Aus dem Institut für Immunologie
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Bacterial modulation by the intestinal roundworm *Ascaris suum*

Inaugural-Dissertation
zur Erlangung des akademischen Grades eines
Doctor of Philosophy (PhD)
in "Biomedical Sciences"
an der Freien Universität Berlin

vorgelegt von
Ankur Midha
Apotheker aus Vancouver, Kanada

Berlin 2024
Journal-Nr.: 4424

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List of Abbreviations

Abbreviation	**Full Term**
ABF	Antibacterial factor
AMP	Antimicrobial peptide
ASABF	*Ascaris suum* antibacterial factor
ASV	Amplicon sequence variant
CTL	C-type lectin
DALY	Disability-adjusted life years
dpi	Days post-infection
ELISA	Enzyme-linked immunosorbent assay
ES	Excretory and secreted
EV	Extracellular vesicles
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
L3	Third-stage larvae
LPS	Lipopolysaccharides
MDA	Mass drug administration
NTD	Neglected tropical disease
OUT	Operational taxonomic unit
PMP	Parasite microbiome project
STH	Soil-transmitted helminth

Th	Helper T cells
Treg	Regulatory T cells
TSLP	Thymic stromal lymphopoetin
WASH	Water, sanitation, hygiene

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1. Introduction

1.1 Biology and life cycle of *Ascaris* parasites

Ascaris lumbricoides and *A. suum* are morphologically indistinguishable parasitic roundworms of humans and pigs, respectively (Dold and Holland, 2011). While both taxa show affinity for their conventional hosts *A. lumbricoides* can infect pigs and *A. suum* can infect humans (Takata, 1951; Anderson, 1995; Crompton, 2001; Nejsum et al., 2005). In fact, genomic and proteomic similarities suggest they might even be the same species. (Zhu et al., 1999; Leles et al., 2012; Xu et al., 2013; Shao et al., 2014; Nejsum et al., 2016). Additionally, the direct life cycle of *Ascaris* is thought to be near identical in humans and pigs (Figure 1). Mice can serve as a model for the early tissue-migratory phase of the *Ascaris* life cycle, as they can also be infected with *Ascaris* though the worm cannot complete its full life cycle (Lewis et al., 2006). Thus, *Ascaris* is of zoonotic importance, humans and pigs can function as reservoirs for one another, and the pig is a physiologically relevant infection model for humans.

Following ingestion of infective eggs containing third-stage (L3) larvae, egg hatching is stimulated by ova-extrinsic factors in the host gut, including temperature, pH, bile, and mechanical stimulation provided by peristalsis (Han et al., 2000; Mkandawire et al., 2022). Free larvae can be isolated from the distal small intestine, cecum, and colon within 3 hours of ingestion (Murrell et al., 1997). By 6 hours, the majority of free larvae are found in the cecum and colon and by 18 hours, larvae are no longer detected in the intestinal contents, indicating that they have either penetrated the intestinal barrier and begun hepato-tracheal migration or have been expelled from the host (Murrell et al., 1997). Following intestinal invasion, the larvae reach the liver via the portal vein, after which they reach the lungs by 6-8 days post-infection (dpi) (Roepstorff and Murrell, 1997). In the lungs, the larvae penetrate the alveoli to then migrate to the pharynx where they can again be swallowed to return to the small intestine, primarily the jejunum, by 8-10 dpi (Roepstorff and Murrell, 1997). In the jejunum, the larvae undergo two more moults to reach sexual maturity (Pilitt et al., 1981). The majority of worms are expelled by the 23rd week of infection (Miquel et al., 2005), but the ones that survive can reside in the intestine for over a year, growing to lengths upwards of 35 cm, mating and shedding hundreds of thousands of eggs per adult female per day (Olsen et al., 1958; Pilitt et al., 1981; Sinniah, 1982; Dold and Holland, 2011; Centers for Disease Control and Prevention, 2019). Notably, *Ascaris* infection is marked by an over-dispersed or aggregated distribution, where most individuals carry light worm burdens and a minority of

the population carries heavy worm burdens (Holland, 2009). Fertilized, unembryonated eggs produced by mature females are shed with the feces into the environment where they undergo embryonation and development to the infective L3 stage and can survive for up to 14 years (Else et al., 2020) while unfertilized eggs are also shed in the feces and can be ingested but are not infective (Centers for Disease Control and Prevention, 2019).

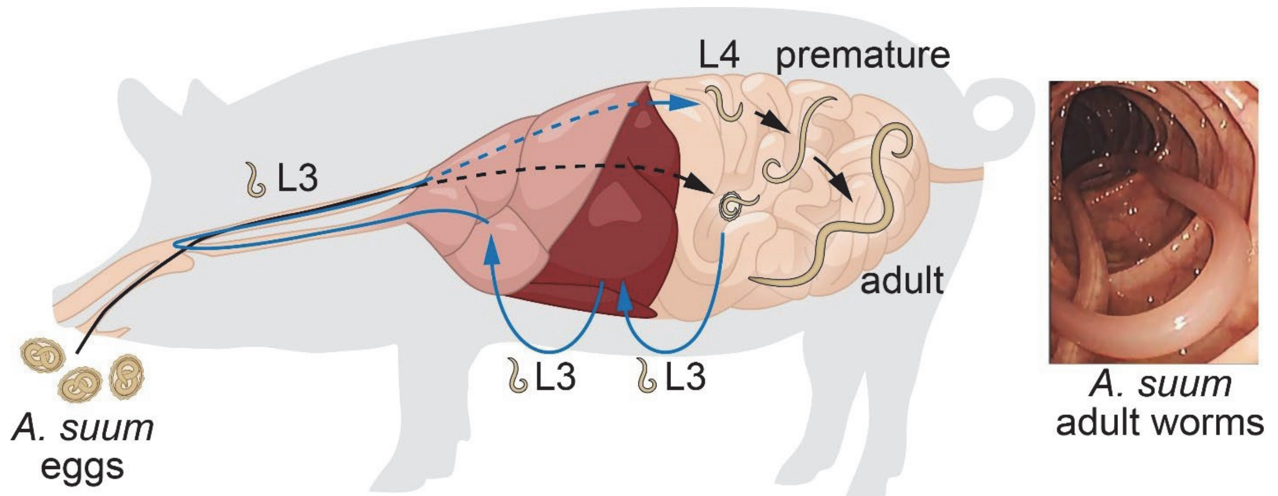

Figure 1 Life cycle of *Ascaris suum.* Infected embryonated eggs containing third-stage larvae (L3) are ingested orally. The eggs hatch in the small intestine and the larvae begin a tissue migratory phase after breaching the intestinal barrier in the cecum and proximal colon. The L3 migrate to the liver via the portal blood before reaching the lungs via the systemic circulation. The larvae then enter the alveolar space and migrate up to the pharynx where they are swallowed to return to the intestine. In the jejunum, they undergo further development. Figure created by Dr. Anne Winkler, Institute of Immunology, Freie Universität Berlin.

1.2 Ascariasis epidemiology and relevance

Recent estimates indicate that soil-transmitted helminths (STHs; parasitic worms) collectively infect more than a billion people globally, primarily in Asia, Latin America, and sub-Saharan Africa (Pullan et al., 2014). STH infections are neglected tropical diseases (NTDs) - a group of conditions caused by a diversity of pathogens that occur primarily in low-income countries and are under- funded on a global scale (World Health Organization, 2023a) - and ascariasis is the most prevalent STH and a very important NTD, infecting upwards of 800 million people (Pullan et al., 2014; Holland et al., 2022). Children are at highest risk of heavy parasite burdens and people in endemic areas experience frequent re-infection (Holland, 2009; Wright et al., 2018). While mortality due to intestinal obstruction is rare (de Silva et al., 1997), disability-adjusted life years (DALY) due to *Ascaris* are estimated at 1 million (Jourdan et al., 2018; Kyu et al., 2018) along with evidence for cognitive impairment and loss of education in STH-infected children (Pabalan et al., 2018). Furthermore, STH infections influence risks of coinfection with other pathogens as well as impaired protective immune responses to vaccines (Schlosser-Brandenburg et al., 2023). In addition to its impact on human health,

ascariasis is also a considerable problem in animal agriculture as *A. suum* is widespread in pig husbandry. Monitoring for helminth infestation can be accomplished by detecting eggs in feces, liver inspection at slaughter, or by assessing seroprevalence using enzyme-linked immunosorbent assay (ELISA) tests. Exemplary of estimates from European pig farms, one study evaluated the presence of *A. suum* in pigs at an industrial slaughterhouse in northern Italy and found all farms to be positive for *Ascaris,* with prevalence among pigs ranging from 3.8% to 98.3% by liver inspection, and 36.4% to 100% by ELISA (Vismarra et al., 2023). Similarly, a study of nine pig farms across four states in the United States assessed *A. suum* prevalence by fecal egg counting and found 88.9% of farms positive for *Ascaris,* with growing and finishing pigs showing the highest eggs per gram in feces, and higher prevalence and egg abundance in organic certified farms compared to non-organic certified farms (Hernandez et al., 2023). *Ascaris* seroprevalence is negatively correlated with farm productivity (Vlaminck et al., 2015) and the overall impact of high *Ascaris* prevalence is economic losses in the hundreds of millions due to reduced growth and feed conversion, potential co-infection with other pathogens, costs of infection control, and losses due to liver condemnation (Stewart and Hale, 1988; Kipper et al., 2011; Knecht et al., 2012; Thamsborg et al., 2013; Vlaminck et al., 2015; Ózsvári, 2018; Joachim et al., 2021). In contrast, strategic deworming is associated with improved performance of pig farms (Van Meensel et al., 2010). Therefore, ascariasis persists as a major problem in global health and an economic burden to animal agriculture.

1.3 Host immune responses and pathology during *Ascaris* infection

Our understanding of *Ascaris*-induced immune responses and pathology comes from studies in humans, pigs, and mice and is further supplemented with data from other helminth infection models. Though ascariasis is largely subclinical and asymptomatic, an estimated 8 - 15% of ascariasis patients demonstrate morbidity (Dold and Holland, 2011). In chronically infected patients, heavy worm burdens can cause partial or complete obstruction of the intestine or gall bladder, manifesting as abdominal pain and discomfort, distension, nausea, anorexia, and intermittent diarrhea, failure to gain weight (Bokhari, 2021; Ballweber, 2022), though the heavily- infected patient with severe ascariasis is rare. More common however are acute symptoms early in infection. During migration, *Ascaris* larvae damage the various tissues they migrate through, inducing inflammatory and wound-healing immune responses in the intestine, liver, and lungs. Damage of the gut barrier induces epithelial and mast cells to release the alarmins thymic stromal lymphopoietin (TSLP), interleukin- (IL) 25, and IL-33 (Allen and Maizels, 2011). Alarmin release leads to activation of an innate immune response involving mast cells, dendritic cells, and type 2 innate lymphoid cells producing the cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 which then promote the development of type 2 T-helper (Th2)

cells and an associated adaptive type 2 response (Figure 2) (Turner et al., 2003; Jackson et al., 2004; Else et al., 2020). IL-4 and IL-13 lead to alternative activation of macrophages while IL-5 leads to recruitment of eosinophils into affected tissues (Allen and Maizels, 2011; Gazzinelli-Guimaraes et al., 2019). This type 2 response is characterized by mucin-producing goblet cell hyperplasia and increased intestinal smooth muscle contractility, culminating in the "weep and sweep" response driving parasite expulsion (Allen and Maizels, 2011; Masure et al., 2013a, 2013b). Larval migration through the liver is responsible for the formation of milk spots: fibrotic hepatic lesions are composed of trapped larval material surrounded by infiltrating immune cells (Perez et al., 2001). Early lesions are composed of hemorrhagic foci surrounded by eosinophils, neutrophils and macrophages which progress to granulomatous lesions which retain the necrotic centre and are surrounded by eosinophils, macrophages, and lymphocytes (Perez et al., 2001). Larval migration through lung tissue can induce respiratory distress - referred to as Löffler's syndrome - an eosinophilic pneumonia characterized by dyspnea, dry coughing, wheezing, hemoptysis, and chest pain in humans and by abdominal breathing, dry cough, and wheezing in pigs (Löffler, 1956; Dold and Holland, 2011; Bokhari, 2021; Ballweber, 2022). Furthermore, murine models have shown that repeated larval migration through the lungs induces robust type 2 responses which simultaneously promote protection and lung pathology characterized by persistent airway hyperresponsiveness (Nogueira et al., 2016; Weatherhead et al., 2018).

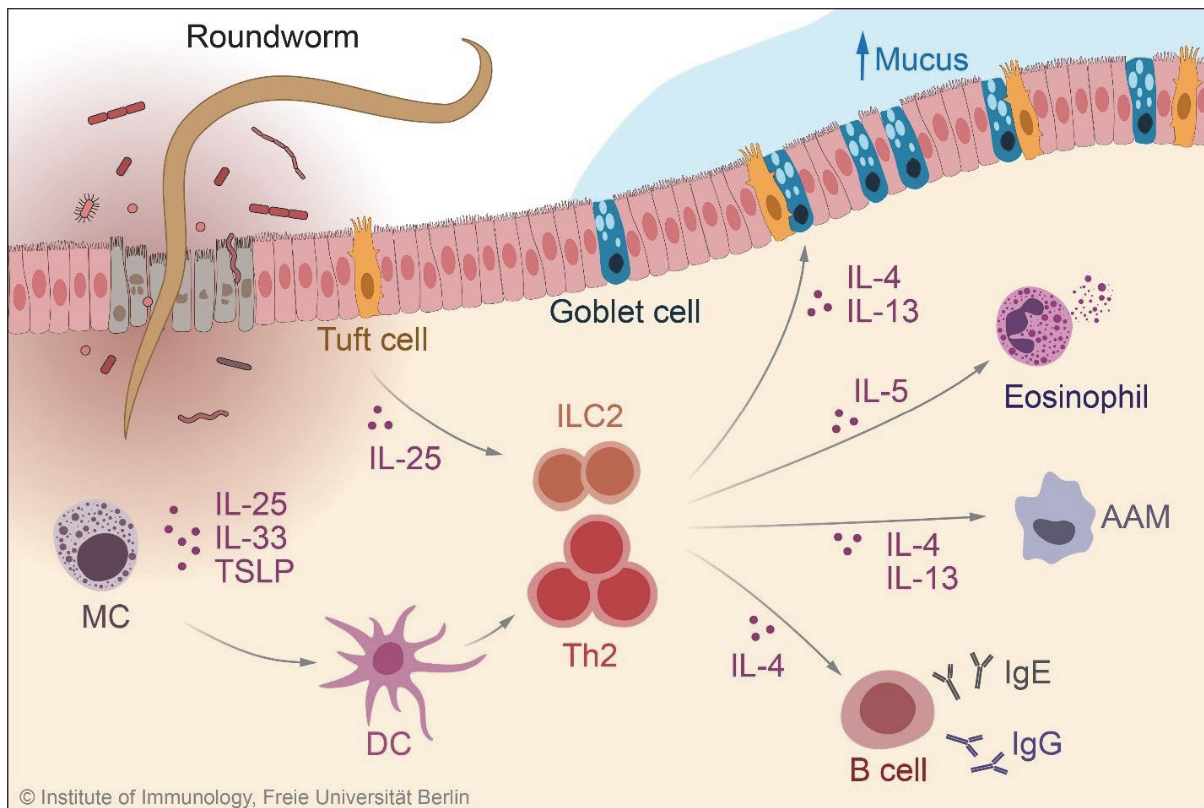

Figure 2 Immune responses to *Ascaris* infection. During tissue invasion, alarmins derived from epithelial, tuft, and mast cells lead to the activation of a type 2 immune response involving the production of the type 2 cytokines, IL-4, IL-5, and IL-13. These cytokines drive effector mechanisms involved in worm expulsion, including increased mucus production via goblet cell hyperplasia, eosinophil recruitment, and antibody responses by B cells. AAM, alternatively activated macrophages; DC, dendritic cell; ILC2, type 2 innate lymphoid cell; MC, mast cell; TSLP, thymic stromal lymphopoetin. Figure adapted from Schlosser- Brandenburg J., Midha A. et al. (2023) Infection with soil-transmitted helminths and their impact on coinfections. Frontiers in Parasitology. 2:1197956. doi: 10.3389/fpara.2023.1197956.

Previous work has established a clear link between parasite-specific immunoglobulin (Ig) E levels and infection intensity; children with high levels of *Ascaris*-specific IgE against the protein antigen ABA-1 had a lower risk for heavy infection intensity compared to children with low levels of *Ascaris*-specific IgE, while IgG levels did not correlate with protection (McSharry et al., 1999). In general, type 2 responses are usually associated with helminth infections and allergies and are typically opposed to type 1 and type 3 responses induced by viral, bacterial, protozoan, and fungal infections (Allen and Maizels, 2011; Eberl, 2016). While type 2 responses are associated with protection against helminths, inflammation needs to be modulated to reign in pathology. In fact, some parasite antigens can modulate myeloid cell function and responsiveness to lipopolysaccharide (LPS) and helminth infections are frequently associated with regulatory T cells (Treg) and IL-10 (Dowling et al., 2011; Favoretto et al., 2014; Titz et al., 2017; Almeida et al., 2018; Gazzinelli-Guimaraes et al., 2019). Thus the clinical presentation of ascariasis is primarily due to tissue migration and corresponding immunopathology.

1.4 Prevention and treatment

As *Ascaris* is widespread and adult females produce hundreds of thousands of robust, long surviving eggs, long-term control and eradication depends on multiple solutions, including improved sanitation, behavioral changes, and mass deworming programs (Dold and Holland, 2011; Else et al., 2020). Improvements in water, sanitation, and hygiene, referred to as WASH initiatives, form a major pillar of helminth control but require time and continued investment in infrastructure to have an impact (Vaz Nery et al., 2019). Meanwhile, the World Health Organization advocates for reducing morbidity by reducing worm burdens through periodic deworming campaigns, primarily by administering the benzimidazoles, albendazole and mebendazole, to preschool and school age children (Bokhari, 2021; World Health Organization, 2023b). Benzimidazoles inhibit tubulin polymerization leading to decreased adenosine triphosphate production, immobilization, and eventual expulsion and death of the helminth (Bokhari, 2021). Additional anthelmintics such as pyrantel pamoate, ivermectin, and piperazine are also effective but have not been as widely used as benzimidazoles (Bokhari, 2021; Ballweber, 2022). Ascariasis control of pig herds includes the use of sanitation procedures in addition to deworming sows with anthelmintics 1 week before farrowing and continuous deworming in 8- week intervals, as repeated treatment is required to improve performance (Boes et al., 2010; Ballweber, 2022). Eradication of *Ascaris* through mass drug administration (MDA) has not been achievable as the efficacy of these campaigns has been mixed in different regions, with some studies showing little to no effect while others have shown reduced worm burdens and prevalence (Chai et al., 2020; Eneanya et al., 2022). Furthermore, MDA can lead to the emergence of drug resistance (Pilotte et al., 2022). Immunity to helminths also appears to be limited as it requires repeated exposure, is only partially protective, and leads only to reduced parasite burdens while re-infections continue to occur (Urban et al., 1988; Nogueira et al., 2016; Gazzinelli-Guimarães et al., 2018; Colombo and Grencis, 2020). Furthermore, development of protective immunity is compromised by the ability of parasites to modulate host responses by activating immune regulatory networks (Loukas et al., 2021). Thus, vaccines against *Ascaris* have not yet been brought into human clinical trials though continued effort is devoted to vaccine development. In summary, prevention and control efforts have had some success but novel treatment modalities are needed.

1.5 Ascaris and microbes

1.5.1 Life in a microbial environment

Ascaris is first exposed to microbes upon hatching in the small intestine prior to invading the cecum and proximal colon (Figure 3) (Roepstorff and Murrell, 1997). Following tissue migration the worms end up primarily in the jejunum where those that survive expulsion will remain for over a year (Dold and Holland, 2011). Most work on the host intestinal microbiome has focused on fecal samples and the colon, with approximately 10^{11} bacterial cells per mL of human colonic ingesta (Sender et al., 2016). The small intestine is also populated with microbes, albeit at a lower concentration ranging from 10^3 cells/mL in the duodenum, increasing along the length of the intestine up to 10^8 cells/mL at the most distal end of the small intestine in the ileum (Sender et al., 2016). These estimates are assumed to be comparable in pigs given the anatomical, physiological, and even microbiota similarities between pigs and humans, in contrast to mice (Rose et al., 2022). Therefore, *Ascaris* spends most of its lifetime in a microbial environment.

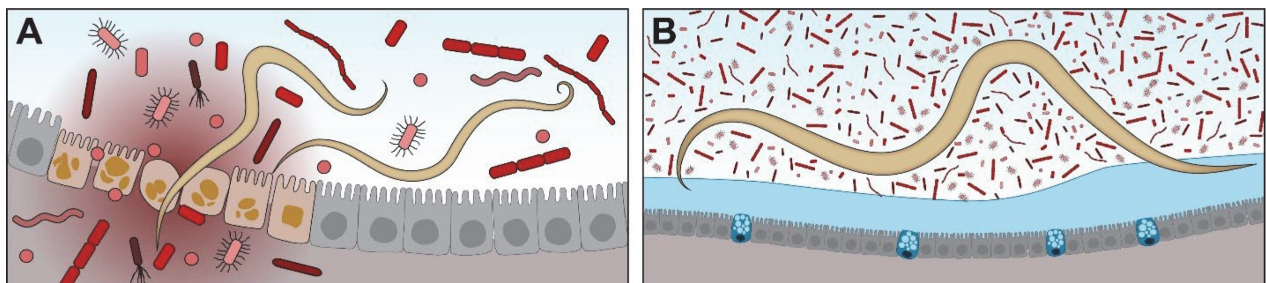

Figure 3 Sites of interactions between *Ascaris* and microbes. **A** The first encounter between *Ascaris* and microbes occurs in the small intestine upon hatching, where larvae breach the intestinal epithelium causing inflammation and possibly translocating gut microbial contents, including microbes, across the intestinal barrier. **B** *Ascaris* live in a microbial environment amongst the host microbiota in the jejunum.

Microbial environments present infectious challenges for all organisms, including nematodes. Due to various limitations, our understanding of parasitic nematode immune systems is in its infancy and most of our understanding of nematode immune defenses is informed by the free-living model nematode *Caenorhabditis elegans,* frequently used as a useful model for host-pathogen interactions (Powell and Ausubel, 2008). Outside of well-defined laboratory conditions where it is typically maintained on cultures of *Eischerichia coli* OP50 (Arata et al., 2020), *C. elegans* worms are found living amidst a diversity of environmental microbes, feeding on bacteria in rotting fruit and herbaceous stems (Félix and Duveau, 2012; Frézal and Félix, 2015). Also unlike laboratory life, worms isolated from their natural habitat are observed in various states of stress such as starvation and infection (Barrière and Félix, 2005). Intriguingly, *C. elegans* acquires an intestinal microbiota, derived but distinct from its environment (Berg et al., 2016; Dirksen et al., 2016; Zhang et al., 2017). As with any other

animal, *C. elegans* worms experience beneficial and detrimental interactions with microbes, where some microbes support nematode growth while others cause infection and induce stress responses (Félix and Duveau, 2012; Samuel et al., 2016). *C. elegans* responds to experimental infections by coordinating pathogen-specific responses using evolutionarily conserved signaling pathways, including p38 mitogen-activated protein kinase and a forkhead box family transcription factor, to activate effector genes, including antimicrobial peptides (AMPs) and other effector molecules (Pukkila-Worley and Ausubel, 2012). AMPs are typically low molecular weight, broad-spectrum antimicrobial agents which can be classified according to their source organism, target organisms, or structural and chemical peculiarities owing to their amino acid profiles (Huan et al., 2020). *Ascaris* also expresses AMPs in response to bacterial challenge (Pillai et al., 2003, 2005). As is the case for *C. elegans*, previous studies have determined that viable bacteria can be cultured from the *Ascaris* intestine (Hsu et al., 1986; Shahkolahi and Donahue, 1993). Despite environmental differences and different evolutionary pressures, *C. elegans* can provide insights into defense mechanisms employed by *Ascaris*.

In contrast to our impressive understanding of nematode-microbe interactions in the case of *C. elegans*, our understanding of helminth-microbe interactions is relatively limited. Hence, in 2017 Dheilly and colleagues proposed the Parasite Microbiome Project (PMP) to bring together researchers to advance parasitology into the microbiome era (Dheilly et al., 2017). The PMP subsequently identified various grand challenges for the community, including the identification of parasite-associated microbiomes (Dheilly et al., 2019). In the case of *Ascaris*, studies in humans and pigs have documented host intestinal microbiome alterations during infection, though they usually sample sites such as the cecum, colon, or feces which are all distal to the site of infection in the jejunum (Williams et al., 2017; Wang et al., 2019; Kupritz et al., 2021; Springer et al., 2022). Additionally, we do not yet understand the mechanisms responsible for these interactions and our knowledge of antimicrobial mechanisms employed by *Ascaris* are rather limited. Finally, previous reports of *Ascaris*-associated microbes relied on culturing rather than modern sequencing-based approaches. While much work has been done on host-microbiota and host-parasite interactions, little is known concerning parasite-microbiota interactions, and even less about the trilateral interactions between parasites, host cells, and microbes. However, data from various experimental helminth infection systems provide glimpses of how such interactions might occur (Table 1).

1.5.2 Coinfections with other pathogens

Helminth endemic areas are also endemic for numerous other viral, bacterial, and protozoan pathogens (Schlosser-Brandenburg et al., 2023). *Ascaris* worms can impact host immunity to co- infecting pathogens during different stages of their life cycle. As larvae migrate through

host tissues, they can influence local immune responses in the intestine, liver, and lung while long- lived adult nematodes produce excreted and secreted (ES) products containing locally and systemically active immunomodulatory molecules (Schlosser-Brandenburg et al., 2023). Regarding intestinal pathogens *A. lumbricoides* appears to impair host immune responses to cholera toxin-B following oral vaccination with attenuated *Vibrio cholerae* while anthelmintic treatment of ascariasis patients can enhance vibriocidal antibody responses (Cooper et al., 2000, 2001). In pigs, there is an association between *Salmonella* seroprevalence and *Ascaris*-liver condemnation (van der Wolf et al., 2001). Data from mice indicate that helminth infections promote coinfection with *Salmonella* and *Citrobacter rodentium* (Weng et al., 2007; Collins et al., 2014; Reynolds et al., 2017; Knuhr et al., 2018; Schramm et al., 2018). Regarding liver pathogens, several studies have documented increased *Plasmodium* burdens in helminth-infected mice as well as altered antibody responses in co-infected patients, suggestive of hepatic or systemic immunomodulation (Schlosser-Brandenburg et al., 2023). The lung is also impacted by *Ascaris* migration and studies have found *Ascaris* as a risk factor for increased pneumococcal carriage density in Ecuadorian children (Law et al., 2021), while data from pigs demonstrates that *A. suum* infection negatively impacts protective immunity to *Mycoplasma hypopneumonaie* vaccination (Steenhard et al., 2009). Furthermore, mice co-infected with *A. suum* and *Pasteurella multocida* experienced more severe pneumonia and septicemia compared to *P. mulcocida* infection alone (Tjørnehøj et al., 1992). Thus, there is ample evidence that *Ascaris* can modulate antimicrobial immune responses leading to increased pathogen burdens in the various organs impacted by *Ascaris* infection.

Table 1 Demonstrated and predicted *Ascaris*-microbiota-host cell interactions

Interaction (experimental system)	Direction[a]	Outcomes
Ascaris-derived antimicrobial peptides and proteins (ASABFs, cecropins, lectins, lysozymes) (*Ascaris suum*)	A➔M A➔H	Microbial killing, microbial neutralization, immunomodulation
Ascaris-derived metabolites (e.g., SCFA, succinate) (*A. suum*)	A➔H, A➔M	Promotion of regulatory immune phenotype, altered microbiome and metabolic environment, influence bacterial motility, growth, and gene
Egg-hatching (*Trichuris muris, Trichuris suis*)	M➔A	Promotion or prevention of egg hatching and infection
Microbiota-derived anthelmintic activity/infection of nematode by microbe (*A. suum*)	M➔A	Hampered larval development, nematode killing
Microbiota-mediated defense of nematodes, promotion of nematode viability (*Caenorhabditis elegans, Heligmosomoides polygyrus*)	M➔A	Providing nutrients, protecting nematode against microbial infection
Bacterial translocation during nematode tissue migration (*A. suum*)	A➔M➔H	Increased risk of microbial infection; compromised anthelmintic immune response
Gut–lung axis (*H. polygyrus, T. suis*)	A/M➔H➔M	Nematode infection alters intestinal microbiome and metabolome, which modulates respiratory immune responses
Host immunomodulation by *Ascaris* (*A. suum*)	A➔H➔M	Compromised immune responses against microbes and nematode, altered microbiome and intestinal metabolome
Host immunomodulation by microbes (*H. polygyrus, Nippostrongylus brasiliensis*)	M➔H➔A	Compromised immune responses against *Ascaris*

a Interactions: **A**, *Ascaris*; **M**, microbiota; **H**, host cells; ➔ indicates sequence and directionality of interactions (e.g., M➔H➔A: microbiota impact host cells which then impact *Ascaris*).

Table adapted from Midha A et al. (2021) Trilateral Relationship: *Ascaris*, Microbiota, and Host Cells. Trends in Parasitology. 37(3):251-262. https://doi.org/10.1016/j.pt.2020.09.002.

1.6 Aims

Ascariasis is a neglected tropical disease contributing considerable morbidity to the hundreds of millions of people currently infected with it. Furthermore, ascariasis remains a costly challenge in pig farming. Efforts to control *Ascaris* infections have seen some success; mass drug administration campaigns are effective for reducing parasite burdens but eradication of the pathogen has not been possible and there are concerns about the emergence of anthelmintic resistance. Currently, prevention via vaccination is also not an option. Therefore, novel strategies to control helminth infections are needed and could be unveiled by understanding these pathogens within the context of their microbial environments. The pig serves as a human-relevant model for this purpose. The primary aims of this thesis are:

1. To determine if *Ascaris* nematodes release antimicrobial compounds in their excreted and secreted products.
2. To characterize the antimicrobial activities of these products.
3. To characterize the *Ascaris* microbiome.
4. To identify the primary determinants of Ascaris microbiome composition.

2. The Intestinal Roundworm Ascaris suum Releasezs Antimicrobial Factors Which Interfere With Bacterial Growth and Biofilm Formation

Ankur Midha, Katharina Janek, Agathe Niewienda, Petra Henklein, Sebastian Guenther, Diego O. Serra, Josephine Schlosser, Regine Hengge, Susanne Hartmann

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2.1 Author Contributions

Project designed by AM, SH, and JS. Microbiological experiments designed by AM, SG, RH, and DS. Mass spectrometry analysis performed by KJ and AN. Peptides for mass spectrometry analysis synthesized by PH. AM performed all experiments. All authors interpreted data. Manuscript was written by AM and SH with input from the other authors. All authors gave final approval for manuscript publication.

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The Intestinal Roundworm *Ascaris suum* Releases Antimicrobial Factors Which Interfere With Bacterial Growth and Biofilm Formation

*Ankur Midha[1], Katharina Janek[2], Agathe Niewienda[2], Petra Henklein[3], Sebastian Guenther[4,5], Diego O. Serra[6], Josephine Schlosser[1], Regine Hengge[6] and Susanne Hartmann[1]**

[1] Department of Veterinary Medicine, Institute of Immunology, Freie Universität Berlin, Berlin, Germany, [2] Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Institute of Biochemistry, Shared Facility for Mass Spectrometry, Berlin, Germany, [3] Charité – Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Institute of Biochemistry, Berlin, Germany, [4] Department of Veterinary Medicine, Institute of Animal Hygiene and Environmental Health, Freie Universität Berlin, Berlin, Germany, [5] Department of Pharmaceutical Biology, Institute of Pharmacy, Ernst-Moritz-Arndt-Universität Greifswald, Greifswald, Germany, [6] Institute of Biology/Microbiology, Humboldt-Universität-zu-Berlin, Berlin, Germany

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***Correspondence:**
*Susanne Hartmann
susanne.hartmann@fu-berlin.de*

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Ascariasis is a widespread soil-transmitted helminth infection caused by the intestinal roundworm *Ascaris lumbricoides* in humans, and the closely related *Ascaris suum* in pigs. Progress has been made in understanding interactions between helminths and host immune cells, but less is known concerning the interactions of parasitic nematodes and the host microbiota. As the host microbiota represents the direct environment for intestinal helminths and thus a considerable challenge, we studied nematode products, including excretory-secretory products (ESP) and body fluid (BF), of *A. suum* to determine their antimicrobial activities. Antimicrobial activities against gram-positive and gram-negative bacterial strains were assessed by the radial diffusion assay, while effects on biofilm formation were assessed using the crystal violet static biofilm and macrocolony assays. In addition, bacterial neutralizing activity was studied by an agglutination assay. ESP from different *A. suum* life stages (*in vitro*-hatched L3, lung-stage L3, L4, and adult) as well as BF from adult males were analyzed by mass spectrometry. Several proteins and peptides with known and predicted roles in nematode immune defense were detected in ESP and BF samples, including members of *A. suum* antibacterial factors (ASABF) and cecropin antimicrobial peptide families, glycosyl hydrolase enzymes such as lysozyme, as well as c-type lectin domain-containing proteins. Native, unconcentrated nematode products from intestine-dwelling L4-stage larvae and adults displayed broad-spectrum antibacterial activity. Additionally, adult *A. suum* ESP interfered with biofilm formation by *Escherichia coli*, and caused bacterial agglutination. These results indicate that *A. suum* uses a variety of factors with broad-spectrum antibacterial activity to affirm itself within its microbe-rich environment in the gut.

Keywords: intestinal nematode, ascariasis, helminth, microbiota, antimicrobial peptides, biofilm, lectin

INTRODUCTION

Soil-transmitted helminth infections infect approximately 1.5 billion people worldwide (World Health Organization, 2017) as well as most companion, livestock, and wild animals (Eijck and Borgsteede, 2005; Nganga et al., 2008). The most prevalent helminth infection in people, Ascariasis, is caused by the intestinal roundworm *Ascaris lumbricoides* which infects approximately 800 million people (Brooker and Pullan, 2013) while the closely related *Ascaris suum* is commonly found in pigs raised for pork consumption (Dold and Holland, 2011; Thamsborg et al., 2013; Kreinoecker et al., 2017). The porcine host serves as a valuable infection model for humans for many diseases (Meurens et al., 2012), but particularly for Ascariasis, given the similarities between the human and pig intestinal tract and microbiota in comparison to that of mice (Heinritz et al., 2013) as well as the life cycles, genetic, and proteomic similarities of both *Ascaris* species (Leles et al., 2012; Xu et al., 2013; Shao et al., 2014). Infection begins with the ingestion of embryonated eggs containing L3-stage larvae which hatch in the small intestine before penetrating the intestinal wall of the cecum and colon to start their tissue migratory phase (Murrell et al., 1997). These L3-stage larvae then migrate through the liver before reaching the lungs by 6–8 days post-infection (Roepstorff et al., 1997). From the lungs, the larvae are coughed up and swallowed again, thereby reaching the small intestine where the nematodes will further develop into the L4 and adult stages and remain for approximately 1 year (Dold and Holland, 2011).

The small intestine hosts a microbiota, albeit at a lower density of microbes than that of the colon (Zoetendal et al., 2008; Isaacson and Kim, 2012; Sender et al., 2016). *A. suum* larvae invade host tissues in the distal small intestine, cecum, and proximal colon while adult worms reside in the small intestine; therefore, *A. suum* inhabits a microbial environment. Many studies have explored interactions between intestinal parasites and their hosts (Varyani et al., 2017), as well as hosts and their intestinal microbiota (Hooper et al., 2012); however, our understanding of how intestinal nematodes interact with the host microbiota is very limited. Recently, studies have linked various helminth infections to alterations in the host-intestinal microbiota (Zaiss and Harris, 2016). While host-immune factors and local metabolic factors have been implicated in shaping the microbiota, helminth components involved in the interaction with the microbial environment remain unexplored.

Studies in the free-living model nematode *Caenorhabditis elegans* suggest that these worms acquire an intestinal microbiota, distinct from their environments (Berg et al., 2016; Dirksen et al., 2016; Zhang et al., 2017). Though derived from environmental sources, the composition of the *C. elegans* microbiota was found to be selectively enriched and conserved across diverse sampling origins (Zhang et al., 2017). Additionally, certain microbes have been shown to support nematode growth and proliferation, while others pose infectious threats (Félix and Duveau, 2012; Samuel et al., 2016). Many laboratory-based studies have established *C. elegans* infection model systems with various bacterial pathogens (Couillault and Ewbank, 2002). Furthermore, other studies have also shown differential effects of biofilm-associated bacteria on *C. elegans* physiology (Tan and Darby, 2004; Begun et al., 2007; Smolentseva et al., 2017), demonstrating the diversity and importance of nematode-microbe interactions. Using these models, numerous details of the *C. elegans* antimicrobial defense response have identified detection mechanisms, transcription factors, and inducible effector molecules that form the nematode's innate immune system (Kim and Ewbank, 2015). In contrast in parasitic nematodes not much is known. Previous studies in *A. suum* have described induced transcription of members of two families of antimicrobial peptides (AMPs), *A. suum* antibacterial factors (ASABFs) and cecropins, in response to injection with heat-killed *Escherichia coli* (Pillai et al., 2003, 2005). In these studies, transcripts of some AMPs were also detected in the absence of an overt infectious challenge, suggesting that some defense molecules are produced constitutively. Homologs of ASABFs, called antibacterial factors, have also been described in *C. elegans* (Kato et al., 2002), as well as several other proteins and peptides involved in defense (Tarr, 2012).

Given the importance of interactions with bacteria for *C. elegans* physiology and longevity, as well as the absence of severe systemic inflammation of the host during Ascariasis despite migration of larvae originating in the intestine, we hypothesized a direct interaction of components of the intestinal parasitic nematode *A. suum* with the host gut-microbiota. Understanding the strategies that parasitic nematodes have evolved to control their microbial environments can provide insights into how the microbiota may be intentionally modified for therapeutic purposes, especially since nematodes do this without apparent detriment to their hosts. Herein we aimed to determine if *A. suum* nematodes release antimicrobial proteins and peptides in their excreted and secreted products (ESP) and whether or not these nematode products possess detectable antimicrobial activities.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were conducted in accordance with the principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and ethical approval was obtained from the Landesamt für Gesundheit und Soziales Berlin, Germany (approval numbers H0288/15 and H0005/18).

Parasite Material

Adult *A. suum* worms were obtained from infected pigs at a local slaughterhouse. Upon retrieval, worms were separated by sex and washed several times in a balanced salt solution (BSS), recipe modified from Locke's solution (Chehayeb et al., 2014), containing antibiotics and used as culture media for adult worms (127 mM NaCl, 7.5 mM $NaHCO_3$, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 200 U/mL penicillin, 200 μg/mL streptomycin, 50 μg/mL gentamicin, 2.5 μg/mL amphotericin B), then kept at 37°C with 5% CO_2. Three to five adult worms were kept together in 300 mL of BSS. Media changes were completed daily by transferring worms to fresh bottles containing fresh BSS. To generate ESP for use in our experiments, worms were cultured in antibiotic-free

BSS for several days with daily media changes. Spent media from the first 48 h were not used in microbiological assays. ESP were sterile filtered through a 0.22 μM vacuum-driven filter system and stored at −20°C until further use. For body fluid collection, adult worms were cultured in the absence of antibiotics as described for antibiotic-free ESP collection and body fluid was collected using the method of Chehayeb et al. (2014), sterile filtered using a 0.22 μM syringe-driven filter system and stored at −20°C until further use.

Third stage larvae were generated as previously described (Urban et al., 1981). Unembryonated *A. suum* eggs were collected from cultures of adult female worms, washed multiple times in water and placed in 0.1 N H_2SO_4 for 6–8 weeks at room temperature. Embryonation rates were assessed visually by light microscopy. Embryonated eggs were hatched using 5.25% hypochlorite treatment and incubation with slowly moving glass beads. Hatched third-stage larvae (L3) were cultured at a density of approximately 30,000 larvae/well of a 12-well tissue culture plate, in 1 mL of larval media [RPMI-1640 media (PAN Biotech, Aidenbach, Germany), 50 mM glucose, 200 U/mL penicillin, 200 μg/mL streptomycin, 50 μg/mL gentamicin, 2.5 μg/mL amphotericin B]. After 2 days in culture, worms were washed extensively with antibiotic-free media and then maintained in antibiotic-free larval media with media changes every 24 h for the first 2 days. Spent media from the first 48 h were discarded. Thereafter, supernatants were harvested every 48 h for 10–14 days, sterile filtered through a 0.22 μM syringe-driven filter system, and stored at −20°C until further use.

For tissue migrating larval stages, German Landrace piglets aged 8 weeks were orally infected with 12,000–15,000 embryonated *A. suum* eggs/pig. Pigs were sacrificed at 8 days post-infection for lung-stage larvae, and 16 days post-infection for L4-stage larvae. Lung-stage L3 larvae were retrieved as previously described with minor modifications (Slotved et al., 1997; Saeed et al., 2001). Briefly, harvested organs were ground using a hand-operated meat grinder. Ground organs were mixed with 0.9% NaCl to 300 mL and subsequently mixed with 300 mL of 2% agar solution which had been autoclaved and held at 45°C until use. The tissue-agar mixture was then poured into large glass petri dishes lined with plastic wrap and allowed to solidify, forming tissue gels. Tissue gels were wrapped in 200 μm woven synthetic mesh (Sefar, Edling, Germany), transferred to beakers with 0.9% NaCl, and incubated at 37°C for 3 h to allow worms to migrate into the saline solution. After 3 h, gels were removed and the remaining suspension transferred to Baermann funnels and allowed to sediment for 0.5–1 h. Worms were then collected and washed several times with larval media. Worms were cultured at 37°C with 5% CO_2 with media changes every 24 h. Unfortunately, we were unable to retrieve antibiotic-free lung-stage larvae, so this material was excluded from microbiological assays.

For L4-stage larvae, pigs were sacrificed at 16 days post-infection and the distal small intestine and proximal cecum were removed. Intestinal contents were incubated in pre-warmed NaCl at 37°C for 3 h to allow larval migration away from host tissue and ingesta. This mixture was then poured over a Baermann funnel and allowed to sediment, then collected and washed extensively, and the worms cultured as described for L3-stage larvae, except with approximately 100 larvae per well of a 12-well tissue culture plate in 1 mL of larval media.

For use in the agglutination assay, adult *A. suum* ESP were concentrated using Vivaspin centrifugal concentrators with a 5 kDa molecular weight cut off (Sartorius, Göttingen, Germany) to a final protein concentration of 1 mg/mL. For LC-MS/MS analysis, ESP and BF samples were prepared as previously described (Eberle et al., 2015), with modifications. Oasis HLB Plus cartridges (Waters 186000132, Milford, USA) were rinsed with 2 mL of pure methanol, equilibrated with 3 mL of 0.2% formic acid, and loaded with either 5 mL of *A. suum* ESP or 3 mL of BF. Samples were washed with 5 mL of 0.2% formic acid then eluted with 1 mL of 30% acetonitrile/0.2% formic acid, then 1 mL of 60% acetonitrile/0.2% formic acid, and finally with 1 mL of 80% acetonitrile/0.2% formic acid. Eluates were pooled and dried in a centrifugal evaporator.

Bacterial Strains

The strains used to evaluate antibacterial activities of *A. suum* products in the radial diffusion assay included: *Escherichia coli* IMT19224, *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) ATCC 14028, and *Staphylococcus aureus* IMT29828, all obtained from the strain collection of the Institute of Microbiology and Epizoonotics, Freie Universität Berlin. The strains used to assess the effects of *A. suum* ESP on biofilm formation included the biofilm forming *E. coli* K-12 strains AR3110 and AR115. *E. coli* IMT19224, AR3110, and AR115 were used to assess agglutinating activity of *A. suum* ESP. Strains were selected to include representative gram-negative and gram-positive bacterial strains which may model *A. suum*-microbe interactions or to elucidate anti-biofilm activities of *A. suum* ESP. *E. coli* IMT19224 is a sequence type 131 (ST131) strain; ST131 isolates are commonly multidrug resistant, producing extended-spectrum β-lactamases and resistant to fluoroquinolones (Nicolas-Chanoine et al., 2014). *E. coli* AR3110, derived from *E. coli* K-12 strain W3110 by correcting a single nucleotide polymorphism in the *bcs* operon, produces phosphoethanolamine-modified cellulose and amyloid curli fibers as predominant extracellular matrix components in macrocolony biofilms (Serra et al., 2013; Thongsomboon et al., 2018). *E. coli* AR115 was derived from AR3110 by deleting *wcaE*, a gene involved in colanic acid synthesis (Miajlovic et al., 2014).

Radial Diffusion Assay

Antibacterial activities of ESP were assessed using the radial diffusion assay (Takemura et al., 1996). Overnight cultures were diluted 1:100 in Mueller-Hinton Broth (Carl Roth, Karlsruhe, Germany) and incubated at 37°C with shaking at 250 rpm until reaching an optical density of 0.3–0.4 at 600 nm. The bacteria were centrifuged at 880 x *g* for 10 min at 4°C, washed once with cold sodium phosphate buffer (100 mM, pH 7.4), and resuspended in cold sodium phosphate buffer. Bacteria were suspended in previously autoclaved, warm (50°C) underlay agar (10 mM sodium phosphate buffer, 1% (v/v) Mueller-Hinton broth, 1.5% (w/v) agar), at 4×10^5 colony forming units per mL. 15 mL of underlay agar was poured into 120 mm square petri dishes and allowed to solidify. Using the blunt ends of P10 pipet tips, evenly spaced 5 mm wells were punched into the agar

into which 5 μL of treatments and controls were added. Adult and larval growth media were included as negative controls. The *A. suum* AMP Cecropin P1 (Sigma-Aldrich, St. Louis, USA) was also included in the analysis. Plates were then incubated at 37°C for 3 h and then overlaid with 15 mL of overlay agar (4.2% (w/v) Mueller-Hinton broth, 1.5% (w/v) agar). The plates were incubated for 18 h at 37°C and the growth inhibition zones around each of the wells were measured. Antibacterial activity is herein represented as the diameter of the inhibition zone (mm) beyond the well.

Crystal Violet Assay

The influence of *Ascaris* ESP on biofilm formation was assessed using the microtiter dish biofilm formation assay (O'Toole, 2011). The biofilm forming *E. coli* K-12 strains AR3110 and AR115 were grown overnight in liquid salt-free Luria-Bertani (LB) medium at 37°C. The overnight culture was diluted in 2X LB medium (9×10^8 colony forming units per mL) for use in the biofilm assay. Hundred microliter of this bacterial suspension was used per well of a 96-well tissue-culture plate (Corning, New York, NY, USA) in replicates of four. The final volume per well was 200 μL with the remaining volume made up of controls and treatments at the concentrations indicated in the text. The plates were incubated for 24 h at 37°C. After incubation, cell suspensions were removed and the wells washed twice with phosphate buffered saline (pH 7.2) and stained for 15 min at room temperature with 0.1% (w/v) crystal violet solution (Sigma-Aldrich). The wells were then washed twice with distilled water and air-dried. For quantification, 125 μL of 30% acetic acid were added to each well and the plate incubated at room temperature for 15 min. The solubilized stain was transferred to a fresh flat-bottom 96-well plate and the absorbance read at 550 nm. Statistical analyses were performed using GraphPad Prism 7.0a to conduct 2-way ANOVA followed by Tukey's multiple comparison tests. *P*-values less than 0.05 were considered significant.

Macrocolony Biofilm Assay

The influence of *Ascaris* ESP on the morphology of biofilms was assessed using the macrocolony biofilm model (Serra and Hengge, 2017). Experiments were carried out using the same strains as for the crystal violet biofilm formation assay. Cells were grown overnight in salt-free LB medium at 37°C. 5 μL of the overnight culture was spotted on salt-free LB agar plates containing Congo red 40 μg/mL and Coomassie brilliant blue 20 μg/mL. 35 mm petri dishes (Sarstedt, Nümbrecht, Germany) were used to grow one colony per plate. After autoclaving and cooling to 42°C, agar was prepared with controls and treatments at the indicated final concentrations. Colonies were incubated at 28°C for up to 5 days. Macrocolonies were visualized at 10X magnification with a Stemi 2000-C stereomicroscope (Zeiss, Oberkochen, Germany) and photographed with an AxioCamICC3 digital camera (Zeiss).

Agglutination Assay

Agglutination activity of nematode products was assessed as previously described (Gasmi et al., 2017), using *E. coli* IMT19224. Bacteria were collected at mid-logarithmic phase by centrifugation at 880 × g for 5 min, washed then resuspended in BSS at approximately 10^9 cells/mL. 20 μL of bacteria were mixed with 20 μL of treatments in the presence and absence of 10 mM $CaCl_2$ and incubated for 1 h at room temperature on a glass slide. Concanavalin A from *Canavalia ensiformis* (Con A) and Lectin from *Triticum vulgaris* (Wheat germ agglutinin; WGA, both from Sigma-Aldrich) were included as positive controls. Samples were then visualized and photographed at 40X magnification on a Leica DM750 microscope equipped with an ICC50HD digital camera (Leica Microsystems, Wetzlar, Germany).

In-solution Tryptic Digestion and LC-MS/MS Analysis

Dried protein samples were resuspended in 50 μL of 50 mM ammonium bicarbonate in 5:95 (v/v) acetonitrile/water (digestion buffer) and reduced with 8 μL of 45 mM dithiothreitol in digestion buffer at 60°C for 30 min. After cooling to room temperature 8 μL iodoacetamide solution (100 mM in digestion buffer) were applied and the sample was kept in the dark for 30 min. Subsequently the samples were diluted with 190 μL digestion buffer and digested with 0.15 μg trypsin at 37°C for 4 h. The reaction was stopped with 2.5 μL of 10% (v/v) trifluoroacetic acid in water. The samples were concentrated to approximately 50 μL and desalted with μC18-ZipTips (Millipore, Darmstadt, Germany), dried and reconstituted in 0.1% (v/v) trifluoroacetic acid in 2:98 (v/v) acetonitrile/water. LC-MS/MS analyses of peptides were performed on an Ultimate 3000 RSLCnano system online coupled to an Orbitrap Q Excative Plus mass spectrometer (Thermo Fisher Scientific). The system comprised a 75 μm i.d. × 250 mm nano LC column (Acclaim PepMap C18, 2 μm; 100 Å; Thermo Fisher Scientific). Mobile phase (A) was 0.1% formic acid in 2:98 (v/v) acetonitrile/water and (B) 0.1% formic acid in 80:20 (v/v) acetonitrile/water. The gradient was 3–40% B in 85 min. Full MS spectra (350–1,600 m/z) have been acquired at a resolution of 70.000 (FWHM) followed by a data-dependent MS/MS fragmentation of the top 10 precursor ions (resolution 17.500; 1+ charge state excluded, isolation window of 1.6 m/z, normalized collision energy of 27%). The maximum ion injection time for MS scans has been set to 50 ms and for MS/MS scans to 80 ms.

Database Searching and Sequence Analysis

Protein identifications were performed with Mascot software version 2.6.1 (Matrix Science Ltd., London, UK). Data were searched against an *A. suum* protein database from nematode.net (http://nematode.net/NN3_frontpage.cgi?navbar_selection=speciestable&subnav_selection=Ascaris_suum), 17,843 sequences, 2017_05), *A. suum* proteins from Uniprot (9,213 sequences, 2017_05), the antimicrobial peptide database (http://aps.unmc.edu/AP/main.php, 2,338 sequences; 2017_05), SwissProt (555,100 sequences, 2017_07) and a contaminant database (247 sequences). The following parameters were set: enzyme: trypsin/P with one missed cleavage, static modification: carbamidomethylation (C), variable modifications: oxidation (M) and pyro-glu (Q), mass tolerances for MS and MSMS: 5 ppm and 0.02 Da. Proteins were accepted as identified if at

least two unique peptides with $p < 0.01$ were detected. Proteins identified only by one peptide were verified by comparison of their peptide fragment pattern with those of synthetic analogs. These reference peptides were synthesized in-house using Fmoc solid phase chemistry as previously described (Venken et al., 2011). In case of the common peptide (ISEGIAIAIQGGPR) of cecropin P1 and P2 an identification threshold of $p < 0.00001$ was set. Protein sequences were analyzed for the presence of classically secreted proteins containing signal peptides using SignalP 4.1 (Petersen et al., 2011) and for non-classically secreted proteins using SecretomeP 2.0 (Bendtsen et al., 2004).

RESULTS

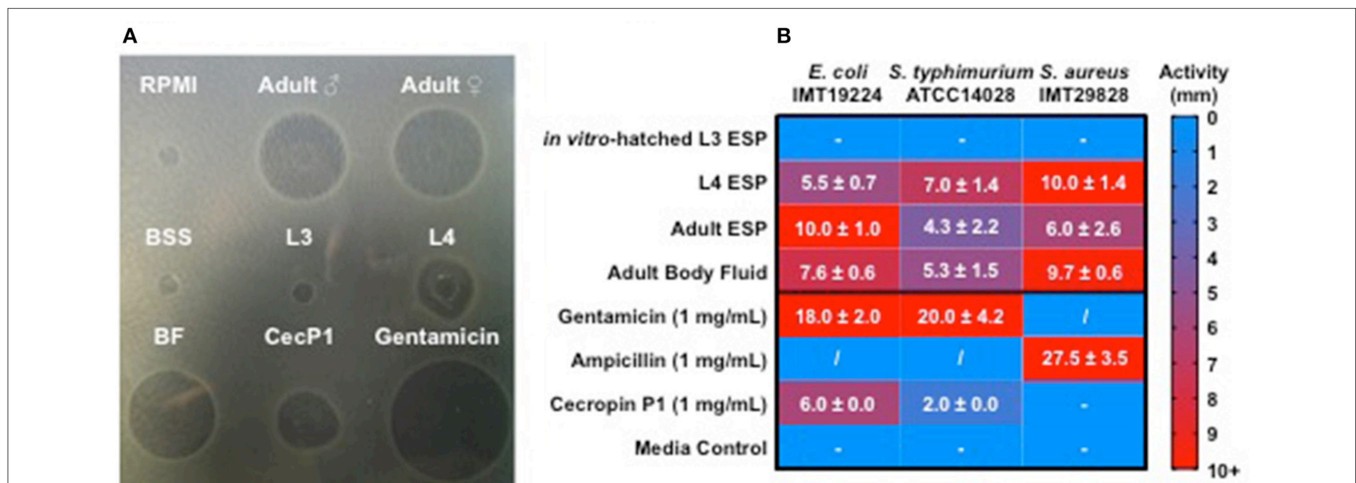
Ascaris suum ESP Possess Antibacterial Activity

As intestinal parasitic nematodes inhabit a microbe rich environment, they are likely to experience microbial challenges while dwelling in the intestine. These challenges would need to be managed in order for the parasite to establish itself and survive during a long-term infection. We used the radial diffusion assay to test the antibacterial activity of native, unconcentrated secreted products (ESP) of different *A. suum* life stages and body fluid (BF) of adult male worms. The activities of nematode products against *E. coli* ST131 IMT19224, *S. aureus* IMT29828, and *S. typhimurium* ATCC 14028 were assessed. Adult ESP were obtained from 3 to 5 adult worms kept in 300 mL of culture medium (BSS), L3-stage material was harvested from the pooled supernatants of 30,000 larvae/well of a 12-well plate in 1 mL of larval media, while L4-stage material was harvested from the pooled supernatants of 100 larvae/well of a 12-well plate. BF was pooled from 5 adult males per batch. Treatments of ESP and BF were applied to proliferating bacteria and the resulting growth inhibition zones measured in comparison to BSS and larval culture media as controls (**Figure 1**). *Ascaris* ESP from *in vitro*-hatched L3-stage larvae resulted in no observable antibacterial activity. In contrast, ESP harvested from L4-stage larvae were very active, resulting in growth inhibition zones comparable to synthetic cecropin P1 against *E. coli*, and considerably more active than cecropin P1 against *S. typhimurium*. Interestingly, cecropin P1 had no detectable activity against *S. aureus.* Adult ESP were active against all strains tested and no considerable difference was detected between male and female ESP, thus they were considered together as "Adult ESP." BF from adult males demonstrated activity comparable to that of L4-stage larval ESP. Thus, these results show that native parasite material harvested directly from *A. suum,* including ESP and BF, possess considerable antibacterial activity. ESP from the intestinal L4 and adult life stages were most active, whereas ESP from *in vitro*-hatched L3 larvae did not show antibacterial activity.

Ascaris suum ESP Impair Bacterial Biofilm Formation

Many species of bacteria live in communities known as biofilms in which cells are embedded in an extracellular matrix of self-produced polymers. In addition to representing the preferred lifestyle in nature for many bacteria, biofilms are often of medical relevance for infectious diseases (Hall-Stoodley et al., 2004; Flemming et al., 2016). In the case of free-living *C. elegans* nematodes, biofilms have been shown to be harmful, contributing to the pathogenicity of *Staphylococcus epidermidis* against the worm (Begun et al., 2007), whereas biofilm formation by *Bacillus subtilis* enhances nematode stress resistance (Smolentseva et al., 2017). Therefore, as biofilms may also be of importance to intestinal nematodes, we evaluated the effects of *A. suum* ESP on biofilm formation using the submerged biofilm model (O'Toole, 2011) and the macrocolony biofilm model (Serra et al., 2013). We used the biofilm-forming *E. coli* K12 strain AR3110, which is a W3110 derivative with restored capacity to produce phosphoethanolamine-modified cellulose (pEtN-cellulose). AR3110 produce pEtN-cellulose along with amyloid curli fibers as key components of the extracellular matrix in biofilms (Serra et al., 2013; Thongsomboon et al., 2018). PEtN-cellulose production has been restored by repairing a single nucleotide polymorphism that resulted in a stop codon in the *bcs* operon (Serra et al., 2013). As adult worms can survive for approximately 1 year in the intestine, growing between 15 and 25 cm in length (Dold and Holland, 2011), they may present surfaces on which biofilms can form in the small intestine. Hence, we used adult material to study the impact of *A. suum* ESP on biofilm formation. In the submerged biofilm assay, bacterial suspensions were mixed with *A. suum* ESP in a volume-dependent manner as indicated and inoculated into the wells of flat-bottom 96-well tissue culture plates and grown for 18 h at 37°C. The same concentrations of adult culture media, BSS, were used as media controls. Biofilm formation was assessed by crystal violet staining of the biomass that had formed on the submerged wall and bottom of the wells, thereby staining bacterial cells as well as extracellular matrix components. *A. suum* adult ESP demonstrate a dose-dependent inhibition of bacterial biofilm formation for both strains tested, in comparison to control (**Figure 2**).

In the macrocolony biofilm assay, a dose-dependent disruption of colony growth was observed (**Figure 3**). With the AR3110 strain, the overall size of the resultant colony was decreased in the presence of *A. suum* adult ESP. Importantly, with 25% of ESP, *E. coli* responded to the treatment by producing large amounts of a white viscous substance (white shiny colony sectors; **Figure 3**). Since this substance was not formed in the *wcaE* mutant AR155, it can be ascribed to colanic acid, a mucoid exopolysaccharide that is typically produced in response to cell envelope stress and can confer resistance to antimicrobial insults and desiccation (Detweiler et al., 2003; Laubacher and Ades, 2008). This indicates that at least some of the ESP constituents act on the *E. coli* cell envelope, causing stress. However, also for the colanic acid-free mutant AR155, growth was not completely abolished suggesting that bacteria still resist the treatment by alternative mechanisms other than the production of colanic acid. Thus, these results show that the bacteria are able to adapt and survive to *A. suum* adult ESP, albeit while displaying signs of considerable stress. Notably, treatment with *A. suum* adult ESP did not interfere with curli and pEtN-cellulose production, since colonies of reduced size were still wrinkled as is particularly



	E. coli IMT19224	S. typhimurium ATCC14028	S. aureus IMT29828
in vitro-hatched L3 ESP	-	-	-
L4 ESP	5.5 ± 0.7	7.0 ± 1.4	10.0 ± 1.4
Adult ESP	10.0 ± 1.0	4.3 ± 2.2	6.0 ± 2.6
Adult Body Fluid	7.6 ± 0.6	5.3 ± 1.5	9.7 ± 0.6
Gentamicin (1 mg/mL)	18.0 ± 2.0	20.0 ± 4.2	/
Ampicillin (1 mg/mL)	/	/	27.5 ± 3.5
Cecropin P1 (1 mg/mL)	6.0 ± 0.0	2.0 ± 0.0	-
Media Control	-	-	-

FIGURE 1 | *Ascaris suum* excretory/secretory products and body fluid possess antimicrobial activity. Five microliter of nematode products were applied to agar plates with proliferating bacteria for 18 h at 37°C and growth inhibition zones measured in millimeters. *Ascaris* products tested include native excreted and secreted products (ESP) from adult worms kept in culture for 24 h, body fluid (BF) from adult males, native ESP from approximately 30,000 L3-stage larvae hatched *in vitro*/mL media, native ESP from approximately 100 L4-stage larvae/mL media, and a synthetic form of the *A. suum* antimicrobial peptide, cecropin P1. Larval (RPMI) and adult worm media (BSS) were included as controls. **(A)** Representative agar plate of a radial diffusion assay, with nematode products tested against *E. coli*. **(B)** Activity shown as diameter (mm) of inhibition zones on agar plates. Results are expressed as means ± standard deviations obtained from 2 to 3 independent experiments with multiple batches of *A. suum* products (L3 $n = 3$, L4 $n = 2$, adult ESP and body fluid n = 3). "–" represents no detected activity. "/" = not tested.

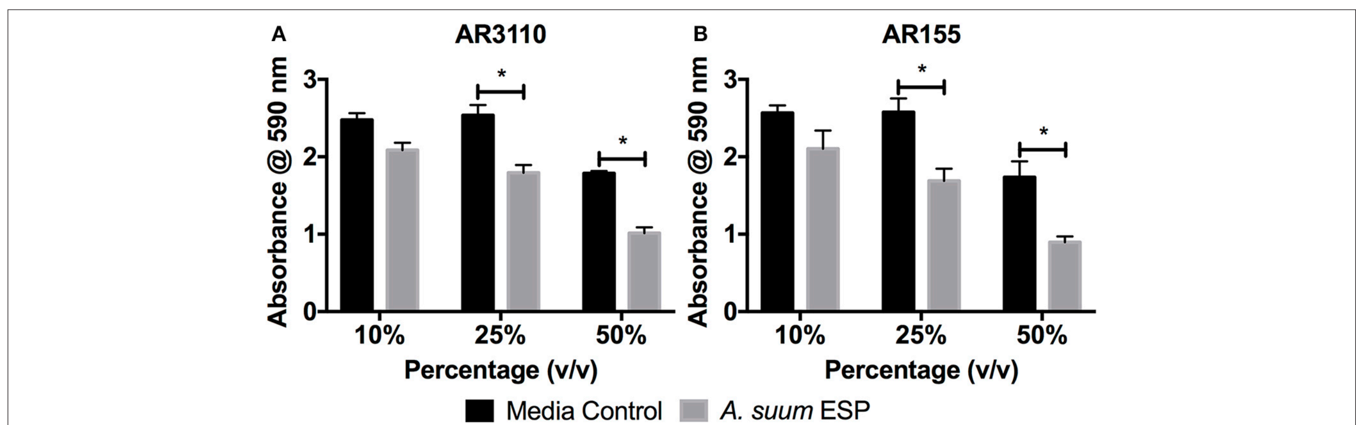


FIGURE 2 | *Ascaris suum* excretory/secretory products decrease biomass of submerged biofilms. Biofilm forming *E. coli* K-12 strains **(A)** AR3110 and **(B)** AR115 (a *wcaE* derivative of AR3110) were grown in 96-well cell culture plates in salt-free LB medium for 18 h at 37°C in the presence of adult *A. suum* excreted/secreted products (ESP) or adult worm media (BSS) as a control. Treatment doses were added as a percentage (v/v) of final culture volume (total = 200 µL per well). Results represented as the mean of three independent experiments ± SEM. Significance determined by 2-way ANOVA with Tukey's multiple comparison tests, $^*p < 0.05$.

visible in the absence of the large amount of viscous colanic acid with strain AR155 (**Figure 3**).

Ascaris suum ESP Possess Agglutinating Activity

Having demonstrated growth-inhibiting and biofilm-disrupting capabilities of *A. suum* ESP, we sought to determine if the nematodes could defend themselves against microbial threats without overtly killing bacteria. In addition to the inhibition of bacterial growth in the radial diffusion and macrocolony assays, we observed that also in our submerged biofilm assays some bacteria were still able to survive the treatment and reasoned that there may be non-lethal defense mechanisms employed by the worms such as neutralization via agglutination. In order to test the agglutinating activity of *A. suum* ESP, we treated *E. coli* ST131 IMT19224 with adult *A. suum* ESP (1 mg/mL) in the presence and absence of $CaCl_2$ (10 mM) and observed calcium-dependent agglutinating activity (**Figure 4**). The calcium-dependence implies the activity of C-type lectin domain-containing (CTLD) proteins which require calcium in order to exert their agglutinating and glycan-binding activities (Mayer et al., 2017). Similar results were obtained for the biofilm-forming *E. coli* K12 AR3110 and AR115 strains (**Supplementary Figures 1**, **2**).Thus, in addition to inhibiting

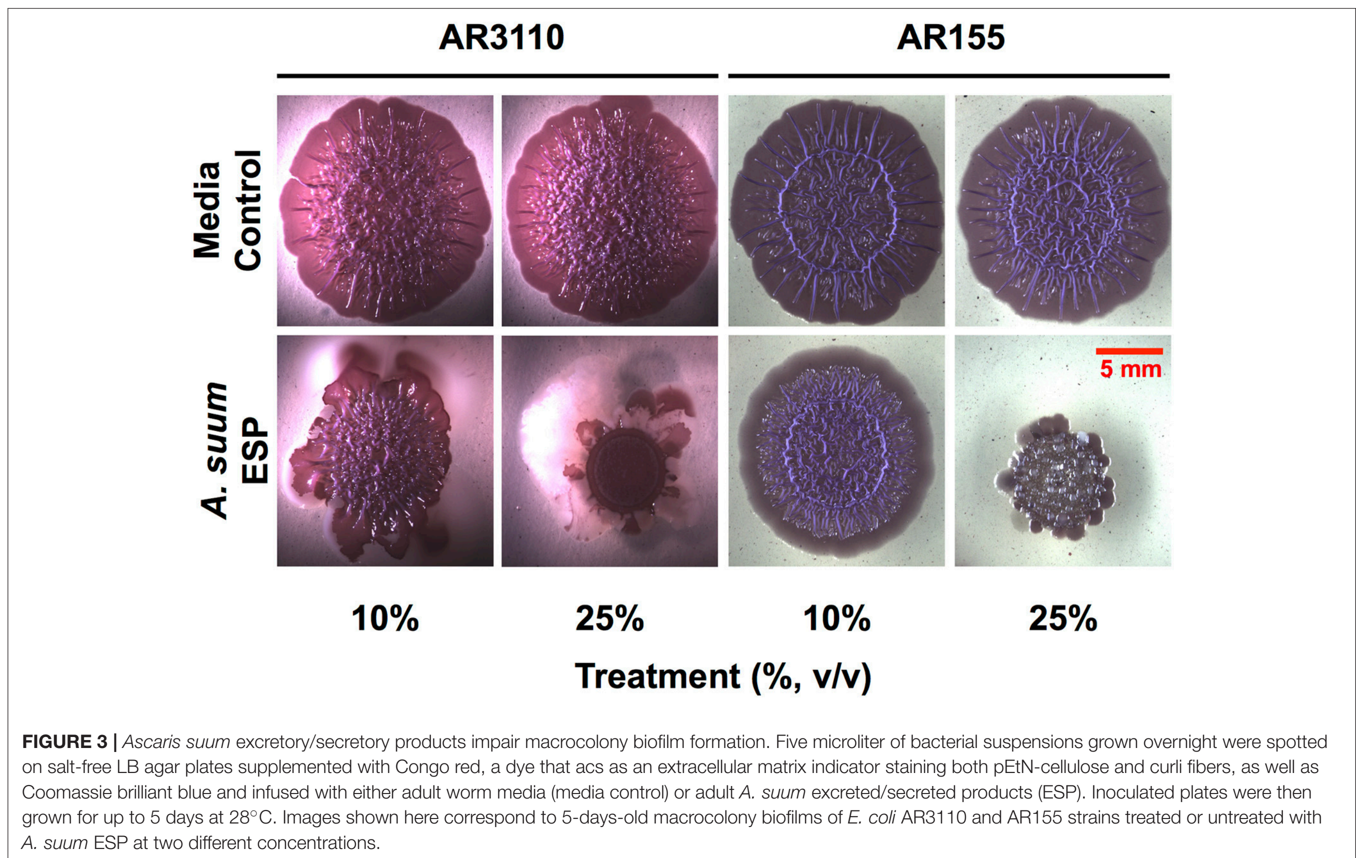


FIGURE 3 | *Ascaris suum* excretory/secretory products impair macrocolony biofilm formation. Five microliter of bacterial suspensions grown overnight were spotted on salt-free LB agar plates supplemented with Congo red, a dye that acs as an extracellular matrix indicator staining both pEtN-cellulose and curli fibers, as well as Coomassie brilliant blue and infused with either adult worm media (media control) or adult *A. suum* excreted/secreted products (ESP). Inoculated plates were then grown for up to 5 days at 28°C. Images shown here correspond to 5-days-old macrocolony biofilms of *E. coli* AR3110 and AR155 strains treated or untreated with *A. suum* ESP at two different concentrations.

bacterial growth and disrupting bacterial biofilm formation, *A. suum* adult ESP are also capable of neutralizing infectious threats by agglutinating bacteria.

Ascaris suum ESP and Body Fluid Contain Proteins and Peptides With Known and Predicted Antimicrobial Activities

In order to characterize ESP and body fluid of *A. suum* with respect to defense strategies that the nematode may employ in its microbial environment, we used native nematode material and omitted ultrafiltration-based concentration and trichloroacetic acid-mediated precipitation steps during our sample preparation which would have removed key antimicrobial components such as antimicrobial peptides from the final sample. By LC-MS/MS analysis, we assessed the protein and peptide constituents of ESP from different larval stages, including *in vitro*-hatched L3, lung-stage L3, intestinal-stage L4, and from adults, as well as BF obtained from adult males. The analysis revealed the presence of several proteins and peptides with known and predicted roles in nematode defense (**Table 1**), including galectins, C-type lectin domain-containing (CTLD) proteins, AMPs, a lysozyme (GH family 25 lysozyme 2), and a cysteine protease inhibitor (cystatin). Adult male and female ESP did not seem to differ in antimicrobial contents and are therefore shown together. Interestingly, we detected all of the aforementioned antimicrobial proteins and peptides in the ESP of adult nematodes, whereas we detected none of the proteins of interest in the ESP of lung-stage L3 larvae. ASABF-alpha, -beta, and –epsilon were detected only in adult ESP. In contrast, members of the cecropin family were detected in adult ESP and body fluid as well as in L4-larval ESP as well as cecropin P1 or P2 *in vitro*-hatched L3 ESP. While a significant and distinct peptide was detected and attributed to cecropin P1 in adult, L4, and *in vitro*-hatched L3 ESP as well as in BF, the same peptide could be attributed to cecropin P2; therefore, it is unclear if only cecropin P2 was detected or both cecropin P1 and P2. Cecropin P3 was detected in adult ESP and BF, as well as L4-stage ESP while cecropin P4 was detected only in adult ESP and BF, but not in larval material. The aforementioned lysozyme was detected only in adult ESP. Lectins, including CTLD proteins and galectins, were detected only in adult ESP but not in adult body fluid or in larval material. We detected seven unique CTLD proteins (including the three uncharacterized proteins, all of which contain CTLDs), though two of the seven did not contain signal peptides. Similarly, both galectins detected also did not contain signal peptides and were not predicted to be non-classically secreted using SecretomeP. Cystatin was detected in adult and *in vitro*-hatched L3 ESP. Cystatins from chickens and humans possess antibacterial activity (Blankenvoorde et al., 1998; Wesierska et al., 2005; Ganeshnarayan et al., 2012) whereas helminth cystatins, including from *Ascaris*, have a well-established role in modulating host immunity (Hartmann and Lucius, 2003; Mei et al., 2014; Coronado et al., 2017). Whether nematodes use cystatins to modulate the gut microbiota in

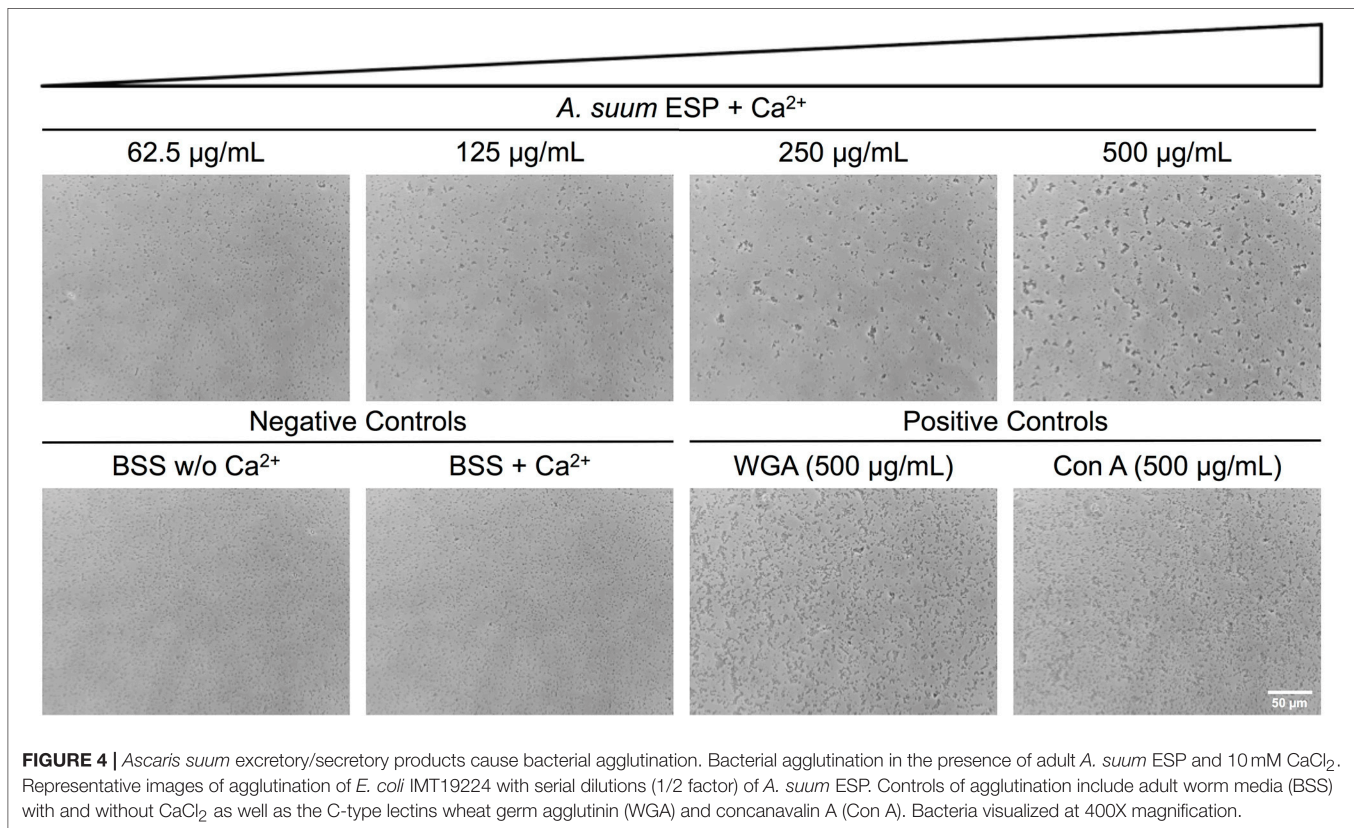


FIGURE 4 | *Ascaris suum* excretory/secretory products cause bacterial agglutination. Bacterial agglutination in the presence of adult *A. suum* ESP and 10 mM $CaCl_2$. Representative images of agglutination of *E. coli* IMT19224 with serial dilutions (1/2 factor) of *A. suum* ESP. Controls of agglutination include adult worm media (BSS) with and without $CaCl_2$ as well as the C-type lectins wheat germ agglutinin (WGA) and concanavalin A (Con A). Bacteria visualized at 400X magnification.

addition to host immune cells requires further study. The results of the LC-MS/MS analysis demonstrate that *A. suum* secretes diverse antimicrobial proteins and peptides which explain the various antibacterial activities we have observed. Furthermore, these factors likely act together to shape the nematode's microbial environment within the intestine of its host.

DISCUSSION

Intestinal parasites inhabit a microbe-rich environment. Diverse interactions between environmental microbes and free-living nematodes have been described, and similarly, the microbes in the host-gut may present benefits and risks for parasitic nematodes as they establish themselves in the niche of the intestine and migrate through the host tissue without eliciting overt inflammation. How *A. suum* survives in the small intestine of its porcine host has thus far been studied with a focus on host-pathogen interactions, whereas the interactions between *Ascaris* and the host-gut microbiota remain largely unexplored. Secreted products of helminths play various roles during the establishment of nematode infections, including invasion, migration, immune avoidance and immune modulation (Coakley et al., 2016). Hence, examining the role of secreted nematode products in nematode-microbe interactions is necessary to gain insights into the intricate trilateral interplay between the parasite, the host and the intestinal microbes during *A. suum* infection.

In this study, we demonstrated that *A. suum* ESP from different life stages possess antimicrobial activity against gram-negative and gram-positive bacteria (**Figure 1**). Interestingly, detectable antibacterial activity was limited to samples obtained from intestine-dwelling life stages, namely ESP from fourth larval-stage and adult worms, as well as body fluid from adult worms. Several proteins and peptides with known and predicted roles in antimicrobial defense were detected in these *A. suum* ESPs. In the nematode secreted products and BF samples, we detected members of the ASABF and cecropin AMP families (**Table 1**), previously shown to possess broad-spectrum antimicrobial activity (Pillai et al., 2003, 2005), accounting for observed antibacterial activities. Adult ESP also contained the highest diversity of potential antimicrobial components, including lectins, cystatin, and a lysozyme, GH family 25 lysozyme 2. To our knowledge, antibacterial activities of *Ascaris* lectins, cystatin, and lysozyme have not been reported previously; however, adult female BF has been reported to possess lysozyme-like and agglutinating activities, though specific factors were not identified (Kato, 1995). We were unable to detect antibacterial activity of ESP from *in vitro*-hatched L3 larvae and from lung-stage L3 larvae. Third-stage larvae hatch from infectious eggs protected by the L2 cuticle before migrating through host tissues (Douvres et al., 1969). As the liver is continuously exposed to microbial antigens from the gut, hepatic immune cells are particularly primed to deal with incoming threats (McNamara and Cockburn, 2016). L3-stage larvae may therefore be protected from microbial threats by cuticle barriers for the few hours in the intestine before entering the host, and by the host-antimicrobial immune system responding to any microbes that may be

TABLE 1 | Proteins and peptides with known and predicted antimicrobial activities detected in excreted/secreted products and body fluid of *A. suum*[a].

Protein name uniprot[b]	Protein mass (Da[b])	Signal peptide[c]	Accession number uniprot
C-type lectin domain-containing protein 160	41,886	+	F1L7R9
C-type lectin domain-containing protein 160	47,612	+	F1L4K4
C-type lectin domain-containing protein 160	43,174		F1L8I9
C-type lectin protein 160	60,173	–	F1L0R7
32 kDa beta-galactoside-binding lectin	32,483	–	F1L893
32 kDa beta-galactoside-binding lectin	31,791	–	F1LAD2
GH family 25 lysozyme 2	24,644	+	F1LE63
GH family 25 lysozyme 2	21,687	–	F1LEA7
Cystatin	13,961	+	F1LHQ3
ASABF-alpha	9,843	+	P90683
ASABF-beta	9,219	+	Q8MMG8
ASABF-epsilon	7,037	+	Q8IAC9
Cecropin-P1	7,876	+	P14661
Cecropin-P2	9,760	+	Q5H7N6
Cecropin-P3	8,381	+	Q5H7N5
Cecropin-P4	8,424	+	Q5H7N4
ADULT MALE BODY FLUID			
Cecropin-P1 and/or Cecropin-P2	7,876/9,760	+	P14661/Q5H7N6
Cecropin-P3	8,381	+	Q5H7N5
Cecropin-P4	8,424	+	Q5H7N4
L4-STAGE LARVAE			
Cecropin P1	7,876	+	P14661
Cecropin-P2	9,760	+	F1LBL1
Cecropin-P3	8,381	+	Q5H7N5
***IN VITRO*-HATCHED L3 LARVAE**			
Cecropin-P1 or Cecropin-P2	7,876	+	P14661/Q5H7N6
Cystatin	13,961	+	F1LHQ3

[a]*Extended version of table available in* ***Supplementary Material****.*
[b]*Protein name and mass from Uniprot database (https://www.uniprot.org).*
[c]*Identified proteins predicted to contain secretory signal peptide (+) or not (–) using SignalP.*

carried with the larvae as they penetrate through the intestinal tissue to the liver. While microbial threats are abundant in the intestine, tissue migration presents other unique challenges for the nematode larvae. Cystatin likely plays an important role in the interaction between migratory *A. suum* larvae and host immune cells; if it possesses antimicrobial activity as shown for cystatins from chickens and humans (Blankenvoorde et al., 1998; Wesierska et al., 2005; Ganeshnarayan et al., 2012) remains to be determined. Thus, our data indicate tissue migratory third-stage larvae may not produce high quantities of antimicrobials. In contrast, we detected considerable antibacterial activity in cecropin-containing ESP from L4-stage larvae which have undergone further development after re-entering the intestine and thereby facing the presence of the intestinal microbiota. Furthermore, material harvested from adult nematodes, which have to contend with the host microbiota for the majority of the worm's lifespan, also showed considerable antibacterial activity. To counteract a diversity of potential threats originating from the microbiota, *Ascaris* is armed with several antimicrobial factors resulting in broad-spectrum antibacterial activity.

Studies in *C. elegans* have demonstrated the importance of biofilms in bacterial-nematode interactions. Within biofilms, bacteria are bound together within an extracellular matrix composed of exopolysaccharides, proteins, and nucleic acids (Hall-Stoodley et al., 2004) which provides support and protection, allowing bacteria to withstand higher concentrations of antibiotics (Dufour et al., 2010). Biofilm exopolysaccharides have been shown to enhance virulence of *S. epidermidis* during colonization of the *C. elegans* intestine in addition to enhancing bacterial resistance to nematode antimicrobial factors (Begun et al., 2007). Interestingly, biofilm forming *B. subtilis* promote oxidative stress resistance, thermotolerance, and upregulated expression of a lysozyme leading to enhanced resistance to worm killing by the pathogenic *Pseudomonas aeruginosa* (Smolentseva et al., 2017). Though the experimental settings differ, these studies highlight the importance of the biofilm lifestyle to nematode health. Thus, as biofilms might also influence parasitic nematode physiology, we studied the impact of *Ascaris* ESP on biofilm formation by *E. coli* K-12 strain AR3110. ESP from adult worms clearly resulted in a dose-dependent reduction in biomass accumulation in the submerged biofilm model (**Figure 2**). *E. coli* AR3110 also form macrocolony biofilms with pEtN-cellulose and amyloid curli fibers as key components of the extracellular matrix (Serra et al., 2013; Thongsomboon et al., 2018). In the presence of adult *A. suum* ESP, macrocolony formation was considerably disrupted and was accompanied

by the production of the complex exopolysaccharide colanic acid, while the production of pEtN-cellulose and curli fibers (reflected by colony wrinkling) was not affected (**Figure 3**). Colanic acid production, which is under the control of the RcsC/RcsB phosphorelay cascade (Majdalani et al., 2005) and is induced in response to cell envelope stress (Laubacher and Ades, 2008), confers resistance to antimicrobial peptides (Detweiler et al., 2003). Hence, nematode antimicrobial factors present in the *A. suum* ESP, especially AMPs, represent extracytoplasmic stress and, by inducing production of colanic acid, modify bacterial biofilm formation. While the inability of *E. coli* to produce colanic acid increased the growth inhibitory effects of *A. suum* ESP, a portion of the bacterial population was still able to survive the treatment (**Figure 3**). Similarly, resistance to AMPs allows *S. typhimurium* to persist in the intestine of *C. elegans* (Alegado and Tan, 2008); while nematodes release factors to defend themselves against bacterial threats, some can withstand these assaults. While bacteria are able to colonize the intestine of *Ascaris*, as determined by culture-based methods (Nalin and McLaughlin, 1976; Hsu et al., 1986; Shahkolahi and Donahue, 1993), the role of biofilms in microbial colonization of *A. suum* and interplay with the host microbiota during ascariasis require further study.

In addition to bactericidal factors such as ASABFs and Cecropins, we also detected lectins, including C-type lectin domain-containing (CTLD) proteins and galectins (**Table 1**). CTLD proteins recognize and bind to carbohydrate ligands and are critical in immunity (Brown et al., 2018). CTLD proteins can be transmembrane proteins, functioning as cell surface receptors, or can be secreted. A previous study isolated three CTLD proteins from the murine intestinal nematodes *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* (Harcus et al., 2009). The authors reported that these lectins are primarily expressed in the intestine-dwelling adult stages; however, bacterial binding functions were not assessed in their study. *C. elegans* possesses an estimated 283 CTLD (*clec*) genes, the majority of which are thought to be secreted (Pees et al., 2016). Previous studies have demonstrated that during infection with *S. marcescens*, *clec-39,−49,* and *-50* are upregulated and worms deficient in *clec-39* are more susceptible to infection with *S. marcescens* (Mallo et al., 2002; Engelmann et al., 2011; Miltsch et al., 2014). Additionally, recombinant CLEC-39 and−49 were shown to bind *S. marcescens* without killing the bacteria (Miltsch et al., 2014). We demonstrated calcium-dependent agglutinating activity of adult *A. suum* ESP (**Figure 4**), likely due to the CTLD proteins we detected. Both mammalian and non-mammalian hosts use lectins to shape the intestinal microbiota (Pang et al., 2016), an effect which could be compounded by secreted *A. suum* CTLD proteins. Galectins are β-galactoside-binding proteins also thought to function in host defense (Vasta, 2009). *C. elegans* deficient in the galectin LEC-8 were more susceptible to infection with *Bacillus thuringiensis* (Ideo et al., 2009). Interestingly, galectins do not typically contain secretory signal peptides but many localize extracellularly and are thought to be non-classically secreted (Barondes et al., 1994; Hughes, 1999). The galectins reported in our study were not predicted to contain signal peptides or to be secreted through non-classical pathways. However, though their presence in the *A. suum* ESP may contribute to agglutinating activity, their roles in nematode defense and in shaping the porcine intestinal microbiota need further investigation.

In this study we described diverse impacts of *A. suum* ESP on bacterial species from direct antimicrobial activity, disrupted biofilm formation, and neutralization by agglutination. These observations correlated with proteins and peptides detected in the ESP by mass spectrometry analysis and suggest that intestinal nematodes employ multiple strategies in their interactions with bacteria. Studies in infection models of *C. elegans* reveal pathogen and tissue-specific gene expression changes (Engelmann et al., 2011) along with differentially synthesized proteins in response to different microbial pathogens (Bogaerts et al., 2010a,b). These studies identified a diversity of upregulated factors including antimicrobial peptides, lectins, and lysozymes, all of which we detected in *A. suum* ESP. These multiple factors would then act in concert with one another to endow nematodes with a broad-spectrum defense system to allow survival in a microbial environment, as faced by *A. suum* in the porcine intestine. While we focused on the protein components of *Ascaris* ESP, it is important to note that helminth ESP also contain RNAs (Buck et al., 2014) and metabolites such as short-chain fatty acids (Zaiss et al., 2015) which in addition to modulating host immunity, may also impact the microbiota. Further study is required to determine the role of non-protein contents in shaping the microbiota; however, antibacterial activity described in our study due to combination effects of the various constituents of *A. suum* ESP have been accounted for by our use of native material.

In summary, our findings suggest that intestine-dwelling life stages of *A. suum* employ diverse antimicrobial strategies to establish themselves amongst the host microbiota. Our results provide a first indication of the direct impact of an intestinal nematode on its immediate microbial environment. Furthermore, our results suggest that the antimicrobial potential of nematode products differ depending on the parasite life-stage and corresponding host-environments. While metabolic and host immune factors would also contribute to an altered microbiome during helminth infection, we propose that nematodes themselves also have a direct role in shaping the microbiota as they establish themselves in the host gut, involving the secreted products and antimicrobial activities described herein. These changes would be more pronounced with a high worm burden as the local concentration of nematode antimicrobials would likely be higher. The defense strategies discussed in this study involve killing and non-killing mechanisms exerted by several different secreted factors acting in combination, as exemplified by the constitution and diverse activities of *A. suum* ESP. Together, these factors allow nematodes to carve out a niche to survive within a microbial environment and while doing so, may be partially responsible for changes to the intestinal microbiome during helminth infection.

AUTHOR CONTRIBUTIONS

All authors gave final approval for manuscript publication. Project designed by AM, SH, and JS. Microbiological experiments

designed by AM, SG, RH, and DS. Mass spectrometry analysis performed by KJ and AN. Peptides for mass spectrometry analysis synthesized by PH. AM performed all experiments. All authors interpreted data. Manuscript was written by AM and SH with input from the other authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2018.00271/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary Material

The intestinal roundworm *Ascaris suum* releases antimicrobial factors which interfere with bacterial growth and biofilm formation

Ankur Midha[1], Katharina Janek[2], Agathe Niewienda[2], Petra Henklein[3], Sebastian Guenther[4,5] Diego O. Serra[6], Josephine Schlosser[1], Regine Hengge[6], Susanne Hartmann[1*]

[1]Institute of Immunology, Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany

[2]Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Institute of Biochemistry, Shared Facility for Mass Spectrometry, Chariteplatz 1, D-10117 Berlin, Germany

[3]Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Institute of Biochemistry, Chariteplatz 1, D-10117 Berlin, Germany

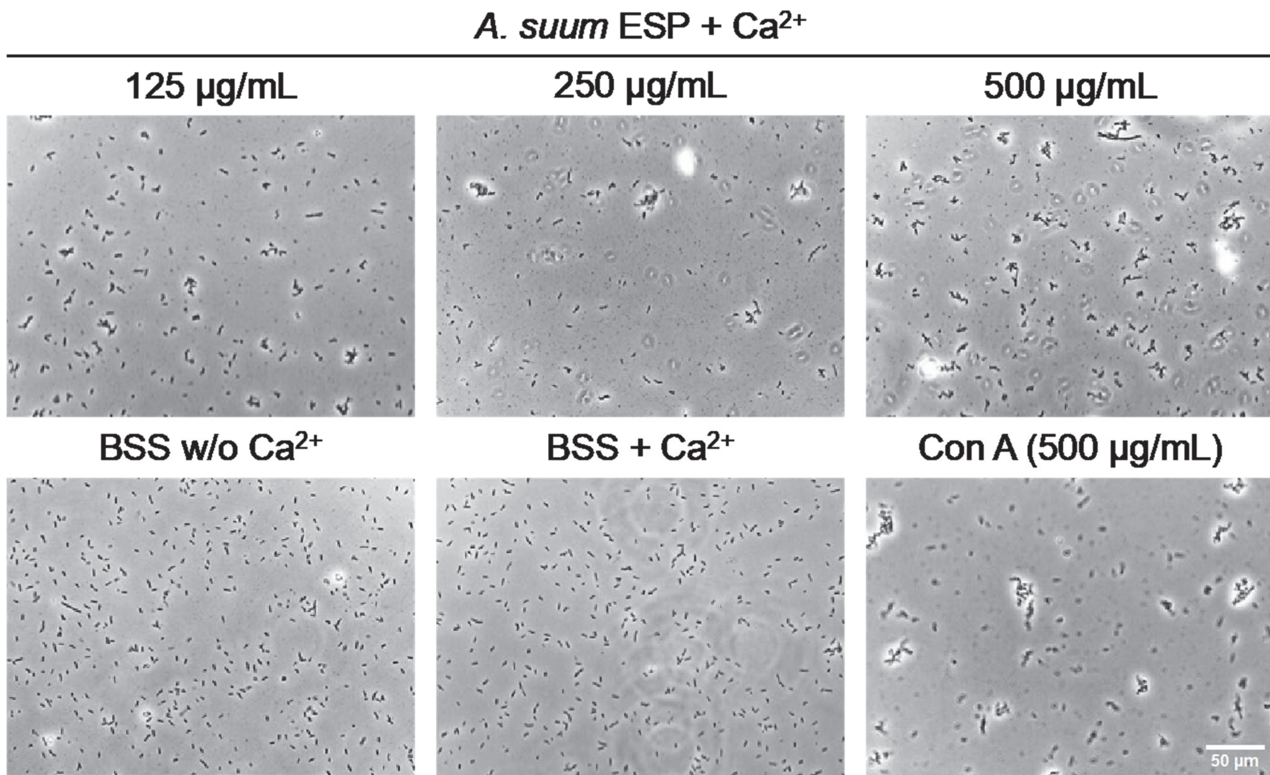
[4]Institute of Animal Hygiene and Environmental Health, Department of Veterinary Medicine, Freie Universität Berlin, Berlin, Germany

[5]Institute of Pharmacy, Department of Pharmaceutical Biology, Ernst-Moritz-Arndt-Universität Greifswald, Greifswald, Germany

[6]Institute of Biology / Microbiology, Humboldt-Universität-zu-Berlin, Berlin, Germany

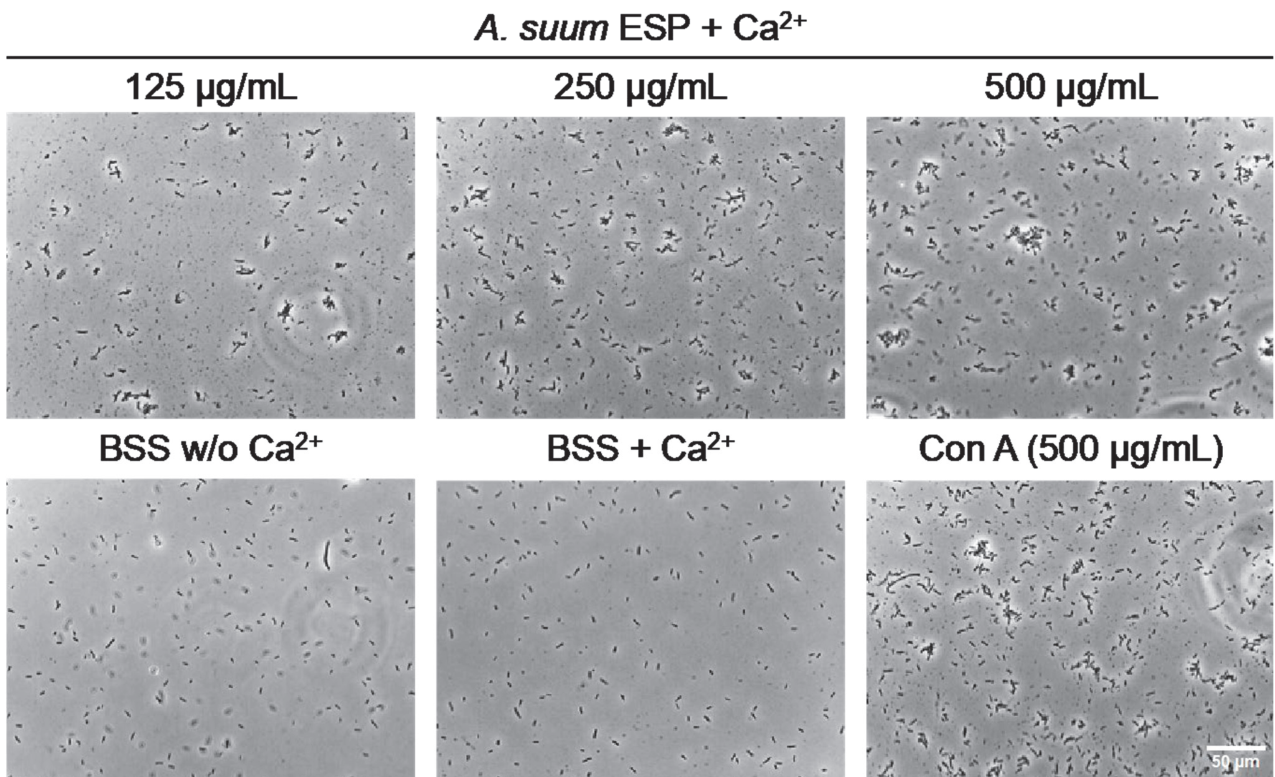
*** Correspondence:**
Susanne Hartmann
susanne.hartmann@fu-berlin.de

1.1 Supplementary Figures and Tables



Supplementary Figure 1. *Ascaris suum* excretory/secretory products cause bacterial agglutination of biofilm-forming *E. coli* AR3110.

Bacterial agglutination in the presence of adult *A. suum* ESP and 10 mM $CaCl_2$. Representative images of agglutination of *E. coli* K12 AR3110 with serial dilutions (1/2 factor) of *A. suum* ESP. Controls of agglutination include adult worm media (BSS) with and without $CaCl_2$ as well as the C-type lectin concavalin A. Bacteria visualized at 400X magnification.



Supplementary Figure 2. Ascaris suum excretory/secretory products cause bacterial agglutination of biofilm-forming E. coli AR155.

Bacterial agglutination in the presence of adult *A. suum* ESP and 10 mM $CaCl_2$. Representative images of agglutination of *E. coli* K12 AR155 with serial dilutions (1/2 factor) of *A. suum* ESP. Controls of agglutination include adult worm media (BSS) with and without $CaCl_2$ as well as the C- type lectin concavalin A. Bacteria visualized at 400X magnification.

Supplementary Table 1. Proteins and peptides with known and predicted antimicrobial activities detected in excreted/secreted products and body fluid of *A. suum.*

[a]https://www.uniprot.org

[b]Identified proteins predicted to contain secretory signal peptide (+) or not (-) using SignalP.

[c]The proteins were identified by mass spectrometry with accession numbers from the databases: 1, http://nematode.net/NN3_frontpage.cgi?navbar_selection=speciestable&subnav_selection=Ascaris_suum; 2, http://aps.unmc.edu/AP/main.php; 3, Swissprot 4, Uniprot *Ascaris suum*

[d]Mascot

[†]Closest match: uncharacterized protein (*Toxocara canis*) containing two C-type lectin domains

[§]Identity was confirmed by comparison of the MS/MS spectrum with the fragmentation pattern of a synthetic reference peptide.

[#]same sequence for Cecropin P1 and P2

[*]$p<0.00001$

Protein Characteristics				Identification by Mass Spectrometry						
Protein Name Uniprot[a]	**Protein Mass (Da) Uniprot**	**SP[b]**	**Accession Number Uniprot**	**Accession Number of Identified Protein**	**Database[c]**	**Protein Mass (Da)**	**Protein Score[d]**	**Number of significant distinct sequences (p<0.01)**	**Number of unique sequences**	**Sequence Coverage**
Adult, male or female										
uncharacterized protein	51771	-	A0A183VED4[†]	AS00034	1	23257	87	3	3	18
C-type lectin domain-containing protein 160	41886	+	F1L7R9 (aa 1-364)	AS03243	1	41584	768	14	8	86
C-type lectin domain-containing protein 160	47612	+	F1L4K4 (aa 31-259)	AS02827	1	30453	787	8	7	45
C-type lectin domain-containing protein 160	43174	+	F1L8I9 (aa 13-397)	AS01800	1	24619	335	7	7	46
uncharacterized protein	85734	+	A0A0M3HP70	AS02732	1	32062	357	7	7	33
C-type lectin protein 160	60173	-	F1L0R7 (aa 139-302)	AS04212	1	18321	338	4	2	34
			F1L0R7 (aa 227-430)	AS10343	1	23961	768	14	8	86
			F1L0R7 (aa 348-531)	AS02748	1	22787	464	10	7	65
32 kDa beta-galactoside-binding lectin	32483	-	F1L893	F1L893	2	32483	385	17	17	57
32 kDa beta-galactoside-binding lectin	31791	-	F1LAD2	F1LAD2	2	31791	546	12	11	53
GH family 25 lysozyme 2	24644	+	F1LE63	AS00167	1	23880	567	8	8	37
GH family 25 lysozyme 2	21687	-	F1LEA7	AS00467	1	10338	94	5	4	38
uncharacterized protein	19086	+	A0A0M3HT95	AS00263	1	17357	471	4	4	45
Cystatin	13961	+	F1LHQ3	AS02342	1	14281	343	5	5	50

ASABF-alpha	9843	+	P90683	AP01523	3	8408	121	5	4	37
ASABF-beta	9219	+	Q8MMG8	AS01881	1	10403	52	2	1[§]	20
ASABF-epsilon	7037	+	Q8IAC9	AS02651	1	7457	78	2	2	38
Cecropin-P1	7876	+	P14661	P14661	4	7876	71	1[#*]	0	20
Cecropin-P2	9760	+	Q5H7N6	Q5H7N6	4	9760	96	2	1[§]	36
Cecropin-P3	8381	+	Q5H7N5	Q5H7N5	4	8381	68	2	2	35
Cecropin-P4	8424	+	Q5H7N4	Q5H7N4	4	8424	42	1	1[§]	18
Adult male body fluid										
Cecropin-P1 and/or Cecropin-P2	7876 / 9760	+	P14661 / Q5H7N6	P14661 / Q5H7N6	4	7876 / 9760	54	1[#*]	0	20
Cecropin-P3	8381	+	Q5H7N5	CECP3_AS CSU	4	8376	29	1	1[§]	14
Cecropin-P4	8424	+	Q5H7N4	CECP4_AS CSU	4	8418	30	1	1[§]	19
L4-stage larvae										
Cecropin P1	7876	+	P14661	P14661	4	7871	66	1[#*]	0	20
Cecropin-P2	9760	+	F1LBL1	F1LBL1_AS CSU	4	9760	71	2	1[§]	36
Cecropin-P3	8381	+	Q5H7N5	Q5H7N5	4	8381	78	1	1[§]	22
***in vitro*-hatched L3 larvae**										
Cecropin-P1 or Cecropin-P2	7876	+	P14661 / Q5H7N6	P14661 / Q5H7N6	4	7871	41	1[#*]	0	20
Cystatin	13961	+	F1LHQ3	AS02342	1	14281	149	3	3	28

3. Lectin-Mediated Bacterial Modulation by the Intestinal Nematode Ascaris suum

Ankur Midha, Guillaume Goyette-Desjardins, Felix Goerdeler, Oren Moscovitz, Peter H. Seeberger, Karsten Tedin, Luca D. Bertzbach, Bernd Lepenies, Susanne Hartmann

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3.1 Author Contributions

Conceptualization: AM, BL, and SH; methodology: AM, GG-D, FG, and LDB.; resources: OM, PHS, KT, BL, and SH; formal analysis: AM, GG-D, and FG; writing—original draft: AM, GG-D, and FG; writing—review and editing: OM, PHS, KT, BL, and SH All authors have read and agreed to the published version of the manuscript.

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Article

Lectin-Mediated Bacterial Modulation by the Intestinal Nematode *Ascaris suum*

Ankur Midha [1], Guillaume Goyette-Desjardins [2], Felix Goerdeler [3,4], Oren Moscovitz [3,4], Peter H. Seeberger [3,4], Karsten Tedin [5], Luca D. Bertzbach [6,7], Bernd Lepenies [2] and Susanne Hartmann [1,*]

1. Institute of Immunology, Freie Universität Berlin, 14163 Berlin, Germany; ankur.midha@fu-berlin.de
2. Institute for Immunology & Research Center for Emerging Infections and Zoonoses (RIZ), University of Veterinary Medicine Hannover, 30559 Hannover, Germany; guillaume.goyette-desjardins@tiho-hannover.de (G.G.-D.); bernd.lepenies@tiho-hannover.de (B.L.)
3. Biomolecular Systems, Max Planck Institute of Colloids and Interfaces, 14476 Potsdam, Germany; felix.goerdeler@mpikg.mpg.de (F.G.); oren.moscovitz@mpikg.mpg.de (O.M.); peter.seeberger@mpikg.mpg.de (P.H.S.)
4. Department of Biology, Chemistry, Pharmacy, Freie Universität Berlin, 14195 Berlin, Germany
5. Institute of Microbiology and Epizootics, Freie Universität Berlin, 14163 Berlin, Germany; karsten.tedin@fu-berlin.de
6. Institute of Virology, Freie Universität Berlin, 14163 Berlin, Germany; luca.bertzbach@leibniz-hpi.de
7. Department of Viral Transformation, Leibniz Institute for Experimental Virology (HPI), 20251 Hamburg, Germany

* Correspondence: susanne.hartmann@fu-berlin.de

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Abstract: Ascariasis is a global health problem for humans and animals. Adult *Ascaris* nematodes are long-lived in the host intestine where they interact with host cells as well as members of the microbiota resulting in chronic infections. Nematode interactions with host cells and the microbial environment are prominently mediated by parasite-secreted proteins and peptides possessing immunomodulatory and antimicrobial activities. Previously, we discovered the C-type lectin protein AsCTL-42 in the secreted products of adult *Ascaris* worms. Here we tested recombinant AsCTL-42 for its ability to interact with bacterial and host cells. We found that AsCTL-42 lacks bactericidal activity but neutralized bacterial cells without killing them. Treatment of bacterial cells with AsCTL-42 reduced invasion of intestinal epithelial cells by *Salmonella*. Furthermore, AsCTL-42 interacted with host myeloid C-type lectin receptors. Thus, AsCTL-42 is a parasite protein involved in the triad relationship between *Ascaris*, host cells, and the microbiota.

Keywords: *Ascaris*; helminths; intestinal nematode; microbiota; lectin; *Salmonella*; glycan array; C-type lectin; C-type lectin receptor

1. Introduction

Intestinal parasitic nematode and other helminth infections are widespread in humans, companion animals, livestock, and wildlife. Ascariasis, caused by *Ascaris lumbricoides* in humans and the closely related *Ascaris suum* in pigs, is one of the most common nematode infections worldwide [1,2]. In humans, ascariasis in children with high worm burdens can lead to malnutrition, developmental deficits, and death [3–5]. In pigs, *Ascaris* causes major production losses due to reduced feed conversion and growth rates as well as liver condemnation [6]. Worm burdens vary between individuals, and the majority of the worm burden is carried by a minority of the infected population [7]. The parasite life cycle is thought to follow a similar trajectory in both host species; eggs containing third-stage larvae hatch within hours of ingestion followed by invasion of the cecum and proximal colon [8]. Then, the larvae begin their tissue migration through the liver, reaching the lungs by 6–8 days post-infection (dpi) [9]. The larvae get coughed up and swallowed arriving in the small intestine where they mature into adults, which can reside there

for at least 1 year [6]. Pigs are a powerful model for human infectious diseases due to the anatomical, physiological, and genetic similarities between pigs and humans [10], especially in the case of ascariasis where the intestinal tracts and microbiota are more comparable as opposed to widely available mouse models [11]. Furthermore, *Ascaris* is also a zoonotic pathogen, and the porcine gut may represent a reservoir for additional bacterial pathogens such as *Salmonella*, the second most common food-borne pathogen in the European Union [12,13]. Despite the close coexistence of *Ascaris* with numerous microbes, little is known concerning the reciprocal interactions of the nematodes with the microbiota. It has previously been reported that nematode infections lead to changes in intestinal microbial composition [14,15]. One study reported increased alpha diversity in the acute phase at 14 days post-infection (dpi) [14] while another documented decreased diversity in chronically infected pigs at 54 dpi [15]. In both studies, altered microbial compositions were most apparent in the proximal colon, a site with high bacterial loads in contrast to the small intestine where the parasite resides.

Interactions between *Ascaris*, the microbiota, and host cells are mediated in part by the release of excreted and secreted (ES) products [16]. Characterization of the *Ascaris* ES proteome has revealed developmental, life stage-dependent differences in ES content [17]. In addition to structural proteins and proteins involved in molting, motor activity, and metabolism, ES components also contain proteins and peptides with known or predicted antimicrobial and immunomodulatory activities, including antimicrobial peptides, lysozymes, chitinases, cystatins, and lectins [17,18]. Lectins, carbohydrate-binding proteins with numerous functions, are abundant in nematodes [19]. Recently, we discovered several C-type lectin (CTL) domain-containing proteins in the ES of adult *A. suum* nematodes [18]. *A. suum* total ES proteins induce calcium-dependent bacterial agglutination, indicative of CTL-mediated activity [18]. Interestingly, lectin-containing ES from the murine helminth *Heligmosomoides polygyrus* also exhibits calcium-dependent bacterial agglutination [20]. The mammalian lectin RegIIIγ possesses antibacterial activity and maintains segregation between the intestinal microbiota and host epithelium in mice [21]. Furthermore, CTLs are involved in the defense of the free-living nematode *Caenorhabditis elegans* against microbial threats [22–24] as well as the maintenance of gut microbiome homeostasis in mosquitoes [25]. Thus, nematode CTLs may defend worms against infection [24] or alternatively may modulate host immune responses [26].

We hypothesized that CTLs from *A. suum* might have microbiota-modulating properties. Therefore, we aimed to determine whether a prominent CTL protein found in *A. suum* ES (hereafter referred to as AsCTL-42) has the potential to modulate the intestinal microbiota. Here, we expressed a recombinant, 42 kilodalton (kDa), signal peptide-containing CTL protein that we had detected in intestine-dwelling adult *A. suum* (UniProt name: C-type lectin domain-containing protein 160, UniProt accession number: F1L7R9) [18]. As host defense molecules can be multi-functional, possessing antimicrobial and immune-modulating activities [27], we tested AsCTL-42 for its effects on the viability of host and bacterial cells, probed for potential binding partners for the protein, and assessed the impact of AsCTL-42 on the invasion of host epithelial cells by the pathogen *Salmonella enterica* subsp. enterica serovar Typhimurium (*S.* Typhimurium).

2. Results

2.1. Eukaryotic Expression of AsCTL-42

AsCTL-42 and a control protein GH family 25 lysozyme 2 (herein denoted AsGH) were both recombinantly expressed using the eukaryotic *Leishmania tarentolae* expression system (Figure 1A) [28]. For AsCTL-42, we observed a band at a molecular weight between 35 and 55 kDa as well as additional bands of a lower molecular weight. The additional bands were confirmed to be derived from AsCTL-42 by mass spectrometry (Figure S1). To ensure recombinant proteins were free of lipopolysaccharide (LPS) contamination, we used the Endosafe endotoxin testing system as described in the methods. Proteins used in this study were found to have LPS levels below 0.1 ng/mL (less than 1 endotoxin unit per

mL). To confirm the presence of post-translational modifications, we cultured *L. tarentolae* in the presence of tunicamycin (10 µg/mL) to inhibit N-glycosylation [29] and observed mobility shifts expected of a glycosylated protein (Figure 1B). To confirm these findings and further assess additional post-translational modifications, AsCTL-42 was treated with a protein deglycosylation enzyme mixture, including PNGase F, O-Glycosidase, α2-3,6,8 Neuraminidase, β1-4 Galactosidase, and β-N-Acetylglucosaminidase. We subjected the products of this reaction to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and once again observed mobility shifts indicative of glycosylation patterns (Figure 1C). Images of original, uncropped gels are available in the supplementary material (Figure S2).

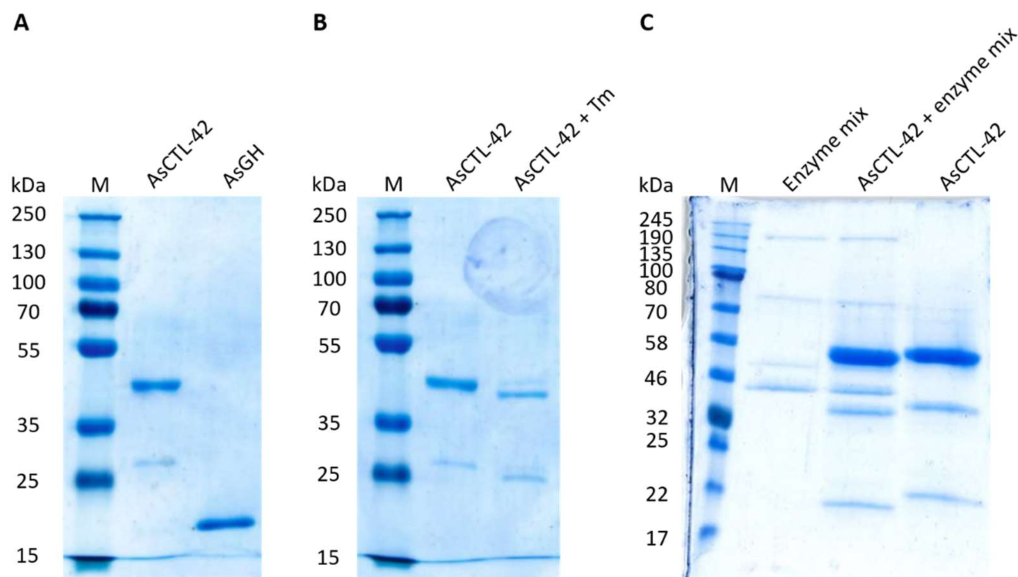

Figure 1. Coomassie-stained SDS-PAGE gels of recombinantly expressed *Ascaris suum* proteins and glycosylation patterns of AsCTL-42. (**A**) 1 µg of protein loaded onto 12% SDS-polyacrylamide gels, stained with Coomassie G-250 dye. (**B**) *Leishmania tarentolae* were cultured in the presence (right; AsCTL-42 + Tm) or absence (left; AsCTL-42) of tunicamycin (10 µg/mL). 1 µg of protein loaded onto 12% SDS-polyacrylamide gels, stained with Coomassie G-250 dye. (**C**) AsCTL-42 was treated with a protein deglycosylation enzyme mixture and the products of this reaction were loaded onto 14% SDS-polyacrylamide gels, stained with Coomassie G-250 dye.

2.2. AsCTL-42 Agglutinates Salmonella

Nematodes can neutralize microbial threats using CTL proteins [24]. Having shown previously that lectin-containing *A. suum* ES products agglutinate bacteria [18], we sought to determine whether recombinant AsCTL-42 could recapitulate this observation. To test the agglutinating activity of AsCTL-42, we treated *S.* Typhimurium 4/74 with AsCTL-42 in the presence and absence of $CaCl_2$ (10 mM) and observed dose- and calcium-dependent agglutinating activity (Figure 2). Interestingly, we also observed reduced motility in agglutinated samples (Supplemental Videos). Thus, recombinant AsCTL-42 is capable of neutralizing potential infectious threats by agglutination.

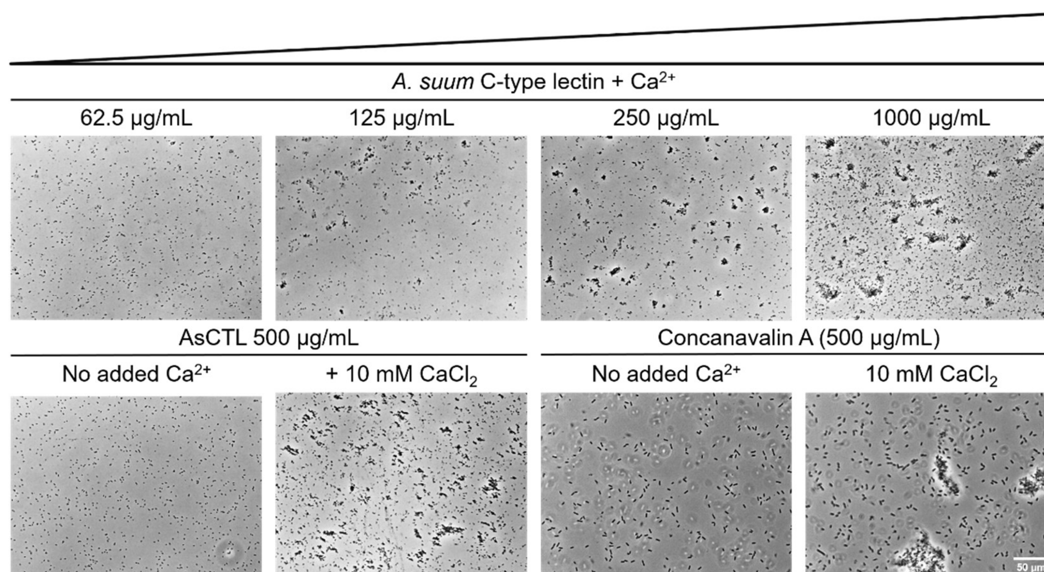

Figure 2. AsCTL-42 agglutinates *Salmonella* in the presence of calcium. Representative images of agglutination of *S.* Typhimurium with increasing concentrations of AsCTL-42. Controls include buffer (tris-buffered saline) without added calcium as well as the C-type lectin concanavalin A with and without added calcium. Bacteria visualized at 400× magnification. Data are representative of two independent experiments performed with independent batches of AsCTL-42.

2.3. AsCTL-42 Does Not Inhibit Bacterial Growth

As *Ascaris* nematodes inhabit a rich microbial environment, they need to modulate not only the microbiota of the host's intestine but also their own microbiota. Microbiota modulation may be achieved via the release of factors with antimicrobial activity. We have previously shown that *A. suum* ES products can inhibit bacterial growth [18]. Amongst the factors we detected in the ES products, we identified several CTL proteins. Lectins have been implicated in shaping the microbiota, in some cases by killing bacteria [25]. We therefore tested whether recombinant AsCTL-42 inhibits the growth of different bacterial strains in comparison to the antimicrobial peptide pexiganan in radial diffusion assays. Treatment with AsCTL-42 did not inhibit the growth of the Gram-positive or Gram-negative bacterial strains that we tested, including *Enterococcus faecium* DSM20477, *Staphylococcus aureus* IMT29828, *Escherichia coli* IMT19224, and *S.* Typhimurium 4/74 (Table 1), all of which are species that can be found in the porcine intestine [30–33]

Table 1. Bacterial growth inhibition activity [1] of AsCTL-42 in the radial diffusion assay.

	***E. faecium* DSM20477**	***S. aureus* IMT29828**	***E. coli* IMT19224**	***S.* Typhimurium 4/74**
AsCTL-42 (1 mg/mL)	-	-	-	-
Pexiganan (1.25 µg/mL)	5.0	12.0	11.0	11.0
PBS	-	-	-	-

[1] Activity reported as diameter of inhibition zone (mm) produced by treatments (*n* = 3 independent batches of AsCTL protein). "-" indicates no detectable activity. Data are representative of two independent experiments.

2.4. AsCTL-42 Does Not Bind to Bacterial Glycans

In order to shed light on the interactome of AsCTL-42, we examined potential binding to different glycan structures via a synthetic glycan array. The glycan array slide contained 140 structurally diverse glycans from bacteria, protozoans, fungi, mammals, and plants as listed in Table S1 [34]. The plant lectin concanavalin A was used as a positive control. However, AsCTL-42 failed to recognize any of the printed structures, even at high protein

concentrations (Table S1), while concanavalin A expectedly bound strongly to glycans containing mannose and glucose (Figure S3) [35].

2.5. AsCTL-42 Decreases Invasion of Porcine Intestinal Epithelial Cells by Salmonella

We further assessed the impact of AsCTL-42 treatment on the invasion of intestinal porcine epithelial cells (IPEC-J2) by *Salmonella* using an in vitro invasion assay [36]. We recovered significantly fewer intracellular *Salmonella* from the IPEC-J2 cells in the presence of AsCTL-42 (Figure 3). In order to determine whether the AsCTL-42 reduced bacterial invasion by acting on host or bacterial cells, we performed the experiment by adding the *Ascaris* protein to the culture medium at the same time as the bacteria, or by pre-treating either host or bacterial cells with AsCTL-42 for 30 min prior to infection. We observed a dose-dependent decrease in epithelial cell invasion by *S.* Typhimurium, an effect that was particularly evident when we pre-treated the bacteria prior to infection (Figure 3). Colony-forming unit (CFU) counts from individual experiments can be found in Table S1. Thus, AsCTL-42 is able to reduce the invasion of porcine intestinal epithelial cells by *Salmonella* by acting on bacterial rather than host cells.

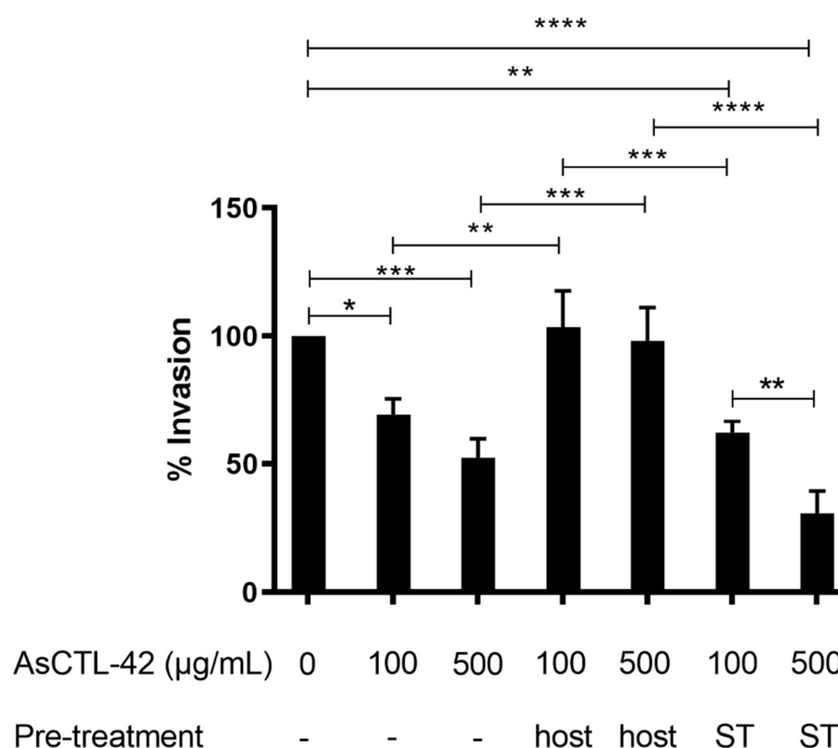

Figure 3. AsCTL-42 impairs porcine intestinal epithelial cell invasion by *Salmonella*. Treatments (AsCTL-42 or PBS as a control) were added to IPEC-J2 cells at the time of infection, or host and bacterial (ST) cells were incubated with treatments for 30 min prior to infection. IPEC-J2 cells were infected by *S.* Typhimurium 4/74 and intracellular CFU were determined. Columns represent mean % invasion (with PBS-treated cells set to 100%) from three independent experiments ± SEM. Significance determined by one-way ANOVA with Tukey's multiple comparison tests, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$, **** $p < 0.0001$. For clarity, only significant differences have been annotated. All missing comparisons are not statistically significant.

2.6. AsCTL-42 Does Not Interfere with Host Cell Viability

As *Ascaris* nematodes dwell in the lumen of the porcine intestine, their ES products may interact with microbes as well as host epithelia. Having determined that AsCTL-42 does not inhibit the growth of various bacterial strains, we sought to determine whether it interferes with host cells. We assessed cell viability of IPEC-J2 cells by the colorimetric MTT assay that involves the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a formazan product by mitochondrial NAD(P)H-dependent reductases [37]. The formazan product is quantified by absorbance and reflects the viability and metabolic health of the cells. As shown in Figure 4, AsCTL-42 does not inhibit the viability of IPEC-J2 cells.

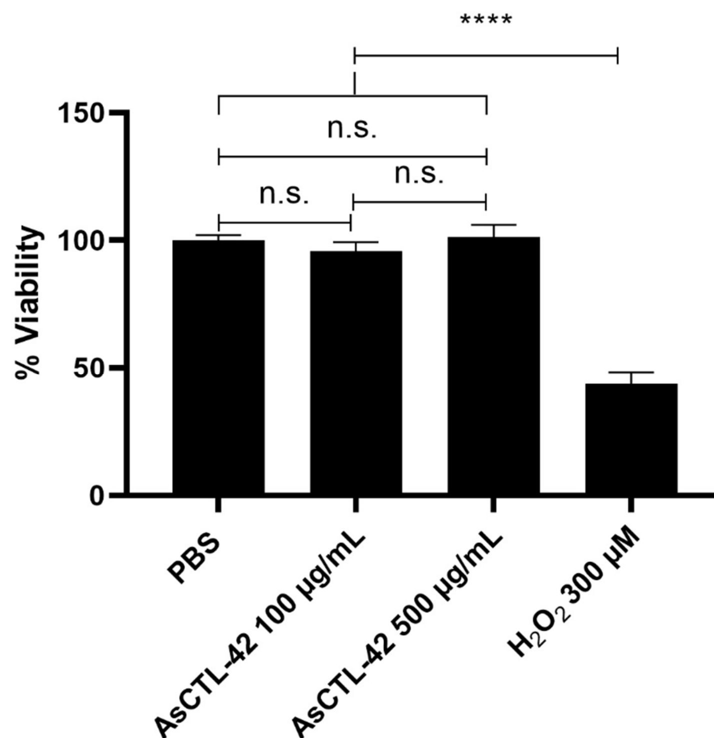

Figure 4. AsCTL-42 treatment does not reduce viability of IPEC-J2 cells. Cells were treated for 24 h with PBS (vehicle control), AsCTL-42 (100 µg/mL, 500 µg/mL), or H_2O_2 (300 µM) as a positive control for reduced viability. Cell viability was assessed using the MTT assay. Columns represent mean viability from four independent experiments ± SEM. Significance determined by one-way ANOVA with Tukey's multiple comparison tests, n.s. = not statistically significant, **** $p < 0.0001$.

2.7. AsCTL-42 Binds Selected Mammalian C-Type Lectin Receptors

In order to assess the potential for AsCTL-42 to bind to host cells, we screened for interactions between AsCTL-42 and C-type lectin receptors (CLR) from humans and mice. We found that AsCTL-42 binds to selected human and murine myeloid CLRs (Figure 5). To verify the specificity of lectin binding, we used another similarly expressed and purified recombinant control protein from Ascaris, AsGH, which did not demonstrate strong binding to myeloid CLRs (Figure 5). To further rule out non-specific effects due to the expression system, we included an *L. tarentolae* medium that did not exhibit notable binding compared to AsCTL-42 (Figure 5).

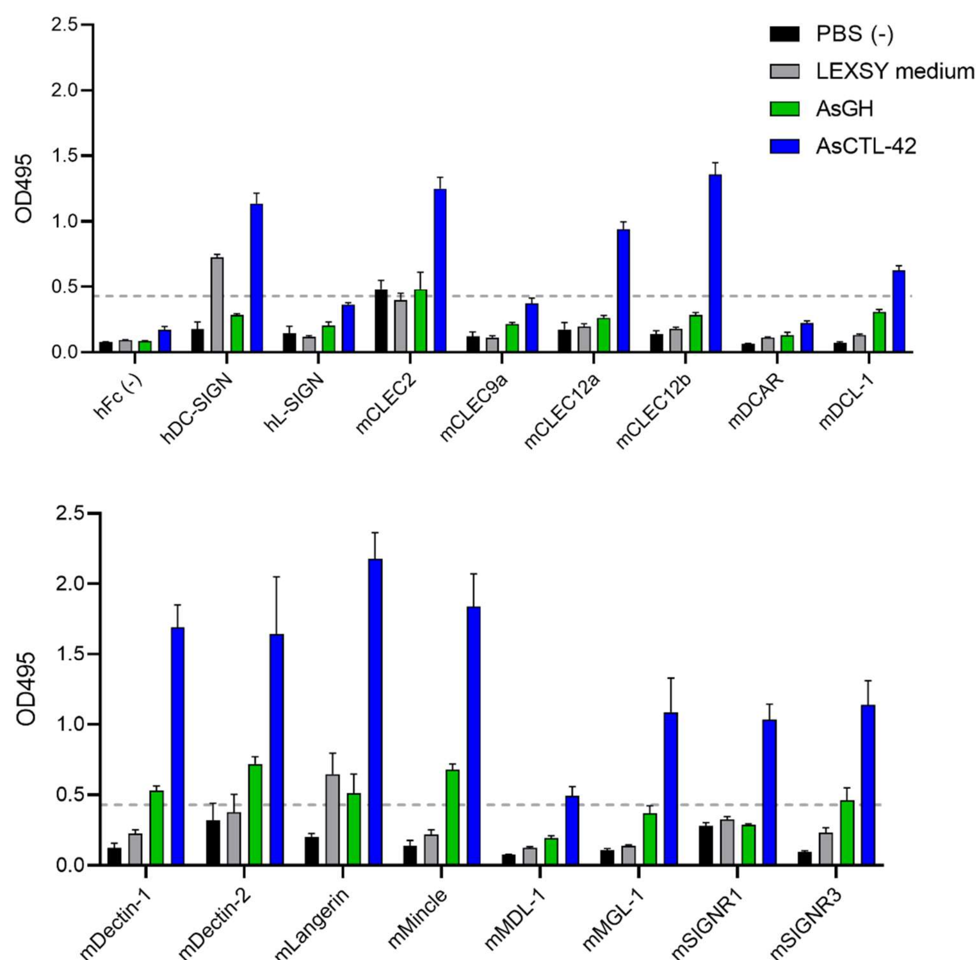

Figure 5. AsCTL-42 binds to selected human (h) and murine (m) C-type lectin receptors. ELISA plates were coated with treatments (0.5 μg) and screened for binding to CLR-hFC fusion proteins. Binding was detected using horseradish peroxidase (HRP)-conjugated goat anti-human IgG to generate absorbance readings at 495 nm with an ELISA plate reader. Spent LEXSY cultivation medium from *L. tarentolae* was included as a control to rule out contribution from *Leishmania* proteins while AsGH was included as an expression system control. Data are presented as mean absorbance readings from three independent experiments ± SEM. The dashed line represents the threshold for CLR binding, defined as four times the average OD values for the hFc control.

Interestingly, AsCTL-42-CLR binding appeared to be calcium-dependent, as binding tended to decrease in the presence of EDTA (Figure 6).

Particularly prominent binding was observed for Dectin-1, Dectin-2, Langerin, and Mincle. These data indicate that AsCTL-42 has the potential to interact with host cells and may have immunomodulating activities via CLRs. The corresponding porcine CLRs can be found in Table 2.

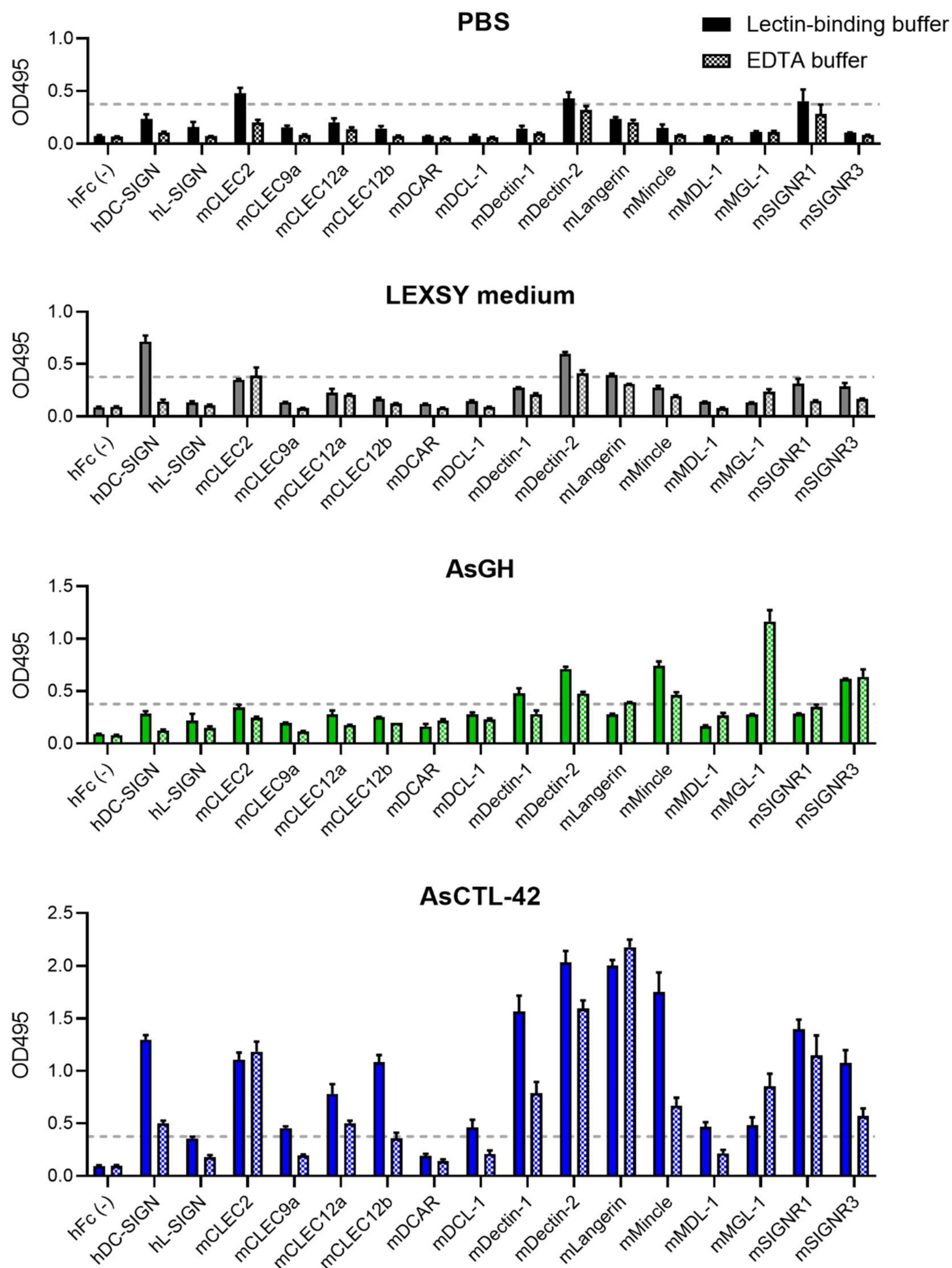

Figure 6. Binding of AsCTL-42 to C-type lectin receptors is calcium-dependent. ELISA plates were coated with treatments (0.5 μg) and screened for binding to CLR-hFC fusion proteins in the presence of calcium-containing lectin binding buffer (solid bars) or EDTA buffer (checkered bars). Binding was detected using horseradish peroxidase (HRP)-conjugated goat anti-human IgG to generate absorbance readings at 495 nm with an ELISA plate reader. Spent LEXSY cultivation medium from *L. tarentolae* was included as a control to rule out contribution from *Leishmania* proteins while AsGH was included as an expression system control. Data are presented as average absorbance readings from three independent experiments ± SEM. The dashed line represents the threshold for CLR binding, defined as four times the average OD values for the hFc control.

Table 2. Human and murine C-type lectin receptors tested in this study and their corresponding receptors in pigs [1].

Human (h) or Murine (m) Protein	Human or Murine Gene	Corresponding Porcine Protein	Corresponding Porcine Gene
hDC-SIGN	*CD209/CLEC4L*	CD209	*CD209*
hL-SIGN	*CD209L/CLEC4M*	CD209	*CD209*
mCLEC2	*Clec1b*	CLEC1b	*CLEC1B*
mCLEC9a	*Clec9a*	CLEC9a	*CLEC9A*
mCLEC12a	*Clec12a*	CLEC12a	*CLEC12A*
mCLEC12b	*Clec12b*	CLEC12b	*CLEC12B*
mDCAR	*Clec4b1*	CLEC4A	*CLEC4A*
mDCL-1	*Clec2i*	CLEC2D	*CLEC2D*
mDectin-1	*Clec7a*	CLEC7A	*CLEC7A*
mDectin-2	*Clec6a*	No corresponding protein	No corresponding gene
mLangerin	*Cd207*	CLEC4K	*CD207*
mMincle	*Clec4e*	CLEC4E	*CLEC4E*
mMDL-1	*Clec5a*	CLEC5A	*CLEC5A*
mMGL-1	*Clec10a*	Asialoglycoprotein receptor 1	*ASGR1*
mSIGNR1	*Cd209b*	CD209	*CD209*
mSIGNR3	*Cd209d*	CD209	*CD209*

[1] Corresponding gene and protein names obtained using protein BLAST functions on Uniprot (https://www.uniprot.org, accessed on 28 June 2021) and National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 28 June 2021). Where appropriate, the closest match in BLAST searches were assigned as the corresponding porcine proteins and genes.

3. Discussion

Intestinal nematodes inhabit a rich microbial environment. In addition to confronting host immunity, these organisms must contend with microbial cohabitants including potential pathogens. Studies of these multi-lateral interactions have demonstrated that helminths can sense host microbes and also rely on them for proper development, infectivity, and fecundity [20,38,39]. Parasite-driven immune responses can alter the production of host defense molecules and mucin resulting in alterations to the microbiota [40]. Furthermore, interactions between bacteria and helminths can be mutually beneficial as was shown for *Lactobacillus taiwanensis* and *H. polygyrus* where both species promote each other in the murine gut [41]. While numerous studies have documented microbiome alterations associated with nematode infections, the underlying mechanisms can be quite complex and difficult to decipher as interactions between host, microbes, and parasites can be direct and indirect as well as multi-directional.

Nematode ES products include a cocktail of proteins and peptides possessing antimicrobial and immunomodulatory activities [18,20,42]. Lectin domain-containing proteins, including CTLs and galectins, were prominent in the ES products of intestine-dwelling adult *Ascaris* worms [18]. Lectins are best known for their glycan-binding properties and perform multiple biological functions. The *A. suum* genome encodes at least 78 lectin domain-containing sequences, including 36 CTLs [19]. Secreted lectins may be cytotoxic, as was shown for the CTL CEL-1 from the sea cucumber *Pseudocnus echinatus* (formerly *Cucumaria echinata*), which exhibits cytotoxicity against numerous cell lines [43]. In this study, we demonstrated that the secreted lectin AsCTL-42 from *A. suum* does not directly impact the viability of host or bacterial cells. There was no detectable influence of AsCTL-42 on host cell viability using the porcine intestinal epithelial cell line IPEC-J2 (Figure 4) that is representative of the host cells in the immediate vicinity of *Ascaris.* Lectins are also under investigation for their diverse antimicrobial activities [44]; however, we did not detect any influence on the viability of different bacterial strains in this study (Table 1). Unlike the bactericidal mammalian lectin RegIIIγ, nematode lectins have thus far not shown bactericidal activity. This is consistent with our data showing that AsCTL-42 may play a non-lethal role in modulating microbial populations, as has also been observed for lectins from *C. elegans* where selected CTLs released by the nematode in response to bacterial exposure are able to bind the bacteria without killing them [22–24].

Although our data show that AsCTL-42 is not bactericidal, it exhibits a non-toxic antimicrobial activity. We detected calcium-dependent bacterial agglutination by AsCTL-42 (Figure 2). Our previous work showed that ES products from *A. suum* and *H. polygyrus* agglutinate bacteria in a calcium-dependent manner [18,20]. Interestingly, CTLs are upregulated in response to microbial threats in *C. elegans* [22,23] and recombinant *clec-39* and *-49* bind bacteria without killing them in a calcium-independent manner [24]. Although we did not identify glycan binding partners in the glycan arrays, the presence of a lectin domain does not assure sugar-binding. Previously, glycan array screening using *clec-39* and *-49* from *C. elegans* did not reveal carbohydrate binding partners [24]. Furthermore, CTLs may also bind to non-glycan ligands [45], and only eight of the 36 CTLs encoded in the *A. suum* genome are predicted to bind carbohydrate ligands by hidden Markov modeling [19]. The agglutinating activity we detected confirms that AsCTL-42 does indeed interact with bacterial cells. Together, these observations suggest that secreted nematode lectins may neutralize bacterial threats.

In addition to interactions with microbial cells, we also demonstrated the potential for AsCTL-42 to interact with mammalian cells. As myeloid CLRs can sense microbes such as Gram-negative bacteria, fungi, *Mycobacterium* spp., trematodes, and viruses [46,47], their modulation has implications for intestinal microbial communities. We found that AsCTL-42 interacts with selected human and murine myeloid CLRs (Figure 5). Interestingly, these interactions were calcium-dependent, as the addition of EDTA tended to reduce binding (Figure 6). CLR modulation has been documented for different helminth species. DC-SIGN is a receptor for egg antigens from the trematode *Schistosoma mansoni* [48] while Dectin-1 on macrophages was found to be a target of immunomodulation by the sheep liver fluke *Fasciola hepatica* [49]. While we did not assess porcine CLRs, the porcine parasite *A. suum* and the human parasite *A. lumbricoides* are both capable of infecting pigs and humans [12]. Notably, paleoparasitological and genetic evidence indicate that *A. suum* and *A. lumbricoides* are the same species [50]. Thus, interactions between *A. suum* and human receptors are insightful for both human and porcine ascariasis. In addition, human, murine, and porcine CLRs overlap considerably (Table 2). Our observations suggest a potential for *Ascaris* lectins to directly influence host myeloid cells but downstream consequences for host microbiota modulation remain to be determined.

Having determined that AsCTL-42 can interact with both bacteria and host cells, we sought to determine the functional consequences of such interactions. It has been shown previously that helminth infections can modulate immune responses against intracellular pathogens [51]. Thus far, there have been no reports of how *Ascaris* might influence immune responses against *Salmonella* even though both pathogens are prevalent in pigs, are of considerable zoonotic importance, and there exists an association between high *Ascaris* exposure and *Salmonella* prevalence in pigs [52]. Thus, we studied the relationship between *Ascaris*, *Salmonella*, and host cells using an in vitro porcine epithelial cell invasion assay. We found that AsCTL-42 reduced the invasion of intestinal epithelial IPEC-J2 cells by *Salmonella* by acting on the bacteria rather than on host cells (Figure 3). Pre-treating host cells prior to infection did not reduce epithelial cell invasion while pre-treating bacterial cells did; hence, we attribute our observations to agglutination and the reduced motility of *Salmonella* in the presence of AsCTL-42. Interestingly, a previous study found that *H. polygyrus* infection altered the metabolomic environment of the murine intestine and that these metabolomic alterations promoted coinfection of mice with *Salmonella* [53]. We have demonstrated the potential for one particular lectin protein to decrease epithelial cell invasion, though *Ascaris* ES products contain numerous other factors, including metabolites. Notably, *A. suum* can produce short-chain fatty acids [54] that can have mixed effects on *Salmonella* virulence, growth, and motility [55–57]. Though our data point to potentially meaningful interactions between these two pathogens, further study is warranted to determine the outcomes and mechanisms underlying interactions between *Ascaris* and *Salmonella* in vivo.

A potential limitation of our work is posed by the high concentrations of AsCTL-42 used in some of the experiments. While we focused on one particular CTL in vitro, it is

likely that multiple lectins may act synergistically in vivo and we have previously identified several lectin domain-containing proteins in *A. suum* ES products [18]. Furthermore, helminths including *Ascaris* are frequently found aggregated together in the host intestine [58,59] where sexually mature adult worms must be in close proximity to mate. *A. suum*, also referred to as the 'large roundworm', is indeed quite large—individual worms can weigh up to 7 g and measure up to 30 cm in length [60]. In severe cases, this aggregation can obstruct the intestine [6]. Notably, parasite burdens of well over 100 worms per host have been observed in humans and pigs [7,15]. We speculate that in a natural system, numerous worms aggregating in the rather narrow confines of the jejunum could collectively produce lectin-containing ES products in considerable concentrations. Thus, we consider the concentrations used herein as insightful, particularly in the case of individuals with high worm burdens, due to the specific microenvironment that *Ascaris* adults are found in and the composition of their ES products.

In summary, our findings suggest that secreted CTLs considerably aid the establishment of *A. suum* in the porcine intestine. Others have speculated on a role for helminth CTLs in parasite–host interactions [61,62]. Previous studies have also pointed to host CLRs as important modulators of the host immune response against helminths [48,49]. Here, we provide support for these observations having shown that AsCTL-42 can interact with myeloid CLRs. We have identified several potential binding candidates, which warrant further study. Furthermore, we have demonstrated a role for AsCTL-42 in directly modulating microbes through its interactions with *Salmonella*. Future studies should be carried out to elucidate the mechanistic underpinnings of AsCTL-42–bacterial interactions, in particular to determine the bacterial binding partner of AsCTL-42. Further investigation may place lectins amongst a handful of other well-studied helminth immunomodulators, such as cystatins, helminth defense molecules, and transforming growth factor beta mimic proteins [63–65]. Considered in context, the multiple lectins produced by *Ascaris* would have evolved to ensure parasite survival within the host, perhaps by binding to multiple targets.

4. Materials and Methods

4.1. Recombinant Expression of AsCTL-42 and Protein Analysis

AsCTL-42 and AsGH were both recombinantly expressed using the eukaryotic *Leishmania* expression system (LEXSY; Jena Bioscience, Jena, Germany) as described previously [28,66]. The nucleotide sequences of AsCTL-42 and AsGH without their specific signal sequences were cloned into the pLEXSY-sat2 plasmid of the LEXSYcon2 Expression kit. Following manufacturer's instructions, a monoclonal LEXSY cell strain expressing and secreting the desired target protein with a hexa-histidine tag was developed. Purification of the protein was performed via affinity chromatography using HisTrap™excel columns and the ÄKTA™ pure chromatography system (GE Healthcare Bio-Science AB, Uppsala, Sweden) using imidazole as a competitive eluent in a non-denaturing protocol. Purified proteins were dialyzed against PBS, sterile filtered, and protein concentrations were determined using the Pierce™ BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). LPS contamination was assessed using Endosafe® PTS cartridges (Charles River Laboratories, Charleston, VA, USA). Protein mass was assessed by SDS-PAGE on 12% agarose gels followed by Coomassie staining. We confirmed the identity of the observed bands by LC-MS/MS analysis. Briefly, bands were removed from the gel and protein was retrieved by in-gel tryptic digestion followed by reconstitution in 0.1% trifluoroacetic acid in 2:98 acetonitrile/water. LC-MS/MS analysis and protein identifications of the peptides were performed on an Ultimate 3000 RSLCnano system online coupled to an Orbitrap Q Excative Plus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) followed by database searching using Mascot software version 2.6.1 (Matrix Science Ltd., London, UK) against an internal database (359 sequences), SwissProt 2017_11 (556,196 sequences), and a contaminant database (247 sequences) as described previously [15].

4.2. Bacterial Strains

The bacterial strains used to evaluate antibacterial activity of AsCTL-42 in the radial diffusion assay included: *Enterococcus faecium* DSM20477 (kindly provided by Dr. Markus Heimesaat, Institute of Microbiology, Infectious Diseases and Immunology, Charité—Universitätsmedizin Berlin), *Escherichia coli* IMT19224, *Staphylococcus aureus* IMT29828, and *Salmonella enterica* subsp. enterica serovar Typhimurium 4/74, all obtained from the strain collection of the Institute of Microbiology and Epizootics, Freie Universität Berlin. *S.* Typhimurium 4/74 was used to assess agglutinating activity of AsCTL-42 and in epithelial cell invasion assays.

4.3. Radial Diffusion Assay

Bacterial growth inhibition activity of AsCTL-42 was assessed using the radial diffusion assay as described previously [18,20]. Overnight bacterial cultures were diluted 1:100 in Mueller–Hinton broth (Carl Roth, Karlsruhe, Germany) and incubated at 37 °C with shaking at 250 rpm until reaching an optical density of 0.3–0.4 at 600 nm. Bacteria were then centrifuged at 880× *g* for 10 min at 4 °C, washed once, and resuspended with cold sodium phosphate buffer (100 mM, pH 7.4). Bacteria were resuspended in 50 °C sterile underlay agar (10 mM sodium phosphate, 1% (*v*/*v*) Mueller-Hinton broth, 1.5% (*w*/*v*) agar) at 4×10^5 colony forming units (CFU) per mL. Fifteen milliliters of underlay agar were poured into 120 mm square petri dishes. After the agar solidified, evenly spaced wells (5 mm) were formed using the blunt end of P10 pipet tips. Treatments were added to the wells (5 µL/well) and the plates incubated at 37 °C for 3 h before being overlaid with 15 mL of double-strength Mueller–Hinton agar (4.2% (*w*/*v*) Mueller–Hinton broth, 1.5% (*w*/*v*) agar). Petri dishes were incubated at 37 °C for 18 h and growth inhibition zones around each well were measured. Growth inhibition is represented as the diameter of the inhibition zone (mm) beyond the well. PBS and the antimicrobial peptide pexiganan (kindly provided by Prof. Jens Rolff, Institute of Biology, Freie Universität Berlin) were used as negative and positive controls, respectively.

4.4. Cell Culture and Growth Conditions

Porcine intestinal epithelial cells (IPEC-J2 cell line) were cultured as monolayers in DMEM/Ham's F-12 (1:1) medium supplemented with 10% fetal calf serum (both from PAN-Biotech, Aidenbach, Germany) under standard tissue culture conditions (37 °C, 5% CO_2). Experiments were performed within five passages after seeding the original frozen stocks. *Salmonella* invasion assays were performed in the presence of 5 mM $CaCl_2$.

4.5. Cell Viability Testing

For cell viability assays, IPEC-J2 cells were seeded at 5×10^3 cells/well in 96-well tissue culture plates and grown until ~80% confluence prior to treatment. Cells were incubated with PBS (vehicle control), different concentrations of AsCTL-42 diluted in PBS, or 300 µM H_2O_2 (positive control [67]) for 24 h. Viability was assessed using the MTT cell proliferation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Briefly, after 24 h treatments, 10 µL of MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) were added to each well for 4 h followed by overnight solubilization of formazan crystals in the incubator with 100 µL of solubilization solution (10% SDS in 0.01 M HCl). Absorbance was measured in a Biotek Synergy H1 Hybrid microplate reader at 570 nm. Cell viability was calculated by normalizing treatment groups to PBS-treated cells as 100% viability controls. Statistical analyses were performed using GraphPad Prism 9.0.1 to conduct a one-way ANOVA followed by Tukey's multiple comparison tests. *p*-values less than 0.05 were considered significant.

4.6. Glycan Array

The array contained 140 different synthetic glycans (0.2 mM), printed in the lab on *N*-hydroxyl succinimide ester-activated slides as described previously (Table S1) [34].

Glycans were immobilized on slides using a piezoelectric spotting device (S3; Scienion, Berlin, Germany) in a pattern of 16 individual subarrays. After 24 h in a humid chamber at room temperature, the slides were quenched using 50 mM aminoethanol solution (pH 9) for 1 h at 50 °C and a final ddH_2O wash before storage. Next, 16-well microplate holders were assembled onto the slides and each well was blocked with 100 µL of HEPES buffer (50 mM HEPES pH 7.2, 5 mM $CaCl_2$, 5 mM $MgCl_2$) with 1% BSA for 1 h. After washing blocked wells with HEPES buffer without BSA, 75 µL of AsCTL-42 at different concentrations (5, 10, 50, 100, 200 µg/mL) and concanavalin A fluorescein (25 µg/mL, Vector Labs, Burlingame, USA) were added to each well followed by 1 h incubation. Each concentration was tested in duplicates. The wells were washed three times with HEPES buffer + 0.05% Tween and incubated with 75 µL of 6xHis tag monoclonal antibody FITC (1:200, Invitrogen) for 1 h in a dark, humidified chamber. The wells were washed once with HEPES buffer + 0.05% Tween. Then the microplate holder was removed and the whole slide was washed twice with the HEPES buffer + 0.05% Tween and once with the HEPES buffer without detergent. The slide was dried by centrifugation (300× *g*, 3 min) and directly scanned using a Glycan Array Scanner Axon GenePix® 4300A (Molecular Devices, San Jose, CA, USA). Results were analyzed using GenePix Pro7 (Molecular Devices).

4.7. C-Type Lectin Receptor Screening

The generation of the CLR-hFc fusion protein library was described previously [34,68–70]. Treatments were diluted to 10 µg/mL in PBS, then 50 µL (0.5 µg) were added to each well of a medium binding half-area 96-well ELISA plate (Greiner Bio-One, Kremsmünster, Austria). Plates were left overnight at 4 °C. The next day, plates were washed three times with PBS containing 0.05% (*v*/*v*) Tween 20 (PBST) then blocked with the addition of 150 µL of PBS containing 1% (*w*/*v*) BSA for 2 h. After washing, 50 µL (0.25 µg) of CLR-hFc fusion proteins, diluted at 5 µg/mL in either lectin-binding buffer (50 mM HEPES, 5 mM $CaCl_2$, 5 mM $MgCl_2$ (pH 7.4)) or EDTA buffer (50 mM HEPES, 10 mM EDTA (pH 7.4)), was added to each well for 1 h. After washing, the plates were incubated for 1 h with 50 µL of a horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Fcγ fragment specific; Jackson Immunoresearch West Grove, USA) diluted 1:5000 in PBST containing 1% BSA. The enzyme reaction was developed by the addition of 50 µL of o-phenylenediamine dihydrochloride (OPD; Thermo Fisher Scientific), stopped by the addition of 50 µL of 2.5 M H_2SO_4, and the absorbance was read at 495 nm with an ELISA plate reader. Spent LEXSY cultivation medium from *L. tarentolae* was included to rule out contribution from *Leishmania* proteins. AsGH was included as an expression system control. Potential binding with the specified CLR was defined as an OD value greater than four times the OD of hFc negative controls.

4.8. Agglutination Assay

Agglutinating activity of AsCTL-42 was assessed as described previously [18,20], using *S.* Typhimurium strain 4/74. Bacteria grown in Luria–Bertani (LB) medium were collected at mid-logarithmic phase by centrifugation at 880× *g* for 5 min. They were then washed and re-suspended in tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) at approximately 10^9 cells/mL. Twenty microliters of bacterial suspension were mixed with 20 µL treatments (diluted in TBS) with or without added calcium (10 mM $CaCl_2$) and incubated for 1 h at room temperature on a glass slide. Concanavalin A from *Canavalia ensiformis* (Sigma-Aldrich, St. Louis, MO, USA) was included as a positive control. Samples were visualized and photographed using the 40× objective (final 400× magnification) on a Leica DM750 microscope equipped with an ICC50HD digital camera (Leica Microsystems, Wetzlar, Germany).

4.9. Salmonella Invasion Assay

For invasion assays, IPEC-J2 cells were grown to a density of ~5 × 10^4 cells/well in 48-well tissue culture plates and infected at multiplicities of infection (moi) of 1–5.

Salmonella was grown in an LB medium with aeration at 37 °C to late log/early stationary phase (optical density of 2–3 at 600 nm) and collected from 1 mL of culture suspension by centrifugation and resuspended in 1 mL LB medium. Optical density was determined, and dilutions were made to provide the final moi. Treatments were either added at the time of infection or separately to pre-treat host and bacterial cells 30 min prior to infection, as indicated. Cells were infected for 30 min, then the culture medium was changed and supplemented with 50 µg/mL gentamicin (PAN-Biotech) to kill extracellular bacteria and the cells were incubated for 2 h. Cells were then washed twice with PBS and lysed by the addition of 0.1% (*v*/*v*) Triton X-100 in distilled water. Dilutions of the resulting lysates were plated on LB agar plates for the determination of intracellular CFU. Invasion was determined by the ratio of intracellular CFU to the CFU of the original infecting bacterial suspension. Invasion was calculated by normalizing treatment groups to PBS-treated cells as 100% invasion controls. Statistical analyses were performed using GraphPad Prism 9.0.1 to conduct a 1-way ANOVA followed by Tukey's multiple comparison tests. *p*-values less than 0.05 were considered significant.

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Supplementary Material

Lectin-Mediated Bacterial Modulation by the Intestinal Nematode *Ascaris suum*

Ankur Midha, Guillaume Goyette-Desjardins, Felix Goerdeler, Oren Moscovitz, Peter H. Seeberger, Karsten Tedin, Luca D. Bertzbach, Bernd Lepenies, Susanne Hartmann

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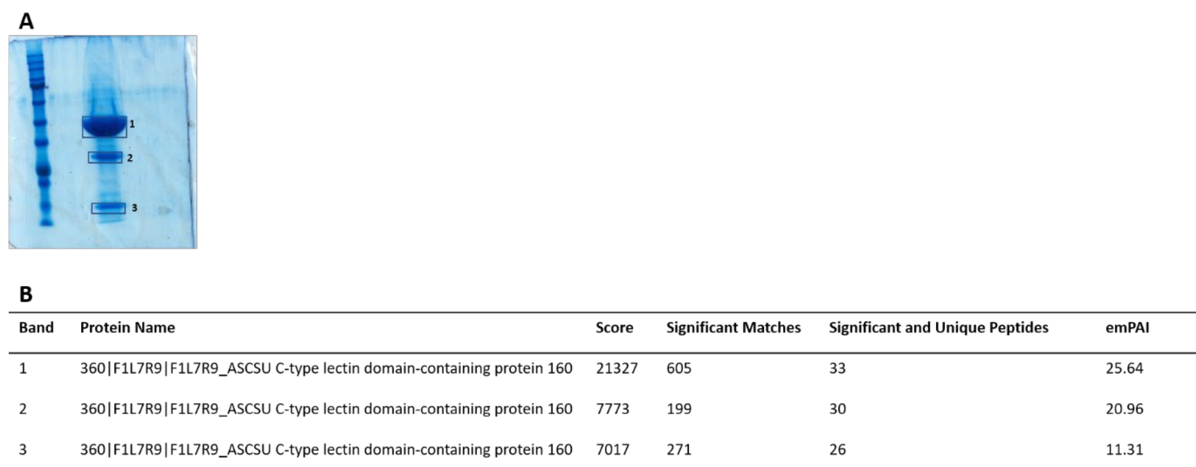
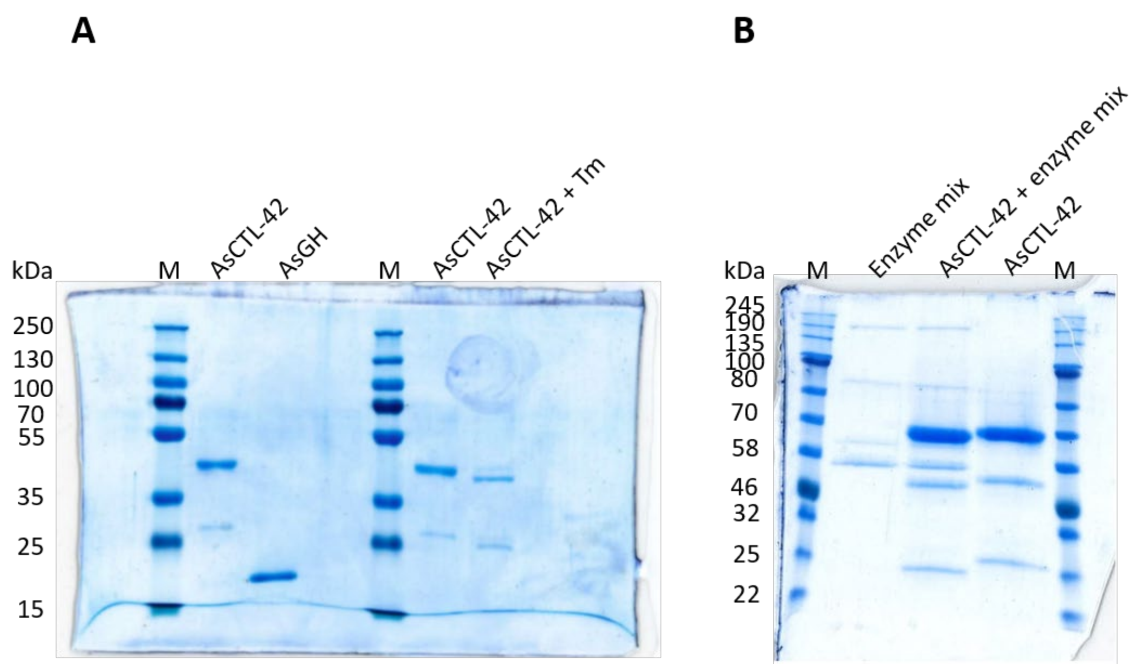
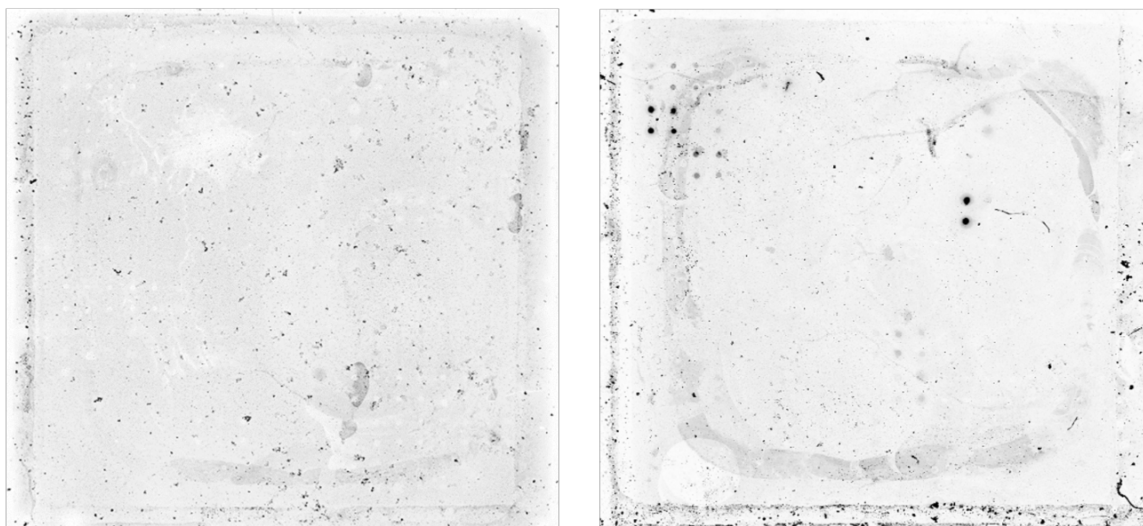


Band	Protein Name	Score	Significant Matches	Significant and Unique Peptides	emPAI
1	360|F1L7R9|F1L7R9_ASCSU C-type lectin domain-containing protein 160	21327	605	33	25.64
2	360|F1L7R9|F1L7R9_ASCSU C-type lectin domain-containing protein 160	7773	199	30	20.96
3	360|F1L7R9|F1L7R9_ASCSU C-type lectin domain-containing protein 160	7017	271	26	11.31

Figure S1 A. Coomassie-stained SDS-PAGE gel of recombinantly expressed AsCTL-42. **B.** Mass spectrometry based-confirmation of protein identity.



Figures S2. Coomassie-stained SDS-PAGE gels of recombinantly expressed *Ascaris suum* proteins and glycosylation patterns. Full gel images from Figure 1. **A.** 1 µg of protein loaded onto 12% SDS-polyacrylamide gels, stained with Coomassie G-250 dye. **B.** Leishmania tarentolae were cultured in the presence (right; AsCTL-42 + Tm) or absence (left; AsCTL-42) of tunicamycin (10 µg/mL). 1 µg of protein loaded onto 12% SDS-polyacrylamide gels, stained with Coomassie G-250 dye. (C) AsCTL-42 was treated with a protein deglycosylation enzyme mixture and the products of this reaction were loaded onto 14% SDS-polyacrylamide gels, stained with Coomassie G-250 dye.



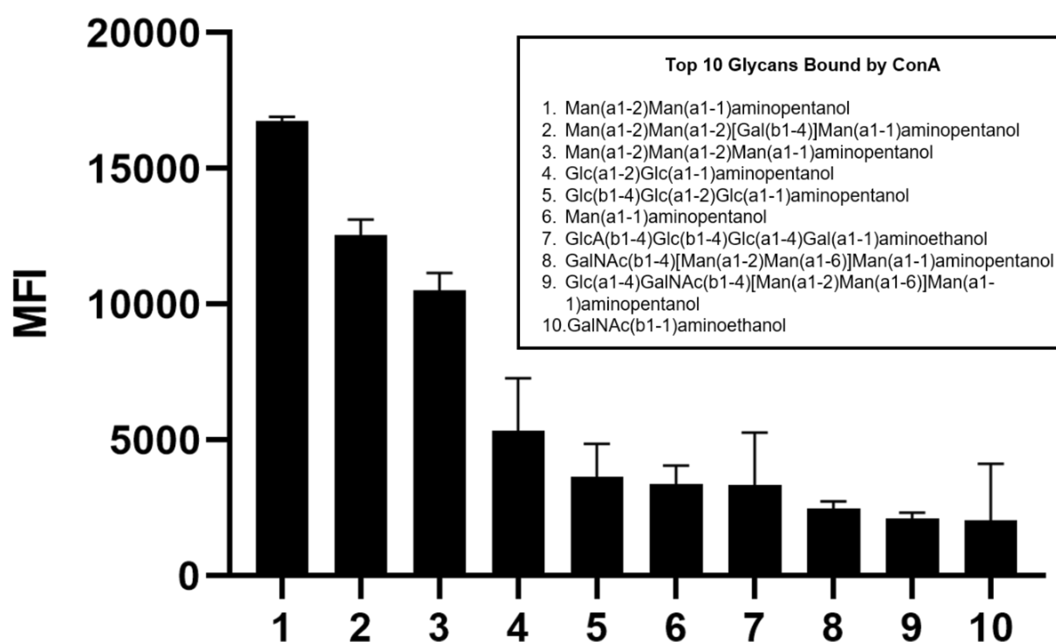


Figure S3. Binding of AsCTL-42 and Concanavalin A (ConA) to compounds on a glycan array. **A.** Representative field images of glycan arrays. **B.** Bar chart of the top ten glycans bound by ConA. Each value is the mean ± SEM of two spots from a glycan array experiment. Inset shows the top ten glycans by name.

Table S1. AsCTL-42 impairs porcine epithelial cell invasion by *Salmonella*.[1]

	Experiment 1		**Experiment 2**		**Experiment 3**	
	Inoculum (Mean CFU)	**Number of Epithelial Cells/Well**	**Inoculum (Mean CFU)**	**Number of Epithelial Cells/Well**	**Inoculum (Mean CFU)**	**Number of Epithelial Cells/Well**
	179,000	50,000	82,000	50,000	121,667	50,000
	Multiplicity of Infection (moi) = 4		**Multiplicity of Infection (moi)** = 2		**Multiplicity of Infection (moi)** = 2	
Treatment	**Mean (CFU/mL)**	**% Invasion Rel. to Control**	**Mean (CFU/mL)**	**% Invasion Rel. to Control**	**Mean (CFU/mL)**	**% Invasion Rel. to Control**
AsCTL-42 0 µg/mL	12,600	100	48,133	100	9,800	100
AsCTL-42 100 µg/mL	8,467	67	36,733	76	6,333	65
AsCTL-42 500 µg/mL	6,607	48	29,333	61	4,733	48
AsCTL-42 100 µg/mL + IPEC pre-treatment	14,533	115	51,733	107	8,600	88
AsCTL-42 500 µg/mL + IPEC pre-treatment	14,000	111	47,400	98	8,333	85
AsCTL-42 100 µg/mL + ST pre-treatment	8,400	67	29,922	62	5,667	58
AsCTL-42 500 µg/mL + ST pre-treatment	2,800	22	19,000	39	3,000	31

[1]Data presented here as CFU counts from the individual experiments collated in Figure 3.

Table S2. Synthetic glycans tested for AsCTL-42-glycan binding in glycan array[1].

Glycan	Microbial origin (if applicable)	Mean Fluorescence Intensity (MFI) for ConA[2]
Neu5Ac(a2-6)Gal(b1-4)GlcNAc(b1-3)Gal(b1-4)Glc(b1-1)aminohexanol		337,5
Neu5Ac(a2-3)Gal(b1-3)GlcNAc(b1-3)Gal(b1-4)Glc(b1-1)aminohexanol		224
Fuc(a1-3)[Neu5Ac(a2-3)Gal(b1-4)]GlcNAc(b1-3)Gal(b1-4)Glc(b1-1)aminohexanol		0
Neu5Ac(a2-6)Gal(b1-4)Glc(b1-1)aminohexanol		188,5
Neu5Ac(a2-3)Gal(b1-4)Glc(b1-1)aminohexanol		251,5
Neu5Ac(a2-6)Gal(b1-4)GlcNAc-6-sulfate(b1-1)aminohexanol		0
Gal(b1-4)Glc(b1-1)aminohexano		1116,5
Gal(b1-4)GlcNAc-6-sulfate(b1-1)aminohexanol		0
Araf(a1-5)Araf(a1-1)aminopentanol	*Mycobacterium tuberculosis*	1628,5
Araf(a1-5)Araf(a1-3)[Araf(a1-5)Araf(a1-5)]Araf(a1-5)Araf(a1-1)aminopentanol	*Mycobacterium tubercolosis*	1807
Araf(a1-3)[Araf(a1-5)]Araf(a1-1)aminopentanol	*Mycobacterium tuberculosis*	1815,5
Araf(a1-5)Araf(a1-5)Araf(a1-5)Araf(a1-5)Araf(a1-5)Araf(a1-5)aminopentanol	*Mycobacterium tuberculosis*	1890
Col(a1-3)[Col(a1-6)]Glc(a1-4)Gal(a1-3)GlcNAc(b1-1)aminopentanol	*Escherichia coli* 0111	367
ManNAc(b1-3)FucNAc(a1-3)GalNAc(a1-4)Gal(a1-1)aminopentanol	*Streptococcus pneumoniae*	208,5
GalNAc(a1-4)Gal(a1-1)aminopentanol	*Streptococcus pneumoniae*	1649
GalNAc(b1-4)Gal(a1-1)aminopentanol		1492,5
FucNAc(a1-3)GalNAc(a1-4)Gal(a1-1)aminopentanol	*Streptococcus pneumoniae*	244
FucNAc(b1-3)GalNAc(a1-4)Gal(a1-1)aminopentanol		250

GalNAc(b1-1)aminoethanol		2051,5
FucNAc(a1-1)aminopentanol		622
Man(a1-2)Man(a1-2)[Gal(b1-4)]Man(a1- 1)aminopentanol	*Leishmania donovani*	12566
Man(a1-2)Man(a1-2)Man(a1-1)aminopentanol	*Leishmania donovani*	10525,5
Gal(b1-4)Man(a1-1)aminopentanol	*Leishmania chagasi*	1940
Man(a1-2)Man(a1-1)aminopentanol		16754
Glc(b1-1)aminoethanol		0
GlcNAc(a1-2)Hep(a1-3)Hep(a1-5)Kdo(a2-1)aminopentanol	*Neisseria meningitidis*	491
Hep(a1-3)Hep(a1-5)Kdo(a2-1)aminopentanol	*Neisseria meningitidis*	539,5
Hep(a1-3)Hep(a1-5)[L-Ara4N(b1-8)]Kdo(a2-1)aminopentanol	*Proteus spp.*	216,5
Hep(a1-7)Hep(a1-3)Hep(a1-5)Kdo(a2-1)aminopentanol	*Yersinia pesti*	168,5
Hep(a1-2)Hep(a1-3)Hep(a1-5)Kdo(a2-1)aminopentanol	*Haemophilus influenzae*	695
Hep(a1-5)Kdo(a2-1)aminopentanol		879
Hep(a1-7)Hep(a1-3)Hep(a1-1)aminopentanol	*Yersinia pesti*	437,5
Kdo(a2-8)Kdo(a2-4)Kdo(a2-1)aminopentanol	*Chlamydia spp.*	551
Kdo(a2-1)aminopentanol		103,5
Hep(a1-1)aminopentanol		866,5
Glc(b1-1)aminopentanol		344
D-FucNAc(b1-1)aminopentanol		620,5
FucNAc(b1-1)aminopentanol		296
Glc(b1-3)D-FucNAc(b1-1)aminopentanol	*Pseudomonas aeruginosa*	367,5
Glc(b1-3)FucNAc(b1-1)aminopentanol	*Pseudomonas aeruginosa*	635,5
Gal(b1-3)GalNAc(a1-1)aminopentanol		1080,5
Fuc(a1-3)[Gal(b1-4)]GlcNAc(b1-1)aminopentanol		14,5
Neu5Ac(a2-6)GalNAc(a1-1)aminopentanol		118,5
Gal(b1-4)[Gal(b1-4)Glc(b1-6)]GlcNAc(b1- 1)aminopentanol	*Streptococcus pneumoniae*	555
Fuc(a1-3)[Fuc(a1-2)Gal(b1-4)]GlcNAc(b1-1)aminopentanol		457
Gal(b1-3)[Fuc(a1-4)]GlcNAc(b1-1)aminopentanol		341

Fuc(a1-2)Gal(b1-3)[Fuc(a1-4)]GlcNAc(b1-1)aminopentanol		0
Gal-2,3-Pyruvate(a1-1)aminopentanol (mixture of R/S pyruvate)	*Streptococcus pneumoniae*	239,5
Gal(a1-3)Gal(b1-4)Glc(b1-1)aminopentanol		248,5
Gal(a1-3)Gal(b1-4)GlcNAc(b1-1)aminopentanol		117,5
Gal(a1-3)Gal(b1-4)GlcNAc(b1-3)Gal(b1-4)Glc(b1-1)aminopentanol		0
Gal(b1-4)GlcNAc(b1-3)Gal(b1-4)Glc(b1- 1)aminopentanol	*Streptococcus pneumoniae*	0
Fuc(a1-2)Gal(b1-3)GlcNAc(b1-3)Gal(b1-4)Glc(b1-1)aminopentanol		1767
6 Gal(b1-3)GlcNAc(b1-3)Gal(b1-4)Glc(b1-1)aminopentanol		303,5
Rha(a1-1)aminopentanol		0
Rha(a1-3)Glc(b1-1)aminopentanol		1504,5
Glc(a1-2)Glc(a1-1)aminopentanol	*Clostridium difficile*	5359
Glc(b1-4)Glc(a1-2)Glc(a1-1)aminopentanol	*Clostridium difficile*	3637
Rha(a1-3)Glc(b1-4)Glc(a1-1)aminopentanol	*Clostridium difficile*	636,5
Gal(b1-3)GalNAc(b1-3)Gal(a1-4)Gal(b1-4)Glc(b1-1)aminopentanol		1205,5
Neu5Ac(a2-8)Neu5Ac(a2-3)[GalNAc(b1-4)]Gal(b1-4)Glc(b1-1)aminopentanol		162
Gal(a1-4)Gal(b1-4)Glc(b1-1)aminopentanol		487,5
GalNAc(a1-1)AminoLinker2		989
Fuc(a1-3)[Gal(b1-4)]GlcNAc(b1-1)AminoLinker2		735
GlcNAc(a1-2)Hep(a1-3)Hep(a1-1)aminopentanol	*Neisseria meningitidis*	558,5
Hep(a1-3)Hep(a1-1)aminopentanol		267,5
Gal(b1-4)Glc(b1-1)aminopentanol		0
GalNAc(b1-4)Gal(b1-4)Glc(b1-1)aminopentanol		1193,5
Neu5Ac(a2-3)Gal(b1-4)Glc(b1-1)aminopentanol		127,5
GalNAc-4-sulfate(b1-1)aminopentanol		19
IdoA-2,4-disulfate(a1-1)aminopentanol		528
IdoA(a1-3)GalNAc-4-sulfate(b1-1)aminopentanol		237
IdoA-2-sulfate(a1-3)GalNAc-4-sulfate(b1-1)aminopentanol		262

IdoA(a1-3)GalNAc(b1-1)aminopentanol		210,5
GlcA(b1-4)Glc(b1-3)GlcA(b1-4)Glc(b1-1)aminoethanol	*Streptococcus pneumoniae*	378
Glc(b1-3)GlcA(b1-4)Glc(b1-1)aminoethanol	*Streptococcus pneumoniae*	1456
GalNAc(a1-1)Thr-Linker		10,5
Glc(b1-3)Glc(b1-3)[Glc(b1-6)]Glc(b1-3)Glc(b1-1)aminopentanol	*Candida spp.*	132,5
Glc(b1-3)Glc(b1-3)[Glc(b1-6)]Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-1)aminopentanol	*Candida spp.*	236
Glc(b1-3)Glc(b1-3)[Glc(b1-6)]Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-1)aminopentanol	*Candida spp.*	100,5
Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-3)Glc(b1-1)aminopentanol	*Candida spp.*	127
L-PneNAc(a1-2)GlcA(b1-3)FucNAc(a1-3)D-FucNAc(b1-1)aminopentanol	*Streptococcus pneumoniae*	24,5
Mixture of: D-6d-xylHexpNAc-4-ulo(b1- 1)aminopentanol (Sugp(b1-1)aminopentanol) and D- FucNAc(b1-1)aminopentanol	*Streptococcus pneumoniae*	845,5
Mixture of: FucNAc(a1-3)D-6d-xylHexpNAc-4-ulo(b1-1)aminopentanol and FucNAc(a1-3)D-FucNAc(b1-1)aminopentanol	*Streptococcus pneumoniae*	164
FucNAc(a1-3)D-FucNAc(b1-1)aminopentanol	*Streptococcus pneumoniae*	301
GlcA(b1-4)FucNAc(a1-1)aminopentanol	*Streptococcus pneumoniae*	336
Glc(b1-3)FucNAc(a1-1)aminopentanol	*Streptococcus pneumoniae*	324,5
L-PneNAc(a1-2)GlcA(b1-1)aminopentanol	*Streptococcus pneumoniae*	670
L-PneNAc(a1-1)aminopentanol	*Streptococcus pneumoniae*	279,5
L-PneNAc(b1-1)aminopentanol	*Streptococcus pneumoniae*	276,5
Gal(b1-4)[Glc(b1-6)]GlcNAc(b1-3)Gal(b1- 1)aminopentanol	*Streptococcus pneumoniae*	289,5

Glc(a1-4)Gal(a1-4)GlcA(b1-4)Glc(b1-1)aminoethanol	*Streptococcus pneumoniae*	1735,5
Glc(a1-4)Gal(a1-1)aminoethanol	*Streptococcus pneumoniae*	919,5
GlcA(b1-4)Glc(b1-4)Glc(a1-4)Gal(a1-1)aminoethanol	*Streptococcus pneumoniae*	3351
Glc(a1-4)Gal(a1-4)GlcA(b1-4)Glc(b1-1)aminopentanol	*Streptococcus pneumoniae*	836,5
Gal(a1-4)GlcA(b1-4)Glc(b1-4)Glc(a1-1)aminopentanol	*Streptococcus pneumoniae*	60
GlcA(b1-4)Glc(b1-4)Glc(a1-4)Gal(a1-1)aminopentanol	*Streptococcus pneumoniae*	567
Glc(b1-4)Glc(a1-4)Gal(a1-4)GlcA(b1-1)aminopentanol	*Streptococcus pneumoniae*	511,5
Xyl(b1-4)Xyl(b1-1)aminopentanol		642
Xyl(b1-4)Xyl(b1-4)Xyl(b1-4)Xyl(b1-1)aminopentanol		4,5
Xyl(b1-4)Xyl(b1-4)Xyl(b1-4)Xyl(b1-4)Xyl(b1-4)Xyl(b1-1)aminopentanol		0
Xyl(b1-4)Xyl(b1-4)Xyl(b1-4)Xyl(b1-4)Xyl(b1-4)Xyl(b1-4)Xyl(b1-4)Xyl(b1-1)aminopentanol		0
Glc(b1-4)Glc(b1-4)Glc(b1-4)Glc(b1-1)aminopentanol		85
Glc(b1-3)GlcA(b1-4)Glc(b1-1)aminopentanol	*Streptococcus pneumoniae*	1175
GlcA(b1-4)Glc(b1-1)aminoethanol	*Streptococcus pneumoniae*	402,5
Glc(b1-3)GlcA(b1-1)aminoethanol	*Streptococcus pneumoniae*	282,5
ManNAc(b1-3)FucNAc(a1-3)GalNAc(a1-4)Gal-2,3-pyruvate(a1-1)aminopentanol	*Streptococcus pneumoniae*	134
GlcA(b1-1)aminoethanol		1185,5
Glc(a1-4)GalNAc(b1-4)Man(a1-1)aminopentanol	*Toxoplasma gondii*	436
Glc(a1-4)GalNAc(b1-4)[Man(a1-2)Man(a1-6)]Man(a1-1)aminopentanol	*Toxoplasma gondii*	2121,5
Glc(a1-4)GalNAc(b1-4)[Man-6-PEtN(a1-2)Man(a1-6)]Man(a1-1)aminopentanol	*Toxoplasma gondii*	1237,5
GalNAc(b1-4)Man(a1-1)aminopentanol		447,5

GalNAc(b1-4)[Man-6-PEtN(a1-2)Man(a1-6)]Man(a1-1)aminopentanol		804
GalNAc(b1-4)[Man(a1-2)Man(a1-6)]Man(a1-1)aminopentanol		2487
GalNAc(b1-4)[Man-6-PEtN(a1-2)Man(a1-6)]Man-2-PEtN(a1-1)aminopentanol		784
GalNAc(b1-4)Man(a1-1)aminododecanol		163,5
GalNAc(b1-4)Man(a1-1)p-aminocyclohexanol		0
GlcA(b1-4)Glc(b1-3)GlcA(b1-1)aminoethanol	*Streptococcus pneumoniae*	19
GlcA(b1-4)Glc(b1-3)Glc(b1-4)Glc(b1-1)aminoethanol and/or Glc(b1-4)Glc(b1-3)GlcA(b1-4)Glc(b1-1)aminoethanol		60,5
Man(a1-1)aminopentanol		3367,5
GlcNAc-6-P-phosphoaminopentanol(a1-3)GlcNAc-6-P-phosphoaminopentanol(a1-2)glyceric acid	*Clostridium difficile*	283
GlcA(a1-3)Gal(a1-3)ManNAc(b1-4)Glc(b1-4)Glc(a1-1)aminopentanol	*Streptococcus pneumoniae*	459
GlcA(a1-3)Gal(a1-3)ManNAc-6-acetate(b1-4)Glc(b1-4)Glc(a1-1)aminopentanol	*Streptococcus pneumoniae*	0
GlcA(a1-3)Gal(a1-1)aminopentanol	*Streptococcus pneumoniae*	425,5
Glc(b1-4)Glc(a1-1)aminopentanol		1195,5
ManNAc(b1-4)Glc(b1-4)Glc(a1-1)aminopentanol	*Streptococcus pneumoniae*	208,5
GalNAc(b1-3)GalNAc(b1-1)aminopentanol		80
Glc(b1-4)Gal(b1-4)Glc(b1-1)aminopentanol		116,5
Rha(a1-3)[Rha(a1-3)Glc(b1-4)]Glc(a1-2)Glc(a1-1)aminopentanol	*Clostridium difficile*	893
GlcNAc(a1-3)GlcNAc-6-P-phosphoaminopentanol(a1-2)glyceric acid	*Clostridium difficile*	175
GlcNAc(a1-3)GlcNAc[(a1-2)glyceric acid](6-P-6)GlcNAc(a1-3)GlcNAc-6-P-phosphoaminopentanol(a1-2)glyceric acid	*Clostridium difficile*	1288
Man(a1-2)Man(a1-2)[Gal(b1-4)]Man(a1- 1)aminoethanol	*Leishmania donovani*	1910,5
Glc(b1-3)Gal(b1-4)Man(a1-1)aminopentanol	*Leishmania chagasi*	304
Rha(a1-2)Rha(a1-2)Rha(a1-1)aminopentanol	*Klebsiella pneumoniae*	1507,5

GalNAc-2,3-0xazolidinone(a1-4)GalNAc-2,3-0xazolidinone(a1-1)aminopentanol		273
Glc(a1-2)Glc(a1-3)[FucNAc(a1-3)GalNAc(b1-4)]ManNAcA(b1-1)aminopentanol	*Streptococcus pneumoniae*	1078,5
Glc(a1-2)Glc(a1-3)[Gal(a1-3)FucNAc(a1-3)GalNAc(b1-4)]ManNAcA(b1-1)aminopentanol	*Streptococcus pneumoniae*	15
Araf(a1-3)[Araf(a1-5)]Araf(a1-5)Araf(a1- 1)aminopentanol	*Mycobacterium tuberculosis*	1706,5
Man(a1-5)Araf(a1-3)[Man(a1-5)Araf(a1-5)]Araf(a1-5)Araf(a1-1)aminopentanol	*Mycobacterium tuberculosis*	1593,5
GalNAc-3,4-diacetate(a1-4)GalNAc-3-acetate(a1-4)GalNAc-3-acetate(a1-4)GalNAc-3-acetate(a1-1)aminopentanol		300,5

[1]Glycan and microbial origin data for this table retrieved from supplemental material in ref [27].

[2]Concanavalin A (ConA) was used as a positive control in this glycan array. 0nly values for ConA are shown as no signals were detected for AsCTL-42 (see Fig. S3A).

4. Guts within guts: the microbiome of the intestinal helminth parasite Ascaris suum is derived but distinct from its host

Ankur Midha, Víctor Hugo Jarquín-Díaz, Friederike Ebner, Ulrike Löber, Rima Hayani, Arkadi Kundik, Alessio Cardilli, Emanuel Heitlinger, Sofia Kirke Forslund, Susanne Hartmann

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4.1 Author Contributions

AM, FE, and SH conceived of and designed the experiments. AM and FE performed the experiments. VHJD performed the library preparation. UL and VHJD processed sequence data. VHJD, AC, EH, and SKF carried out data analysis. VHJD carried out data visualization. AM and VHJD wrote the manuscript. RH and AK prepared larval material and assessed for the presence of an inherited larval microbiome. EH, SKF, and SH supervised the work. SH acquired financial funding for the project and reviewed and edited the manuscript. FE, UL, EH, and SKF reviewed and edited the manuscript. The authors read and approved the final manuscript. AM and VHJD contributed equally to this work.

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Guts within guts: the microbiome of the intestinal helminth parasite *Ascaris suum* is derived but distinct from its host

Ankur Midha[1†], Víctor Hugo Jarquín-Díaz[2,3,4,5,6†], Friederike Ebner[1], Ulrike Löber[2,3,4], Rima Hayani[1], Arkadi Kundik[1], Alessio Cardilli[2,3,4], Emanuel Heitlinger[5,6*], Sofia Kirke Forslund[2,3,4,7,8,9*] and Susanne Hartmann[1*]

Abstract

Background: Intestinal helminths are extremely prevalent among humans and animals. In particular, intestinal roundworms affect more than 1 billion people around the globe and are a major issue in animal husbandry. These pathogens live in intimate contact with the host gut microbiota and harbor bacteria within their own intestines. Knowledge of the bacterial host microbiome at the site of infection is limited, and data on the parasite microbiome is, to the best of our knowledge, non-existent.

Results: The intestinal microbiome of the natural parasite and zoonotic macropathogen, *Ascaris suum* was analyzed in contrast to the diversity and composition of the infected host gut. 16S sequencing of the parasite intestine and host intestinal compartments showed that the parasite gut has a significantly less diverse microbiome than its host, and the host gut exhibits a reduced microbiome diversity at the site of parasite infection in the jejunum. While the host's microbiome composition at the site of infection significantly determines the microbiome composition of its parasite, microbial signatures differentiate the nematodes from their hosts as the *Ascaris* intestine supports the growth of microbes that are otherwise under-represented in the host gut.

Conclusion: Our data clearly indicate that a nematode infection reduces the microbiome diversity of the host gut, and that the nematode gut represents a selective bacterial niche harboring bacteria that are derived but distinct from the host gut.

Introduction

The gastrointestinal ecosystem contains a diverse community of viral, prokaryotic (bacteria & archaea), and eukaryotic (helminths & protozoa) components, the latter being recognized mainly as parasites. Understanding host-parasite interactions in this complex environment requires knowledge on the dynamics between these community members. Bacteria and parasites share the same environment in the gut in which they alter host physiology and metabolism and at the same time provide crucial signals for the development and function of the host intestinal immune system [1–3]. Intestinal nematode infections are extremely widespread in humans as well as companion animals, livestock, and wildlife. Studies

[†]Ankur Midha and Víctor Hugo Jarquín-Díaz contributed equally to this work.

*Correspondence: emanuel.heitlinger@hu-berlin.de; Sofia.Forslund@mdc-berlin.de; Susanne.hartmann@fu-berlin.de

[1] Department of Veterinary Medicine, Center for Infection Medicine, Institute of Immunology, Freie Universität Berlin, Robert-von-Ostertag-Straße 7, 14163 Berlin, Germany
[6] Research Group Ecology and Evolution of Molecular Parasite-Host Interactions, Leibniz-Institute for Zoo and Wildlife Research (IZW), Alfred-Kowalke-Straße 17, 10315 Berlin, Germany
[9] DZHK (German Centre for Cardiovascular Research), Partner Site Berlin, Berlin, Germany
Full list of author information is available at the end of the article

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suggest that helminths may modify host-associated bacterial communities to modulate host immunity to promote their own successful establishment in the gut [4, 5]. Despite the close coexistence of helminths with numerous microbes, little is known concerning the reciprocal interactions of intestinal helminths with the microbiota and underlying mechanisms, and in particular, nothing is known about how parasite-associated microbiomes interact with the host microbiome and the host itself.

Several studies report alterations in the gut microbial composition of experimental and naturally helminth-infected murine, human, porcine, and other hosts [6, 7]; however, the consequences of these alterations are not elucidated. Dheilly et al. [8] proposed that parasites might benefit from modifications of host-associated microbiomes, which leads to immune modulation that reduces the resistance to infection. We showed earlier that infections with the murine nematode *Heligmosomoides polygyrus* alter the composition of the host-gut microbiota, and that the nematodes benefit from microbiota-induced immunomodulation [9, 10]. Others have also shown that alterations in bacterial composition during murine *H. polygyrus* and *Nippostrongylus brasiliensis* infection led to the induction of regulatory immune responses [11, 12], while increased short-chain fatty acid (SCFA) production was observed during numerous helminth infections, including in *Ascaris suum*-infected pigs [11]. Hence, microbes and their metabolites are involved in shaping the adaptive immune response directed against nematodes [10, 11]. Notably, intestinal nematodes also have a gut and are themselves colonized by bacteria [13, 14]; knowledge of the microbial inhabitants of intestinal helminths and their symbiotic and antagonistic relationships, however, remains elusive, despite recognition of parasite microbiomes as a key research target [15, 16]. There is an intimate trilateral interaction between intestinal nematodes, their microbial environment, and host cells [17], but almost nothing is known regarding parasite-associated microbiomes, and there is currently no human-relevant parasite microbiome available.

Ascaris is one of the most common and widespread intestinal parasites in humans and livestock. In tropical countries, the prevalence exceeds 10% of the population, causing around 60,000 deaths per year, malnutrition, and developmental deficits in children [18–21]. In pig husbandry, *Ascaris* leads to significant economic losses due to reduced feed conversion and liver condemnation at slaughter [22]. Although *Ascaris* exhibits a tissue migratory phase, it spends most of its lifetime in the gut sharing its environment with host-associated microbes that might present infectious threats or be beneficial by providing key nutrients, protecting against infections [23], promoting fecundity, or modulating host responses against *Ascaris* [17]. While there is some knowledge on alterations of the fecal microbiome of *Ascaris*-infected humans [24–26] as well as the porcine colonic and fecal microbiome during *Ascaris* infection [27, 28], the microbiome of *Ascaris* itself has not yet been studied. *Ascaris* produces various antimicrobial proteins and peptides which likely shape the microbiome in the immediate vicinity of, and within, the nematode itself [29]. Further insights into parasite-associated microbiomes could unveil novel strategies to control helminth infections [30]. Thus, we aimed to unravel the parasite microbiome and its interdependence with the host microbiome in which it exists. Our study indicates for the first time that a parasitic nematode's microbiome is derived from microbes in its immediate vicinity but distinct in composition from the microbiome of the host.

Methods

Animals, infection trials, sampling, and DNA extraction

Intestinal content and worm samples were derived from two independent *A. suum* infection trials as well as non-infected animals. Infective *A. suum* eggs were collected and prepared as previously described [29]. In brief, slaughterhouse-derived adult female worms were cultured overnight, and released eggs were collected from culture fluid, washed, and incubated at room temperature in the dark for 6–8 weeks until > 90% embryonation rates were observed.

German Landrace pigs (*Sus scrofa*) from a conventional breeder aged 6 weeks were kept in separate groups and orally infected with 2000 (exp. 1) or 4000 (exp. 2) embryonated *A. suum* eggs/pig.

At 56 days post infection (DPI), pigs were sedated using ketamine hydrochloride and azaperone (20 mg/kg body weight [BW]; Ursotamin; Serumwerk Bernburg AG and 2 mg/kg BW; Stresnil; Janssen-Cilag GmbH, Germany) and euthanized by intracardial injection of T61 (10 mg/kg BW of tetracaine hydrochloride, mebezonium iodide, and embutramide, Intervet, Germany).

Luminal content samples were collected from several intestinal regions (duodenum, jejunum, ileum, cecum, colon) and snap-frozen in liquid nitrogen before being transferred to −80 °C until further processing. To assess the worm burden and the *A. suum* microbiome, adult worms were collected from the entire gut, counted, and morphologically separated by sex (Fig. 1A). A subset was then dissected after washing in 0.9% NaCl; their intestines harvested and snap-frozen in liquid nitrogen before storage at −80°C. Samples were homogenized with the MP FastPrep-24 homogenizer (MP Biomedicals, Eschwege, Germany), and DNA was extracted using the Macherey-Nagel NucleoSpin soil DNA extraction kit

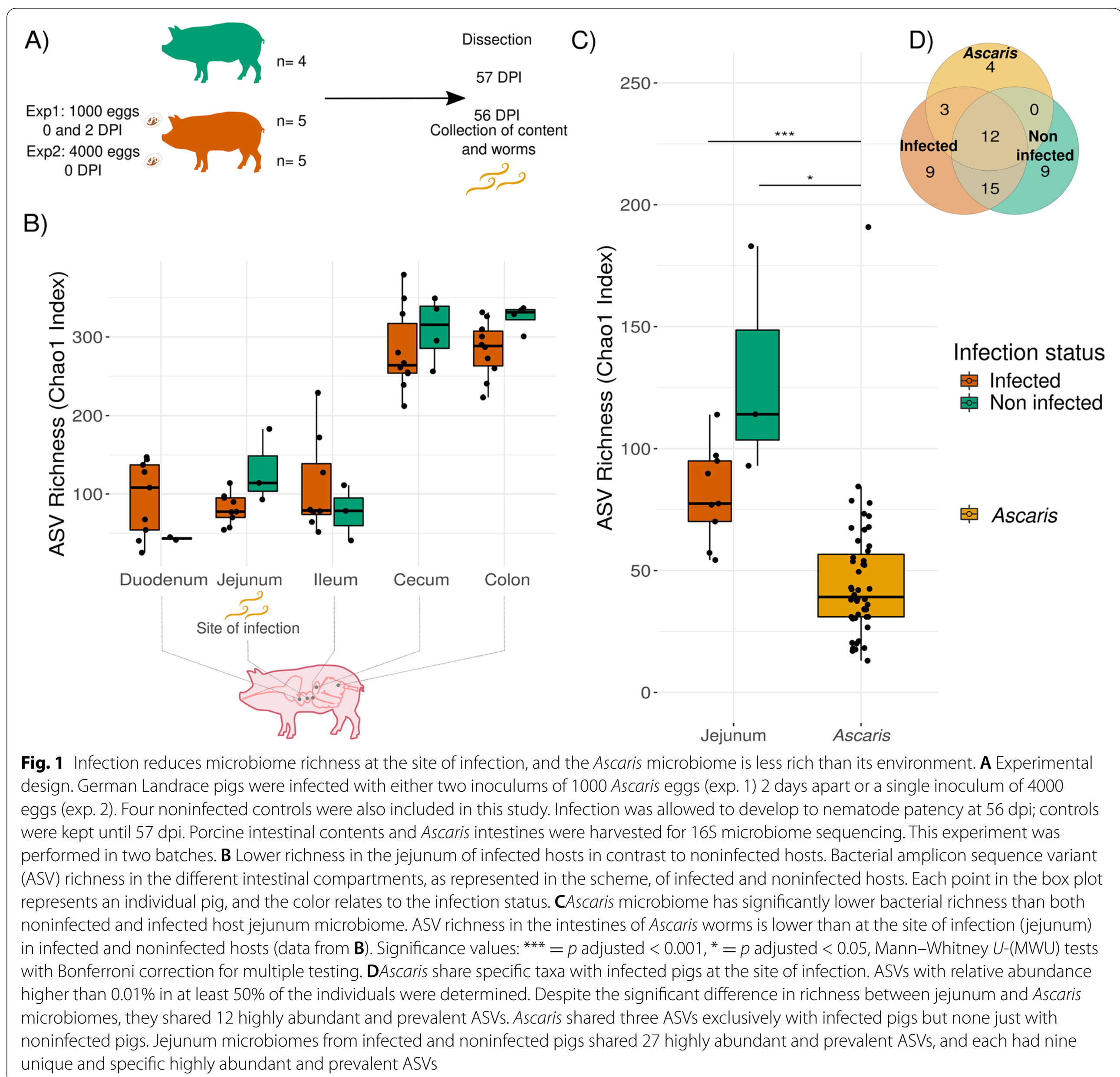


Fig. 1 Infection reduces microbiome richness at the site of infection, and the *Ascaris* microbiome is less rich than its environment. **A** Experimental design. German Landrace pigs were infected with either two inoculums of 1000 *Ascaris* eggs (exp. 1) 2 days apart or a single inoculum of 4000 eggs (exp. 2). Four noninfected controls were also included in this study. Infection was allowed to develop to nematode patency at 56 dpi; controls were kept until 57 dpi. Porcine intestinal contents and *Ascaris* intestines were harvested for 16S microbiome sequencing. This experiment was performed in two batches. **B** Lower richness in the jejunum of infected hosts in contrast to noninfected hosts. Bacterial amplicon sequence variant (ASV) richness in the different intestinal compartments, as represented in the scheme, of infected and noninfected hosts. Each point in the box plot represents an individual pig, and the color relates to the infection status. **C** *Ascaris* microbiome has significantly lower bacterial richness than both noninfected and infected host jejunum microbiome. ASV richness in the intestines of *Ascaris* worms is lower than at the site of infection (jejunum) in infected and noninfected hosts (data from **B**). Significance values: *** = p adjusted < 0.001, * = p adjusted < 0.05, Mann–Whitney U-(MWU) tests with Bonferroni correction for multiple testing. **D** *Ascaris* share specific taxa with infected pigs at the site of infection. ASVs with relative abundance higher than 0.01% in at least 50% of the individuals were determined. Despite the significant difference in richness between jejunum and *Ascaris* microbiomes, they shared 12 highly abundant and prevalent ASVs. *Ascaris* shared three ASVs exclusively with infected pigs but none just with noninfected pigs. Jejunum microbiomes from infected and noninfected pigs shared 27 highly abundant and prevalent ASVs, and each had nine unique and specific highly abundant and prevalent ASVs

(Macherey-Nagel, Düren, Germany) according to manufacturer's instructions.

To assess for the presence of an inherited microbiome in larvae, embryonated eggs were hatched in vitro by mechanical disruption using 5 mm glass beads and shaking. Viable larvae were purified and separated from unhatched eggs by allowing them to migrate through a cell strainer. DNA was extracted from four independent batches of *A. suum* larvae (50,000 larvae per sample) using the Macherey-Nagel NucleoSpin soil DNA extraction kit as above, with the addition of 300 g of washed and autoclaved sand (60.08 g/mol).

To estimate bacterial load in hatched L3 compared to gastrointestinal content and *Ascaris* adults, qPCR was performed using the Applied Biosystems QuantStudio 3 system (Thermo Fisher Scientific, Darmstadt, Germany) as described before [31]. In brief, amplification and detection were performed in 96-well optical plates (Applied Biosystems) with SYBR-Green (Applied Biosystems). All amplifications were performed in triplicates in a final volume of 10 μL containing 5 μL of a 10× SYBR Green PCR Master Mix including ROX as a passive reference (Applied Biosystems), 10 μM of each primer (Univ 337 F 5′-ACTCCTACGGGAGGCAGCAGT-3′ and Univ

518 R 5′-GTATTACCGCGGCTGCTGGCAC-3′), and 1.2 μL of template DNA (1.5 μg/μL).

For amplification, the standard protocol of the Applied Biosystems QuantStudio 3 system was followed, i.e., an initial cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 1 min at 60 °C. To check for specificity, melting curve (Tm) analysis was performed, increasing the temperature from 60 to 95 °C at a rate of 0.2 °C per second with the continuous monitoring of fluorescence.

Standard curves for quantification consisted of 10-fold serial dilutions in the range of 10^9–10^2 copies of the 16S rRNA gene of the *E. coli* (Invitrogen, C404010) amplified with primers 27 F (5′-GTTTGATCCTGGCTCAG-3′) and 1492 R (5′-CGGCTACCTTGTTACGAC-3′). Copy numbers per ng DNA were calculated for each. Additionally, DNA samples from larvae were subjected to the two-step PCR method for library preparation described below.

Library preparation and sequencing

DNA extracted from pig gastrointestinal (GI) tract contents and from *Ascaris* intestines were subjected to PCR amplification of the V3-V4 (~460 bp) hypervariable region of the 16S rRNA gene. The primers of Klindworth et al. [32] were modified to contain universal adaptor sequences for later addition of indexing barcodes as follows: forward Klin0341-19: [***ACACTGACGACATGGTTCTACA***]CCTACGGGNGGCWGCAG and reverse Klin0785_CR: [***TACGGTAGCAGAGACTTGGTCT***]GACTACHVGGGTATCTAATCC.

PCR target-specific amplification was performed with the S7 Fusion High-Fidelity DNA polymerase (Biozym Scientific GmbH, Germany) in 25 μL final volume of reaction with primers at a final concentration of 0.2 μM and 25 ng of extracted DNA under the following conditions: 95° for 30 s, followed by 35 cycles of 95° for 30 s, 60° for 30 s, 72 °C for 30 s, and a final extension of 72° for 5 min. PCR amplicons were cleaned using the magnetic beads MagBio HighPrep Clean-up kit (MagBio, USA) following the instructions of the manufacturer and eluted in 40 μL of elution buffer (10 mM Tris pH 8.5). The layout of samples over microtiter plates was randomized, including extraction controls (allowing detection of contamination during DNA extraction), non-template controls (allowing detection of contaminant DNA introduced during library preparation and PCR amplification), and a standard mock-community DNA as positive control (Zymo Research, USA). Negative and positive controls were processed and sequenced alongside the biological samples. A second PCR using 5 μL of the purified PCR products was performed employing Access Array indexing primers (Fluidigm, USA). The second PCR was run at 95° for 3 min followed by 8 cycles of 95° for 30 s, 60° for 30 s, 72° for 30 s, and a final extension at 72° for 10 min. Indexed amplicons were purified with magnetic beads and quantified using a Qubit 2.0 fluorometer with the dsDNA high sensitivity assay kit (Thermo Scientific, USA). Libraries were created by pooling each sample in equimolar concentrations. Quality and integrity of the final library were verified using the Agilent 2200 TapeStation with D1000 ScreenTapes (Agilent Technologies, USA). The pooled library was sequenced at the Berlin Center for Genomics and Biodiversity Research (BeGenDiv) on the Illumina MiSeq platform using MiSeq v2 (500 cycles) reagent kit for 2 × 250 bp paired-end reads.

16S sequence processing

Sequences were preprocessed to infer amplicon sequence variants (ASVs) following the pipeline of DADA2 v1.18 [33]. In brief, the raw forward and reverse reads (fastq) were truncated at base 240, as quality scores dropped at this point. Primer sequences were trimmed, and other filtering parameters were kept at default settings. Only fragments between 410 and 440 bp (expected amplicon size is ~426 bp) were further processed by removing PCR chimera sequences. Taxonomic annotation was done using the naive Bayesian classifier [34] as implemented in DADA2 with SILVA SSU database v138.1. Species were assigned for exact matches of the 16S fragments. All ASVs, metadata, and taxonomic information were compiled into a single object for further analysis using the package Phyloseq v1.22.3 [35].

Microbiome statistical analysis

Data preprocessing

Further cleaning was performed as follows: (1) removing samples with zero or low read counts (less than 2000 reads), (2) discarding ASVs of nonbacterial origin or unassigned at phylum level to ensure off-target amplicon removal, and (3) removing low prevalent ASVs that do not appear more than five times in more than 10% of the samples. Samples were further analyzed according to their host (pig individual) and intestinal (duodenum, jejunum, ileum, cecum, colon) origin (Table S1). *Ascaris* microbiomes were treated as independent samples, irrespective of their eventual origin from the same host. This merged and filtered dataset was rarefied to the minimum library size and used for further alpha diversity estimation (see next section). Finally, the data was normalized by transforming ASV proportions by sample to an even depth (10^6) for beta diversity estimations.

Estimation of alpha and beta diversity and dominant taxa

The Chao1 index was used as a metric for richness. Alpha diversity was calculated using the package Microbiome v1.13.8 [36]. Alpha diversity was compared (1) between

gastrointestinal compartments for infected and noninfected hosts, (2) between infected hosts and their parasites, and (3) between *Ascaris* from different origin or sex. Mann–Whitney *U*-(MWU) tests with Bonferroni correction for multiple testing were computed to assess significance with the package rstatix v0.7.0 [37]. Beta diversity was assessed using Bray-Curtis dissimilarity index between samples and computed using vegan v2.5-7 [38]. Comparisons of distance between individual hosts and parasite microbiomes were tested using MWU tests. Multivariate analysis was carried out using nonmetric multidimensional scaling (NMDS) with the vegan package v2.5-7 [38], also employing Bray-Curtis distance metrics as above.

Bacterial community type (enterotype) classifications were performed from the ASV abundance matrix using the Dirichlet multinomial mixture (DMM) method described in [39] and implemented in the R package *DirichletMultinomial*.

Statistical analysis

PERMANOVA and ANOSIM tests for multivariate effect were done using the adonis and anosim function, respectively, from the vegan package v2.5-7 [38], stratified by experimental batch. PERMANOVA tests whether Bray-Curtis dissimilarity distance differs between groups and assesses marginal effects of variables, while ANOSIM tests whether distances between groups are greater than within groups. Dominant genera were defined as those with the highest relative abundance in at least one sample. The composition of ASVs belonging to those genera were then compared between different sample types (parasites, hosts, worm sexes).

Generalized linear mixed models (GLMM) were used to test whether host microbiomes at the site of infection (jejunum) and parasite microbiomes (at this site) were more similar when they came from the same host individual than from different hosts and more similar than microbiomes from other compartments. These models included only distances between microbiomes from infected hosts and worms. Bray-Curtis dissimilarity was used as the response; the sample identity of each microbiome in the paired comparison was included as a random effect to control for pseudoreplication. Paired comparisons were categorized as matching the following conditions (two predictors):

1. Both microbiomes come from the same host (yes or no).
2. Both microbiomes shared the site of infection (jejunum; yes or no; this is "yes" for *Ascaris* and the jejunum microbiome of the host, "no" for *Ascaris* and other compartments).

We also tested a statistical interaction effect between 1 and 2 to assess additivity vs. effects beyond additivity between those factors.

For each comparison, the unmatched distances were grouped as *different host*, *different compartment*, or *different individual and compartment*, respectively. Models were compared by likelihood ratio test (LRT) to determine whether each parameter was significant.

GLMM tests were also employed to test whether *Ascaris* microbiomes at the site of infection were more similar to each other when both were collected from the same pig host than when they come from different hosts and whether two *Ascaris* microbiomes were more similar when both worms had the same sex. All the models and statistical analysis are summarized by research question in Table 1.

Impact of dominant taxa on jejunum-*Ascaris* microbiome

To investigate whether composition variation between the site of infection and parasites is driven by the most dominant bacteria (see section above, 'Estimation of alpha and beta diversity and dominant taxa'). Jejunum-*Ascaris* microbes were analyzed using the microbial (ASV level) composition restricted to dominant taxa. Bray-Curtis dissimilarity was estimated, and further PERMANOVA and ANOSIM analysis was done using individual pig and host or parasite assignment as predictors.

Identification of differentially abundant bacterial group microbiomes

ASV enrichment was tested as a function of (1) host jejunum against *Ascaris* microbiomes and (2) *Ascaris* female against male microbiomes. DESeq2 package v1.30.1 was used for the assessment; this pipeline uses negative binomial distribution models that account for differences in library sizes to test for differential abundance between testing conditions using the Wald statistics test [39]. Raw counts were used, and the pipeline ran under default settings. The *q*-values were calculated with the Benjamini-Hochberg procedure [40] to correct *p*-values and control for false discovery rates. All significant ASVs were additionally checked using NCBI BLAST searches against the NCBI nr database to confirm their identity.

Results

The parasite and host microbiome sequencing data contained 3,004,508 total reads with an average of 12,677 reads/sample, ranging between 2090 and 57,121 reads. A total of 7934 amplicon sequence variants (ASV) were derived, with an average of 125 ASVs/sample. A total of 172 genera were detected across all samples. We found no evidence of bacterial DNA in larval samples (Fig. S1)

Table 1 Statistical modeling

Question	Statistical approach	Response	Predictor(s)	Random effect
1) How closely does the microbiome composition of the worm resemble that of its immediate environment, the jejunum?	GLMM	Pig-*Ascaris* Bray-Curtis dissimilarity	Same host (yes, no) Location (jejunum, other compartment) Location: same host (interaction)	Individuals (pig ID and *Ascaris* ID)
2) How either host or parasite origin determine the composition of their microbiomes when only dominant microbes are taken into account?	PERMANOVA	Jejunum-*Ascaris* BC dissimilarity	Origin (host or parasite) Individual host (pig IDs)	-
3) What is the impact of the host, worm sex, and dominant bacteria on microbiome variation from pigs and *Ascaris*?	PERMANOVA	Pig-*Ascaris* BC dissimilarity	Host (pig IDs) Worm sex (male, female) Dominant bacteria (*Clostridium sensu stricto* 1 *Lactobacillus*, *Escherichia-Shigella*, *Prevotella*, *Streptococcus*, and *Romboutsia*)	-
4) What is the impact of the host of origin and worm sex on microbiome variation among *Ascaris* individuals?	GLMM	*Ascaris-Ascaris* BC dissimilarity	Same host (yes, no) Same sex (yes, no)	Individuals (*Ascaris* IDs)
5) Is the infection status a relevant factor driving the differences in microbial composition between host and parasites?	GLMM	Pig-pig BC dissimilarity	Same host (yes, no) Same compartment (yes, no) Same infection status (yes, no)	Individuals (pig IDs)

and thus focused our analyses on adult parasites and host contents.

The *Ascaris* microbiome is less rich than that of its porcine host

In order to decipher the microbiome of the parasite gut we first asked, is an *Ascaris* infection beneficial or detrimental for the microbial diversity of the host gut, and does *Ascaris* influence the host microbiome along the entire gut? To locate the effects of *Ascaris* infection on the host microbiota, we assessed the alpha diversity and compared the richness among different intestinal compartments of infected and noninfected pigs. We observed a general and progressive increase in the richness from the small intestinal compartments further down the gut to the colon, independent of the infection status. Interestingly, a notably lower richness was detected in the jejunum of infected hosts in contrast to noninfected hosts, independently from the batch of the experiment (Fig. 1B). The extent of richness decrease at the site of infection was not correlated with individual worm burden (Fig. S2), suggesting a decrease in resident host bacteria between microbiomes of infected and noninfected hosts independent of infection intensity.

Next, we analyzed the microbiome richness of the parasites and compared it to the microbiome richness of the host (from Fig. 1B). At the site of infection in the jejunum, we observed that resident *Ascaris* worms presented a significantly lower bacterial richness than both noninfected and infected host intestines (Fig. 1C), implying that the *Ascaris* gut microbiome is less diverse compared to its host. This observation prompted us to determine whether *Ascaris* and its host share specific bacteria and which groups characterize each microbiome.

The search for the core microbiome involves determining which taxa, if any, are shared among two or more microbial communities. To infer whether hosts and parasites have a core microbiome, we aimed to explore the shared highly abundant and prevalent ASVs between noninfected and infected pigs as well as *Ascaris* worms. We defined the highly abundant and prevalent ASVs for jejuna from noninfected and infected pigs and *Ascaris* (microbial taxa shared by most of the different studied microbiomes) as the group of ASVs with relative abundance higher than 0.01% in at least 50% of the individuals. Those shared ASVs between sample types were considered as "core microbiome" (Fig. 1D; Table S2). Infected jejuna in our study exhibited a total of 39 highly abundant and prevalent ASVs, while the noninfected jejunum presented 36 highly abundant and prevalent ASVs. Infected and noninfected host jejunum microbiomes shared 27 highly abundant and prevalent ASVs;

however, while 12 were also in *Ascaris* microbiomes, 15 were exclusively detected in jejunum microbiomes. Exclusively shared microbes between infected and non-infected host microbiomes suggest a set of taxa of a jejunum core microbiome. In contrast, *Ascaris* microbiomes had just four unique ASVs and shared three exclusively with infected pigs (ASV4 — *Escherichia-Shigella*; ASV29 — *Lactobacillus pontis*, and ASV40 — *Lactobacillus*) but none just with noninfected pigs.

In conclusion, an *Ascaris* infection leads to a loss of microbial diversity of the host gut, though only at the site of *Ascaris* infection in the small intestine. The parasite microbiomes differ drastically in diversity from their host environments, being less diverse while sharing specific taxa with infected pigs at the site of infection.

Ascaris microbiomes are similar to their host microbiome at the site of infection

Our next question was where is the parasite microbiome derived from? Having observed a less diverse worm microbiome, we now assessed how closely the microbiome composition of the worm resembles that of its immediate environment, the jejunum. To compare microbiome composition between intestinal compartments from infected hosts and their worms, we used permutational analysis of variance (PERMANOVA) based on Bray-Curtis dissimilarities. Nonmetric multidimensional scaling (NMDS) analysis shows that *Ascaris* microbiomes cluster closer to microbiomes from the upper intestinal compartments (Fig. 2A). In particular, the *Ascaris* microbiome is more similar to the microbiome of the jejunum and duodenum and more different to the colon microbiome. ANOSIM results showed that both host-parasite differentiation and intestinal compartment are the significantly influential parameters explaining the clustering of the samples ($ANOSIM_{compartment}$: $R = 0.455$, $p < 0.01$; $ANOSIM_{host\text{-}parasite}$: $R = 0.378$, $p < 0.01$). The differentiation between the host or parasite microbiome (PERMANOVA, $R^2 = 0.157$, $p < 0.01$) and intestinal compartment (PERMANOVA, $R^2 = 0.137$, $p < 0.01$) explained almost 30% of the variation (Table S4), suggesting a higher impact from the worms' microbiomes in the clustering than the infection status per se. In addition, enterotype clustering and the best Dirichlet multinomial mixture model (DMM, $\pi = 0.284$, $\theta = 247.873$, $k = 2$, Laplace: 175799.8 BIC: 198685.3 AIC: 188431.3) confirmed the similarity of *Ascaris* microbial communities to the upper GI tract by classifying the samples into two enterotypes (Fig. S3). The first enterotype contained all samples from the upper gastrointestinal tract (GI; duodenum, jejunum, and ileum) and most of the *Ascaris* microbiomes ($N = 46/47$), while a second enterotype included all lower GI samples and one *Ascaris* sample.

To shed light on the origin of *Ascaris* microbiomes, we tested whether the parasite microbiome composition was more similar to a specific gut compartment (the jejunum, the infection site) and whether an individual *Ascaris* microbiome was more similar to that of the individually infected host than to that of other hosts. To test this, we compared the host-parasite microbiome dissimilarity (Bray-Curtis). We observed that host and parasite microbiomes were significantly more similar when they came from the same host individual (LRT: $\chi^2 = 52.349$, $df = 1$, $p < 0.001$; Table 2, Fig. 2B, left). In addition, microbiomes from the shared gut compartment (host microbiomes from the jejunum and those of *Ascaris*) are only more similar when they additionally come from the same host individual (LRT: $\chi^2 = 33.821$, $df = 1$, $p < 0.001$; Table 2,

(See figure on next page.)

Fig. 2 Characterization of microbial communities between hosts and *Ascaris* worms **A** *Ascaris* microbiome composition is closer to the upper gastrointestinal tract microbiome than to the colon and cecum. Nonmetric multidimensional scaling (NMDS) showing differences in microbial composition among gastrointestinal compartments from infected individuals: duodenum, jejunum and ileum (upper GI tract), cecum and colon (lower GI tract), and *Ascaris* worms. Each triangle in the graph represents an infected individual, circles represent individual *Ascaris*, and distances between points are proportional to their biological dissimilarity, calculated with the Bray-Curtis index. The color of the points and the dotted lines surrounding them represents the clusters by compartment. NMDS shows general proximity among *Ascaris* microbiomes and those from upper GI tract compartments, particularly to jejunum and duodenum, the jejunum being the distinct site of infection. Arrows represent the top ASVs (genus level) linked to NMDS axes; their length reflects the relative importance of the ASV on the respective axes. **B** Host and parasite microbiome compositions are closer when both come from the same individual and the infection site. Dissimilarity among bacterial communities derived from *Ascaris* to those from the infected hosts is shown. Host and parasite microbiomes were significantly more similar when they came from the same host individual (LRT: $\chi^2 = 52.349$, $df = 1$, $p < 0.001$; left). Microbiomes from the shared gut compartment (host microbiomes from the jejunum and those of *Ascaris*) are only more similar when they additionally come from the same host individual (LRT: $\chi^2 = 33.821$, $df = 1$, $p < 0.001$; right). If the host individual was not taken into account, only the kind of compartment (jejunum) did not significantly explain microbiome similarity (LRT: $\chi^2 = 0.441$, $df = 1$, $p = 0.507$; middle). The dashed line highlights that the median microbial dissimilarity in host-parasite microbiomes from *different individual*, *different compartment*, and *different individual and compartment* is above 0.75. **C** The majority of *Ascaris* microbiomes are similar to their host's jejunum microbiome. Bray-Curtis dissimilarity values to assess the similarity between worms within the same hosts. In six out of eight pigs, the jejunum-*Ascaris* microbial dissimilarity has a median below the overall median of host-parasite dissimilarity (0.75; dashed line). This is a graphical representation of the significantly higher similarity between host-parasite pairs from the same host than host-parasite pairs from different hosts (GLMM; Table 2). This also points to residual variability in the similarities, so that (only) few worms' microbiomes are still less similar to their own hosts microbiome than to that of the other hosts. The closer the values are to zero, the more similar the microbiome compositions

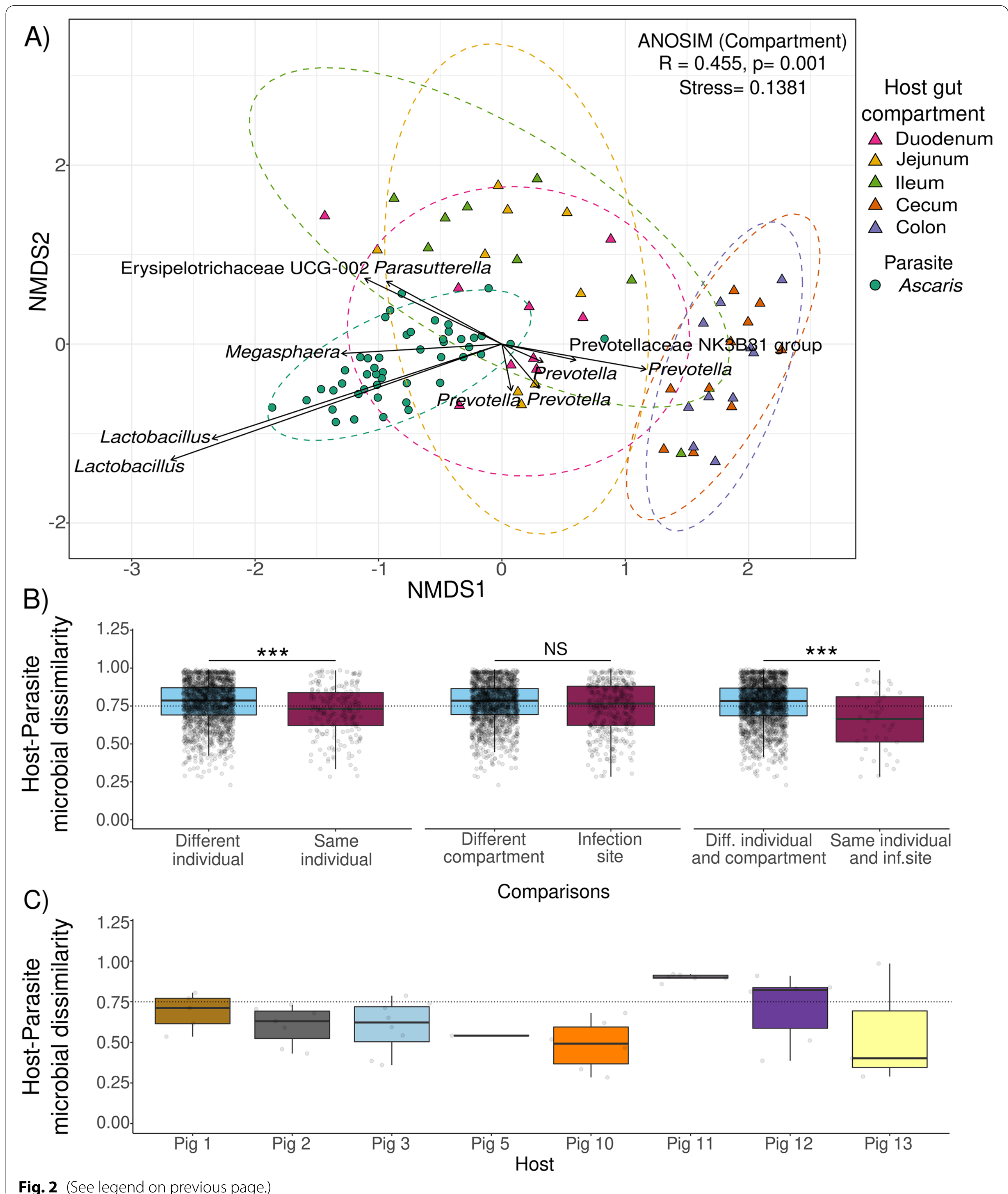


Fig. 2 (See legend on previous page.)

Table 2 GLMM to assess microbial dissimilarity among host-parasite microbiomes

	Estimate	SE	*t*-value	Chisq	*P*-value
Model: host-parasite microbial dissimilarity					
Intercept	0.7737	0.0188	41.201	-	-
Same individual	−0.0351	0.0060	−5.873	52.349	**< 0.001****
Same site of infection	−0.0221	0.0332	−0.665	0.441	0.507
***Same individual and site of infection*(statistical interaction effect)**	−0.0793	0.0136	−5.840	33.821	**< 0.001*****

SE standard error, *t-value* t-test statistic, *Chisq* likelihood ratio chi-squared statistic and *p*-value

Significance codes, ***0.001, **0.01, *0.05

Fig. 2B, right). If the host individual was not taken into account, only the kind of compartment (jejunum) did not significantly explain microbiome similarity (LRT: χ^2 = 0.441, $df = 1$, $p = 0.507$; Table 2, Fig. 2B, middle).

Considering that host-parasite microbiome similarity increases when both come from the infection site within the same host, we specifically analyzed the bacterial composition of the jejunum (site of infection) and worm microbiomes by the individual origin of the sample (individual pig) to determine whether the majority of worms within the same environment showed the same degree of similarity in all hosts. We observed that worm and jejunum microbiomes from the same host presented a trend of high similarity (values closer to zero, median below 0.75) for six out of eight pigs (Fig. 2C), suggesting a general close similarity of worm microbiome with the microbiome of their respective host at the site of infection (values closer to one). Most (but not all) individual worms are more similar to their individual host than the average similarity between host-parasite pairs. The discrepancy of few individual worm microbiomes showing lower similarity to their hosts might be attributed to differences in their genotype or developmental stage, indicating worm microbiome individuality. Pig 4 and pig 14 were not included in this analysis as they lack either *Ascaris* or jejunum microbiome information, respectively. We found that the individual pig of origin explains 45% of variation (PERMANOVA, R^2 = 0.454, p < 0.01; ANOSIM, R = 0.431, p < 0.01) while being jejunum or worm (host-parasite parameter) accounts for less than 10% (PERMANOVA, $R^2 = 0.093$, $p < 0.01$; ANOSIM, R = 0.463, p < 0.01) (Table S4), suggesting a close proximity of both jejunum and *Ascaris* microbiomes within the same individual.

In addition, for the entire GI tract, nonmetric multidimensional scaling (NDMS) shows the differences in bacterial composition among GI compartments, but the derived configuration is not necessarily linked to the infection status (Fig. S4). Our data show a clear differentiation between the upper GI tract compartments (duodenum, jejunum, and ileum) and compartments of the large intestine (caecum and colon) driven by some ASVs annotated as *Anaerosporobacter*, *Lactobacillus*, and *Parasutterella* but not by infection status (Fig. S4A). Within the same intestinal compartment, the Bray-Curtis distance between infected and uninfected hosts does not significantly differ from that between infected or between uninfected hosts. This confirms the minor effect of infection status on microbial composition (Fig. S3B). We observed that overall variation of the GI microbiome composition varies with intestinal compartment (R^2 = 0.314, p < 0.01). Overall, the infection status ($R^2 = 0.069$, p < 0.01) and the individual pig (R^2 = 0.246, p < 0.01) had significant but smaller effects (Tables S5 and S6). Analysis of similarity (ANOSIM) confirmed this effect of compartment (R = 0.475, p < 0.01), infection status, (R = 0.066, p < 0.01), and individual host (R = 0.231, p < 0.01) on microbiome dissimilarities. Despite the apparent decrease in richness between infected and noninfected pigs, there is a stronger effect of the compartment on the microbial composition than the effect of infection status.

Taken together, our data show that the less diverse *Ascaris* microbiome is similar to its immediate host microbial environment, rather than containing a random subset of host microbiomes.

Differences in microbial composition are driven by dominant bacteria

We now asked, is there a detectable core microbiota in the parasite gut? After finding the worm microbiome to be highly similar to the microbiome of the individual host at the site of infection, we aimed to investigate whether this similarity between the parasite and its host microbiome is driven by ASVs belonging to the most dominant bacterial genera. For this purpose, we defined the dominant bacteria as the taxa at genus level with the highest relative abundance within any one of the microbial communities. Six genera are the most dominant in jejunum and *Ascaris* microbiomes: *Clostridium sensu stricto* 1 (29 samples), *Lactobacillus* (17 samples), *Escherichia-Shigella*

(3 samples), *Romboutsia* (4 samples), *Prevotella* (1 sample), and *Streptococcus* (1 sample). The most dominant bacterial genus in the jejunum is *Lactobacillus* (in 4 out of 8 pigs). *Ascaris* microbiomes derived from those pigs have microbiomes dominated by either *Lactobacillus* or *Clostridium sensu stricto* 1 (13 and 28 worms, respectively) (Fig. 3A). *Escherichia-Shigella* and *Streptococcus* are dominant in just three and one *Ascaris* microbiome, respectively. *Prevotella* dominates in just one jejunum microbiome, while *Romboutsia* dominates in each of two *Ascaris* and pig samples.

To determine the impact of the dominant bacteria on microbiome variation, we restricted jejunum and *Ascaris* compositions to ASVs taxonomically assigned to the six dominant genera observed in 3A. We observed that 45% of the variation in microbiomes with dominant-restricted compositions was explained by individuals of origin, linked to the distribution of *Ascaris* samples closer to jejunum from the same pig rather than to a different host (Fig. 3B). The observed variation explained by individuals in the dominant-restricted compositions did not increase compared to the whole bacteria composition. This might point to abundance-independent differences between host and parasite microbiomes: either highly or lowly abundant taxa can make the difference between hosts. The microbiome subset in worms is derived from their respective individual hosts without preference for either lowly or highly abundant (dominant) bacteria, but dominant bacteria are primary drivers of microbiome composition due to their sheer abundance. Additionally, the distribution of samples along NMDS axes relates to the relative importance of the ASV assigned to *Clostridium sensu stricto* 1, *Lactobacillus*, *Prevotella*, *Streptococcus*, and *Romboutsia* (arrows in Fig. 3B), while the origin of the microbiome, either being jejunum or worm (Host-Parasite parameter), explains less than 10% of the variation (PERMANOVA, $R^2 = 0.454$, $p < 0.01$; ANOSIM, $R = 0.397$, $p < 0.01$) (Table 3) as when including nondominant taxa (Table S4).

Lastly, each dominant-restricted microbiome was categorized based on the genus with the highest relative abundance within each sample. By indicating the highest dominant taxa in the microbiomes, we confirmed that jejunum and *Ascaris* clustering is driven by the individual host and *Clostridium sensu stricto* 1 *Lactobacillus*, *Escherichia-Shigella*, *Prevotella*, *Streptococcus*, and *Romboutsia*. A comparison of Bray-Curtis distances between samples among dominant groups confirmed the significantly high dissimilarity (ANOSIM, $R = 0.776$, $p < 0.01$). When matched to individual identification from Fig. 3B, the jejunum microbiome from pigs 2, 3, 5, 12, and 13 shared the dominant taxa with at least one worm microbiome collected from them, and in three pigs (1, 10, and 11), this was not the case (Fig. 3C).

Taken together, these data indicate that the host of origin and dominant taxa of the infected host respectively are the main drivers of the differences in microbial composition among *Ascaris* samples.

Parasite sex does not considerably impact microbiome composition in worms

The next major question was as follows: are gut microbes distinct in female parasites compared to males? Female *Ascaris* worms are significantly larger than males, and individual females release over 200,000 eggs per day [41]. As female worms may depend on particular microbial metabolites for their excessive reproduction, we investigated whether the sex of worms is associated with differences in the diversity or specific composition of microbes. We compared the alpha diversity (ASV richness) of *Ascaris* worms depending on their sex. We did not observe differences in ASV richness linked to the sex of the worms (Fig. 4A). The lack of sex difference with regard to the bacterial composition in worms was independent of the experimental batch effects, as for the richness in infected and noninfected pigs (Fig. 1B). We compared microbial composition between worms from different sexes. Our data indicate that sex plays a minor role as a driver of the bacterial composition in the worm and did not achieve significance ($PERMANOVA_{sex}$: $R^2 = 0.009$, $p > 0.05$; $ANOSIM_{Sex}$: $R = 0.091$, $p > 0.05$) (Fig. 4B), while the dominant bacteria of the individual host is the

(See figure on next page.)

Fig. 3 Host of origin and dominant taxa are the main drivers of differences in microbial composition among host and *Ascaris*. **A** Bacterial composition in *Ascaris* and its host jejunum microbiome. Composition of worms and host-associated microbiomes do not show a clear pattern of relative abundance linked to the host; nevertheless, we observed six dominant bacteria represented by *Clostridium sensu stricto* 1, *Escherichia-Shigella*, *Lactobacillus*, *Prevotella*, *Romboutsia*, and *Streptococcus* as bacteria with the higher relative abundance, dominating the communities. All nondominant taxa were shown as a single group. **B** The similarity of *Ascaris* and jejunum microbiome compositions is determined by the individual of origin. Microbial composition restricted to the six dominant taxa among host jejunum, and the microbiome from *Ascaris* worms infecting them shows differences detectable via nonmetric multidimensional scaling (NMDS). Individual pigs explain most of the variation (45%) PERMANOVA, $R^2 = 0.454$, $p < 0.01$; ANOSIM, $R = 0.421$, $p < 0.01$). Arrows represent the top ASVs (genus level) linked to NMDS axes; their length reflects the relative importance of the ASV on the respective axes. **C** The jejunum and *Ascaris* samples clustered based on their dominant bacteria. Detecting dominant bacteria (most dominant genus within each community) showed worms and jejunum belonging to the same dominant bacteria cluster (ANOSIM, $R = 0.776$, $p < 0.01$). Together with (**B**), it was possible to confirm that the individual host and the dominant bacteria are the most relevant factors linked to the clustering of the samples

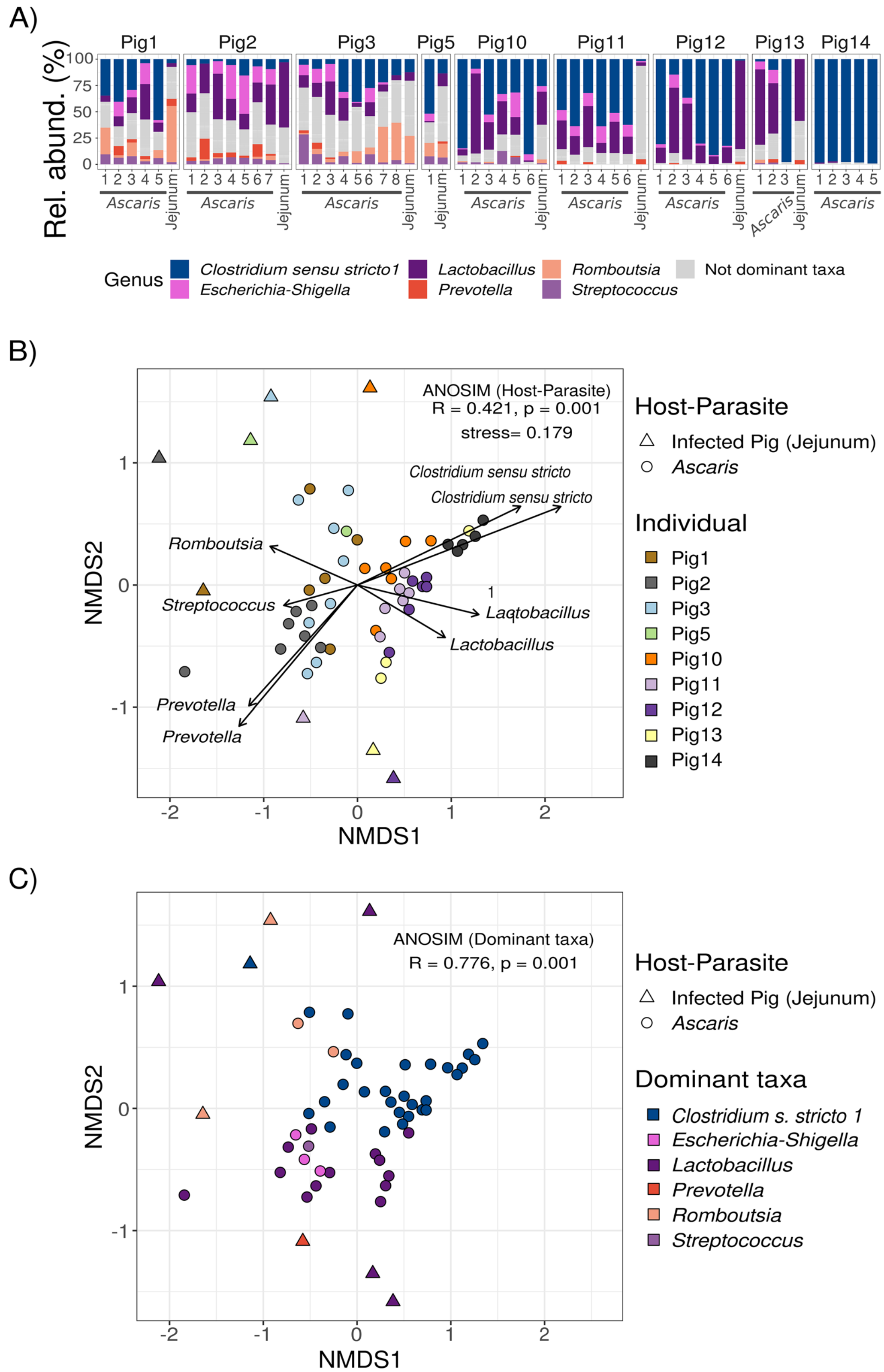


Fig. 3 (See legend on previous page.)

Table 3 Permutational analysis of variance for dominant bacterial taxa composition in jejunum and *Ascaris* from infected pigs

	Df	Sums of squares	Mean sqs.	*F*-model	R^2	Pr (> *F*)
Host or parasite	1	1.0203	1.0203	9.282	0.093	0.001***
Host individual	8	4.9599	0.620	5.640	0.454	0.001***
Residuals	45	4.9467	0.110	-	0.453	-
Total	54	10.9269	-	-	1.000	-

Df degrees of freedom, *F-model pseudo-F*-test statistic, R^2 variance explained and *p*-value based on 999 permutations

Significance codes, ***0.001, **0.01, *0.05

most relevant driver of the bacterial composition in the worm ($PERMANOVA_{\text{dominant bacteria}}$: $R^2 = 0.244$, $p < 0.01$; $ANOSIM_{\text{dominant bacteria}}$: $R = 0.759$, $p < 0.01$; $PERMANOVA_{\text{Individual}}$: $R^2 = 0.532$, $ANOSIM_{\text{Individual}}$: $R = 0.485$, $p < 0.01$) (Table 4). To confirm the drivers in *Ascaris* bacterial composition, we compared parasite-parasite Bray-Curtis microbial dissimilarity using a GLMM approach. We confirmed that coming from the same host (LRT: $\chi^2 = 113.61$, $df = 1$, $p < 0.001$) better explained microbiome composition proximity between *Ascaris* worms (Table 5). Microbiomes from worms of the same sex are not more similar than those of worms with different sexes (LRT: $\chi^2 = 0.105$, $df = 1$, $p = 0.746$).

Having found similar ASV richness between male and female worms, we sought to assess compositional differences between worms of different sexes. We did not observe a difference in the abundance of the four major phyla: Actinobacteoriota, Bactoriodota, Firmicutes, and Proteobacteria between female and male worms (Fig. 4C). Despite there being no overall community level differences between worm sexes (alpha and beta diversity not significantly altered), we found a few ASVs differentially abundant between males and females. Male worms presented 15 differentially abundant ASVs compared to females that presented five ASVs (Fig. 4D; Table S7). Males have *Prevotella* or members of the family Prevotellaceae as the most represented ASVs, in contrast to females in which the majority of the enriched ASVs belong to *Clostridium sensu stricto* 1. The few taxa showing differences do not impact the overall composition, as observed in Fig. 4A–C, if they are not relevant to the microbiome structure (keystones). Microbial composition of worms by sex (Fig. 4E) generally shows similarly abundant genera of bacteria; *Clostridium sensu stricto* 1, *Escherichia-Shigella*, *Lactobacillus*, *Prevotella*, *Romboutsia*, and *Streptococcus* have the highest relative abundance compared to the rest and dominate the communities in both female and male worms. Given the lack of sex effect in the overall bacterial composition of the worm intestines, the few differentially abundant ASVs can probably not be linked to any worm physiological function. In conclusion, these results further highlight the importance of the host of origin and dominant host genera as essential determinants of the *Ascaris* microbiome.

Different bacterial groups are enriched between pigs and *Ascaris* microbiomes

Finally, we asked, do the intestines of the parasites show a depletion or enrichment of specific bacterial species? Having ascertained that dominant ASVs at the site of infection in the host of origin serve as the primary determinants of the *Ascaris* microbiome, we now characterized the constituents of the *Ascaris* microbiome in relation to the jejunum. To test whether specific bacteria are enriched or depleted in the microbiomes of worms compared to their hosts, we performed an analysis of differential abundance. In jejunum samples from infected pigs, 17 ASVs were enriched, compared to 21

(See figure on next page.)

Fig. 4 Characterization of microbial communities in *Ascaris* worms. **A** Female and male *Ascaris* microbiomes do not differ in richness. ASV richness from *Ascaris* worms was not linked to the sex of worms. The lack of sex difference in ASV richness was independent of experimental batch effects. **B** *Ascaris* microbiome composition is not determined by worm sex. Worm sex plays a minor and nonsignificant role in clustering of worm microbiomes ($PERMANOVA_{\text{sex}}$: $R^2 = 0.009$, $p > 0.05$; $ANOSIM_{\text{Sex}}$: $R = 0.091$, $p > 0.05$) compared to the dominant bacteria or the host of origin. **C** The abundance of main phyla in *Ascaris* microbiomes is not different between worm sexes. A nonsignificant difference in abundance of the main bacterial phyla between *Ascaris* worms of both sexes was detected. However, only a trend for Firmicutes abundance in females and Proteobacteria abundance in males was detected. **D** Differential bacterial ASVs in female and male *Ascaris*. Despite there being no overall community differences, particular bacterial taxa (ASVs) were differentially abundant between male and female worms. Each point depicts $\log_2$ fold differential abundance values (x-axis) and $-\log_{10}$ of the adjusted *p*-values (odds ratio). Values > 0 represent enrichment of the 15 ASVs in the males compared to females which included *Bifidobacterium* and *Lactobacillus*. Values < 0 reflect differential abundance of 5 ASVs in the females compared to males, including *Clostridium sensu stricto* 1 and members from the family Prevotellaceae. **E** Microbial composition at genus level of worms by sex. Relative abundance at genus level is presented for each individual worm collected from infected pigs. Genus with less than 1% relative abundance are binned as *Taxa less represented*

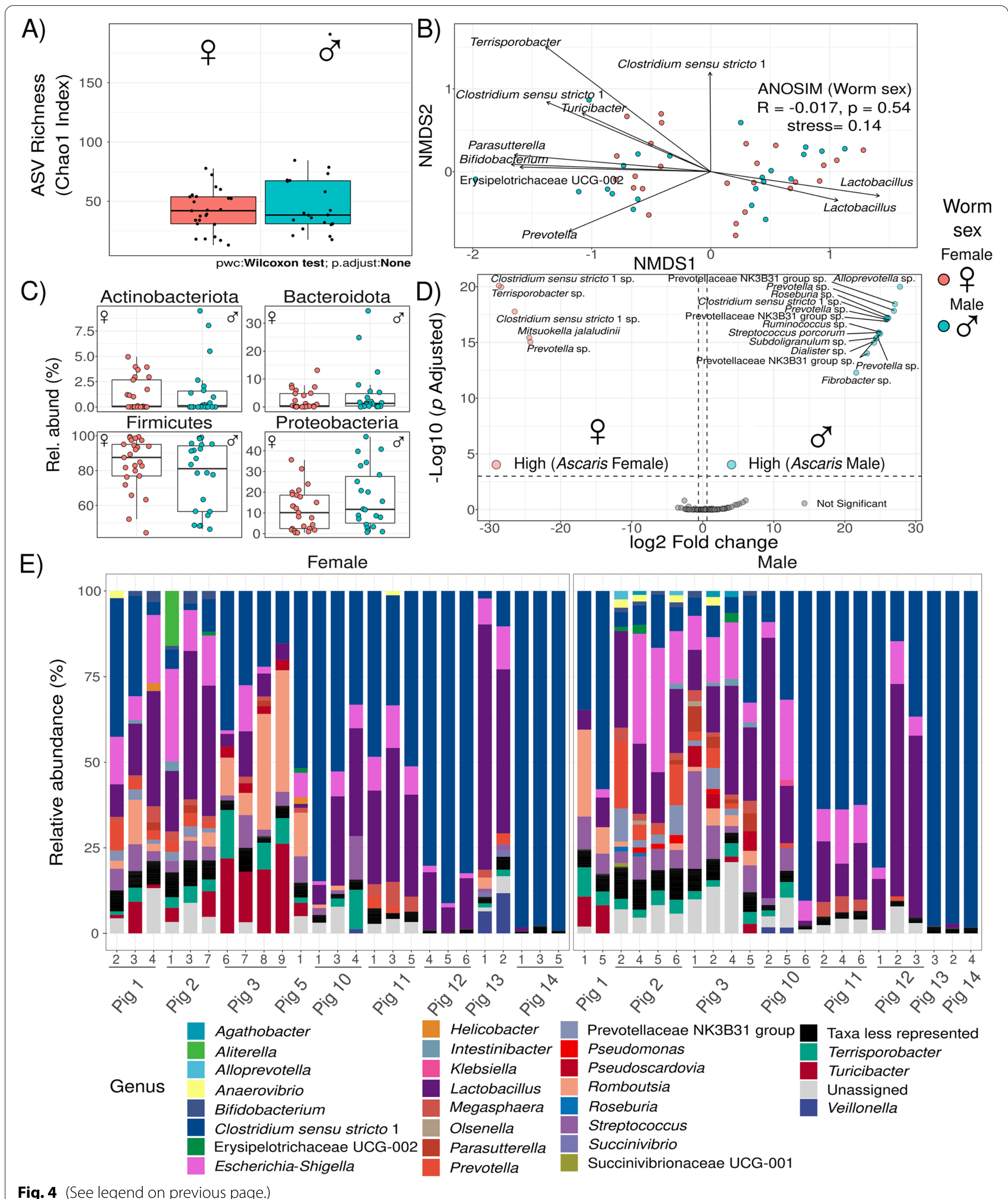


Fig. 4 (See legend on previous page.)

ASVs enriched in *Ascaris* (Fig. 5A, Table S8). In jejunum microbiomes, those include seven ASVs belonging to the dominant taxa *Lactobacillus* (ASV203, ASV400, and ASV430), *Clostridium sensu stricto* 1 (ASV462, ASV513), and *Prevotella* (ASV112 and ASV169) and ten more to nondominant taxa *Bifidobacterium* (ASV197, ASV266,

Table 4 Permutational analysis of variance for bacterial taxa composition in *Ascaris* from infected pigs

	Df	Sums of squares	Mean sqs.	*F*-model	R^2	Pr (> *F*)
Host individual	8	4.8524	0.607	10.209	0.532	0.001***
Worm sex	1	0.0811	0.081	1.364	0.009	0.225
Dominant bacteria	4	2.2273	0.557	9.372	0.244	0.001***
Residuals	33	1.9606	0.059	-	0.215	-
Total	46	9.1214	-	-	1.000	-

Df degrees of freedom, *F-model pseudo-F*-test statistic, R^2 variance explained and *p*-value based on 999 permutations

Signif. codes, 0***, 0.001**, 0.01*, 0.05'.', 0.1' ', 1

Table 5 GLMM to assess microbial dissimilarity proximity among parasite-parasite microbiomes

	Estimate	SE	*t*-value	Chisq	*P*-value
Model: parasite-parasite microbial dissimilarity					
Intercept	0.6085	0.0205	29.703	-	-
Same host	−0.2114	0.0193	−10.964	113.61	**< 0.001*** **
Same sex	0.0033	0.0103	0.324	0.105	0.746

SE standard error, *t-value t*-test statistic, *Chisq* likelihood ratio chi-squared statistic and *p*-value

Significance codes: ***0.001, **0.01, *0.05

and ASV426), *Peptococcus* (ASV226), *Pseudoscardovia* (ASV1328), *Asaccharospora* (ASV455), and *Megasphaera* (ASV248) and from the families Prevotellaceae (ASV119 and ASV134) and Coriobacteriaceae (ASV350). In *Ascaris* gut microbiomes, eight ASVs belonged to the dominant taxa *Clostridium sensu stricto* 1 (ASV15, ASV156, and ASV259), *Prevotella* (ASV82, ASV228), *Lactobacillus* (ASV212), *Streptococcus* (ASV505), and *Escherichia-Shigella* (ASV4) and 13 more to nondominant taxa mainly from the family Prevotellaceae (ASV73, ASV111, ASV118, ASV141) and to the genus *Alloprevotella* (ASV116), *Agathobacter* (ASV84), *Anaerosporobacter* (ASV315), *Dialister* (ASV155), *Lachnospira* (ASV66), *Pseudomonas* (ASV171), *Roseburia* (ASV124), *Ruminococcus* (ASV215), and *Staphylococcus* (ASV367).

The distribution of *p*-values and fold changes in abundance generates clear, distinct microbe groups for hosts and parasites (Fig. 5A). Despite the microbial composition being driven by some dominant genera like *Lactobacillus*, *Prevotella*, and *Clostridium sensu stricto* 1, less abundant microbes (relative abundance below 0.1%) primarily characterize each of the communities (Fig. 5B). *Alloprevotella*, *Agathobacter*, *Anaerosporobacter*, *Dialister*, *Lachnospira*, *Roseburia*, *Ruminococcus*, and *Staphylococcus* are low abundant genera (less than 0.09% relative abundance) but distinctive in *Ascaris* microbiomes compared to host jejunum microbiota. In addition, *Pseudomonas* is an example of an exclusive, highly prevalent (> 50% prevalence) microbe in the *Ascaris* gut microbiome. The ASV belonging to *Escherichia-Shigella* was prevalent in both community types, slightly differentially abundant but significant in *Ascaris* microbiomes. Interestingly, jejunum and *Ascaris* microbiomes had differential and exclusive ASVs from *Clostridium sensu stricto* 1, *Lactobacillus*, and *Prevotella*, suggesting characteristic microbes in worm microbial communities.

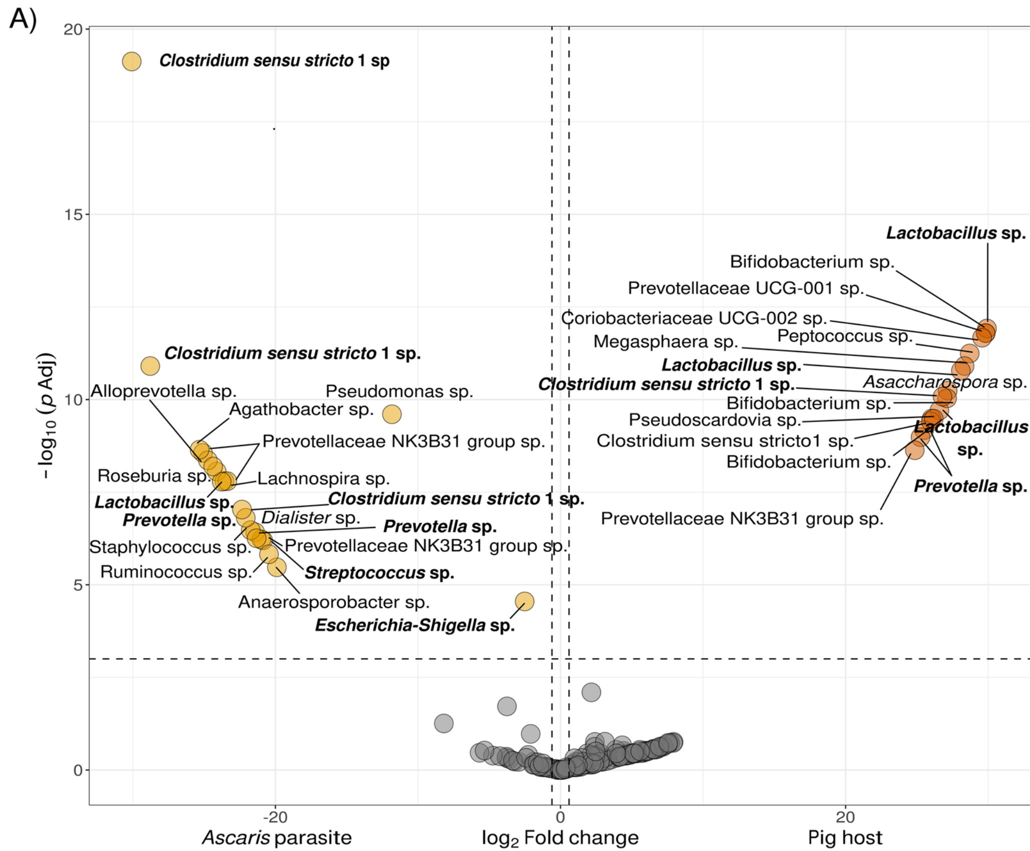
In conclusion, though host microbes from the immediate surroundings are primary determinants of nematode microbiomes, we provide evidence of bacteria that characterizes either the local microbiome at the site of infection of the host or the inner microbiome of the *Ascaris* worms inhabiting it (summarized as a graphical overview in Fig. 6). An enrichment of specific ASVs in the *Ascaris* gut suggests that the *Ascaris* intestine is a unique niche which may support the growth of microbes that are otherwise under-represented in the host gut.

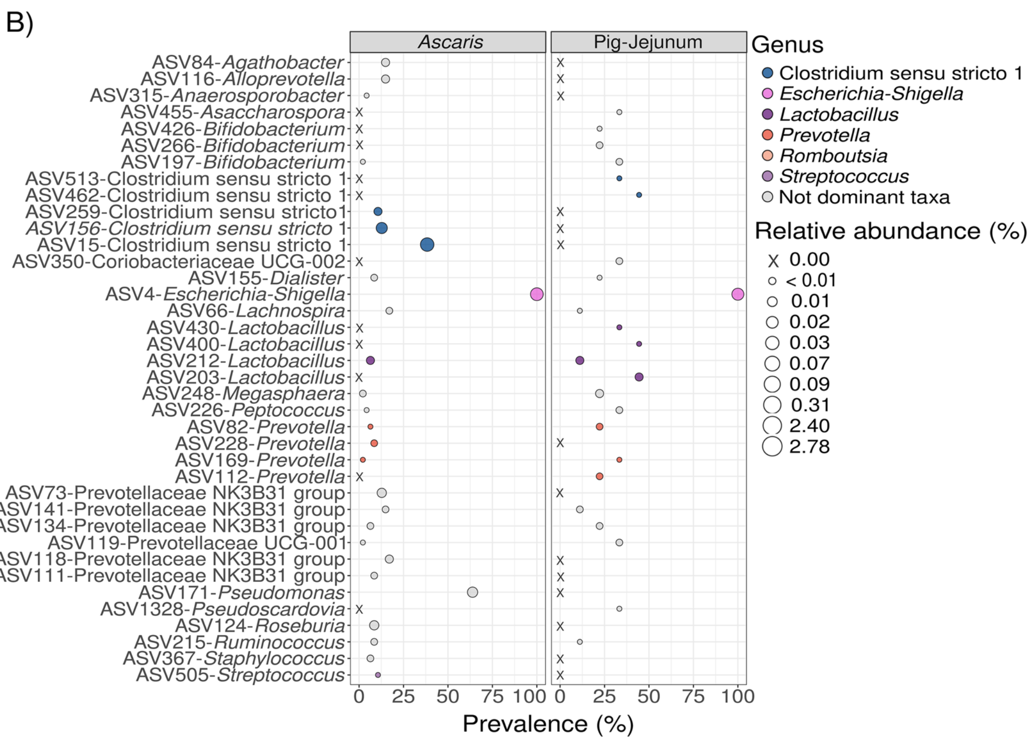
Discussion

Despite numerous studies showing that intestinal nematode infections lead to alterations in the gut microbiome of the host, the helminths' microbiomes and their

(See figure on next page.)

Fig. 5 Bacterial groups are enriched between pigs and *Ascaris* microbiomes. **A** Differentially abundant bacteria between *Ascaris* and jejunum. Significantly enriched ASVs stratified by host and parasite microbiomes (infected jejunum and *Ascaris* from infected individuals). Each point represents $\log_2$ fold enrichment values. Values > 0 represent enrichment of the ASV in the pig jejunum microbiome compared to the *Ascaris* microbiome from each comparison. Values < 0 reflect enrichment of the taxon in the *Ascaris* microbiome compared to the pig jejunum microbiome. Taxa in bold indicate ASVs that belong to dominant genera. **B** Out of the differentially abundant bacteria, specific ASVs are exclusive to *Ascaris* or host microbiomes. General prevalence either in *Ascaris* microbiome or pig jejunum microbiome of all significant differentially abundant ASVs in (**A**). Size of the circles indicates the relative abundance in the respective microbiomes; crosses (X) indicate the absent ASVs in either one or the other microbiome type. Those ASVs belonging to dominant taxa are colored accordingly





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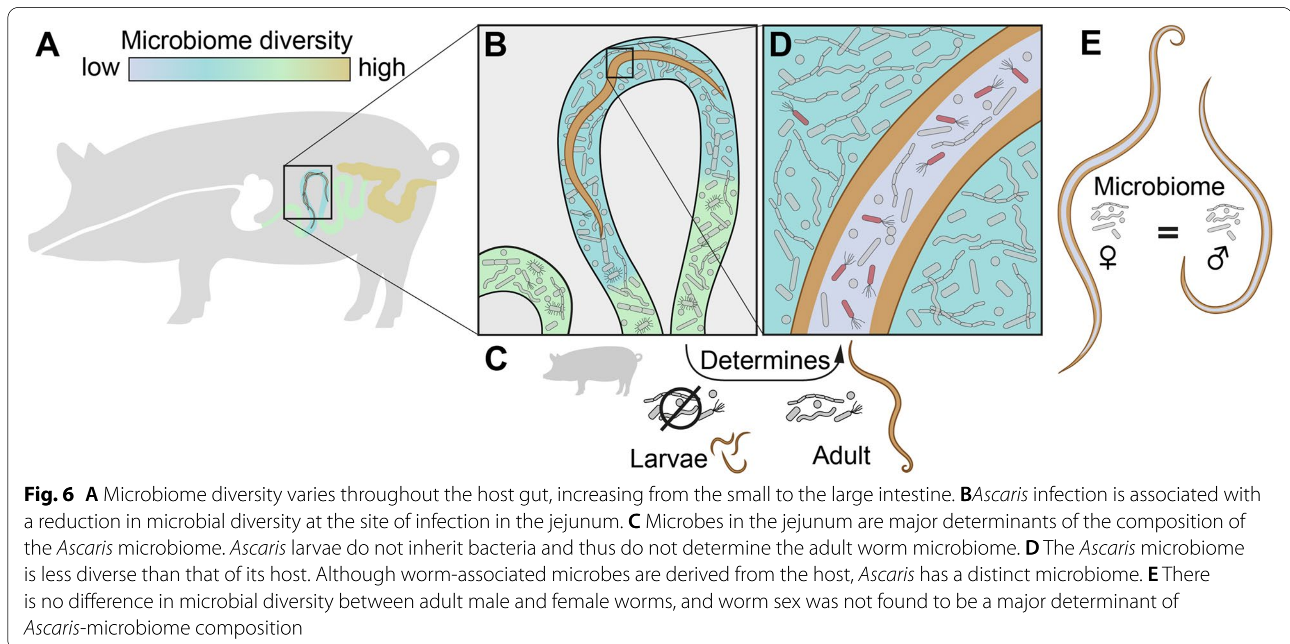

Fig. 6 **A** Microbiome diversity varies throughout the host gut, increasing from the small to the large intestine. **B** *Ascaris* infection is associated with a reduction in microbial diversity at the site of infection in the jejunum. **C** Microbes in the jejunum are major determinants of the composition of the *Ascaris* microbiome. *Ascaris* larvae do not inherit bacteria and thus do not determine the adult worm microbiome. **D** The *Ascaris* microbiome is less diverse than that of its host. Although worm-associated microbes are derived from the host, *Ascaris* has a distinct microbiome. **E** There is no difference in microbial diversity between adult male and female worms, and worm sex was not found to be a major determinant of *Ascaris*-microbiome composition

relationship with host microbes are still an understudied aspect of the parasite-host relationship [15, 16]. Previous work has shown that live bacteria can be retrieved and cultured from the intestine of adult ascarids [13, 14]; however, a culture-free assessment of the microbial composition of the *Ascaris* intestine has not yet been reported. Our work characterizes for the first time the microbiome of this parasitic nematode with respect to its porcine host. We assessed diversity differences between hosts and worms. We report that an *Ascaris* infection reduces microbiome diversity at the site of infection, the jejunum, and that the *Ascaris* microbiome is less diverse than its environment. Importantly, we elucidated the parasite gut microbiome and investigated factors which determine its composition. We highlight that bacteria dominant at the site of infection within the individual host are critical determinants of *Ascaris* microbiome composition in contrast to host bacteria in more distant sites. Our data also indicate that worm microbiome composition is independent of worm sex and parasite burden within the host. While we detected shared bacteria between the parasite microbiome and its microbial environment within the host, we also identified bacteria that differentiate bacterial communities of hosts and parasites. Thus, it appears that the parasite intestine is itself a unique environmental niche better suited for the growth of bacterial communities which are otherwise under-represented in the host gut.

In different parasite-host systems, the effects on gut microbiota diversity, intestinal metabolic environment, and even microbiota-induced immunomodulation during helminth infections have been discussed. Previous studies have assessed the impact of *A. suum* on the porcine fecal microbiome. Williams et al. observed increased diversity in the colon at 14 dpi [27], while Wang et al. reported reduced microbial diversity in the *Ascaris*-infected colon at 54 dpi [28]. Our observations concerning host microbiomes are not directly comparable to these two studies as the others studied distal gut regions and feces while we focused directly on the site of infection and compared this to distal gut regions, which were found to be significantly different from the site of infection, the jejunum. We did however observe a trend towards decreased alpha diversity in the infected jejunum. In agreement with Wang et al., reduction in microbiome diversity was not quantitatively correlated with worm burden. This indicates that worms do not actively compete for host bacteria that they take up from the environment. In accordance with Wang et al. who found significant differences between naive and *Ascaris*-infected gut microbiomes [28], we observed a similar trend towards decreased ASV richness in the jejuna of *Ascaris*-infected pigs. Interestingly, Wang et al. reported enrichment of OTUs assigned to *Lactobacillus*, *Megasphaera*, and *Prevotella* [28], while Williams et al. reported a considerable enrichment of *Succinivibrio* [27]. In our study, the infected jejunum was significantly enriched in ASVs assigned to *Lactobacillus*, *Megasphaera*, and *Prevotella* while *Succinivibrio* was found to be one of the top drivers of the observed enterotypes. These findings are consistent with a meta-analysis of human helminth

studies which found *Prevotella* and *Succinivibrio* to be strongly associated with *Ascaris* infection [42]. Interestingly, *Prevotella* may be linked to intestinal dysbiosis and mucosal inflammation [43], while *Succinivibrio* are dominant within the intestinal microbiome of Behçet's syndrome patients with uveitis [44]. Whether the association of *Prevotella* and *Succinivibrio* with *Ascaris* infection has a pathological consequence for the host or simply serves as a microbial signature of an *Ascaris* infection remains to be determined. While all these studies are restricted to genus-level characterization of prokaryotes, these data suggest that when certain genera are present in the host gut, their relative abundances will change in predictable ways. Future studies employing shotgun metagenomic sequencing and metabolomic profiling will build upon these findings by characterizing the functional potential of the microbial communities present within the *Ascaris* and host intestines.

Previous work demonstrated important roles of gut microbes in the hatching of helminth eggs [45], for the establishment [46], and development and fecundity of helminths [10] within the host intestine. Furthermore, we have previously reported diverse antimicrobial and bacterial modulating activities of excreted and secreted products of helminths in vitro, including *A. suum* [10, 29, 47]. Studies in the free-living nematode *Caenorhabditis elegans* demonstrate that in addition to facing infectious challenges in their immediate environment [48, 49], these roundworms have coevolved microbes and also acquire and shape their own intestinal microbiota, a process strongly influenced by their surroundings [50, 51].

The *Ascaris* intestine is indeed a niche for microbes as ex vivo cultured worms treated with antibiotics still retain living bacteria [14]. Through a culture-free approach, we found that the *Ascaris* intestine possesses a less rich bacterial biome than the jejunal environment. Our findings indicate that the main determinants of the *Ascaris* microbiome are the microbial communities residing in the upper intestinal tract of the host of origin, in particular the dominant bacteria in the host's jejunum. Previous studies suggest worm sex-specific differences in intestinal physiology [52]. Thus, we looked at whether these physiological differences are reflected in the intestinal microbiome of male and female ascarids. Though certain ASVs were differentially enriched between male and female nematodes, akin to the coding RNA findings from Gao and colleagues, male and female worms did not differ in bacterial taxa richness, and worm sex was not a significant driver of *Ascaris* microbiome composition.

Microbial communities within the *Ascaris* intestine are most similar to those found in the duodenum and jejunum of the host of origin, such that the bacterial compositions could be classified into two main enterotypes: those of the small intestine of the host and *Ascaris* and those of the cecum and colon of the respective host. The jejuna of a majority of infected pigs were found to be dominated by *Lactobacillus*, a genus also well represented in the *Ascaris* intestine though we found different variants of *Lactobacillus* between the host and parasite. Furthermore, the *Ascaris* intestine was found to be dominated by six main genera: *Clostridium sensu stricto* 1, *Escherichia-Shigella*, *Lactobacillus*, *Prevotella*, *Romboutsia*, and *Streptococcus*. In partial agreement with a previous study in which bacteria from the *Ascaris* intestine were cultured [13], we also detected *Staphylococcus*, *Streptococcus*, *Escherichia-Shigella*, and *Pseudomonas*. Seventeen ASVs were enriched in the infected jejunum, while 21 unique ASVs were enriched in *Ascaris*. Interestingly, a meta-analysis aimed at defining the core microbiota of the pig gut reported *Prevotella*, *Clostridium*, *Alloprevotella*, and *Lactobacillus*, among others as shared by > 90% of microbiota samples from commercial swine [52]. Thus, the *Ascaris* microbiome is most similar to its host upper intestine but notably distinct from it. As a first report of the *Ascaris* intestinal microbiome, our findings highlight observable differences between the bacteria in the nematode in contrast to those in the host intestine. These data suggest that *Ascaris* recruits its intestinal microbiome from the available microbes in its immediate surroundings. Thus, it seems likely that the helminth intestine itself is a unique environmental niche for specific nematode-microbe relationships, ranging from mutualism to parasitism, as seen for *C. elegans* [23]. The extent to which the nematode regulates the environment of its own intestine and the microbes present therein is an exciting avenue for further research.

Walk and colleagues found that while adult *H. polygyrus* worm-associated microbes were similar to the infected host ileum and dominated by *Lactobacillaceae*, infective larvae-associated microbes were unique and dominated by *Pseudomonadaceae* [53]. The similarity between adult worms' and host microbiomes is in line with our observations that the *Ascaris* microbiome is closely related to its immediate environment in the jejunum as opposed to more distal gut regions. Whereas *Ascaris* larvae get in contact with host-associated bacteria upon egg hatching inside the host, *H. polygyrus* larvae hatch in the environment where they can acquire microbes independently of the host. In native free-living *C. elegans* worms, the nematode microbiome was found to be highly variable, less diverse than, and largely influenced by, its surroundings as well as by individual bacterial taxa but with a shared small core community between worms [51]. Genetic diversity among worms may also contribute to the individuality of microbiomes in *C. elegans* [50, 51, 54]. In addition, *C. elegans* worms

isolated from the wild and enriched for 3 weeks on agar plates with *E. coli* retain similar microbiota to freshly isolated worms [50]. Interestingly, *Ascaris* females can simultaneously mate with multiple males leading to high genetic diversity [55] which may also contribute to microbiome variability between worms. Taken together, these observations raise critical questions about the stability of nematode and helminth microbiomes. Is there a "core" *Ascaris* microbiome, or is it constantly in flux and dependent on the respective life stage and environment? Well-controlled kinetic experiments would be required to determine how the *Ascaris* microbiome changes with the different life stages and their migration. How is the helminth microbiome impacted by dietary changes in the host? Another interesting question is whether the microbiome of the parasite changes with advancing nematode age during this chronic infection where the worm dwells in the host gut for months and years.

Whether helminth microbiomes harbor bacteria that help the worm to grow and survive in its host environment is still not fully understood; nonetheless, previous studies refer to increased immunoregulatory SCFA in the host intestine associated with helminth infections, including those with *A. suum* [11]. While *Ascaris* might produce these metabolites directly ([46], unpublished observations), *Ascaris* infection promotes the outgrowth of SCFA-producing bacteria such as *Lactobacillus*. In the murine small intestine, *Lactobacillus* promotes the establishment of *H. polygyrus* via elevated regulatory T-cell frequencies that might be linked to bacterial immunoregulatory molecules [46]. Our results show that the *Ascaris* microbiome may harbor microbes that participate in host immunomodulation to promote helminth persistence. Thus, helminth infection may support the growth of microbes which promote a less inflammatory gut environment through the production of systemically active metabolites with ramifications for immune pathologies such as allergies and rheumatic diseases. The considerable abundance of various SCFA-producing bacteria such as *Clostridium*, *Lactobacillus*, and *Streptococcus* in the infected host gut and within the nematode presents potential benefits of helminth infection for the host and deserves deeper investigation.

As a niche, there is the potential that the *Ascaris* intestine may retain or carry potential pathogens, for itself and for the host. *Ascaris lumbricoides* obtained from cholera patients was shown to be colonized by *Vibrio cholerae* [57]. Certain genera detected in the nematode intestine, including *Escherichia-Shigella*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus*, tempt us to ask if the *Ascaris* intestine may serve as a niche for potential porcine and human pathogens. Work in *C. elegans* has shown that human-relevant pathogens like *Salmonella* can infect the nematode intestine and serve as a valuable infection model [58]. At present, nothing is known about microbial pathogens of helminths and whether, like *Salmonella*, certain pathogens might infect both the host and the helminth. We can speculate that in cases where commonly co-occurring zoonotic pathogens such as *Campylobacter* and *Salmonella* have also colonized the porcine intestine, the *Ascaris* intestine may provide protection from host immunity and antibiotics. While we have observed key genera present in the *Ascaris* intestine, experimental analysis of the stability of the *Ascaris* microbiota may reveal species and strains that are essential for helminth survival. Ex vivo antibiotic treatment could be used to disrupt the microbiota [59] allowing for studies with experimental microbiomes [50]. Thus, we may discover commensalistic parasite-microbe relationships as well discovering potential microbial pathogens of helminths. Such findings would open the door for novel therapies focused on parasite control via manipulation of the microbiota.

Conclusions

Our work presents the first characterization of the microbiome of a zoonotic macroparasite in relation to its host. This provides a starting point towards understanding the complex multilateral relationships between helminth parasites, microbes, and their hosts. Our findings suggest that *Ascaris* selectively acquires its own microbiome from the available pool of microbes in its environment within the upper intestinal tract. Furthermore, our data lead us to intriguing new research questions important for further study. An in-depth characterization of the *A. suum* microbiome across different life stages would shed light on the stability of the microbiome of a body-migratory and long-lived parasitic nematode such as *Ascaris*. Future studies should assess the potential of the helminth intestine to serve as a protective niche for different microbes, along with determining which microbes are beneficial and harmful to the worm. The characterization of helminth microbiomes is a crucial step towards disentangling the mechanisms driving microbiome variation in infected hosts. Understanding parasite-microbiome interactions may aid in predicting disease outcomes and designing novel parasite control strategies.

Abbreviations

ASV: Amplicon sequence variant; BW: Body weight; Chisq: Likelihood ratio chi-squared statistic; Df: Degrees of freedom; DMM: Dirichlet multinomial mixture; DPI: Days post infection; Exp: Experiment; GI: Gastrointestinal; GLMM: Generalized linear mixed models; Inf: Infected; LRT: Likelihood ratio test; MWU: Mann–Whitney *U*; NMDS: Nonmetric multidimensional scaling; Non:

Non-infected; PCR: Polymerase chain reaction; PERMANOVA: Permutational analysis of variance; SE: Standard error; SCFA: Short-chain fatty acids.

Supplementary Information

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Additional file 1: Figure S1. *Ascaris* larvae lack an inherited microbiome. **Figure S2.** The extent of alterations in richness at the site of infection is not dependent on worm burden. **Figure S3.** Enterotype classification based on the Dirichlet multinomial mixture model. **Figure S4.** Bacterial composition in different gastrointestinal compartments from infected and non-infected pigs.

Additional file 2: Table S1. Individual animals, parasite burden, samples per region, and *Ascaris* intestines included in the microbiome analysis. **Table S2.** Core ASVs by sample type. **Table S3.** Permutational analysis of variance for bacterial taxa composition in different gastrointestinal compartments from *Ascaris* infected pigs. **Table S4.** Permutational analysis of variance for bacterial taxa composition in jejunum and *Ascaris* from infected pigs. **Table S5.** Permutational analysis of variance for bacterial taxa composition in different gastrointestinal compartments from *Ascaris* infected and non-infected pigs. **Table S6.** GLMM to assess impact of infection status on microbial dissimilarity among host microbiomes. **Table S7.** Significant differentially abundant ASV between male and female worms.**Table S8.** Significant differentially abundant ASV between Hosts and parasites (*Ascaris*).

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Authors' contributions

AM, FE, and SH conceived of and designed the experiments. AM and FE performed the experiments. VHJD performed the library preparation. UL and VHJD processed sequence data. VHJD, AC, EH, and SKF carried out data analysis. VHJD carried out data visualization. AM and VHJD wrote the manuscript. RH and AK prepared larval material and assessed for the presence of an inherited larval microbiome. EH, SKF, and SH supervised the work. SH acquired financial funding for the project and reviewed and edited the manuscript. FE, UL, EH, and SKF reviewed and edited the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

The sequence data supporting the conclusions of this article are available in the SRA database under the BioProject PRJNA822897: *Ascaris*-pig-microbiome. Code for generating figures and the analysis is available at https://github.com/VictorHJD/ascaris-pig-microbiome.

Declarations

Ethics approval and consent to participate

This study does not involve the use of human subjects. Infection studies were performed in accordance with the principles outlined in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes and the German Animal Welfare Law. Ethical approval was obtained from the State Office of Health and Social Affairs Berlin, Germany (Landesamt für Gesundheit und Soziales Berlin, Germany, approval numbers G0278/18, T0002/17, and H0005/18).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Department of Veterinary Medicine, Center for Infection Medicine, Institute of Immunology, Freie Universität Berlin, Robert-von-Ostertag-Straße 7, 14163 Berlin, Germany. [2]Experimental and Clinical Research Center, a cooperation between the Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association and the Charité — Universitätsmedizin Berlin, Berlin, Germany. [3]Charité — Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Lindenberger Weg 80, 13125 Berlin, Germany. [4]Max-Delbrück-Center for Molecular Medicine in the Helmholtz Association (MDC), Berlin, Germany. [5]Department of Molecular Parasitology, Institute for Biology, Humboldt-Universität zu Berlin, Philippstraße 13, 10115 Berlin, Germany. [6]Research Group Ecology and Evolution of Molecular Parasite-Host Interactions, Leibniz-Institute for Zoo and Wildlife Research (IZW), Alfred-Kowalke-Straße 17, 10315 Berlin, Germany. [7]Structural and Computational Biology Unit, European Molecular Biology Laboratory, Meyerhofstraße 1, 69117 Heidelberg, Germany. [8]Berlin Institute of Health, Berlin, Germany. [9]DZHK (German Centre for Cardiovascular Research), Partner Site Berlin, Berlin, Germany.

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Supplementary Material

Guts within guts: the microbiome of the intestinal helminth parasite *Ascaris suum* is derived but distinct from its host

Ankur Midha, Víctor Hugo Jarquín-Díaz, Friederike Ebner, Ulrike Löber, Rima Hayani, Arkadi Kundik, Alessio Cardilli, Emanuel Heitlinger, Sofia Kirke Forslund & Susanne Hartmann

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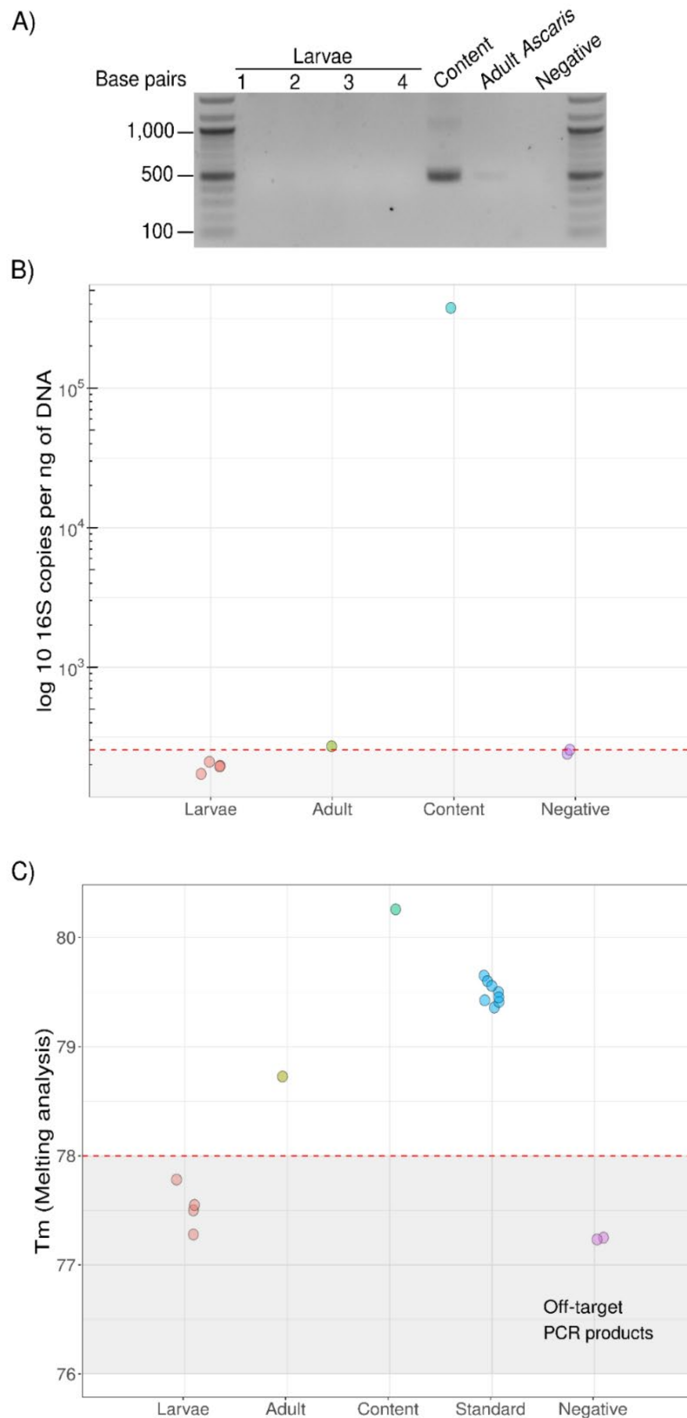

Figure S1. Hatched *Ascaris L3* larvae lack an inherited microbiome. A) DNA from *in vitro* hatched larvae was also subjected to the two step PCR protocol (16S-specific amplification + barcoding) used for library preparation and loaded together with DNA isolated from jejunal content (Content), DNA isolated from adult *A. suum* (Adult) and non-template control (Negative). Bacterial DNA was not observed in samples from larvae. **B)** Absolute quantification by qPCR did not detect 16S bacterial DNA from *in vitro* hatched larvae. All quantifications were comparable to off-target noise also detected in the negative controls and far below the levels observed for an *Ascaris* adult or jejunum content. **C)** Melting curve analysis confirmed that residual amplification products in larvae are non-specific and also observed in the negative controls. The latter shows the absence of bacterial DNA in *Ascaris* larvae.

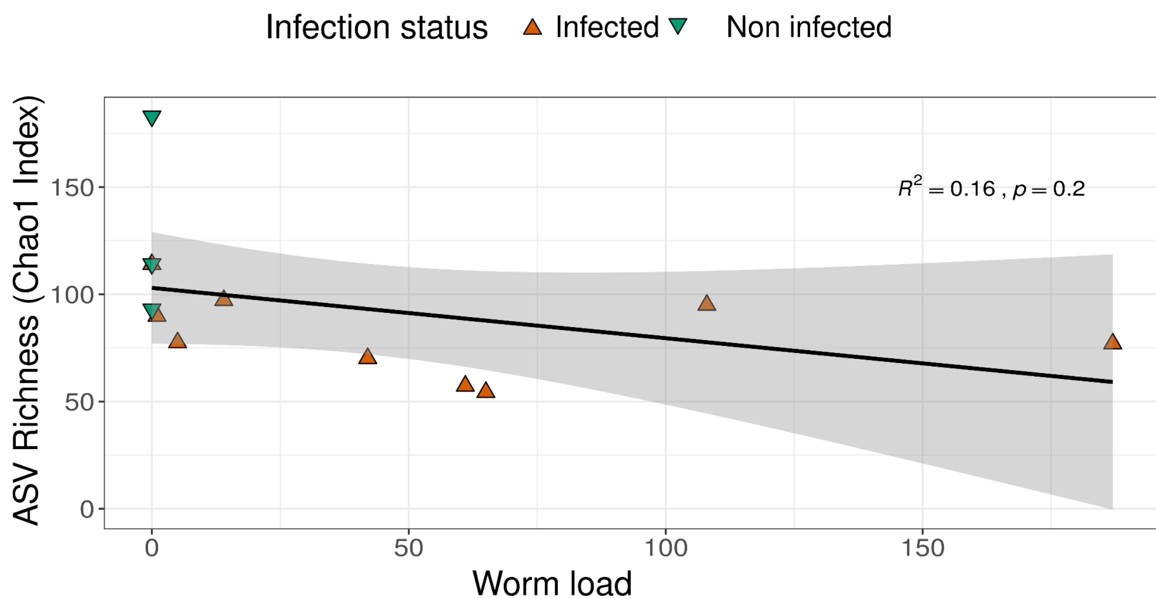

Figure S2. The extent of alterations in richness at the site of infection is not dependent on worm burden. Linear regression was used to predict Chao's richness index (alpha diversity measurement) based on worm load. Worm load did not explain a significant amount of the variance in alpha diversity (F_{Chao} (1,10)=, p= 0.2, R^2= 0.16 , R^2_{adj}= 0.08). Though not statistically significant, the regression coefficient indicates that an increase in one unit of worm load represented on average to a decrease in alpha diversity (β_{Chao}= -0.235).

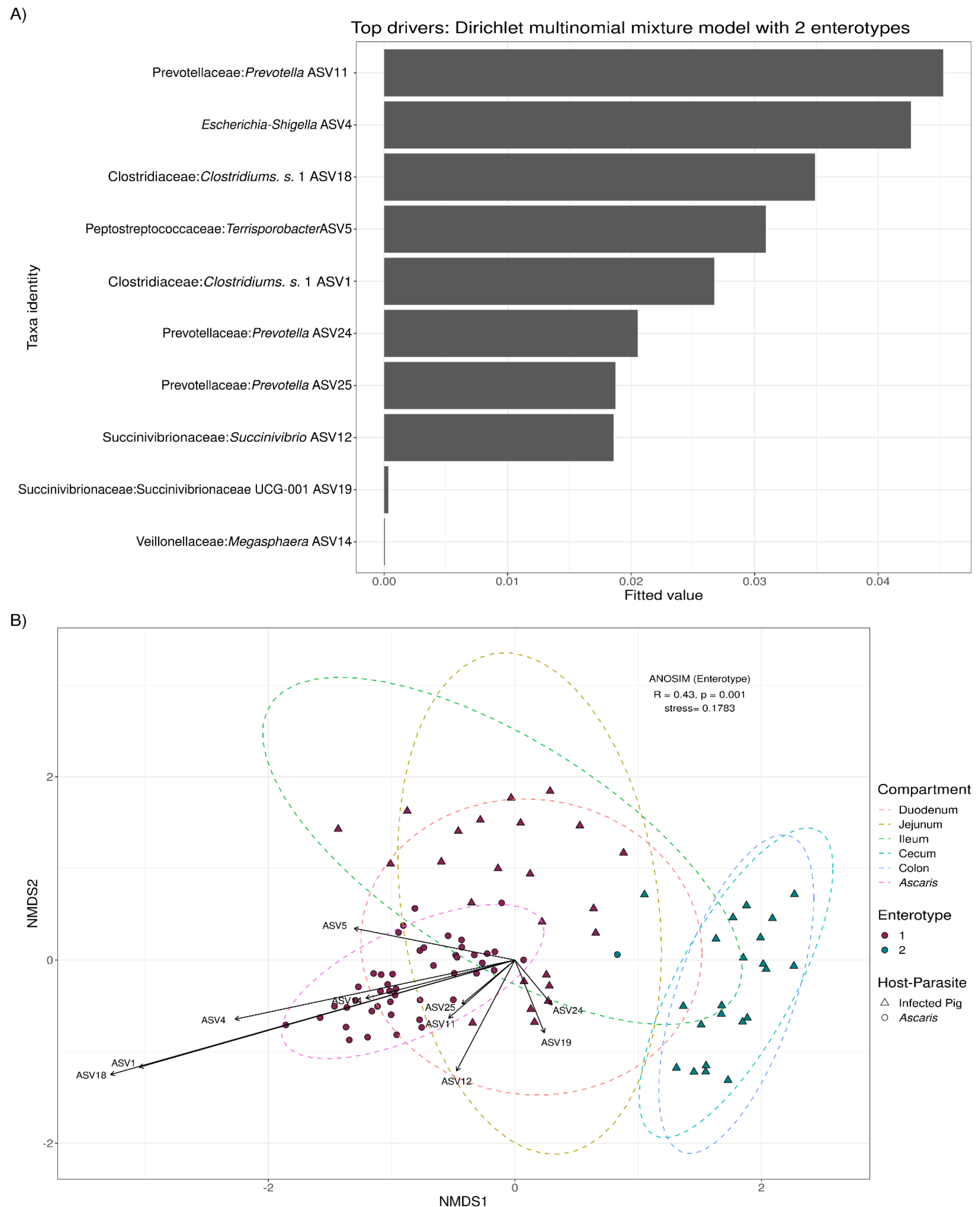

Figure S3. Enterotype classification based on the Dirichlet multinomial mixture model. Dirichlet multinomial mixtures (DMM) modeling was applied to the dataset, including infected pigs and their different compartments and *Ascaris* worms. The entire dataset formed two distinct clusters based on the lowest Laplace approximation. **A)** Contribution of each taxonomic group (ASV) to the DMM model with two enterotypes. **B)** Nonmetric multidimensional scaling (NMDS) visualization of DMM clusters using Bray–Curtis distance of gut bacteria. Duodenum, jejunum and ileum (upper GI tract), and cecum and colon (lower GI tract). Each triangle in the graph represents an individual host, while circles represent individual worms, and distances between points are proportional to their biological

dissimilarity. Color of the points indicates the enterotype and the dotted lines surrounding them represent the clusters by compartment.The ANOSIM statistic R closer to 1 with <0.05 *p*-value suggests significant separation of microbial community structures into one enterotype for upper gastrointestinal tract and *Ascaris*, and a second enterotype for lower gastrointestinal tract. The stress value being lower than 0.2 indicates a good representation in reduced dimensions.

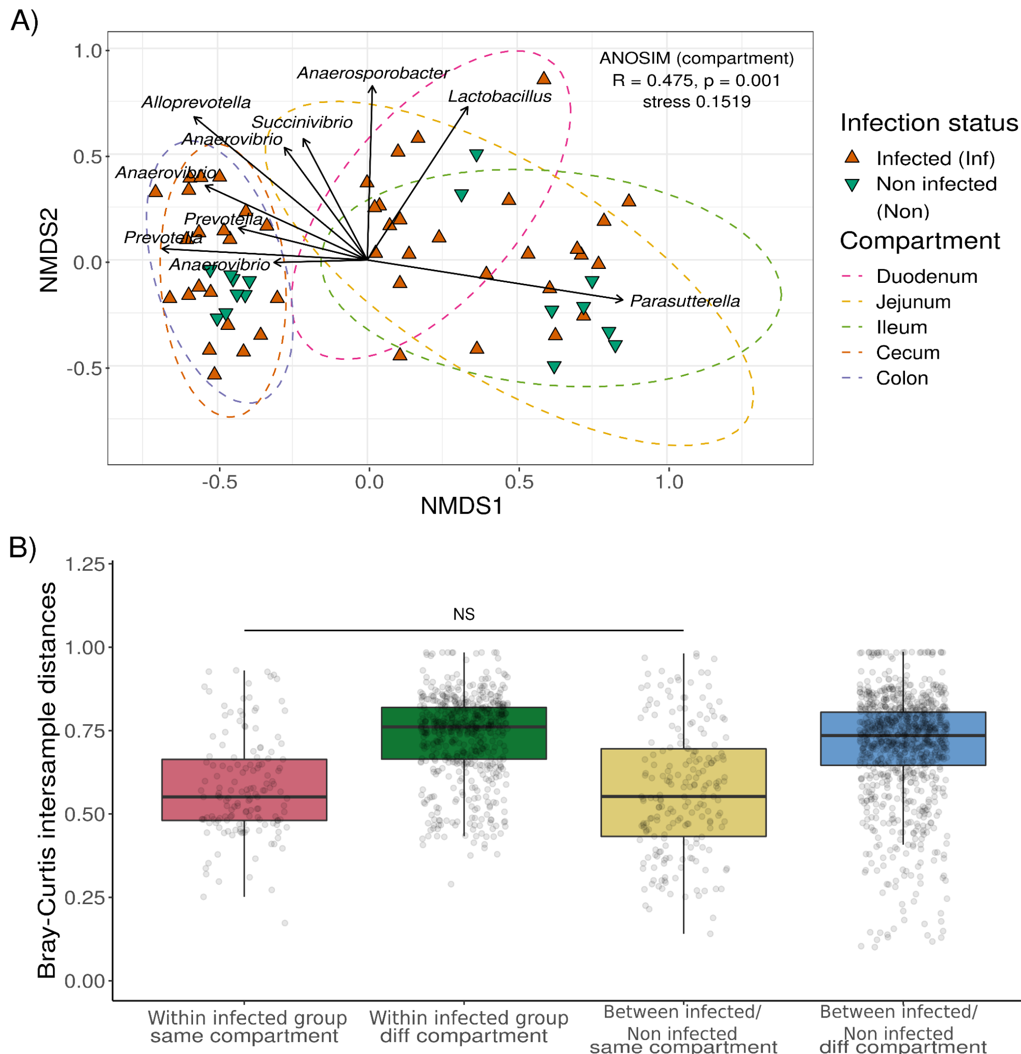

Figure S4. Bacterial composition in different gastrointestinal compartments from infected and non-infected pigs A) Nonmetric multidimensional scaling (NDMS) showing differences in microbial composition among gastrointestinal compartments: duodenum, jejunum and ileum (upper GI tract), and cecum and colon (lower GI tract). Each triangle in the graph represents an individual, and distances between triangles are proportional to their biological dissimilarity, calculated with the Bray-Curtis index. Color and shape of the triangle indicates the infection status and the dotted lines surrounding them represent the clusters by compartment. **B)** Pairwise comparison of intersample Bray-Curtis distances within the same compartment shows no difference between infected (Inf) and non-infected (Non) pigs. Every dot represents the distance between a pair of samples. **C5:** Bray-Curtis dissimilarity between infected and non-infected pigs from the same or different compartments. Bray-Curtis dissimilarity within infected or non-infected, from the same or different compartments.

Table S1. Individual animals, parasite burden, samples per region, and *Ascaris* intestines included in the microbiome analysis.

Animal ID	Infection	Worm burden*	Duodenum	Jejunum	Ileum	Cecum	Colon	*Ascaris* intestines
Pig 1	+	5	2	1	2	4	3	5
Pig 2	+	187	2	4	0	4	3	7
Pig 3	+	42	3	4	2	4	3	8
Pig 4	+	0	2	3	2	3	3	0
Pig 5	+	1	3	2	2	3	3	1
Pig 6	-	-	0	0	2	3	3	0
Pig 7	-	-	0	4	2	3	3	0
Pig 8	-	-	1	4	0	3	3	0
Pig 9	-	-	3	4	2	3	3	0
Pig 10	+	65	3	3	3	3	3	6
Pig 11	+	61	2	1	3	3	3	6
Pig 12	+	108	1	3	3	3	3	6
Pig 13	+	14	0	1	0	3	2	3
Pig 14	+	28	1	0	3	3	3	5

***At the day of dissection. [1]**

Table S2. Core ASVs by sample type

Tax ID ASV No: Higher taxonomic annotation	Jejunum Infected	Jejunum Non infected	*Ascaris*
ASV1:*Clostridium sensu stricto* 1	X	X	X
ASV2:*Lactobacillus*	X	X	X
ASV4:*Escherichia-Shigella*	X		X
ASV5:*Terrisporobacter*	X	X	X
ASV6:*Streptococcus*	X	X	X
ASV7:*Romboutsia*	X	X	X
ASV9:*Turicibacter sanguinis*	X	X	X
ASV11:*Prevotella*	X	X	X
ASV12:*Succinivibrio*	X	X	
ASV14:Megasphaera	X	X	X
ASV17:*Streptococcus alactolyticus*		X	
ASV18:*Clostridium sensu stricto* 1			X
ASV19:Succinivibrionaceae	X	X	
ASV21:*Clostridium sensu stricto* 1			X
ASV22:*Lactobacillus*	X	X	X
ASV24:*Prevotella*	X	X	
ASV25:*Prevotella copri*	X	X	X
ASV26:*Anaerovibrio*		X	
ASV27:Prevotellaceae	X	X	
ASV28:*Parasutterella*		X	
ASV29:*Lactobacillus pontis*	X		X
ASV30:*Lactobacillus reuteri*	X	X	X
ASV31:*Lactobacillus*	X	X	
ASV32:*Prevotella*	X		
ASV34:Prevotellaceae	X	X	
ASV35:*Megasphaera*	X	X	X
ASV37:*Clostridium s. s.* 1 *butyricum*	X	X	
ASV38:*Anaerovibrio*	X	X	
ASV40:*Lactobacillus*	X		X
ASV41:*Intestinibacter*	X	X	
ASV42:*Prevotella*	X		
ASV44:Prevotellaceae	X		
ASV49:Rickettsiales			X

ASV53:Anaerovibrio	X		
ASV54:Prevotella	X	X	
ASV56:*Anaerovibrio*	X	X	
ASV58:Parasutterella		X	
ASV59:Prevotella	X		
ASV61:Prevotellaceae	X	X	
ASV62:Escherichia-Shigella			X
ASV63:Subdoligranulum	X	X	
ASV64:Lachnospiraceae	X		
ASV71:*Parasutterella*		X	
ASV74:Prevotellaceae	X		
ASV76:*Prevotella*	X	X	
ASV78:*Prevotella*	X		
ASV88:*Blautia*	X	X	
ASV91:*Lactobacillus*	X		
ASV131:Clostridia		X	
ASV133:*Subdoligranulum*		X	
ASV191:*Parasutterella*		X	
ASV197:*Bifidobacterium*		X	

Table S3. Permutational analysis of variance for bacterial taxa composition in different gastrointestinal compartments from Ascaris infected pigs

	Df	Sums of squares	Mean Sqs	*F*-Model	R^2	Pr(>*F*)
Host-Parasite	1	3.8043	3.804	26.286	0.157	0.001***
Compartment	4	3.3525	0.838	5.791	0.138	0.001***
Individual	9	5.8167	0.646	4.466	0.240	0.001***
Residuals	78	11.2888	0.145	-	0.465	-
Total	92	24.2623	-	-	1.000	-

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1, Df: Degrees of freedom, *F*-Model: *pseudo* *F*-test statistic, R^2: Variance explained and *p* value based on 999 permutations.

Table S4. Permutational analysis of variance for bacterial taxa composition in jejunum and *Ascaris* from infected pigs

	Df	Sums of squares	Mean Sqs	*F*-Model	R^2	Pr(>*F*)
Host-Parasite	1	1.1339	1.133	9.212	0.093	0.001***
Individual	8	5.5563	0.695	5.643	0.454	0.001***
Residuals	45	5.5388	0.123	-	0.453	-
Total	54	16.2289	-	-	1.000	-

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1, Df: Degrees of freedom, *F*-Model: *pseudo F*-test statistic, R^2: Variance explained and *p* value based on 999 permutations.

Table S5. Permutational analysis of variance for bacterial taxa composition in different gastrointestinal compartments from *Ascaris* infected and non-infected pigs

	Df	Sums of squares	Mean Sqs	*F*-Model	R^2	Pr(>*F*)
Compartment	4	4.790	1.198	9.330	0.314	0.001***
Infection Status	1	1.046	1.046	8.153	0.069	0.001***
Individual	12	3.749	0.312	2.434	0.246	0.001***
Residuals	44	5.648	0.128	-	0.371	-
Total	61	15.233	-	-	1.000	-

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1, Df: Degrees of freedom, *F*-Model: *pseudo F*-test statistic, R^2: Variance explained and *p* value based on 999 permutations.

Table S6. GLMM to assess impact of infection status on microbial dissimilarity among host microbiomes.

	Estimate	SE	*t*-value	Var explained	Chisq	*P*-value
Model: Host-Host microbial dissimilarity						
Intercept	0.7258	0.0188	38.63	-	-	-
Same compartment	-0.1478	0.0087	-17.084	14.7%	271.14	**<0.001****
Same individual	-0.0797	0.0157	-5.068	1.7%	25.25	**<0.001****
Same infection status	-0.0342	0.0144	-2.381	0.3%	4.69	**0.03***

Significance codes: '***' 0.001, '**' 0.01, '*' 0.05, SE: Standard error, *t*-value: *t*-test statistic, Chisq: Likelihood ratio Chi-squared statistic and *p*-value

Table S7. Significant differentially abundant ASV between male and female worms

	log_2Fold Change	Phylum	Family	Genus/Species	
ASV116	27.96	Bacteroidota	Prevotellaceae	*Alloprevotella*	Ascaris Males
ASV79	27.24	Bacteroidota	Prevotellaceae	*Prevotellaceae* NK3B31 group	Ascaris Males
ASV78	26.74	Bacteroidota	Prevotellaceae	*Prevotella*	Ascaris Males
ASV124	26.10	Firmicutes	Lachnospiraceae	*Roseburia*	Ascaris Males
ASV259	26.04	Firmicutes	Clostridiaceae	*Clostridium sensu stricto 1*	Ascaris Males
ASV228	25.98	Bacteroidota	Prevotellaceae	*Prevotella*	Ascaris Males
ASV73	25.85	Bacteroidota	Prevotellaceae	*Prevotellaceae* NK3B31 group	Ascaris Males
ASV210	24.90	Firmicutes	Streptococcaceae	*Streptococcus porcorum*	Ascaris Males
ASV108	24.85	Firmicutes	Ruminococcaceae	*Ruminococcus*	Ascaris Males

ASV141	24.78	Bacteroidota	Prevotellaceae	*Prevotellaceae*	Ascaris Males
ASV128	24.42	Bacteroidota	Prevotellaceae	*Prevotella*	Ascaris Males
ASV133	24.41	Firmicutes	Ruminococcaceae	*Subdoligranulum*	Ascaris Males
ASV155	24.05	Firmicutes	Veillonellaceae	*Dialister*	Ascaris Males
ASV166	23.25	Bacteroidota	Prevotellaceae	*Prevotellaceae NK3B31 group*	Ascaris Males
ASV147	21.73	Fibrobacterota	Fibrobacteraceae	*Fibrobacter*	Ascaris Males
ASV156	-28.84	Firmicutes	Clostridiaceae	*Clostridium sensu stricto 1*	Ascaris Females
ASV51	-28.43	Firmicutes	Peptostreptococcaceae	*Terrisporobacter*	Ascaris Females
ASV297	-26.67	Firmicutes	Clostridiaceae	*Clostridium sensu stricto 1*	Ascaris Females
ASV138	-24.52	Firmicutes	Selenomonadaceae	*Mitsuokella jalaludinii*	Ascaris Females
ASV82	-24.17	Bacteroidota	Prevotellaceae	*Prevotella*	Ascaris Females

Table S8. Significant differentially abundant ASV between Hosts and parasites (*Ascaris*)

	log_2 Fold Change	Phylum	Family	Genus	
ASV203	30.00	Firmicutes	Lactobacillaceae	*Lactobacillus*	Host
ASV197	29.72	Actinobacteriota	Bifidobacteriaceae	*Bifidobacterium*	Host
ASV119	29.71	Bacteroidota	Prevotellaceae	*Prevotellaceae UCG-001*	Host
ASV350	29.45	Actinobacteriota	Atopobiaceae	*Coriobacteriaceae UCG-002*	Host
ASV226	28.84	Firmicutes	Peptococcaceae	*Peptococcus*	Host
ASV248	28.28	Firmicutes	Veillonellaceae	*Megasphaera*	Host
ASV400	28.08	Firmicutes	Lactobacillaceae	*Lactobacillus*	Host
ASV455	27.34	Firmicutes		*Asaccharospora*	Host
ASV266	26.96	Actinobacteriota	Bifidobacteriaceae	*Bifidobacterium*	Host
ASV462	26.78	Firmicutes	Clostridiaceae	*Clostridium sensu stricto 1*	Host
ASV430	26.49	Firmicutes	Lactobacillaceae	*Lactobacillus*	Host
ASV513	26.13	Firmicutes	Clostridiaceae	*Clostridium sensu stricto 1*	Host
ASV1328	26.11	Actinobacteriota	Bifidobacteriaceae	*Pseudoscardovia*	Host
ASV426	26.00	Actinobacteriota	Bifidobacteriaceae	*Bifidobacterium*	Host
ASV112	25.62	Bacteroidota	Prevotellaceae	*Prevotella*	Host
ASV169	25.34	Bacteroidota	Prevotellaceae	*Prevotella*	Host
ASV134	24.80	Bacteroidota	Prevotellaceae	*Prevotellaceae NK3B31 group*	Host
ASV15	-30.00	Firmicutes	Clostridiaceae	*Clostridium sensu stricto 1*	Ascaris
ASV156	-28.60	Firmicutes	Clostridiaceae	*Clostridium sensu stricto 1*	Ascaris

ASV84	-25.12	Firmicutes	Lachnospiraceae	*Agathobacter*	Ascaris
ASV118	-24.95	Bacteroidota	Prevotellaceae	*Prevotellaceae NK3B31 group*	Ascaris
ASV141	-24.63	Bacteroidota	Prevotellaceae	*Prevotellaceae NK3B31 group*	Ascaris
ASV116	-24.37	Bacteroidota	Prevotellaceae	*Alloprevotella*	Ascaris
ASV124	-24.16	Firmicutes	Lachnospiraceae	*Roseburia*	Ascaris
ASV73	-23.73	Bacteroidota	Prevotellaceae	*Prevotellaceae NK3B31 group*	Ascaris
ASV212	-23.69	Firmicutes	Lactobacillaceae	*Lactobacillus*	Ascaris
ASV66	-23.30	Firmicutes	Lachnospiraceae	*Lachnospira*	Ascaris
ASV259	-22.52	Firmicutes	Clostridiaceae	*Clostridium sensu stricto 1*	Ascaris
ASV228	-22.15	Bacteroidota	Prevotellaceae	*Prevotella*	Ascaris
ASV155	-21.58	Firmicutes	Veillonellaceae	*Dialister*	Ascaris
ASV367	-21.48	Firmicutes	Staphylococcaceae	*Staphylococcus*	Ascaris
ASV82	-21.18	Bacteroidota	Prevotellaceae	*Prevotella*	Ascaris
ASV111	-21.11	Bacteroidota	Prevotellaceae	*Prevotellaceae NK3B31 group*	Ascaris
ASV505	-21.06	Firmicutes	Streptococcaceae	*Streptococcus*	Ascaris
ASV215	-20.40	Firmicutes	Ruminococcaceae	*Ruminococcus*	Ascaris
ASV315	-19.77	Firmicutes	Lachnospiraceae	*Anaerosporobacter*	Ascaris
ASV171	-11.77	Proteobacteria	Pseudomonadaceae	*Pseudomonas*	Ascaris
ASV4	-2.65	Proteobacteria	Enterobacteriaceae	*Escherichia-Shigella*	Ascaris

5. Discussion

Helminths are amongst the most prevalent infectious agents of humans and animals. There are currently no vaccinations available against helminth infections, and drug resistance is a growing concern (Pilotte et al., 2022). Ascariasis is a highly prevalent NTD, with *A. lumbricoides* infecting more than 800 million people (Pullan et al., 2014; Holland et al., 2022). The closely related *A. suum* is commonly found on pig farms and is responsible for financial losses in the hundreds of millions annually (Stewart and Hale, 1988). *Ascaris* worms live amongst host-intestinal microbes, and themselves possess an intestine containing microbes. While much progress has been made in understanding host-parasite interactions as well as microbiota-host interactions, these interactions do not occur in isolation but rather, there is a continuous interplay between *Ascaris*, microbes, and host cells (Midha et al., 2021). In this thesis, we focused on parasite-microbe interactions by characterizing antimicrobial activities of *A. suum* ES products and by assessing the microbiome of the *Ascaris*-infected porcine gut as well as the microbiome of *Ascaris* worms.

5.1 The effect of *Ascaris* infection on the porcine microbiome

Previous studies have shown that *A. suum* infection alters the porcine intestinal microbiome (Williams et al., 2017; Wang et al., 2019; Springer et al., 2022). In our work, we found lower bacterial richness in the jejuna of infected pigs relative to uninfected controls (Chapter 4, Figure 1C). Williams and colleagues observed an increased diversity in the porcine colon at 14 dpi (Williams et al., 2017); however, this study is not directly comparable to ours as we assessed the microbiome post-patency at 56 dpi. Furthermore, Williams and colleagues took samples from the colon, which is distal to the site of infection in the jejunum. In another study, Wang and colleagues reported a worm burden-independent decrease in microbial diversity in the porcine colon at 54 dpi (Wang et al., 2019). While Wang and colleagues also assessed fecal samples, they reported findings mostly from the colon. We also found that the decreased richness at the site of infection did not correlate with worm burden (Chapter 4, Figure S2). A third study by Springer and colleagues also found that a single infection with *A. suum* reduces microbial diversity in the cecum after the worms have established themselves in the intestine at 21 and 35 dpi (Springer et al., 2022). Interestingly, in this study they did not see any differences in the jejunum, possibly due to self-reported difficulties in procuring jejunal samples, and also reported a reversion by 49 dpi, where there were no detectable differences in microbial diversity between infected and uninfected pigs. In addition, Springer and colleagues did not observe differences in the diversity of fecal samples, similar to findings in *A. lumbricoides*-infected humans, where there appear to be no differences when

fecal samples are assessed (Klomkliew et al., 2022). Thus, *Ascaris* infection does seem to reduce microbial diversity and such differences are more likely to be detected at, or closer to, the site of infection rather than in feces.

In addition to impacting the diversity of the host microbiome, *Ascaris* infection was associated with the enrichment of 17 bacterial taxa in the infected host jejunum (Chapter 4, Figure 5A, Table S8). These included ASVs belonging to *Lactobacillus, Clostridium sensu stricto 1, Prevotella, Bifidobacterium, Peptococcus, Pseudoscardovia, Asaccharospora,* and *Megasphaera*. Most of these ASVs were either not highly prevalent in, or completely absent from, the *Ascaris* intestine. Other studies of *A. suum*-infected pigs have found similar results; Wang et al. found an enrichment of *Lactibacillus, Megasphaera,* and *Prevotella* at 54 dpi (Wang et al., 2019) while Williams et al. reported an enrichment of *Succinivibrio* at 14 dpi (Williams et al., 2017). Klomkliew and colleagues found that fecal samples from *A. lumbricoides*-infected human hosts had a high abundance of *Prevotella,* Ruminococcaceae, and Lachnospiraceae (Klomkliew et al., 2022). A meta-analysis of human helminth studies reported a strong association between *Ascaris* infection and enrichment of *Prevotella* and *Succinivibrio* (Kupritz et al., 2021).

Whether these changes in the host gut are driven by *Ascaris* or a product of the host immune response to *Ascaris* needs to be determined. Also, the contribution of particular microbes to the establishment of *Ascaris* in the gut of its host is worth considering. Reynolds and colleagues reported that the abundance of *Lactobacillus* is positively correlated with regulatory T cells and Th17 responses as well as susceptibility to the murine parasite *Heligomosomoides polygyrus* (Reynolds et al., 2014). *H. polygyrus* infection in turn led to the outgrowth of *Lactobacillus* in the mouse gut. These findings could be recapitulated by administering the primary *Lactobacillus* species identified, *L. taiwanensis*; mice administered *L. taiwanensis* had elevated regulatory T cell frequencies and worm burdens (Reynolds et al., 2014). Might the enrichment of genera such as *Prevotella* be associated with similar interactions with *Ascaris*? Studies have linked *Prevotella* abundance to augmented Th17 responses and mucosal inflammation or even enhanced Treg responses in humans and mice (Larsen 2017). Thus, *Prevotella* might direct local immune responses away from an antihelminthic type 2 response.

Notably, the experimental design in our study utilized two different infection regimens: a single infection dose of 4,000 eggs and 2 infections with 1,000 eggs (Chapter 4, Figure 1) while Wang and colleagues used 300 eggs daily for 3 days (Wang et al., 2019). The natural pattern of infection likely involves repeated infection due to environmental contamination and continuous re-exposure to embryonated eggs. One approach to modeling a more natural course of infection involves trickle infections, wherein a lower infection dose is administered

regularly throughout the experiment. Springer and colleagues compared a single infection dose of 10,000 eggs with 1,000 eggs administered daily for 10 days, then the pigs were kept until slughet for 21, 35, and 49 dpi (Springer et al., 2022). While the authors referred to this as a trickle infection, a true trickle infection should include infections throughout the duration of the experiment (Masure et al., 2013b). Interestingly, in feces the single infection was associated with decreased diversity compared to uninfected controls while the trickle-like infection was associated with higher diversity throughout the trial (Springer et al., 2022). For intestinal contents from various gut regions, microbiota composition was significantly different between the single-infection and control groups while such differences were not observed in the trickle infection group. Fecal microbial interaction networks were more similar between single-infection and control groups than the trickle-infection group (Springer et al., 2022). Thus, the mode of infection can play a major role in influencing gut microbial composition.

While our study as well as that by Wang and colleagues reported that decreased host microbial diversity was independent of worm burden, Klomkliew and colleagues reported differences in *A. lumbricoides*-infected humans by infection intensity (Klomkliew et al., 2022). They reported significantly higher Chao1 richness in heavily infected individuals compared to lightly or moderately infected individuals (Klomkliew et al., 2022). Furthermore, Bray-Curtis distance separated heavily infected individuals from the other groups (Klomkliew et al., 2022). While very interesting, it is hard to draw firm conclusions from these findings. Firstly, human microbiome data are derived from fecal samples which are very far from the site of infection in the jejunum. Secondly, infection intensity was determined by fecal egg counting which can differ greatly from parasite burden, due to low sensitivity at low worm burdens as well as density-dependent fecundity effects (Sithithaworn et al., 1991; Gassó et al., 2015). It would be insightful to compare fecal egg counts with worm burdens and the impact on the host microbiome in *A. suum*-infected pigs where absolute parasite burden can be determined and host ingesta from different gut compartments can be collected. Thus, the limited data to date suggest that microbiome alterations are independent of worm burden in *Ascaris*-infected hosts.

In summary, *Ascaris* infection reduces host microbial diversity at or close to the sight of infection. Microbial alterations appear to be independent of worm burden but influenced by the mode of infection. Infection is associated with an enrichment of particular microbial strains. Notably, short chain fatty acid and lactic acid producing genera (Markowiak-Kopec and Slizewska, 2020) such as *Lactobacillus, Megasphaera, Clostridium, Prevotella,* and *Bifidobacterium* were enriched in *Ascaris*-infected pigs. Of note, lactic acid bacteria are being studied as probiotics as they are thought to promote gut health and colonization resistance against pathogens, including in *C. elegans* (Ikeda et al., 2007; Chelliah et al., 2018;

Jin et al., 2021; Wang et al., 2021). Further study is required to determine the functional relevance of such taxonomic observations; however, the findings reported thus far point to infection-associated microbiome changes with the potential to influence host intestinal physiology and immunity, thereby impacting the parasite's ability to establish itself in the host gut.

5.2 Ascaris harbors microbes in its intestine

Along with changes to host microbiomes in response to parasite infections, parasite-associated microbiomes have been identified as a key research priority to advance the field of parasitology (Dheilly et al., 2019). Previous studies have shown that viable bacteria can be cultured from the intestine of *A. suum* (Hsu et al., 1986; Shahkolahi and Donahue, 1993). Hsu and colleagues identified a variety of aerobes and facultative anaerobes, including genera and species of potential clinical interest such as *Klebsiella pneumoniae, Staphylococcus aureus, Shigella, Pseudomonas, Streptococcus,* and *Listeria monocytogenes*, among others (Hsu et al., 1986). Shahkolahi reported that culturing *Ascaris* with an antibiotic cocktail (penicillin G, streptomycin, and tetracycline) reduced the bacterial content of *Ascaris* intestines, but it took 96 hours of antibiotic treatment to completely eliminate bacteria while worms that were not treated with antibiotics always retained bacteria in their intestines (Shahkolahi and Donahue, 1993). To our knowledge these were the only reports of *Ascaris*-intestinal bacteria until the publication of two concurrent studies in 2022, ours (Chapter 4) and a study that reported the gut bacteriome of *A. lumbricoides* (Klomkliew et al., 2022).

In addition to reducing microbiome richness in the jejunum of infected pigs, we also found that the richness of the *Ascaris* intestine is considerably lower than that of the host (Chapter 4, Figure 1C). Similarly, Klomkliew and colleagues also reported less richness in *Ascaris* worms compared to fecal samples of hosts (Klomkliew et al., 2022). In our study, the primary determinants of *Ascaris* microbiome composition were found to be the dominant taxa at the site of infection in the hosts from whom the worms were obtained (Chapter 4, Figures 2 & 3). While there were some hints towards compositional differences between male and female worms, we did not uncover any major sex differences in our study (Chapter 4, Figure 4). Of the highly abundant and prevalent microbes (abundance higher than 0.01% in at least 50% of individuals) present in the *Ascaris* intestine, most taxa were shared with both infected and non-infected pigs, a few were shared only with infected pigs, but none were shared only with non-infected pigs (Chapter 4, Figure 1D). Furthermore, there were taxa found only in *Ascaris* intestines. Thus, while most taxa were shared, we found that there were taxa that were differentially enriched in *Ascaris* intestines.

The four highly abundant and prevalent ASVs that were exclusively detected in the *Ascaris* intestine included Ricketsialles, *Escherichia-Shigella,* and two classified as *Clostridium sensu stricto* 1. Another three highly abundant and prevalent ASVs were shared with infected jejunum samples and included *Escherichia-Shigella*, *Lactobacillus pontis*, and *Lactobacillus* (Chapter 4, Figure S2). Differential abundance analysis found 21 ASVs enriched in the *Ascaris* intestine including those belonging to *Clostridium sensu stricto* 1, *Prevotella, Lactobacillus, Streptococcus, Escherichia-Shigella, Pseudomonas, Roseburia, Ruminococcus,* and *Staphylococcus*, among others (Chapter 4, Table S2). Interestingly, Klomkliew and colleagues found *Streptococcus* and *Lactococcus* to be the most prevalent OTUs found in the intestines of *A. lumbricoides* (Klomkliew et al., 2022). As with the porcine jejunum, the *Ascaris* intestine is also populated by microbes that produce short chain fatty acids and lactic acid.

As an intestine within an intestine, the *Ascaris* gut, measuring longer than 35 cm for some adult females (Dold and Holland, 2011), may serve as a niche within the porcine host wherein microbes that would not be well represented in the jejunum or other parts of the pig gut could find a home. Thus, *Ascaris* likely has its own commensals which provide key nutrients such as serotonin and vitamin B12 (Zam et al., 1963; Hsu et al., 1986; Shahkolahi and Donahue, 1993). However, the *Ascaris* gut might also retain pathogens for porcine and human hosts. One study found that *A. lumbriocides* from cholera patients was colonized by *Vibrio cholerae* (Nalin and McLaughlin, 1976). We also detected genera which might correspond to porcine and human pathogens, including *Escherichia-Shigella, Pseudomonas, Staphylococcus,* and *Streptococcus* (Chapter 4, Figure 5, Table S8). Residing within *Ascaris,* microbes might be shielded from antibiotics or host immunity. In addition to host pathogens, some microbes will also be pathogens for *Ascaris* itself. Thus far, nothing is known about pathogens of *Ascaris*, and it would be interesting to investigate whether bacteria that are pathogenic to pigs and humans are beneficial for, or harmful to parasitic nematodes. Therefore, further study is required to determine if *Ascaris* may itself be a reservoir or protective niche for pathogens and if antibiotic treatment of hosts might influence the development of antimicrobial resistance of microbes within the nematode gut.

Thus far, a few studies have either recovered or sequenced bacteria from the intestine of *Ascaris*. Our study has suggested mechanistic insights into the acquisition of bacteria by *Ascaris.* The microbes within the *Ascaris* gut are derived but distinct from the host gut. In particular, the parasite microbiome is most similar to that of its immediate surroundings, the jejunum. We can speculate that as with other species, *Ascaris* will also have commensalistic interactions with the bacteria in its intestine. Some of these microbes may contribute nutrients, help defend the worm against invading pathogens and might also influence host immunity. Alternatively, the nematode intestine could also be infected by pathogens. The influence of *Ascaris* on the host microbiome as well as the determinants of its own microbiome are outlined in Figure 3 (Chapter 4, Figure 6).

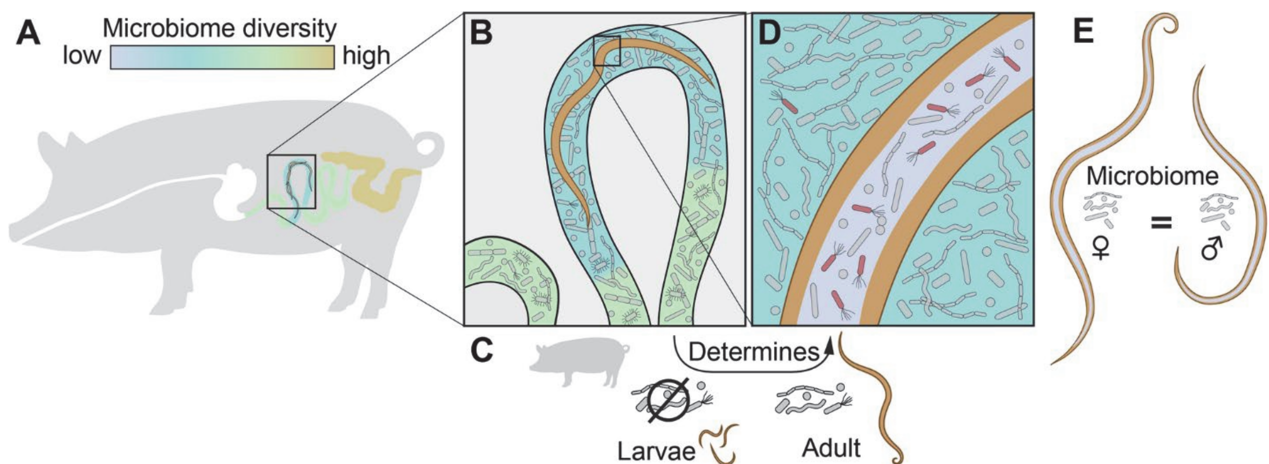

Figure 4 Impact of *Ascaris* on host microbiome and determinants of *Ascaris* microbiome composition. **A** Microbiome diversity varies throughout the host gut, increasing from the small to the large intestine. **B** *Ascaris* infection is associated with a reduction in microbial diversity at the site of infection in the jejunum. **C** Microbes in the jejunum are major determinants of the composition of the *Ascaris* microbiome. *Ascaris* larvae do not inherit bacteria and thus do not determine the adult worm microbiome. **D** The *Ascaris* microbiome is less diverse than that of its host. Although worm-associated microbes are derived from the host, *Ascaris* has a distinct microbiome. **E** There is no difference in microbial diversity between adult male and female worms, and worm sex was not found to be a major determinant of *Ascaris*-microbiome composition. Reprint of Figure 6 from Chapter 4, Midha A. et al. (2022) Guts within guts: the microbiome of the intestinal helminth parasite *Ascaris suum* is derived but distinct from its host. Microbiome. 10:229. doi: 10.1186/s40168-022-01399-5.

5.3 How does Ascaris modulate the microbiome?

Changes to the host microbiome during *Ascaris* infection may come about through multiple parallel mechanisms. These mechanisms can be broadly characterized as 'direct' and 'indirect' mechanisms. Direct mechanisms include direct interactions between *Ascaris* and microbes via antimicrobial or growth-promoting molecules released by the worm as well as interspecies competition between *Ascaris* and microbes. Indirect mechanisms include alterations to host immunity and physiology induced by *Ascaris* which in-turn modulate the microbiome. These broad characterizations are of course conceptual, not mutually exclusive, and occur simultaneously in a dynamic fashion. Furthermore, these mechanisms likely impact the microbial composition of both the porcine host and *Ascaris* itself.

5.3.1 Direct modulation of microbes

Helminths are known to produce numerous secreted compounds including bioactive proteins, lipids, and small molecule metabolites. The excreted and secreted products of *Ascaris* worms include hundreds of proteins and peptides (Wang et al., 2013; Chehayeb et al., 2014) and *Ascaris* also produces immunomodulatory extracellular vesicles composed of proteins and microRNAs (Hansen et al., 2019). While the secreted metabolome of *A. suum* has not yet been characterized, metabolomics analyses have been performed for other helminth parasites including the ascarid *Toxocara canis,* hookworms *Nippostrongylus brasiliensis, Ancylostoma caninum,* and *Necator americanus*, the tapeworm *Dipylidium caninum,* and the whipworm *Trichuris muris* ((Wangchuk et al., 2019, 2020, 2021, 2023; Yeshi et al., 2020). These secreted products mediate nematode- microbe interactions and are thus of considerable importance to understanding defense mechanisms of *Ascaris*.

Moulting animals (Ecdysozoa) such as nematodes and arthropods are likely closely related (Aguinaldo et al., 1997) and have similar immune effectors and mechanisms. Most of our understanding of the nematode immune system comes from *C. elegans*, which can also provide insights into immune mechanisms of *Ascaris*. The genomes of both species encode different antibacterial families, including the antibacterial factors, lectins, lysozymes, and nemapores (Table 2) (Midha et al., 2017). *Ascaris* also produces cecropins, which for nematodes have thus far been described only in ascarids and are otherwise characterized as AMPs of insects (Brady et al., 2019). These effector proteins and peptides are induced by *C. elegans* in response to numerous microbial pathogens and are also thought to shape the nematode's microbiome (Dierking et al., 2016). Similarly, previous studies reported transcriptional upregulation of antimicrobial peptides (*Ascaris suum* antibacterial factors; ASABFs, and cecropins) by adult female *Ascaris* worms injected with heat-inactivated bacteria (Pillai et al., 2003, 2005). Recombinant members of these AMP families have demonstrated antibacterial activity in vitro (Kato and Komatsu, 1996; Pillai et al., 2005).

Table 2 Selected antimicrobial molecules of nematodes

	***Ascaris* spp.**	***Heligmosomoides polygyrus***	***Caenorhabditis elegans***
Antibacterial factors (ABF)	Yes	-	Yes
Cecropins	Yes	-	-
Lectins	Yes*	Yes*	Yes*
Lysozymes	Yes	Yes	Yes
Nemapores	Yes	-	Yes
Nematode products	Tissue extracts, pseudocoelomic fluid, Excreted-secreted products	Excreted-secreted products	-
Yes, detected; -, not detected; *, lectin-like activity detected; gray shading, demonstrated bactericidal activity.			

Table adapted from Midha A et al. (2017) Reciprocal Interactions between Nematodes and Their Microbial Environments. Frontiers in Cellular and Infection Microbiology. 7:144. doi: 10.3389/fcimb.2017.00144. Lectin-like activity and antimicrobial activity of *H. polygyrus* ES products demonstrated in Rausch S, Midha A, et al. (2018) Parasitic Nematodes Exert Antimicrobial Activity and Benefit From Microbiota-Driven Support for Host Immune Regulation. Frontiers in Immunology. 9:2282. doi: 10.3389/fimmu.2018.02282.

We sought to determine if the actively released ES products of Ascaris worms kept in culture possess antibacterial activities and compounds. Native ES products were found to inhibit the growth of gram-positive and gram-negative bacteria (Chapter 2, Figure 1), disrupt biofilm formation (Chapter 2, Figures 2 & 3), and agglutinate bacteria (Chapter 2, Figure 4). Previous reports of proteomic analysis of Ascaris ES products did not report an abundance of antibacterial compounds (Chehayeb et al., 2014; Wangchuk et al., 2023). However, these previous studies were not focused on antimicrobial compounds and employed precipitation and ultrafiltration steps during sample preparation which likely lead to a loss of AMPs which tend to be small and cationic; low molecular weight proteins are less susceptible to precipitation (Baghalabadi and Doucette, 2020) and filter-aided sample preparation uses molecular cutoffs to concentrate higher molecular weight proteins while the filter itself may also bind cationic peptides (Feist and Hummon, 2015). Thus, we omitted these steps in order to retain AMPs in our samples. Using mass spectrometry, we detected proteins and peptides with known and predicted antimicrobial activity in the ES products and body fluid of Ascaris worms, including: AMPs from the ASABF and cecropin families, C-type lectin domain-containing proteins, a lysozyme, and a cystatin protein (cysteine protease inhibitor) (Chapter 2, Table 1). The presence of several proteins and peptides with antimicrobial activity in ES products is consistent with the antibacterial effects observed in our experiments. In particular, the membrane-damaging effects of the cationic AMPs produced by *Ascaris* were apparent in the *E. coli* macrocolony biofilm experiments wherein a dose-dependent disruption of macrocolony growth was observed (Chapter 2, Figure 3). The *E. coli* responded to ES treatment by producing the mucoid exopolysaccharide colanic acid, which is produced in response to cell envelope stress to confer resistance to membrane insults (Laubacher and

Ades, 2008). Furthermore, the presence of several lectin domain-containing proteins in the *Ascaris* ES products is consistent with the agglutinating effects of ES treatments (Chapter 2, Figure 4). Bacterial growth inhibition and agglutination was also demonstrated for ES products from *H. polygyrus* and similarly, *H. polygyrus* also produces lysozymes and lectins (Midha et al., 2017; Rausch et al., 2018). While characterization of the *Ascaris* metabolome and assessment of the antimicrobial potential of these metabolites as well as of extracellular vesicles still needs to be done, the protein and peptide constituents of *Ascaris* ES products are demonstrably antimicrobial (Figure 5) and can therefore contribute to direct shaping of microbial environments by *A. suum*.

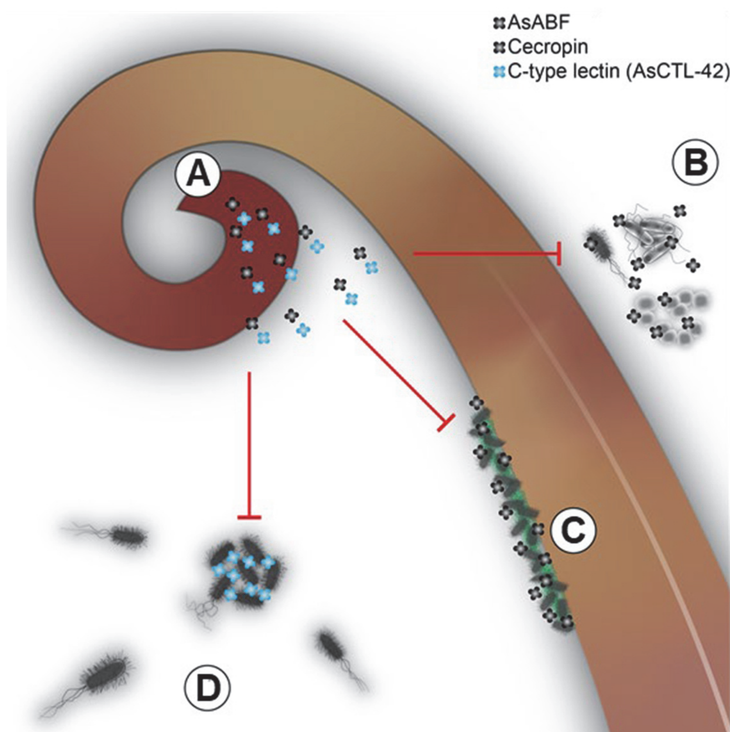

Figure 5 Antimicrobial activities of *A. suum* ES products. **A** *Ascaris* releases proteins and peptides with diverse antimicrobial activies. **B** ES products containing antimicrobial peptides such as *Ascaris suum* antibacterial factors (AsABFs) and cecropins kill bacteria and **C** disrupt biofilm formation**. D** ES products also contain lectins such as AsCTL-42 which agglutinate bacteria.

Recombinant ASABFs and cecropins have been shown to possess antibacterial activity in vitro, though other prominent constituents of *Ascaris* ES products have not been studied in this context, as most work concerning ES products has focused on host-parasite interactions. Thus, we recombinantly expressed two previously undescribed, prominent proteins from different families using the eukaryotic *Leishmania tarentolae* expression system (Breitling et al., 2002). GH family 25 lysozyme 2 (Uniprot Accession number F1LE63; herein denoted AsGH) and C-type lectin domain-containing protein 160 (Uniprot Accession number F1L7R9; herein denoted AsCTL-42) were chosen due to their outsized representation in *Ascaris* ES

products and due to their both containing secretory signal peptides (Chapter 2, Table 1). Both proteins were also reported by other proteomic studies as prominent *Ascaris* ES constituents (Wang et al., 2013; Chehayeb et al., 2014) and AsCTL-42 (annotated GS_08343) was found to be highly abundant in the intestinal transcriptome of adult *Ascaris* worms (Gao et al., 2017). Glycosyl hydrolases, classified into more than 45 families on the basis of amino acid sequence similarities (Davies and Henrissat, 1995), are highly abundant in the ES products of *Ascaris* (Wang et al., 2013) and lysozyme activity is the only known function of enzymes in family 25 (Henrissat, 1991). Lectins are carbohydrate binding proteins, some of which possess bacteriocidal activity (Vaishnava et al., 2011). However, neither AsGH nor AsCTL-42 inhibited bacterial growth (Chapter 3, Table 1). However, AsCTL-42 successfully recapitulated the agglutinating activities of *Ascaris* ES products, in a dose- and calcium-dependent manner (Chapter 3, Figure 2). This neutralizing activity also decreased invasion of porcine intestinal epithelial cells by *Salmonella,* without acting on host cells (Chapter 3, Figure 3). Thus, AsCTL-42 is an example of an effector which can neutralize bacteria without killing them, similar to Clec-39 and Clec-49 which are upregulated in response to bacterial infection in *C. elegans* and have been shown to bind bacteria without killing them (Mallo et al., 2002; O'Rourke et al., 2006; Miltsch et al., 2014).

We were unable to identify a bacterial glycan binding partner for AsCTL-42 using a glycan array (Chapter 3, Table S1). Lipopolysaccharides were notably absent from our glycan array screen and it would be worth investigating whether AsCTL-42 binds LPS. In line with our findings, Miltsch and colleagues also did not find a carbohydrate binding partner for Clec-39 and Clec-49 by glycan array (Miltsch et al., 2014). While this does not rule out a glycan-binding role for AsCTL-42, CTLs can also bind non-glycan ligands (Mitchell and Gibson, 2015) and only a fraction of the CTLs encoded in the *Ascaris* genome are predicted to bind glycans (Bauters et al., 2017). A limitation of this work was that high concentrations of AsCTL-42 had to be used to assess antibacterial activity. However, *Ascaris* ES products contain numerous lectins, likely acting synergistically and some hosts can be very heavily infected and worms can aggregate in such hosts as we and others have observed (Holland et al., 1989; Wang et al., 2019; Midha et al., 2022). Thus, high concentrations of *Ascaris* proteins are attainable. Another interpretation of the high concentrations of AsCTL-42 required is that bacteria are not the intended target of this protein. Hansen and colleagues characterized extracellular vesicles (EVs) from *A. suum* and their evidence suggests that these EVs are directed at host cells and possess immunomodulatory potential (Hansen et al., 2019). Interestingly, these EVs also contain numerous CTLs and cystatin among their protein constituents, suggesting that CTLs may be targeting the host rather than bacteria (Hansen et al., 2019). We therefore also tested the ability of AsCTL-42 to bind to host cells by screening for interactions between AsCTL-42 and mammalian CTL receptors. We found prominent

binding for Dectin-1, Dectin-2, Langerin, and Mincle (Chapter 3, Figure 5). Importantly, these interactions occurred at concentrations well below those required for bacterial modulation. Taken together, these data indicate that AsCTL-42 may have multiple binding partners given its interactions with bacterial cells and host cells, and that the binding partners may be non-glycans given the interactions with mammalian CTL receptor proteins. To better understand its interactions with bacteria, further study is required to determine if AsCTL-42 binds to LPS as it was not part of the glycan array, or if its target is a non-glycan. Additionally, encouraged by its interaction with mammalian CTL receptors, it would be interesting to assess the immunomodulatory potential of AsCTL-42.

In our studies we have focused on the growth-inhibiting activities of *Ascaris* products, with a particular emphasis on proteins and peptides. However, *Ascaris* also produces metabolites and *Ascaris* infection is associated with the outgrowth of certain bacterial genera as evidenced by our study. Future studies should characterize the metabolome of *A. suum* and the microbiome modulating effects of nematode metabolites. Our understanding of inter-species competition between helminths and microbes is also currently limited. It would be very interesting to study the effects of *Ascaris* products on a wider array of bacterial strains to assess for growth-promoting activities in addition to growth limiting effects as *Ascaris* products might also serve as substrates or promoters of bacterial growth. Nevertheless, our observations thus far support the idea that microbiome modulation can be achieved in part by direct interactions between nematodes and microbes.

5.3.2 Indirect modulation of microbes

The immune response elicited during an *Ascaris* infection is influenced by several contributing factors. In general, helminth infections and helminth antigens are associated with a modified type 2 immune response as outlined in the introduction and in the case of *Ascaris*, there is evidence of a mixed type1/2 response, possibly due to microbial antigens introduced during tissue migration, as evidenced by mixed Th1/Th2 associated cytokine expression in the livers of *A. suum*-infected pigs (Dawson et al., 2009). Type 2 immune responses promote parasite expulsion via mucus secretion and enhanced gut motility (Molofsky and Locksley, 2023), in part by increasing ion flux and tissue permeability while reducing epithelial glucose absorption (Shea- Donohue et al., 2001). *Ascaris* ES products can also reduce epithelial nutrient transport, particularly glucose and alanine (Koehler et al., 2021). Altered mucus production and composition could impact mucus-associated genera such as *Ruminococcus* (Suriano et al., 2022) which was enriched in *Ascaris* microbiomes (Chapter 4, Figure 5). Decreased glucose absorption by the host might also alter microbiome composition in favor of microbes which are best equipped for glucose uptake (Jahreis et al., 2008); for example *Escherichia* readily consumes glucose (Beisel and Afroz, 2016) and *Escherichia-Shigella* was

highly abundant and dominant in both the *Ascaris* gut and the *Ascaris*-infected jejunum (Chapter 4, Figure 5). Similarly, alanine can be utilized as a sole carbon source and functions as a chemoattractant for certain bacterial genera such as *Escherichia,* while others such as aspartate are repellent and inhibit growth (Yang et al., 2015). The impact of gut motility can also be considerable; *Prevotella* abundance increases during diarrhea and decreases during constipation in irritable bowel syndrome patients (Downs et al., 2017; Ohkusa et al., 2019; Müller et al., 2020). Additionally, helminth-induced type 2 responses can induce host AMP production, alter microbial compositions, and promote colonization resistance as demonstrated in mouse models (Ramanan et al., 2016). Finally, microbiota changes that occur during *Ascaris* infection might also change bacterial interspecies competition dynamics, further modifying microbial community compositions (Ortiz et al., 2021). Thus, the physiological alterations and immune response induced during *Ascaris* infection can influence microbiome composition without the need for direct interactions between *Ascaris* worms and microbes.

5.4 Conclusions and Outlook

5.4.1 Conclusions

The data presented herein constitutes a considerable portion of what has been published regarding interactions between *Ascaris* and microbes. Seminal prior studies by others, though limited in number, provided the justification and guidance for our work and allowed us to make a significant contribution to the literature. Firstly, we demonstrated that *Ascaris* ES products possess diverse antimicrobial activities, including inhibiting bacterial growth, disrupting bacterial biofilm formation, and agglutinating bacteria. We also detected several proteins and peptides with known and predicted antimicrobial activities by mass spectrometry. Furthermore, each of the antimicrobial activities we observed corresponded to the ES constituents that we detected. Secondly, we characterized a prominent constituent of the ES products, AsCTL-42. We showed that it can recapitulate the agglutinating activity of *Ascaris* ES products and that this agglutination can result in reduced invasion of porcine intestinal epithelial cells by *Salmonella* Typhimurium. We also demonstrated that AsCTL-42 has the potential to interact with host immune cells. Finally and most importantly, we reported for the first time the microbiome of the zoonotic parasite *A. suum* and described the key determinants of *Ascaris* microbiome composition, namely the dominant microbes present at the site of infection. The novel findings presented in this thesis now set the stage for future work to further unravel the complex interplay between host, microbes, and parasites.

5.4.2 Outlook

Our results indicate that we have only just begun to understand the complex and dynamic systems that were the subject of this work and prompt many novel research questions. Many constituents of *Ascaris* ES products remain poorly understood, including AsCTL-42 and its impact on host cells. Furthermore, how nematode antimicrobial peptides and other immune effectors are regulated and whether there exist microbes that are inherently resistant to their actions remains to be determined. Given that *Ascaris* possesses immune defense effectors, what are the infectious threats that it is faced with in its environment? Alternatively, which microbes are beneficial for *Ascaris*? The *Ascaris* metabolome has also not yet been characterized. We and others have reported that *Ascaris* infection alters the host bacterial microbiome; however, we have not assessed the metabolomic implications of these changes. Are the alterations to the microbiome and metabolome harmful or beneficial for the host? Do these changes contribute to colonization resistance or increase the risk of coinfection? Our work focused on the bacterial microbiome of *Ascaris* as well as the host. Future studies should also assess the eukaryotic biome and virome. Furthermore, we can now ask about the influence of other variables on the *Ascaris* microbiome, including: life stage, diet, and antibiotics. We are also inclined to ask if the *Ascaris* gut might serve as a niche for other pathogens or provide protection from antibiotics, thereby contributing to the development of antimicrobial resistance. Thus, our work serves as a foundation for many exciting research questions for the future in dissecting the interplay between parasites, microbes, and host cells.

6. Zusammenfassung

Modulation der Darmmikroben durch den intstinalen Nematoden *Ascaris suum*

Hintergrund: Eine Infektion mit dem Spulwurm *Ascaris* ist eine der häufigsten bodenübertragenen Helmintheninfektionen weltweit und ein erhebliches Problem in der Tierhaltung. Die Infektion wird über den fäkal-oralen Weg verbreitet und erfolgt durch die Aufnahme von Eiern, die infektiöse Larven des dritten Stadiums enthalten. Die aus den Eiern schlüpfenden Larven dringen in den Darm des Wirtes ein und wandern durch die Leber und die Lunge, bevor sie in den Dünndarm zurückkehren. Diese Gewebewanderung führt zu der für *Ascaris* charakteristischen Immunreaktionen, Pathologien und Krankheitssymptomen. Im Jejunum reifen die Würmer heran und leben inmitten der Mikroben des Wirts. Die derzeitigen Methoden zur Bekämpfung von Helmintheninfektionen konnten diese Krankheitserreger bislang nicht ausrotten. Daher werden neue Erkenntnisse über ihre Lebensweise im Wirt benötigt, um neue therapeutische Möglichkeiten zu finden. Über die Interaktionen dieser Erreger mit denen sie umgebenen bakteirellen Mikroben im Darm des Wirtes ist noch wenig bekannt. Ziel dieser Arbeit war es daher, die Wechselwirkungen zwischen *Ascaris* und den bakteriellen Darmmikroben zu bearbeiten, durch die Charakterisierung der antimikrobiellen Aktivitäten der Würmer und dem Entschlüsseln des Ascaris-Mikrobiom und seiner Schlüsseldeterminanten. die Arbeit hatte folgende Ziele:

1. Feststellung, ob *Ascaris*-Nematoden antimikrobielle Moleküle ausscheiden.
2. Charakterisierung der antimikrobiellen Aktivitäten dieser Produkte.
3. Charakterisierung des *Ascaris*-Mikrobioms.
4. Identifizierung der essentiellen Komponenten des Ascaris-Mikrobioms.

Ergebnisse: Es wurde festgestellt, dass die ausgeschiedenen und sekretierten Produkte von *A. suum* eine Vielzahl von Proteinen und Peptiden mit bekannter antimikrobieller Aktivität enthalten, einschließlich antimikrobieller Peptide aus verschiedenen Familien und C-Typ-Lektine. Diese Produkte wiesen verschiedene antimikrobielle Aktivitäten auf, darunter die Hemmung des Bakterienwachstums, die Unterbrechung der bakteriellen Biofilmbildung und die Agglutination. Wir haben eines der von *Ascaris* sezernierten Proteine weiter charakterisiert, ein C-Typ-Lektin- Domäne enthaltenes Protein, AsCTL-42 genannt. Dieses Lektin spiegelte die für *Ascaris*-ES-Produkte beobachtete Agglutination wider und hemmte das Eindringen von *Salmonella* Typhimurium in Darmepithelzellen von Schweinen. Zudem

charakterisierten wir das Mikrobiom von Ascariden, die aus infizierten Schweinen isoliert wurden, und stellten fest, dass das eigene Darmmikrobiom der Nematoden von den im Jejunum des Wirts vorhandenen Mikroben abgeleitet ist, sich aber davon unterscheidet. Die Zusammensetzung des *Ascaris*-Mikrobioms wird in erster Linie von den dominanten Bakterien am Infektionsort bestimmt; bestimmte Gattungen sind jedoch im *Ascaris*-Darm im Vergleich zum Wirt angereichert.

Schlussfolgerungen: Unsere Ergebnisse zeigen, dass das *Ascaris* Mikroben sich ableitet von den Bakterien am Ort der Infektion im Jejunum des Wirts, und dass dieses Mikroben im Wurm im Vergleich zu seinem Wirt sich doch unterscheidet, was darauf hindeutet, dass der Wurm einige Bakterien bevorzugt aufnimmt und andere ausschließt. Unsere Daten charakterisieren potenzielle Mechanismen, durch die der Wurm sein Mikrobiom und das seines Wirts modulieren könnte, indem der Parasit antimikrobielle Moleküle entlässt, die verschiedene antimikrobielle Effektormoleküle enthalten. Unsere Daten bilden die Grundlage für weitere Arbeiten, mit denen die Auswirkungen dieser weit verbreiteten Parasiten und deren Interaktionen mit bakteriellen Mikroben des Wirts, Wirtszellen und deren Auswirkungen auf koinfizierende Krankheitserreger bestimmt werden können, während gleichzeitig bestimmt wird, welche Mikroben für den Wurm nützlich oder schädlich sind.

7. Summary

Bacterial modulation by the intestinal nematode *Ascaris suum*

Background: Ascariasis is one of the most common soil-transmitted helminth infections worldwide and a considerable problem in animal agriculture. The infection is spread via the fecal- oral route and occurs following ingestions of eggs containing infective third-stage larvae. These eggs hatch, releasing larvae which then invade the host intestine and embark on a tissue migration phase which takes them through the host's liver and lungs before returning to the small intestine. This tissue migration leads to the immune responses, pathologies, and symptoms characteristic of ascariasis. In the jejunum, the worms mature and live amongst host microbes. Current efforts to control helminth infections have been somewhat successful but have not been able to eradicate these pathogens. Thus, novel insights into their lifestyle within the host are needed to unveil new therapeutic modalities. Much remains unknown concerning their interactions with microbes in the host intestine. Therefore, this thesis aimed to unravel interactions between *Ascaris* and microbes by characterizing antimicrobial activities of the worms as well as the first description of the *Ascaris* microbiome and its key determinants by completing the following aims:

1. To determine if *Ascaris* nematodes release antimicrobial compounds in their excreted and secreted products.
2. To characterize the antimicrobial activities of these products.
3. To characterize the *Ascaris* microbiome.
4. To identify the primary determinants of *Ascaris* microbiome composition.

Results: The excreted and secreted products of *A. suum* were found to contain a variety of proteins and peptides with known and predicted antimicrobial activity, including antimicrobial peptides from different families and C-type lectins. These products exhibited diverse antimicrobial activities, including bacterial growth inhibition, disruption of bacterial biofilm formation, and agglutination. We further characterized one of the proteins secreted by *Ascaris*, a C-type lectin domain-containing protein we have named AsCTL-42. This lectin recapitulated the agglutination observed for *Ascaris* ES products and inhibited invasion of porcine intestinal epithelial cells by *Salmonella* Typhimurium. Finally, we characterized the microbiome of *Ascaris* nematodes isolated from infected pigs and determined that the nematode's own intestinal microbiome is derived, but distinct from, the microbes present in the host jejunum. Composition of the *Ascaris* microbiome is driven primarily by the dominant

bacteria present at the site of infection; however, certain genera are enriched in the *Ascaris* intestine relative to the host.

Conclusions: Our results demonstrate that *Ascaris* acquires microbes from those present at the site of infection in the host jejunum and that these microbes are present in differential abundances in the worm relative to its host, suggesting that the worm might preferentially take up some bacteria while excluding others. Our data characterize potential mechanisms by which the worm might modulate its microbiome as well as that of its host by demonstrating antimicrobial activities of its excreted and secreted products which contain various antimicrobial effector molecules. Our data provide a foundation for further work which can assess the impacts of interactions between *Ascaris*, microbes, and host cells on co-infecting pathogens while determining which microbes are beneficial or harmful for the worm.

8. References

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9. Publications and Scientific Contributions

9.1 Publications included in this thesis

Midha A, Jarquín-Díaz VH, Ebner F, Löber U, Hayani R, Kundik A, Cardilli A, Heitlinger E, Forslund SK, Hartmann S. (2022). Guts within guts: the microbiome of the intestinal helminth parasite *Ascaris suum* is derived but distinct from its host. *Microbiome,* 10, 229.

Midha A, Goyette-Desjardins G, Goerdeler F, Moscovitz O, Seeberger PH, Tedin K, Bertzbach LD, Lepenies B, Hartmann S. (2021). Lectin-Mediated Bacterial Modulation by the Intestinal Nematode *Ascaris suum*. *International Journal of Molecular Sciences*, 14;22(16):8739.

Midha A, Janek K, Niewienda A, Henklein P, Guenther S, Serra DO, Schlosser J, Hengge R, Hartmann S. (2018). The Intestinal Roundworm *Ascaris suum* Releases Antimicrobial Factors Which Interfere With Bacterial Growth and Biofilm Formation. *Frontiers in Cellular and Infection Microbiology*, 8:271.

9.2 Other publications

Schlosser-Brandenburg J, **Midha A**, Mugo RM, Ndombi EM, Gachara G, Njomo D, Rausch S, Hartmann S. (2023). Infection with soil-transmitted helminths and their impact on coinfections. *Frontiers in Parasitology*, 2:1197956.

Midha A, Ebner F, Schlosser-Brandenburg J, Rausch S, Hartmann S. (2021). Trilateral Relationship: *Ascaris*, Microbiota, and Host Cells. *Trends in Parasitology*, 37(3):251-262.

Bertzbach LD, **Midha A**. (2020). The benefit of science communication and science conference organization for graduate students. *PLoS Blogs*.

Ebner F, Morrison E, Bertazzon M, **Midha A**, Hartmann S, Freund C, Álvaro-Benito M. (2020). $CD4^+$ T_h immunogenicity of the *Ascaris spp*. secreted products. *NPJ Vaccines*, 5:25.

Gordon JA, Noble JW, **Midha A**, Derakhshan F, Wang G, Adomat HH, Tomlinson Guns ES, Lin YY, Ren S, Collins CC, Nelson PS, Morrissey C, Wasan KM, Cox ME. (2019). Upregulation of Scavenger Receptor B1 is Required for Steroidogenic and Nonsteroidogenic Cholesterol Metabolism in Prostate Cancer. *Cancer Research*, 79(13):3320-3331.

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Ebner F, Kuhring M, Radonic A, **Midha A**, Renard BY, Hartmann S. (2018). Silent Witness: Dual- Species Transcriptomics Reveals Epithelial Immunological Quiescence to Helminth Larval Encounter and Fostered Larval Development. *Frontiers in Immunology*, 9:1868.

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Ibrahim F, Sivak O, Wong C, Hopkins P, **Midha A**, Gordon J, Darlington JW, Wasan KM. (2013). Impact of co-administration of prononated nanostructured aluminum silicate (cholesterol absorption inhibitor) on the absorption of lipid soluble vitamins D3 and K1: an assessment of pharmacokinetic and in vitro intraluminal processing. *European Journal of Pharmaceutical Sciences*, 49(2):125-32.

9.3 Conferences and Other Presentations

Talk: Arbeitskreistreffen Veterinärimmunologie (VIA) der Deutschen Gesellschaft für Immunologie, Vienna, Austria, 26.06.-27.06.2023. “Consequences of *Ascaris-Salmonella* co- infection on macrophage functions in the pig”.

Talk: 17th Microbiome Virtual International Forum, online, 17.03.2023. “Guts within guts: the microbiome of the intestinal helminth parasite *Ascaris suum* is derived but distinct from its host”.

Talk: Arbeitskreistreffen Veterinärimmunologie (VIA) der Deutschen Gesellschaft für Immunologie, Hannover, Germany, 06.09.-07.09.2022. “Consequences of *Ascaris-Salmonella* co-infection on macrophage functions in the pig”.

Talk: 28th Annual Meeting of the German Society for Parasitology, Berlin, Germany, 21.03 – 24.03.2018. “Secreted products of the intestinal roundworm *Ascaris suum* impact bacterial growth and biofilm formation”.

Talk: 10th Summer School for Young Parasitologists, German Society for Parasitology, Hamburg, Germany, 07.08 – 11.08.2017. "Antimicrobial strategies of parasitic nematodes".

Poster: Woods Hole Immunoparasitology Meeting, Woods Hole, United States of America, 02.04 – 05.04.2017. "Excretory-secretory products of *Ascaris suum* possess diverse antimicrobial activities".

Poster: 2nd International Symposium on Alternatives to Antibiotics (ATA): Challenges and Solutions in Animal Production, Paris, France, 12.12. – 15.12.2016. "Antimicrobial activities of secreted products of intestinal nematodes".

Poster: 19th Annual Conference, Canadian Society for Pharmaceutical Sciences: From Drug Discovery to Health Outcomes: Population to Patient, Vancouver, Canada, 31.05 – 03.06.2016. "Novel Antimicrobials Derived from Parasitic Nematodes".

Talk: 26th Annual Meeting of the German Society for Parasitology, Göttingen, Germany 09.03 – 12.03.2016. "Antimicrobial activities of secreted products of intestinal nematodes".

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12. Conflict of Interest

In the context of this work, there are no conflicts of interest due to contributions from third parties.

13. Declaration of Independence

I hereby certify that I have prepared this thesis independently. I certify that I have used only the sources and aids indicated. This dissertation has not yet been presented to any other examination authority in the same or a similar form and has not yet been published.

Berlin, den 10.01.2024
Ankur Midha

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