mice were reconstituted in 100 µL minimal media and then samples were pooled together per group (n=4, now samples contain metabolite extracts from 125 mg tissue per mL). Media containing metabolite extracts were sterile-filtered and used to used to grow *S.* Typhimurium Δ*aroA* (strain used in the *in* vivo co-infections in Chapter 2) from a stationary-phase overnight culture.

5.2.6 Growing Salmonella in vitro with isovaleric acid and butyric acid

Differing concentrations of the protonated form of isovalerate (isovaleric acid, Millipore-Sigma) and butyrate (butyric acid, Millipore-Sigma) were added to minimal media, and the pH was adjusted to pH 5 (to match the small intestinal pH) using sodium hydroxide. Control medium was sodium-matched with sodium chloride, to control for effects of salt on bacterial growth. SCFA-enriched and salt-controlled media with metabolite extracts were sterile-filtered, and used to start a culture of *S.* Typhimurium Δ*aroA* from a stationary-phase overnight culture.

5.2.7 Isovalerate-supplemented drinking water

Drinking water was supplemented with 100 mM isovalerate by adding isovaleric acid (Millipore-Sigma), after which the pH was adjusted through addition of sodium hydroxide to match the pH of the control water (around pH 7), thus converting the SCFA to its deprotonated form (isovalerate). Control drinking water was sodium-matched using sodium chloride. Both control and isovalerate-supplemented water were sterile-filtered before administration. Drinking water was replaced once a week. The drinking water was supplemented for 2 weeks prior to *Salmonella* or *H. polygyrus* infection and continued throughout the duration of the infection.

5.2.8 Quantification of SCFA levels

The proximal 6 centimeters of the small intestine were dissected from mice and luminal contents were collected using forceps. Contents were snap-frozen in liquid nitrogen and stored at -80 °C until metabolite analysis.

An internal standard solution containing deuterium-labeled acetic, propionic and butyric acid was prepared in acetonitrile. A mixture of standard substances containing 10 SCFAs was dissolved in 50% acetonitrile at 200 µM. This solution was further diluted step by step in a same ratio of 1 to 4 (v/v) with the same solvent to have working standard solutions.

Each sample was mixed with acetonitrile, at 3 µL per mg raw material and two 3-mm metal beads were added. The samples were homogenized on a MM 400 mill mixer at a shaking frequency of 30 Hz for 2 min, followed by sonication in an ice-water bath for 2 min. The samples were then centrifuged for clarification at 21,000 x *g* and 5 °C for 10 min. Then, 20 µL of each thawed serum sample, the supernatant of each intestinal content sample or each standard solution was mixed in turn with 20 µL of internal standard solution, 40 µL of 200-mM 3-nitrophenylhydrazine HCl solution and 40 µL of 150-mM EDC HCl-6%pyridine solution, both in 60% acetonitrile. The mixtures were allowed to react at 40 °C for 40 min. After reaction, 240 µL of water was added to each solution and mixed well. A 10-µL aliquot of each resultant solution was injected to run LC/MS on an Agilent 1290 UHPLC system coupled to an Agilent 6495B QQQ mass spectrometer equipped with a heated electrospray ionization source and with negative-ion detection. LC separation was carried out on a C18 UPLC column (2.1*100 mm, 1.8 µm) with 0.01% formic acid in water and 0.01% formic acid in acetonitrile as the binary solvents for gradient elution (20% to 60% B in 12 min) according to the procedures previously described [317]. Concentrations of the detected compounds were calculated with internal standard calibration by interpolating the constructed linear-regression curves of individual compounds with the analyte to internal standard peak ratios measured from each sample solution.

5.2.9 Specialized diets

Specialized diets were formulated and manufactured at Envigo in Madison, WI. All diets were made from PicoLab rodent diet 20 (5053, Purina), including the control diet (TD.120321, Envigo), that was diet 5053 re-pelleted at Envigo. Bile acid-supplemented diet (TD.200401, Envigo) contained 0.1% CA (C1129, Sigma-Aldrich) and 0.1% CDCA (C9377, Sigma-Aldrich) and blue food-coloring. Bile acid sequestering diet (TD.200402, Envigo) was supplemented with 2% cholestyramine resin (C4650, Sigma-Aldrich) and red food-coloring. Standard diet 5053 was irradiated before formulation of specialized diet and all diets were stored under sterile conditions.

5.2.10 Quantification of total bile acids levels using a colorimetric assay

Fecal matter and proximal (first 6 cm) small intestinal content was collected from mice, and frozen at -20 °C until metabolite extraction. Metabolites were extracted by adding 1 mL of 75% ethanol to every 50 mg of thawed tissue, samples were incubated at 50 °C for 2 hr and centrifuged for 10 min at 1,050 x *g*. Supernatant was mixed with PBS 1:6, vortexed and assayed using the Crystal Chem mouse total bile acids kit according to manufacturer’s instructions. For determining serum total bile acids, metabolites did not need to be extracted and serum samples could directly be assayed using Crystal Chem mouse total bile acids kit according to manufacturer’s instructions.

5.2.11 Quantification of individual bile acids levels by mass spectrometry

Bile acid analysis was performed at the UVic Genome BC Proteomics Centre. An Agilent 1290 UHPLC system coupled to a 6495B Agilent QQQ mass spectrometer was used. The MS instrument was operated in the multiple reaction monitoring mode with negative-ion detection. A Waters BEH 15-cm long, 2.1-mm I.D. and C18 LC column was used, and the mobile phase was 0.01% formic acid in water and 0.01% formic acid in acetonitrile for binary-solvent gradient elution. Detailed LC and MS operation parameters were the same as we described in [321].

First a mixture of standard substances containing all the targeted bile acids was dissolved in 40% acetonitrile at 10 nmol/mL, as the top standard solution, which was then was further diluted step by step at a same dilution ratio of 1 to 4 (v/v). Then, 100 µL of the standard solutions were mixed with 100 µL of an internal standard solution containing 14 D-labeled bile acids. To run UPLC-MS 10 µL of these solutions was injected. Linear-regression calibration curves were constructed between analyte-to-internal standard peak area ratios (As/Ai) versus molar concentrations (nmol/mL).

Each experimental sample was added with 10 µL/mg of 70% aqueous acetonitrile and two 3-mm metal beads. The samples were then homogenized at a shaking frequency of 30 Hz for 1 min three times on a MM 400 mill mixer, followed by sonication in a water bath for 2 min. After centrifugal clarification at 21,000 x *g* and 10 °C for 10 min. The supernatant was diluted 10 times with 40% acetonitrile. Then, 100 µL of each diluted sample solution was mixed with 100 µL of the internal standard solution, and 10 µL of this was injected for UPLC-MS. Concentrations of detected bile acids were calculated from the internal standard-calibration, linear-regression calibration curves of individual bile acids prepared in duplicate.

Sample analysis for plasma samples was done as follows. First, 20 µL of plasma was mixed with 40 µL of the internal standard solution containing 14 D-labeled bile acids and 40 µL of acetonitrile. After vortex-mixing for 30 s and sonication for 2 min in a water bath, the sample tube was centrifuged at 21,000 g for 10 min at 5 °C. Then, 80 µL of the clear supernatant was taken out and mixed with 920 µL of water. The mixture was loaded onto a 60mg/1mL Waters Oasis HLB cartridge which was activated with 1 mL of methanol and subsequently reconditioned with 1 mL of water before use. Under a positive pressure, the flow-through fraction was discarded. Bile acids were eluted with 800 µL of methanol. The collected fraction was dried under nitrogen and the residue was reconstituted in 64 µL of 40% acetonitrile. 10 µL was injected for UPLC-MS. Concentrations of detected bile acids were calculated from the internal standard-calibration, linear-regression calibration curves of individual bile acids prepared in duplicate.

Moisture content in samples was determined by 100% minus the percentage difference between wet weights (weight of the sample as dissected from the mouse) and dry weight (after lyophilization of the samples).

5.2.12 Statistical analyses

Statistical analyses were performed in GraphPad Prism 7.04. First, normality of the data was assessed with D'Agostino and Pearson normality test. Next, the appropriate statistical test was performed depending on the normality of the data set, the number of experimental groups being compared, and whether correction for multiple hypothesis testing was necessary, as indicated in the figure legends.

5.3 Results

5.3.1 *Metabolites derived from helminths or helminth-infected intestine do not affect* Salmonella *growth in vitro*

Helminths excrete and secrete hundreds of products to modify their environment and promote their survival [2], including metabolites [346], that can contribute to an altered intestinal metabolome. Our work has shown that *H. polygyrus* infection promotes *Salmonella* establishment in the small intestine of mice [7,298] (**Chapter 2**). To test whether helminth ES products could impact *Salmonella* growth, we isolated *H. polygyrus* adult worms from infected mice, and cultured the worms *ex vivo* in minimal media. After removal of worms, this 'worm-conditioned' media was then used to culture *Salmonella*, and we monitored bacterial growth over a 16-hour period. The growth curves showed that *Salmonella* growth was not enhanced in worm-conditioned media compared to control media (**Figure 5.1A**).

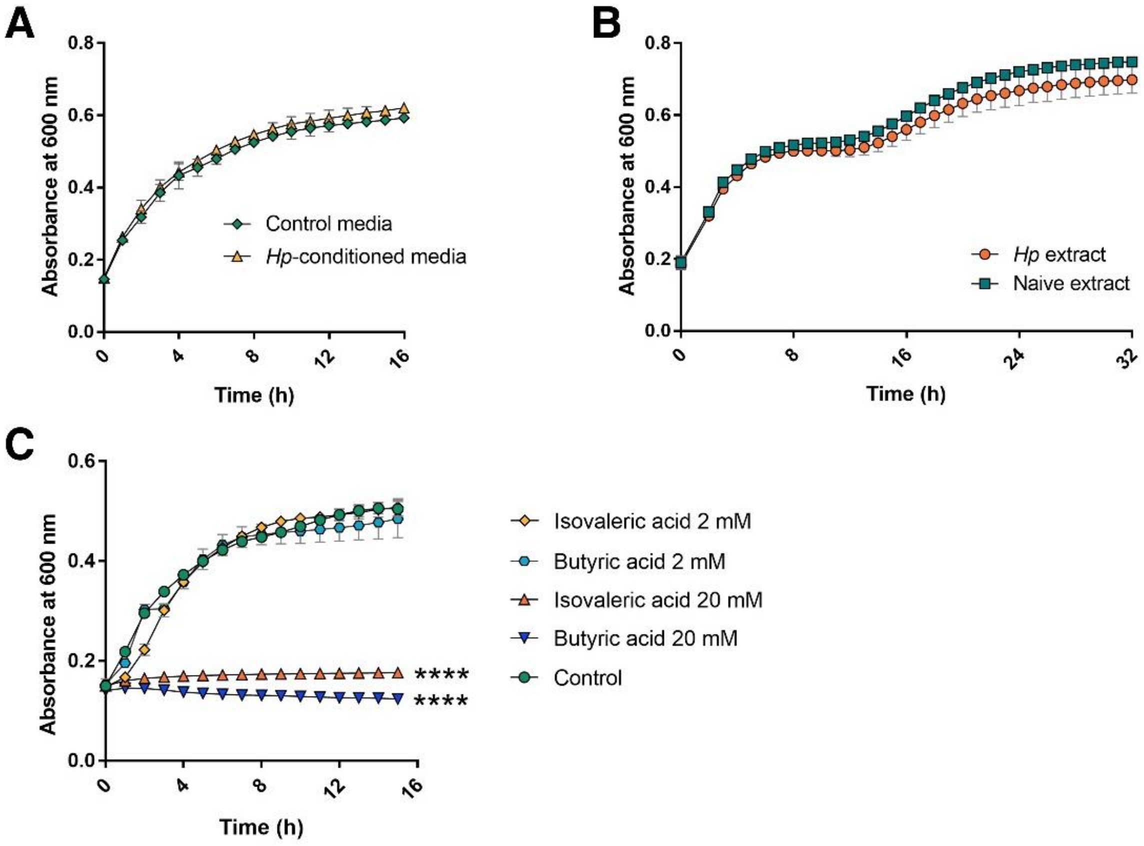

Figure 5.1. The effects of helminth-conditioned media and helminth-modified small intestinal metabolites on *S.* Typhimurium (*S*T) growth *in vitro*. *S*T growth was tracked over 16 or 32 hours by measuring optical density at 600 nm. (**A**) *S*T growth in media that was previously conditioned with live *H. polygyrus* adult worms, and control media. Each curve tracks growth of a *S*T culture in different media, and the error bars denote the standard error of three technical replicates. (**B**) The impact of metabolites extracted from small intestinal content of 14 day-helminth-infected and

naïve female C57BL/6J mice on *Salmonella* growth *in vitro*. Each curve tracks growth of a *S*T culture with pooled metabolites extracted from four mice, and the error bars denote the standard error of three technical replicates. (**C**) The impact of different concentrations of isovaleric and butyric acid on *Salmonella* growth *in vitro*. Each curve tracks growth of a *S*T culture in different media, and the error bars denote the standard error of six technical replicates. Statistical comparisons between two groups were made using multiple t-tests for each time-point followed by multiple hypothesis correction with the FDR method (cut-off for FDR = 5%), and to compare multiple groups a repeated measures (RM) 2-way ANOVA was used followed by a Dunnett's multiple comparisons test (comparing to control media). **** = $p \leq 0.0001$.

It is possible that *H. polygyrus* requires specific substrates that are not present in the culture medium to release all the ESP that it would do *in vivo*. To account for this, we extracted metabolites from the small intestinal contents of naïve and helminth-infected mice, using acetonitrile, and diluted the metabolites in culture media to grow *Salmonella* in. The metabolite extracts from naïve mice caused *Salmonella* to grow in a biphasic growth curve, indicating that additional carbon sources were consumed after the initial preferred substrate ran out (**Figure 5.1B**). However, culturing *Salmonella* with metabolites extracted from *H. polygyrus* infected mice did not offer a growth benefit, suggesting that energy substrates were not more abundant in the extract from infected mice compared to naïve mice. Some metabolites are very volatile, including SCFAs, and may not have been extracted very efficiently using our acetonitrile-based extraction method. To test directly if isovalerate affected *Salmonella* growth, we added isovaleric acid to culture media, alongside some cultures that contained butyric acid, and some cultures without any added SCFAs. Butyrate has been previously shown to inhibit *Salmonella* growth at higher concentrations [253], which is what we observed for high concentrations of butyrate, as well as for high concentrations of isovalerate (**Figure 5.1C**). Additionally, our data here revealed that isovalerate did not promote *Salmonella* growth at a lower concentration of 2mM.

5.3.2 Supplementing drinking water with 200mM isovalerate does not affect bacterial colonization in the small intestine.

Bacterial growth and virulence gene expression can be assessed *in vitro*, however *in vivo* many more factors play a role in whether a bacterial pathogen establishes an infection. Colonization resistance by resident microbiota and innate immune defenses are important barriers against acute infection in the intestine, and successful pathogens must find ways to outcompete other microbes and evade immune responses, which is more challenging to measure *in vitro*. Helminth-modified metabolite availability may give *Salmonella*, which adapts easily to different metabolic requirements, a competitive edge over resident microbiota. Metabolites may also alter immune responses. Additionally, *in vitro* experiments were done in aerobic conditions, which does not mimic the low pO_2 conditions during homeostasis in the small intestine [347]. For these reasons, we assessed the role of isovalerate in *Salmonella* infection in an *in vivo* model. From reviewing previous studies that had investigated SCFAs in inflammatory disease, the following options for *in vivo* supplementation appeared: oral gavage [244,348], SCFA-supplemented drinking water [219,220,246], or a SCFA-enriched diet [240]. Oral gavage is the most controlled method: each mouse will get same dose. However, constant exposure that would replicate an ongoing helminth infection would entail daily oral gavage procedures for 14 days which is likely to cause excessive distress to the mice. An isovalerate-enriched diet is not commercially available and sprinkling regular chow diet with isovalerate solution would be the least controlled method. We decided that the most controlled and feasible option for our purposes was to add isovalerate to the drinking water of mice. We found that adding 100mM isovalerate to the drinking water of mice for 2 weeks resulted in an increase in small intestinal levels of isovaleric acid in naïve mice, similar to the magnitude of increase in isovalerate levels observed following two weeks of *H. polygyrus* infection (**Figure 5.2A,B**). Thus, through supplementing the drinking water of mice with isovalerate two weeks, we could assess whether high isovalerate levels were able to promote *Salmonella* colonization in the small intestine (**Figure 5.2C**). We found that two weeks of supplementing drinking water with 100mM isovalerate did not promote *Salmonella* colonization of the mouse small intestine (**Figure 5.2D**). This suggests that a high abundance of isovalerate in the intestinal lumen by itself does not promote *Salmonella* colonization during helminth infection.

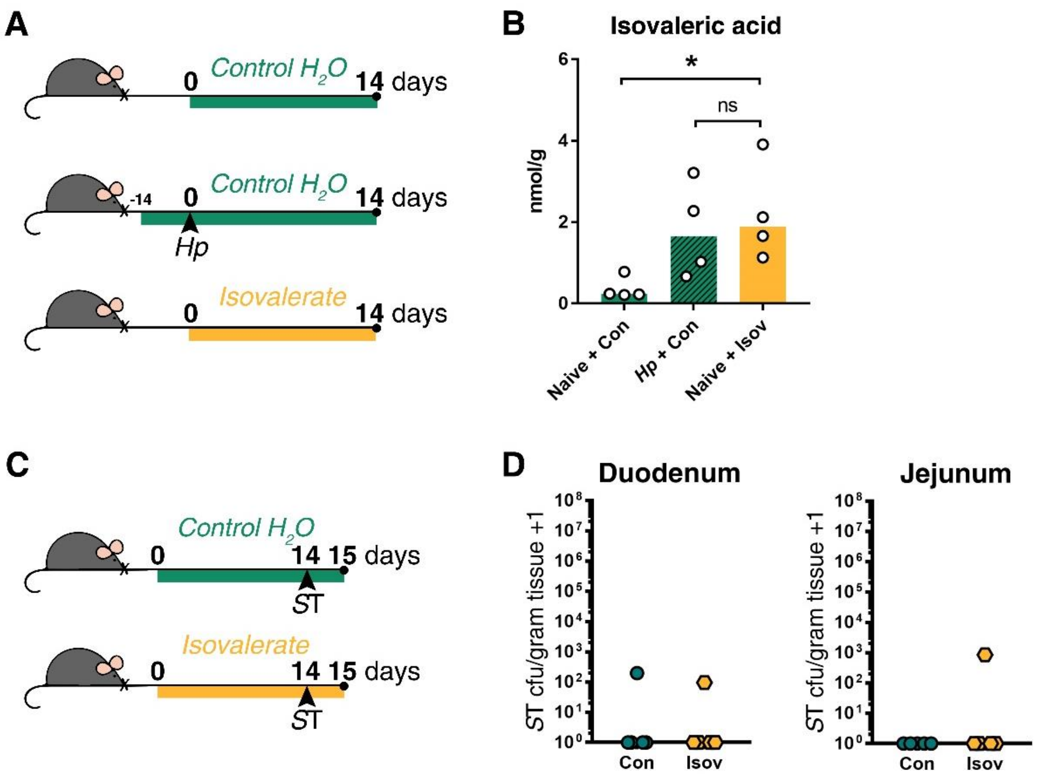

Figure 5.2. Supplementation with 100mM isovalerate does not affect colonization of *Salmonella* in the small intestine. (**A**) Experimental set-up. For two weeks, the drinking water of female C57BL/6J mice was replaced with drinking water supplemented with 100 mM isovalerate, or control water. After two weeks, luminal contents of the proximal small intestine were collected and analyzed for metabolites by UPLC-MS. The levels in the isovalerate-supplemented mice were compared to d14 *H. polygyrus (Hp)*-infected mice (that were on control drinking water 2 weeks prior and during infection; data from the group of mice on isovalerate-supplemented drinking water 2 weeks prior and during *Hp* infection is not shown) (**B**) Levels of isovaleric acid in naïve and *Hp*-infected mice on isovalerate-supplemented water (Isov) or control water (Con) are shown. (**C**) Experimental set-up used to test whether isovalerate supplementation affects *Salmonella* colonization is shown. The drinking water of female C57BL/6J mice was replaced with water supplemented with 100 mM isovalerate, or control water. After two weeks, mice were orally infected with Δ*aroA S.* Typhimurium (*S*T). One day post-ST infection, ST cfu/gram of tissue were determined. (**D**) ST cfu/gram of tissue in the duodenum and jejunum are shown. Data points represent individual mice and data in (**B**) and (**D**) is from two different experiments, each performed once. Statistical comparisons between two groups were made using a Mann-Whitney test, and between three groups with Kruskall-Wallis test followed by a Dunn's multiple comparisons test. *= p ≤ 0.05; ns = non-significant.

5.3.3 Bile acid supplementation during helminth infection does not affect bacterial colonization

Using an additional method that was aimed to restore helminth infection-induced low concentrations of bile acids, we further assessed the role of bile acids in *Salmonella* colonization in helminth-infected mice. The specialized diet used for this aim was enriched with 0.1% CDCA and 0.1% cholic acid CA. In a previous study, mice fed 0.1% CDCA showed an upregulated expression of AMPs in the ileum and limited *Salmonella* colonization at this site [286]. CA is another primary bile acid that is very abundant in naïve mice, while lower in concentration during helminth infection (**Chapter 4**), and feeding of CA has been shown to increase the total bile acid pool size [349]. We first tested whether a 0.1% CDCA- and 0.1% CA-supplemented diet was able to restore bile acid concentrations in helminth-infected mice. We infected mice with *H. polygyrus*, or left the mice naïve, and on day 14 of infection we changed the diet overnight to bile acid-supplemented diet, or a control diet (**Figure 5.5A**). The next morning, we collected small intestinal content and analyzed bile acid levels (**Figure 5.5B**). We found that helminth infection did reduce concentrations of conjugated bile acids, as we had seen before (**Chapter 4**), but we also found increased concentrations of unconjugated bile acids during helminth infection, which we had not seen in our previous experiments. A possible explanation for this discrepancy is that for experiments reported in this chapter, mice were bred at a different facility (Jackson laboratories, as opposed to University of Victoria for bile acid analysis experiments reported in **Chapter 4**), and diets, including control diet, were produced at a different facility as well (Envigo/Purina, instead of LabDiet). Importantly, irrespective of these differing experimental variables, a consistent trend can be observed of a lower concentration of conjugated primary bile acids during helminth infection (**Figure 5.5B, Chapter 4**) and a trend here indicates these lowered concentrations may be restored with a diet supplemented with 0.1% CA and 0.1% CDCA (**Figure 5.5B**). Moreover, across all bile acid categories, bile acid levels of bile acid-fed helminth-infected mice appeared similar to levels in naïve mice on control diet (**Figure 5.5B**).

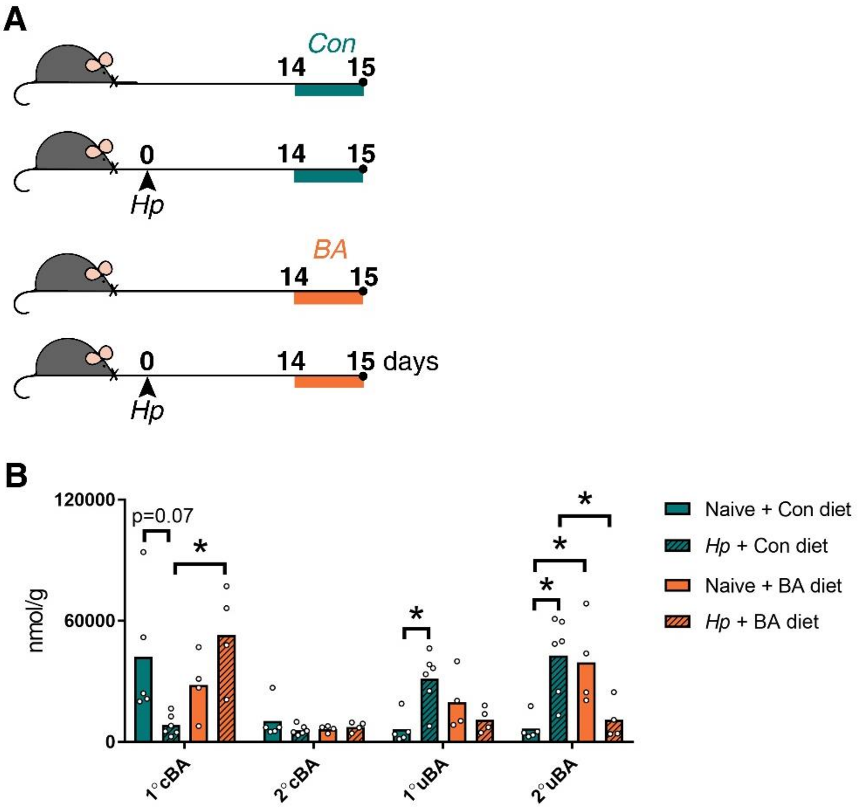

Figure 5.5. Effect of diet enriched with 0.2% CA and 0.2% CDCA on BA levels in the small intestine during helminth infection. (**A**) Experimental set-up. Naïve and *H. polygyrus* (*Hp*)-infected male C57BL6/J mice were switched to a bile acid (BA)-supplemented diet (containing 0.2% CA, 0.2% CDCA) or control diet (Con), fourteen days post-*Hp* infection. The next day, luminal contents of the proximal small were collected and analyzed for metabolites by UPLC-MS. (**B**, **C**) Levels of total primary bile acids (1°) and secondary (2°) bile acids, conjugated bile acids (cBA) and unconjugated bile acids (uBA) are shown. Data points represent individual mice, and all the data is from one experiment. Statistical comparisons between groups for each BA class were made using a two-way ANOVA followed by Tukey's multiple comparisons test. *= $p \leq 0.05$.

Next, we tested whether restoring levels of conjugated primary bile acids in helminth-infected mice affected *Salmonella* colonization in the small intestine. Naïve and *H. polygyrus*-infected mice were switched to a bile acid-supplemented diet, or control diet, overnight on day 14 of helminth infection. The next morning, mice were infected with *Salmonella*, and 24 hrs later, mice were euthanized to determine bacterial burdens (**Figure 5.6A**). We found that as we had reported previously ([298], **Chapter 2**), helminth infection promoted *Salmonella* colonization in the small intestine in mice on a control diet, and this was also true for mice on a bile acid-supplemented diet (**Figure 5.6B**). Overall, this data suggests that lower concentrations of conjugated primary bile acids during helminth infection do not play a significant role in promoting *Salmonella* colonization of the small intestine.

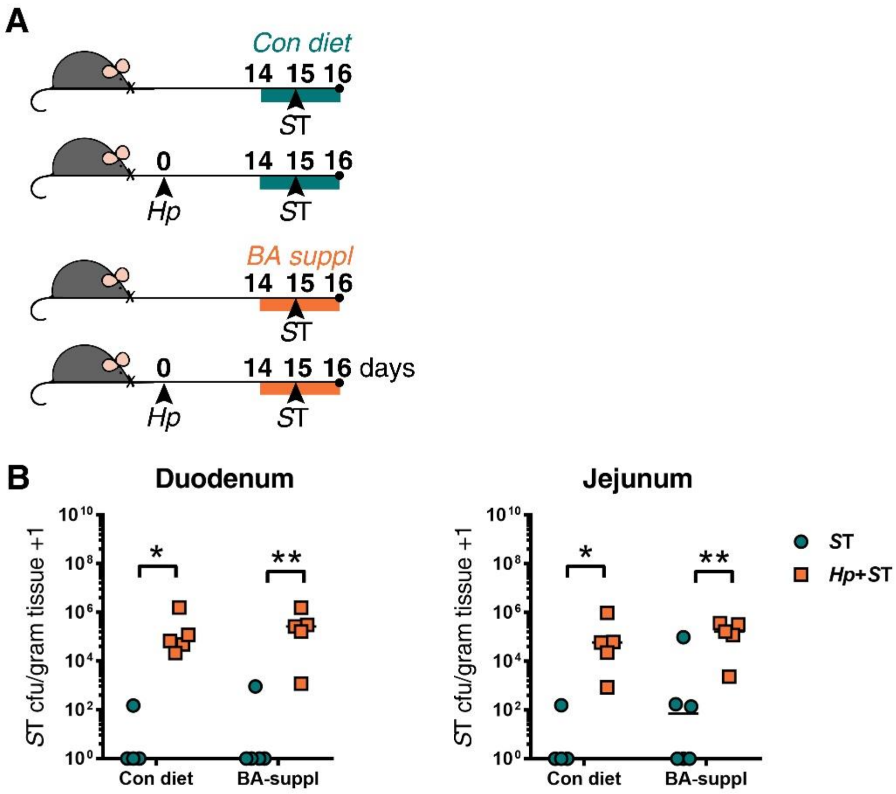

Figure 5.6. Diet enriched with 0.2% CA and 0.2% CDCA does not affect *Salmonella* colonization during *Hp* infection. (**A**) Experimental set-up. Naïve (H_2O) *H. polygyrus* (*Hp*)-infected male C57BL6/J mice switched to a bile acid-supplemented diet (BA-suppl) or control diet (Con diet), fourteen days post-*Hp* infection. The next day, mice were orally infected with Δ*aroA* *S*T. One day post-*S*T infection, *S*T cfu/gram of tissue were determined. (**B**) *S*T cfu/gram of tissue in the duodenum and jejunum are shown. Data points represent individual mice, and data is from one experiment. Statistical comparisons between singly- and co-infected mice on either bile acid-supplemented diet or control diet were calculated with a Mann-Whitney test. *= $p \leq 0.05$; ** = $p \leq 0.01$.

5.4 Discussion

We hypothesized that metabolite shifts during helminth infection may promote pathogenic bacterial colonization. We focused on testing how metabolite shifts during helminth infection affected the growth and establishment of *Salmonella*, given that our work has shown that *H. polygyrus* coinfection promotes *Salmonella* establishment in the small intestinal lumens of mice [7,298] (**Chapter 2**). We first tested this hypothesis *in vitro* and found that culturing *Salmonella* in media supplemented with helminth ES products or metabolites extracted from helminth-infected mice did not confer a growth benefit to the bacteria. Subsequently, we tested hypotheses to specific groups of metabolites that we previously found to be affected by helminth infection in mice. We hypothesized that an increase in isovalerate during helminth infection may promote *Salmonella* growth by providing an additional carbon source, however, isovalerate in bacterial cultures did not promote bacterial growth. We further tested the potential of isovalerate to promote *Salmonella* colonization in the small intestine *in vivo* and found that drinking water supplementation with 100 mM isovalerate does not alter *Salmonella* colonization levels in mice. Furthermore, we tested whether a reduced bile acid concentration in the small intestine would affect *Salmonella* colonization, by adding a bile acid sequestering resin to the diet of the mice. We found that bile acid sequestering did not promote *Salmonella* colonization. We also tested whether reduced levels of bile acids during helminth infection were contributing to higher *Salmonella* burdens in the small intestine, by replenishing the bile acid pool of helminth infected mice via a bile acid-supplemented diet. A bile acid-supplemented diet did not affect bacterial colonization levels. Together, this data did not provide evidence that shifts in isovalerate levels and bile acids during helminth infection can promote *Salmonella* colonization. A limitation of experiments described in this chapter is that experiments including bile acid analysis were performed only once. For conclusive results on the effects of SCFA and bile acid supplementation and sequestering on *Salmonella* colonization, experiments should be repeated.

The bile acid analysis we performed to validate bile acid supplementation revealed different trends to what we had seen in our previous experiments (i.e., the comprehensive bile acid analysis in **Chapter 4**). Where previously we saw an overall reduction in bile acid concentrations during helminth infection, in experiments reported in this current chapter, we found that only primary conjugated bile acids were lower in concentration, and unconjugated bile acids levels were increased during helminth infection. It is possible microbiota differences between experiments are responsible for these discrepancies, as microbial metabolism causes deconjugation of bile acids. Only certain species are capable of deconjugating bile acids [264], and since helminth infection impacts the microbiota composition [187], it is possible that helminths promote bacterial species that contain the enzyme to deconjugate bile acids, leading to an increased ratio of unconjugated bile acids over conjugated bile acids. The impact helminth infection has on the intestinal microbiota is dependent on the pre-infection (baseline) composition of microbial communities in the mice [350]. Genetically identical mice from different vendors can have vastly different microbiota composition, with many factors contributing to these differences, including differences in husbandry (e.g. housing density) and diet (e.g. autoclaved or not) [351]. Because mice in the bile acid experiments in this thesis chapter were ordered directly from Jackson laboratories, instead of being bred in-house at University of Victoria prior to experimentation, and because the specialized diet and control diet were from a different facility as before, it is possible that these environmental factors differentially impacted the microbiota of the mice, and perhaps also the intestinal bile acid composition. This highlights the importance of performing experimental repeats even with expensive analyses like metabolomics, and the need for careful reporting of where mice are purchased and bred, as well as reporting of the environmental conditions the animals were held in during the experiment, for optimal impact and reproducibility of scientific findings.

Although we found no causal links between metabolite shifts and *Salmonella* colonization, it remains possible that other metabolite shifts during helminth infection drive expansion of *Salmonella* during coinfection. Further efforts should explore more groups of metabolites using a hypothesis-based approach of metabolomics and *in vivo* manipulation of the metabolome. It is important to test effects of metabolite shifts on bacterial infection in an animal model as *in vitro* may lack the nuances of the intestinal context of infection. Bacteria use many environmental cues for establishing an infection, metabolite composition [233], but also different pH, osmolarity and levels of O_2 can induce different gene expression programs in bacterial pathogens [261]. These conditions vary along the intestinal tract [347,352] and could even fluctuate locally, and are hard to mimic in a lab environment.

One aspect of intestinal infrastructure that is very challenging to reproduce *in vitro* is the mucosal immune tissue. Metabolites can act as immune messengers and modulate immune responses in the mucosa [32], which in turn may impact immune defenses to *Salmonella* infection. Any change in mucosal immunity may be a result of the host immune response to helminths, for example type 2 inflammation, or a purposeful mechanism of immunomodulation by helminths, which includes expansion of Tregs. Whether metabolite shifts during helminth infection contribute to modulation of local immune responses in the small intestine is yet unknown and studying this may uncover novel interactions in immune control of bacterial and helminth infections. The methods described in this chapter to raise small intestinal isovalerate levels and diets to sequester bile acids and increase bile acids levels during helminth infection will be useful for future studies to explore the impact of helminth-modulated metabolite shifts on host immune functions.

Chapter 6

The impact of the helminth-modified metabolome on helminth fitness and Tregs

6.1 Introduction

Helminths modulate host immune responses to promote their survival in the intestine. Several proteins in the ES products of helminths have been credited with immunomodulatory functions, while the immunomodulatory metabolites produced by the worm or shifts in intestinal metabolites during helminth infection have not been investigated extensively. We hypothesize that certain metabolite shifts during helminth infection improve the ability of the worm to maintain its niche in the intestine.

Previously, we tested whether high isovaleric acid levels during *H. polygyrus* infection act to promote helminth fitness by supplementing drinking water of mice with isovalerate. Isovalerate supplementation did not affect adult worm counts on day 28 of infection, but did increase egg counts in mouse feces from day 14 onwards [85] (**Appendix**). These results suggests that isovalerate supplementation increases *H. polygyrus* fecundity, without changing worm burden.

One of the mechanisms by which helminths modulate immune responses to promote their survival is by inducing Tregs [2]. Maintaining an anti-inflammatory state through promoting Tregs in the intestine is an important aspect of gut homeostasis and metabolite misbalances can lead to inflammatory disease in the intestine [353]. It is therefore of interest to find novel pathways of inducing Tregs that can be targeted for therapeutic purposes. Previously, we hypothesized that elevated levels of isovalerate increased induction of Tregs, which may confer a benefit to the helminths through promoting their fecundity. SCFAs have capacity to induce Tregs [33,181,219], and *H. polygyrus*-driven induction of Tregs during allergic asthma was dependent on a SCFA receptor [23]. Despite this, we found no evidence that isovalerate supplementation in drinking water increases either number or frequency of Tregs in small intestinal tissue (small intestinal lamina propria and Peyer's patches) or MLNs during *H. polygyrus* infection [85] (**Appendix**).

Similar to SCFAs, bile acids play a profound role in regulating mucosal immune homeostasis and intestinal inflammation [34]. It is not yet known whether helminth-driven changes in bile acid availability shape the immune responses in the small intestine during helminth infection. Here, we assessed a potential role of bile acids in the expansion of Tregs during helminth infection, and we tested whether shifts in bile acid levels promote helminth survival in the host.

6.2 Methods

6.2.1 Mice

All experiments were approved by the University of Victoria's Animal Care Committee and were in compliance with the Canadian Council on Animal Care. Wild-type C57BL/6 mice were bought from The Jackson Laboratory (strain 000644, from a maximal barrier room) and used for experiments after a minimum acclimation period of one week, or first bred and maintained under specific-pathogen free conditions at the University of Victoria and then used for experiments. During breeding, prior to and during experiments, mice had access to food and water *ad lib*. All mice were 6-13 weeks old at the beginning of experiments. Only male mice were used for experiments. When bred at the University of Victoria, littermates were randomized between the experimental groups.

6.2.2 Helminth infection

The lifecycle of *H. polygyrus bakeri* (*H. polygyrus*) was maintained in C57BL/6J mice according to an established protocol [299]. For experiments, mice were infected with 200 *H. polygyrus* stage 3 larvae by oral gavage. Control animals that were kept naïve received an oral gavage with the non-sterile water that larvae were kept in but with larvae removed using a 70 μm cell strainer (VWR) and larvae removal was ensured by visual examination using a dissecting microscope. Parasite burdens were tracked by counting *H. polygyrus* eggs released into feces, which were enumerated using a McMaster Counting Chamber slide under a light microscope.

6.2.3 Specialized diets

Specialized diets were formulated and manufactured at Envigo in Madison, WI. All diets were made from PicoLab rodent diet 20 (5053, Purina), including the control diet (TD.120321, Envigo), that was diet 5053 repelleted at Envigo. Bile acid-supplemented diet (TD.200401, Envigo) contained 0.1% CA (C1129, Sigma-Aldrich) and 0.1% CDCA (C9377, Sigma-Aldrich) and blue food-coloring. Bile acid sequestering diet (TD.200402, Envigo) was supplemented with 2% cholestyramine resin (C4650, Sigma-Aldrich) and red food coloring. Standard diet 5053 was irradiated before formulation of specialized diet and specialized diets were stored under sterile conditions.

6.2.4 Isolation of Peyer's patches and MLNs

Peyer's patches were manually dissected from the entire length of the small intestine and incubated for 30 minutes in PBS containing 1 mM EDTA, 5% FBS, and 1 mM DTT in a shaking incubator at 37°C. They were then gently crushed through a 70 μm filter and washed with PBS supplemented with 0.5% bovine serum albumin (BSA; Millipore-Sigma). MLNs were dissected from mice, then gently crushed through a 70 µM filter and washed with PBS supplemented with 0.5% BSA.

6.2.5 Flow cytometry

Cells were first stained with a Fixable Viability Dye (Thermo Fisher Scientific) before staining for surface markers with the following antibodies: PerCP Cy5.5-labelled anti-CD45 (30-F11, BD BioSciences), PeCy7-labelled anti-CD3 (145-2C11, BD BioSciences), BV605-labelled anti-CD8 (SK1, BD BioSciences), BV786-labelled anti-CD4 (30-F11, BD BioSciences), PE-labelled anti-CD103 (M290, BD BioSciences). For detection of FoxP3 transcription factor, cells were fixed and permeabilized using the eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) according to manufacturer's guidelines, and the cells were then stained with Alexa Fluor 647-labelled anti-Foxp3 (MF23, BD BioSciences). Flow cytometry was performed using a CytoFlex Flow Cytometer (Beckman Coulter) and the data was analyzed using CytExpert software (Beckman Coulter).

6.2.6 Statistical analyses

Statistical analyses were performed in GraphPad Prism 7.04. First, normality of the data was assessed with D'Agostino and Pearson normality test. Next, the appropriate statistical test was performed depending on the normality of the data set and the number of experimental groups being compared as indicated in the figure legends.

6.3 Results

6.3.1 Bile acid supplementation does not affect helminth fitness or Treg levels during helminth infection

To test whether helminth-mediated shifts in metabolites affects immunity to helminths in the small intestine, we first tested if a reduced concentration of bile acids during *H. polygyrus* infection promotes helminth fitness. Previously, we found that we can restore reduced bile acid concentrations during helminth infection with a bile acid-supplemented diet (containing 0.2% CA, 0.2% CDCA; **Chapter 5**). To test whether modified bile acid concentrations had an impact on helminth fitness, we put mice on a bile acid-supplemented or a control diet on day 10 of *H. polygyrus* infection, by which time post-infection adult worms have migrated back into the intestinal lumen, and we recorded egg output and number of adult worms present in the small intestine on day 14 of infection (**Figure 6.1A**). We found no differences in worm or egg counts at this timepoint between mice on bile-acid supplemented diet and control diet (**Figure 6.1B**).

We next investigated the impact of bile acid supplementation on small intestinal Treg levels in helminth-infected mice. We followed the same timeline of infection and diet supplementation as above (**Figure 6.1A**). On day 14 of *H. polygyrus* infection, using flow cytometry, we assessed the percentage of Foxp3$^+$ cells amongst the CD3$^+$CD4$^+$ T cell population in the Peyer's patches and the MLNs that drain the intestine. We found no differences in the percentage of Foxp3$^+$ Tregs in helminth-infected mice on a bile acid-supplemented diet compared to infected mice on a control diet (**Figure 6.1C**). Furthermore, we did not observe any differences in Foxp3$^+$ Treg activation as measured by CD103 expression (**Figure 6.1D**).

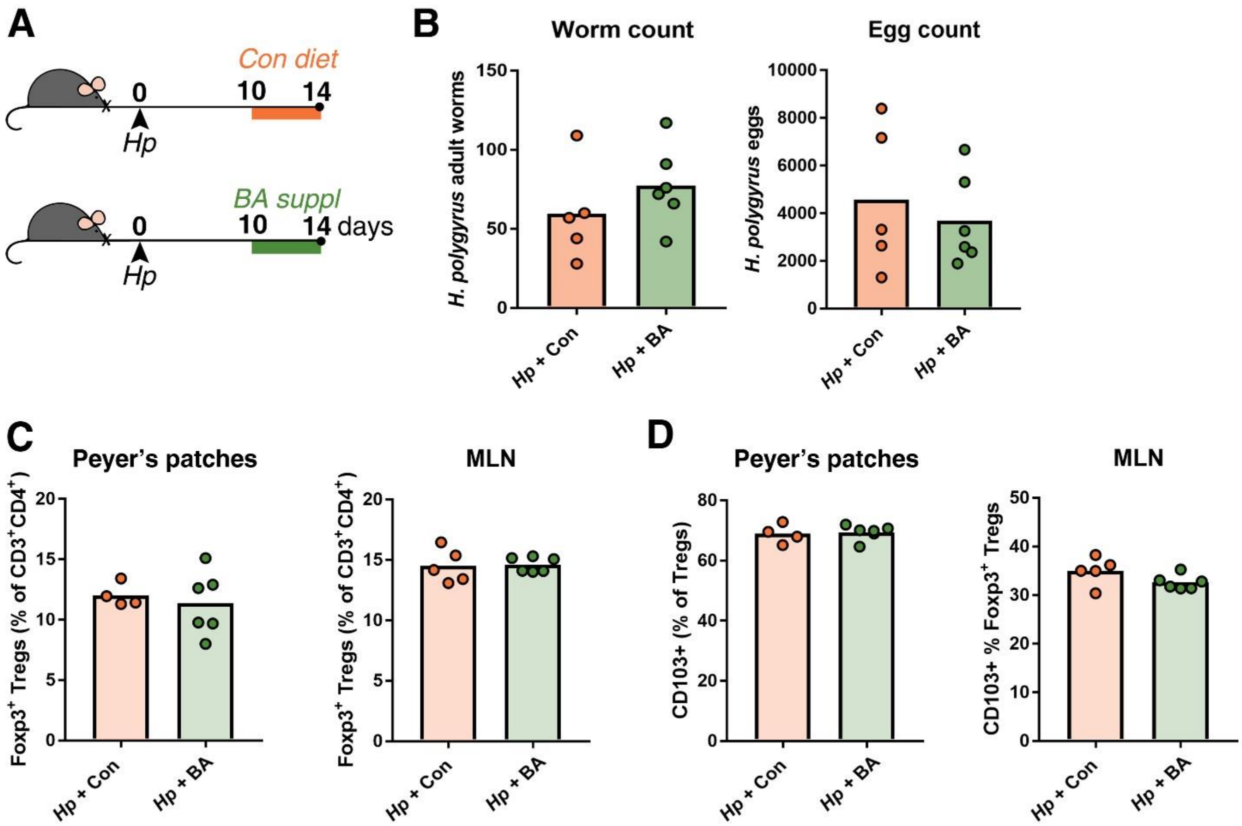

Figure 6.1. Diet enriched with 0.2% CA and 0.2% CDCA does not affect helminth fitness and fecundity or Tregs in the MLNs and Peyer's patches during helminth infection. (**A**) Experimental set-up. *H. polygyrus* (*Hp*)-infected male C57BL6/J mice were switched to a bile acid-supplemented diet (BA) or control diet (Con), ten days post-*Hp* infection. Four days later, on fourteen days post-*Hp* infection, parasite egg counts were determined and adult worms present in the small intestine enumerated (**B**), and cells from Peyer's patches and MLNs were analyzed by flow cytometry (**C, D**). (**C**) The percentages of Foxp3$^+$ Tregs within the CD3$^+$ CD4$^+$ cell population (gated on CD45$^+$ live cells) in the Peyer's patches and MLN are shown. (**D**) The percentages of CD103$^+$ cells within the Foxp3$^+$ Treg population in the Peyer's patches and MLN are shown. Data points represent individual mice and

data is from one experiment. Statistical comparisons between all groups were done using an unpaired t test for parametric data and a Mann-Whitney test for non-parametric data.

6.3.2 *Bile acid sequestering does not affect helminth fitness or Treg levels during helminth infection*

To further investigate the role of bile acid levels in helminth infection, we used an complementary experimental approach to test whether lowering the availability of bile acids in the small intestinal lumen affected helminth fitness. We speculated that lower bile acid concentrations during helminth infection are a way in which *H. polygyrus* can modulate immune responses, therefore, we hypothesized that bile acid sequestering benefits the worm. To test this, mice were switched to a diet containing 2% cholestyramine (bile acid sequestrant) on day 10 of *H. polygyrus* infection, and on day 14 of infection parasite burdens were assessed (**Figure 6.2A**). We found no evidence that 4 days of bile acid sequestering impacted worm counts or egg output (**Figure 6.2B**). Furthermore, we did not observe any effects of 2% cholestyramine supplementation on Foxp3$^+$ expression levels or Foxp3$^+$ Treg activation levels in the Peyer's patches or in the MLNs (**Figure 6.2C,D**). Overall, our data does not support a prominent role of bile acids in impacting helminth chronicity, fecundity, or Treg induction during helminth infection, however as discussed below, future experiments are required to conclusively state that helminth-modification of bile acids does not impact these parameters.

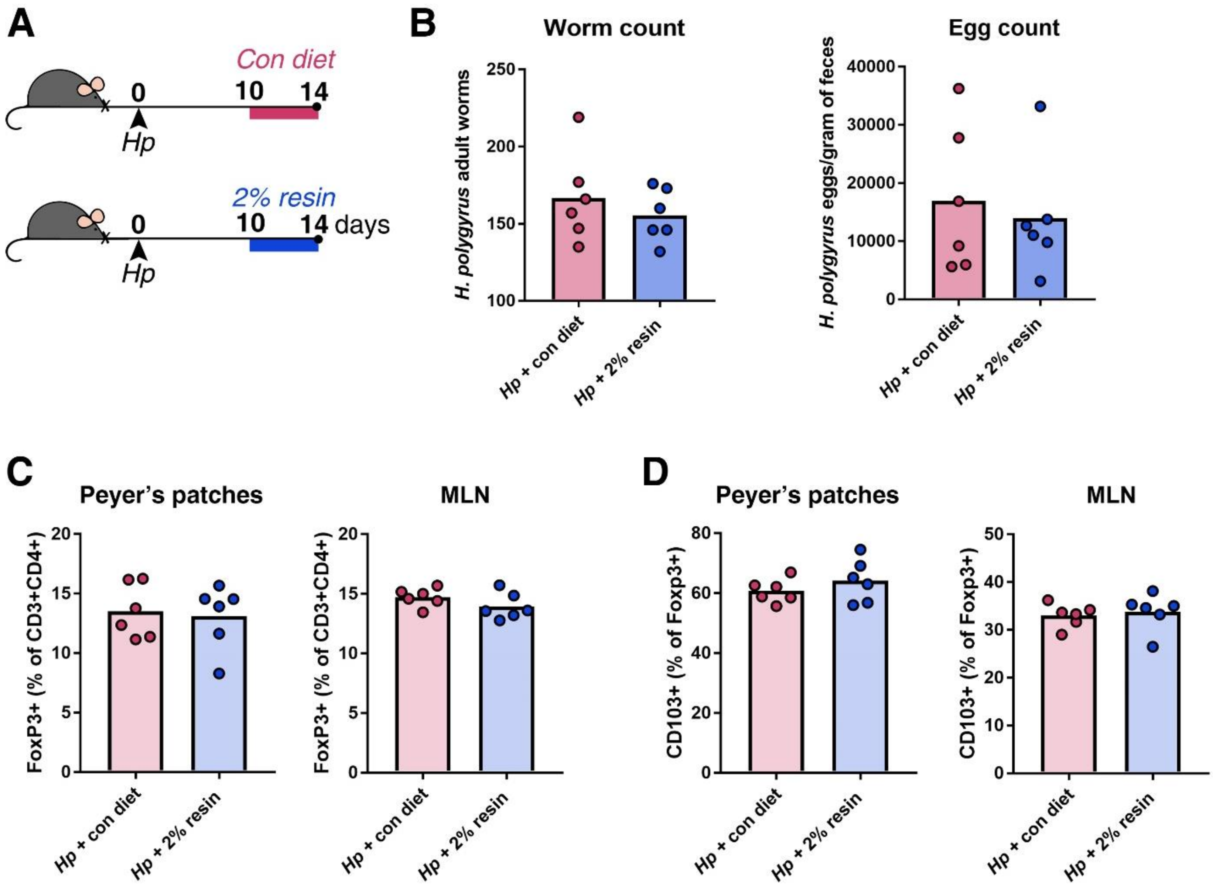

Figure 6.2. Addition of 2% cholestyramine resin to diet does not affect helminth fitness and fecundity or Tregs in the MLNs and Peyer's patches during helminth infection. (**A**) Experimental set-up. *H. polygyrus* (*Hp*)-infected male C57BL6/J mice were switched to a diet containing 2% cholestyramine resin (2% resin) or control diet (con diet), ten days post-*Hp* infection. Four days later, on fourteen days post-*Hp* infection, parasite egg counts were determined, and adult worms present in the small intestine enumerated (**B**), and cells from Peyer's patches and MLNs were analyzed by flow cytometry (**C, D**). (**C**) The percentages of Foxp3$^+$ Tregs within the CD3$^+$ CD4$^+$ cell population (gated on CD45$^+$ live cells) in Peyer's patches and MLN are shown. (**D**) The percentages of CD103$^+$ cells within the Foxp3$^+$ Treg population in Peyer's patches and MLN are shown. Data points represent individual mice and data is from one experiment. Statistical comparisons between all groups were done using an unpaired t test for parametric data and a Mann-Whitney test for non-parametric data.

6.4 Discussion

Helminths produce molecules to promote their survival in the intestine, and several of these products have been characterized [49]. However, metabolites have been relatively ignored in the investigating interactions between helminth and the host immune system. Previously we had uncovered helminth infection-driven shifts in small intestinal levels of specific metabolites, specifically SCFAs and bile acids (**Chapter 3** and **Chapter 4**). Here, we investigated whether reduced concentrations of bile acids had detectable causal impacts on helminth fitness. In addition, we assessed whether these metabolite shifts play a role in Treg expansion during helminth infection. Using dietary supplementation methods, we found that reversing the low abundance of bile acids during helminth infection, or that further decreasing the availability of bile acids, did not affect helminth fitness or Foxp3$^+$ Treg levels.

Bile acid receptor signaling generally seems to have an anti-inflammatory effect on immune responses, including induction of Tregs in the colon [34], however, it is unclear whether bile acids play a similar role in the proximal small intestine. We did not find evidence of bile acid supplementation or sequestering impacting Tregs frequencies in the Peyer's patches on day 14 of *H. polygyrus* infection. We were not able to examine the lamina propria for Treg frequency because of challenges for flow cytometry with this tissue during helminth infection. The inflamed and mucus-rich conditions result in excessive cell death and only a few research groups have been successful in isolating sufficient live cells from the lamina propria during helminth infection for analysis by flow cytometry [54,55]. These groups have published their updated protocols but following these has not yet resulted in improved cell viability in our laboratory. Ongoing efforts should enable sampling and analysis of Tregs in the lamina propria which may reveal localized effects of metabolite shifts in the vicinity of the helminth.

There are other aspects of host immunity that may be altered by changing bile acid concentration; the expression of bile acid receptors is not limited to Tregs, as other T cell subsets express bile acid receptors [269] and are subject to modulation of their function by these metabolites. Inflammatory signaling pathways in innate immune cells are also affected by bile acid receptor signaling [34] which may also play a role in the altered immune environment during helminth infection. Additionally, bile acids can influence gut motility by enhancing smooth muscle contractility [354], thus lowering the concentration of bile acids may be a strategy of helminths to decrease intestinal motility and prevent their expulsion. Overall, several avenues of research are still to be taken to fully comprehend the impact of altered bile acid levels on host immunity during helminth infection.

A limitation of the data presented in this chapter is that experiments were only performed once, and as such the experiments need to be repeated for conclusive answers. In addition, different time points of bile acid supplementation and sequestering need to be tested. In experiments reported in this chapter, sequestering resin was added to the diet of mice from day 10 to day 14 of *H. polygyrus* infection, which may not have been long enough to observe an effect on *H. polygyrus* establishment or fecundity. Therefore, in the future, the effect of a week-long bile acid sequestering treatment (which we have validated increases bile acid excretion in **Chapter 5**) on Treg populations and helminth fitness should be assessed. Bile acid supplementation and sequestering methods should also be applied later on in the infection, as Treg numbers peak around day 28 of *H. polygyrus* infection [355], and changes in the bile acid pool may have the biggest impact at that stage of infection. For this, the timing needs to be carefully optimized, including validation of the treatment by measuring bile acid levels at different times into treatment. Prolonged bile acid supplementation may be counteractive to the goal of enriching the intestinal lumen with bile acids, as host feedback mechanisms may eventually compensate for the added bile acids as bile acid synthesis may be downregulated. One previous study showed that bile acid feeding leads to suppressed liver synthesis gene expression, while addition of a bile acid sequestering resin promotes expression of bile acid synthesis genes [356]; presumably both are host feedback reactions to restore bile acid homeostasis. Interestingly, female and male mice demonstrated different responses in gene expression to supplemented diets [356], which is the reason why in our bile acid supplementation and sequestering studies we chose to used male mice, as in this prior report there was less of a feedback effect of supplementation on the liver enzymes in male mice [356]. These sex differences in bile acid homeostasis mechanisms may underlie differences in immune responses between different sexes in mice and should be further investigated. We assessed sex as a variable in this chapter and found that in this data set it did not significantly impact bile acid levels in naïve or helminth-infected mice, however this was just with one experimental repeat with a small sample size of each sex. Future experiments should continue to evaluate sex as a potential variable.

Overall, these experiments form a preliminary investigation into the effects of bile acid supplementation on host immunity. Our supplementation and sequestering methods during helminth infection provide a starting ground for future research that may reveal novel immunomodulatory pathways by which helminths manipulate the immune system of their hosts. Novel therapeutic methods including diet-based treatment options for inflammatory disease can arise from the comprehensive understanding of the interactions between helminth, the gut microbiota, bile acid metabolism and the host immune system.

Chapter 7

General discussion

Chapter disclaimer: Certain experiments described in the future directions section are the result of discussions with collaborators Dr. Lisa Osborne (University of British Columbia) and Dr. Brian Coombes (McMaster University).

7.1 Concluding remarks

This thesis aimed to investigate how helminth infection can alter bacterial colonization in the intestine, and what the impact is of helminth infection on the intestinal metabolome.

In **Chapter 2** (and [298]) we have shown that helminth co-infection can alter intestinal colonization by a bacterial pathogen. Specifically, we found an increase in luminal colonization of *Salmonella* in the small intestine of mice that were concurrently infected with *H. polygyrus*. We have also shown the impact of anthelmintic treatment on the co-infection outcome in this model: deworming prior to bacterial infection was able to restore small intestinal immunity to *Salmonella*, while deworming after *Salmonella* had already established in the small intestine of co-infected mice did not lead to complete clearance of bacteria from the small intestine.

Furthermore, we have shown that helminth infection can change the availability of certain intestinal metabolites that have immunomodulatory potential. In **Chapter 3** (and [85]) we have presented a comprehensive analysis of SCFAs during helminth infection that revealed a marked increase in small intestinal levels of isovalerate. As we have demonstrated in **Chapter 5**, we developed a supplementation method where isovalerate was administered in the drinking water to raise intestinal levels of isovalerate in uninfected mice to a similar extent as we see during helminth infection. We used this model to investigate whether helminth modulation of isovalerate levels could impact host intestinal immunity. We did not find that modulating isovalerate levels in the absence of worms impacted susceptibility to *Salmonella* infection (**Chapter 5**), however, work stemming from this thesis has found that modulating isovalerate levels can impact helminth fitness [85].

In addition, we have shown that helminth infection lowered the concentration of bile acids in the small intestine of mice (**Chapter 4**). This correlated with alterations in the expression of genes encoding for bile acid transporters in the small intestinal tissue, while genes related to bile acid synthesis remained unaffected by helminth infection. In **Chapter 5** we have demonstrated that bile acid levels in helminth-infected mice could be restored using a diet supplemented with bile acids, and that a lower bile acid concentration may be mimicked by adding a sequestering agent to the diet of mice. These diet-based methods will enable future studies stemming from our work to study the contribution of bile acids in the helminth's modulation of host immunity. This thesis features preliminary experiments looking into the effects of bile acid supplementation and sequestering on Treg levels during helminth infection (**Chapter 6**).

7.2 Pieces of a bigger puzzle

When we consider our own work and other work on helminth co-infections, it is becoming clear that co-infection with helminths and pathogenic bacteria can have many different outcomes. This is likely due to the complexity of factors that are at play, including the particularities of different helminth species and bacterial pathogens, route of infection, host genetics and environmental factors such as diet. Helminth species each have different niches and various ways to modulate host immunity, and different bacterial pathogens have different metabolic requirements, tissue tropisms and immune evasion strategies. The work in this thesis provides an example of a helminth infection *promoting* intestinal colonization with a bacterial pathogen (**Chapter 2**) [298], yet there are also reports of helminths in different contexts providing *protection* against microbial pathogens, mainly when the microbial infection occurred at a non-intestinal site [217,305,306,308,357].

It has not been studied extensively how deworming affects immunity to pathogenic bacteria, and we are the first to demonstrate that deworming promotes small intestinal immunity to a bacterial pathogen (**Chapter 2**). Furthermore, we showed that anthelmintic treatment of helminth-*Salmonella* co-infected mice did not result in complete elimination of *Salmonella* burdens in the small intestine after the helminth had been cleared (**Chapter 2**) [298]. This data may help explain observations in human populations where deworming was not associated with amelioration of TB [122] or a better prognosis of AIDS in individuals previously co-infected with hookworms [120,121]. However, in people that were co-infected with HIV and Ascaris, deworming *did* improve T cell counts [120,121]. This demonstrates that deworming can have different outcomes on co-infecting pathogens. One study of helminth-infected African buffalos highlighted the importance of treatment timing on the outcome of bacterial disease: deworming did not affect the risk of acquiring bovine TB but instead was associated with improved survival rates in buffalos with pre-existing TB [123]. In our study we also observed differential effects on bacterial burdens when deworming treatment preceded bacterial infection or followed it [298], however which timing was beneficial does not match the bovine TB study (**Chapter 2**).

Context is extremely important in understanding the interactions between co-infecting pathogens and in predicting the outcome of deworming. The study of co-infection is challenging in this aspect as many different scenarios need to be studied separately, but these studies are crucial to understand the multitude of factors that can influence the course of infectious disease occurs in human populations. A large proportion of individuals are likely to be exposed to a multitude of pathogens at one moment in time, and this might complicate disease control strategies.

Our metabolomics results contribute to a better understanding of how helminths impact the intestinal metabolome of their host (**Chapter 3** and **Chapter 4**). Metabolites are understudied in the search of molecular pathways in which helminth modulate host immune responses. Rather than merely studying helminth-derived metabolites, we studied helminth-modulated metabolites in the context of the intestinal environment. In characterizing the intestinal metabolome during helminth infection, we are looking at metabolites both used and produced, by helminths as well as by the host and the microbiota. As helminths, the microbiota and their hosts have co-evolved and therefore many interactions between these organisms exist, it is to be expected that metabolite shifts during helminth infection reflect the complexity of these relationships. This context is important in dissecting these relationships such as understanding how helminth modulate host immunity, and sample site and time points need to be chosen carefully to provide the most useful information. In quantifying metabolite shifts during *H. polygyrus* infection we included samples from the small intestine to study localised effects of helminth infection, which for SCFAs were previously overlooked in studies characterizing SCFA levels during small intestine-residing helminth infection [23,315].

It is challenging to study the entire metabolome at once, because the currently available databases do not yet comprehensively cover the entire width of metabolite features that can be detected with mass spectrometry. Reports on untargeted metabolite screens therefore become biased to what information exists in databases. For this reason, in this thesis we chose to take a targeted, hypothesis-based metabolomics approach which provides precise information as to how select groups of metabolites are altered in abundance during helminth infection. Each such hypothesis-based metabolomics study contributes to a collective understanding of metabolite shifts during helminth infection and further studies may decipher specific roles of these metabolites in host-pathogen interactions. Context is important here as well, as metabolites may exert their immunomodulatory function in concert with other metabolites or perhaps protein moieties, and such synergy of immunomodulatory components should be considered.

7.3 Future research directions

As mentioned above, studying co-infection in several contexts is crucial to be able to apply the knowledge gained from defined experimental models to human populations. Therefore, different combinations of helminth species and bacterial pathogens should be investigated to enhance the impact of this current work. For example, future work could include mice infected with a helminth that preferentially establishes infection in the large intestine, such as *T. muris*, and then co-infected with *Salmonella* to see if localised effects on luminal populations of

Salmonella occur in the context of other parts of the intestine during infection with other helminth species.

In the bigger picture, co-infection should be considered in current infectious disease challenges. Importantly, the potential of helminths to offer protection against viral airway infections should be further investigated and should include coronaviruses such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). This research could offer clues that may help disease control of the current coronavirus pandemic and future pandemics in both helminth-endemic areas as well as in world regions where helminths are less abundant.

Furthermore, in-depth studies are needed to inform disease control strategies that aim to harness protective effects of co-infection or that are aimed at counteracting unwarranted effects of co-infection. This thesis work forms a platform to continue further dissection of the impact of *H. polygyrus* infection on *Salmonella* colonization. It will be important to find the underlying molecular mechanism(s) of how *Salmonella* benefits from a helminth-modified small intestinal environment, to be able to apply this knowledge and develop novel ways to target bacterial disease. One promising approach is to assess the role of *Salmonella* virulence factors in this co-infection model which can be done with the use of a *Salmonella* transposon mutant library (such as the library created by Dr. Brian Coombes at the McMaster University). Following infection of mice with helminths and the mutant library, a process of negative selection will follow that allows certain mutants to establish while others are not able to. Sequencing of *Salmonella* strains that succeeded in colonizing the small intestine will reveal which mutants are lost during co-infection, which may indicate which genes are required for *Salmonella* to colonize in a helminth-infected gut. The genome of *Salmonella* is very well-characterized and existing literature will facilitate further analysis of the candidate genes, and many single *Salmonella* mutants are available for further validation. Genes involved in bacterial metabolism should be highlighted since it is feasible that immunomodulatory metabolites produced during helminth infection may have off-target effects and fuel bacterial colonization. One potential pitfall of this mutant library experiment is the so-called bottleneck effect which may result in a random loss of mutants [358]. Therefore, these experiments need to be optimised to minimize bottleneck effects, for example with pre-treatment with streptomycin, and the experiments need to be repeated sufficiently. Overall, *Salmonella* pathogenesis is well-studied and choosing an angle that looks from the bacteria's perspective (i.e. what factors does *Salmonella* require in order to exploit a helminth-modified intestinal environment) may prove very fruitful in discovering ways in which *Salmonella* takes advantage of helminth co-infection.

A different approach to identifying mechanisms by which helminths promote bacterial colonization is to study whether different events in host immunity and intestinal physiology associated with *H. polygyrus* infection revert to a naïve state after deworming treatment, as deworming efficiently restored immunity to *Salmonella* infection. For example, increased mucus production for helminth expulsion may or may not be diminished directly after helminths are cleared, which could be associated with the ability of *Salmonella* to expand in the luminal compartment of the small intestine. Following such association studies, causality should be explored, for example using *Salmonella* mutants and genetic or chemical manipulation of host processes. In the case that such causal relationships between host responses to helminth infection and *Salmonella* colonization are not detectable, results from deworming studies will still be valuable in describing what host immune responses persist after helminths are cleared and thus the impact of a previous helminth infection on host immunity.

In terms of forming a complete picture of how helminth infection alters the intestinal metabolome, further work must be done in comparing the levels of additional groups of metabolites. For example, amino acid metabolism during helminth infection should be taken under a loop, as amino acids such as tryptophan and arginine can be metabolized by the microbiota, and their derivatives exert modulatory functions on the immune system [32]. It is possible that helminth infection-inflicted shifts in the microbiota contribute to changes in amino acid metabolism and metabolites including tryptophan- and arginine-derivatives may in part be responsible for suppressing host inflammation. Future research should also build upon the metabolite shifts described in this thesis, as more work needs to be done to elucidate consequences of increased levels of isovalerate and decreased bile acid concentration on multiple facets of host immunity.

7.4 Impact of this work

Helminths and the microbiota hold enormous potential to modulate host immune responses which can be exploited to treat a variety of inflammatory diseases that are becoming more prevalent each year. There is also hope in the power of the microbiota to counteract bacterial infection as antibiotic resistance is on the rise. As therapeutic helminth infections and fecal microbiota transfers are being introduced into the clinic with mixed results and several safety challenges [359,360], it becomes increasingly clear that these therapeutic options need to be refined. Research that concentrates on finding the molecular mechanisms behind immunomodulation by gut-colonizing microbes and parasites can offer more refined and targeted approaches to manipulate host immune responses. Specifically, metabolites that show great immunomodulatory potential are of interest to further develop into therapeutic agents for human disease. This thesis work shows for the first time the impact of helminth infection on small intestinal levels of SCFAs and bile acids, metabolites that may contribute to the ability of helminths to modulate immune responses in this host tissue. This thesis also investigates immunity to *Salmonella* in the context of helminth co-infection and deworming, which furthers our understanding of the complexity and severity of infectious disease in helminth-endemic areas and informs disease control strategies in these regions. Moreover, at a global level, it explores the question of why certain individuals are more susceptible to bacterial infection, a question that becomes ever so important as personalized medicine and immune-based therapies are on the horizon of disease control.

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Appendix

Appended to this thesis are the three articles that I have published during my PhD, in order of publication date:

1. **Brosschot, T. P.** and Reynolds, L. A. (2018) 'The impact of a helminth-modified microbiome on host immunity', *Mucosal Immunology*.

2. **Brosschot, T. P.**, Lawrence, K. M., Moeller, B. E., Kennedy, M. E., FitzPatrick, R. D., Gauthier, C. M., Shin, D., Gatti, D. M., Conway, K. M. E., Reynolds, L. A., (2021) 'Impaired host resistance to *Salmonella* during helminth co-infection is restored by anthelmintic treatment prior to bacterial challenge', *PLoS Neglected Tropical Diseases*.

3. Kennedy, M. E., **Brosschot, T. P.**, Lawrence, K. M., FitzPatrick, R. D., Reynolds, L. A., (2021) 'Small intestinal levels of the branched short-chain fatty acid isovalerate are elevated during infection with *Heligmosomoides polygyrus* and can promote helminth fecundity', *Infection and Immunity*.