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The impact of nutrition and helminth infection on gut health and the microbiome using a lab-to-wild mouse model

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Rowan Bancroft

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Authorship Declaration

I declare that I am the sole author of this thesis and that it has not been submitted, in whole or part, for any other degree or professional qualification. Except where indicated otherwise by reference or acknowledgement, all writing and analyses herein represent my own work, with input from my main supervisors Amy Pedersen (ABP) and Simon Babayan (SAB).

Data for Chapter 2 and 3 is derived from samples collected during laboratory experiments conducted in 2021 and field studies conducted in 2017 and 2020. Field samples were collected by Amy Sweeny (AS) and Saudamini Venkatesan (SV) in 2017, and by a core field team consisting of myself, Jessica Hall (JH), Agata Delnicka (AD) and Sam Hillman (SH) in 2020 funded by a NERC grant lead by ABP, Andy Fenton (AF) and SAB. I carried out all laboratory work for both chapters, with some sampling assistance from both JH and AD, and some help with DNA extractions from a 4th year honours student, Phoebe Beal. In addition, a master's student, Olivia Flemming helped with some ELISA assays. Caesarean sections, re-derivation and day-to-do colony maintenance was conducted by staff at the Bioresearch and Veterinary Services (BVS), University of Edinburgh under the guidance of BVS Vet Gidona Goodman, JH and ABP in 2018. I have assisted with the management and breeding of the wood mouse colonies described since 2021. All sample processing and subsequent analysis was conducted by me, with guidance from supervisory team: ABP, SAB and AS.

Data for Chapter 4 is derived from samples collected during a field study in 2016 and laboratory study in 2017. All samples were collected by AS with help from SV and associated parasitology laboratory work was conducted by AS with faecal egg count assistance from JH and SV. I conducted all DNA extraction, PCR, and library preparation for sequencing of samples and performed sample processing and analysis, with guidance from ABP, AS, SAB.

I use 'we' throughout my data chapters to account for the collaborative nature of this work as described above, and to remain consistent with the format of scientific papers.

Abstract

The mammalian gastrointestinal tract is a rich ecosystem composed of complex interactions. It is home to a diverse community of bacterial microbes known as the gut microbiota, the preferential niche for many helminth parasites and the largest site of both the immune system and diet-derived nutrient absorption. To date, most studies exploring the relationship between nutrition, helminth infection and immunity, and the gut microbiota have utilised controlled laboratory-rodent models, with intentionally limited genetic, ecological, and behavioural variation. While these controlled studies have been very valuable for understanding mechanisms, they are limited in their ability to extrapolate the outcome of these interactions to natural populations. Using a lab-to-wild mouse model, with both wild and laboratory-reared wood mice (*Apodemus sylvaticus)*, the key aim of my thesis is to use this unique approach to both elucidate the impacts of nutrition and helminth infection on the gut microbiota and health in the lab, and then importantly to test the consequences of these interactions in the wild.

First, I characterised the gut microbiota of our recently derived paired laboratory wood mouse colonies, and found that our formerly wild, but laboratory-reared wood mice had a wild-like gut microbiota (Wild-like: *A. sylvaticus*), similar, although less diverse than wild wood mice. In contrast, our recently caesarean re-derived wood mouse colony, who were fostered by standard laboratory mice (*Mus musculus*) had a more lab-like gut microbiota (Lab-like: *A. sylvaticus*), but also shared many bacterial taxa with other Wild-like:As mice. Then, I investigated how these Wild-like:As and Lab-like:As colonies responded to infection with the gastrointestinal helminth, *Heligmosomoides polygyrus,* which is a natural parasite of wood mice within the wild. I assessed how the diversity and stability of the microbiota composition was altered over the course of infection and determined if this differed between the two colonies. I found that immune responses differed between the two wood mouse colonies and that this was impacted by helminth infection, whereby infection was associated with a decrease in gut-microbiota diversity of Wild-like:As mice, but not Lab-like:As mice.

Previously, we have shown that wood mice given a high-quality supplemented diet, were more resistant to helminth infection and generated stronger immune responses,

in both the wild and our wild-like colony. Here, I expanded upon the findings of this earlier study, by investigating the role of the gut microbiota on the impact of high-quality diet supplementation and improving helminth resistance. I found that nutrition and to a lesser extent helminth infection significantly drive the microbiota composition and diversity in both lab and wild wood mice; and could be, in part, important in driving the impact of nutrition on helminth immunity.

Overall, this thesis both develops an exciting new lab-to-wild mouse model that will enable both mechanistic studies in the lab, and fitness-relevant experiments in the field to better understand the complex interactions between nutrition, infection, the gut microbiota, and health. Importantly, my results show that the gut microbiota is an important player in the gut ecosystem, and my results provide a greater understanding of how the interplay between nutrition, immunity and helminthiasis can impact host health and infection dynamics.

Lay Summary

The digestive system, also known as the gastrointestinal tract (GI), of most mammals, consists of a several tubular organs, including the oesophagus, stomach, small intestine, large intestine, and rectum. These organs work together, to allow the GI tract to carry out its essential functions of digestion and absorbing nutrients from an individual's diet, which then provide the body with energy. The GI tract is also home to a diverse community made up of millions of microbes, mostly bacteria, which is referred to as the gut microbiota. Most bacteria within the gut microbiota are harmless and in fact, facilitate digestion. However, there is a large immune system within the GI tract that interacts with the microbiota and prevents the bacteria here causing infection. In addition, an estimated 1.5 billion people, and many free-living animal populations are infected with gut dwelling parasitic worms known as helminths. A well-described characteristic of helminths is that they can interact with an individual's immune system, to prevent an immune response that would kill and remove them from the body. As such, many helminth infections last for years, with negative health consequences for the individual infection. My thesis focuses on developing our understanding of how the gut microbiota and helminths interact with each other and the immune system, to determine how this affects the health of an individual. I also explore what impact an individual's diet can have on these microbiota and helminth interactions within the gut.

To date, most studies investigating the relationship between nutrition, helminth infection and immunity, and the gut microbiota have utilised laboratory-mouse models. These have been a valuable tool in aiding our understanding of health and disease among mammals and allowed us to develop a number of successful disease treatments and vaccines. However, lab mice are housed under semi-sterile, controlled conditions where they have constant access to food and water and are specifically bred to limit variation, so that different individuals often respond in a similar way to infection or disease. However, this does not truly reflect real-life where both humans and wild-animal populations live in very different environments, have differing diets and are exposed to different infections and bacteria that make up their microbiota. As such, in this thesis I use a lab-to-wild mouse model, with both wild and laboratory-reared wood mice (*Apodemus sylvaticus)*. This is a unique approach, whereby I can

investigate the impacts of nutrition and helminth infection on the gut microbiota in controlled laboratory settings, but also make direct comparisons between these interactions taking place in nature.

First, I characterised the gut microbiota of two distinct cohorts of wood mice, one cohort consisted of mice that were formally wild, living in UK woodlands, but are now laboratory-reared and have a wild-like gut microbiota (Wild-like: *A. sylvaticus*), similar, although less diverse than wild wood mice. Our second cohort consisted of wood mice from this wild-like cohort, but were fostered and reared from birth, by standard laboratory mice (*Mus musculus*) and therefore had a lab-like gut microbiota (Lab-like: *A. sylvaticus*). I found that the gut microbiota of these two mouse cohorts significantly differed in a number of ways, including how diverse their gut microbiota was and what bacteria made up the microbiota (composition). Then, I investigated how these Wild-like:As and Lab-like:As colonies responded to infection with the gastrointestinal helminth, *Heligmosomoides polygyrus,* which is a natural parasite of wood mice within the wild. I assessed how the diversity and stability of the microbiota composition was altered over the course of infection and determined if this differed between the two colonies. I found that immune responses differed between the two wood mouse colonies and that this was impacted by helminth infection, whereby infection was associated with a decrease in gut-microbiota diversity of Wild-like:As mice, but not Lab-like:As mice.

Previously, we have shown that wood mice given a high-quality supplemented diet, were able to generate stronger immune responses to helminth infection, killing the worms and removing them from the gut. This was shown in both wild wood mice and those in our Wild-like:As colony. Here, I expanded upon these findings, by investigating the impact of high-quality diet supplementation on the gut microbiota of mice in the lab and wild, to provide a greater understanding of this may have impacted helminth infection. Here, I found that nutrition and to a lesser extent helminth infection significantly drive the microbiota composition and diversity in wood mice in both environments; and could be, in part, important in driving the impact of nutrition on helminth immunity.

Overall, this thesis both develops an exciting new lab-to-wild mouse model that will enable a greater understanding of interactions within the gut and how these alter can individuals health. Importantly, my results show that the gut microbiota plays an important role in maintaining gut health, especially during infection and my results provide a greater understanding of how the interplay between nutrition, immunity and helminth infection can impact health and infection dynamics.

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Chapter 1

1. General introduction

Section 1

1.1. The gastrointestinal environment: A thriving ecosystem

The mammalian gastrointestinal (GI) tract comprises a system of tubular organs, from the mouth to the rectum that cooperate to extract and absorb nutrients from the diet, providing the body with the energy and metabolites required for essential functions such as cell division, ion transport and tissue repair (Coss-Bu & Mehta 2016; Liao et al. 2009). The gut also acts as one of the body's main lines of defence, by providing a physical barrier to prevent exogenous elements entering circulation or gaining access to the rest of the body, and thus, has an extensive mucosal immune system (Liao et al. 2009; Vancamelbeke & Vermeire 2017). As a result of the GI tract being constantly exposed to the external environment through consumption of food, it is home to a complex and diverse ecosystem. This ecosystem is composed of commensal microorganisms, including bacteria, fungi, protozoa and viruses, known as the gut microbiome, which has co-evolved with its host, fostering a symbiotic relationship (Lozupone et al. 2012). Bacterial communities make up the largest component of the gut microbiota and as such will be the focus of this thesis, henceforth referred to as the gut microbiota (Bhattacharjee et al. 2022).

The advancement of high-throughput sequencing technologies over the last decade has led to detailed characterisation of the microbiota composition of many hosts during health and disease (Lozupone et al. 2012). The composition of the microbiota varies throughout the mammalian digestive system; however, the highest density resides in the large intestine, including the caecum and colon, which can be inhabited by between 300-1000 different species of microbes at any one time (Bhattacharjee et al. 2022; Desselberger 2018; Guarner & Malagelada 2003). Among mammals, the most common types of bacteria found within the microbiota are usually derived from just

five phyla: *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria* and *Fusobacteria* (Bhattacharjee et al. 2022; Kinross et al. 2011; Moszak et al. 2020). The *Firmicutes* alone comprise more than 200 different genera (one order above species), including *Lactobacillus*, *Clostridium,* and *Enterococcus* whereas the *Bacteroidetes* phylum is predominantly made up of bacteria from the *Bacteroides* and *Prevotella* genera (Bhattacharjee et al. 2022; Desselberger 2018).

Colonisation of the GI tract by microbiota begins at birth by vertical transmission from the mother (Donovan 2020). While there is evidence that the mode of delivery and provision of breastmilk can have a marked impact on the early microbiota composition (Cioffi et al. 2020; Keady et al. 2023; Reyman et al. 2019; Rutayisire et al. 2016), the microbial community of most mammals continues to evolve through the first weeks and months of life, and stabilises post-weaning (Cioffi et al. 2020; Donovan 2020; Frese et al. 2015; Ge et al. 2021; Keady et al. 2023; Schloss et al. 2012). Over time, while the gut microbiota becomes stable within individuals, there is significant variation between individuals of the same species, due to considerable ecological, genetic, and environmental variation leading to unique antigen exposure for every individual (Lozupone et al. 2012; Sommer & Bäckhed 2013; Turnbaugh et al. 2009). For instance, in humans, geographical location and cultural practices appear to be key drivers of microbial composition (Gupta et al. 2017; Tasnim et al. 2017; Yatsunenko et al. 2012), whilst in animals, distinct differences can be seen between free-living populations and those reared in captivity (Clayton et al. 2016b; McKenzie et al. 2017a; Suzuki 2017; van Leeuwen et al. 2020). To determine how these GI communities influence host health and disease, it is important to understand which factors cause the gut microbiota to vary within and between species.

1.1. The functional role of the gut microbiota

Extensive evidence from human and model systems indicates that these microbial communities are responsible for a wide range of biological processes which are vital for host health (Bhattacharjee et al. 2022; Kinross et al. 2011; Lynch & Pedersen 2016; Sommer & Bäckhed 2013). The specific role of the gut microbiota in host health and disease has been reviewed comprehensively elsewhere (Bhattacharjee et al. 2022;

Fujimura & Lynch 2015; Kinross et al. 2011; Lynch & Pedersen 2016; Nicholson et al. 2012; Sommer & Bäckhed 2013); here I summarise a select few essential roles.

One key role that gut resident bacteria play is aiding in the breakdown of indigestible dietary plant cell-wall polysaccharides and resistant starch into short-chain fatty acids (SCFAs) which can be utilised for downstream metabolic processes (Nicholson et al. 2012; Parada Venegas et al. 2019; Zhang & Davies 2016). The gut microbiota has also been implicated in energy homeostasis and metabolization of pharmaceutical drugs (Nicholson et al. 2012; Turnbaugh et al. 2006; Wilson & Nicholson 2009), as well as vitamin synthesis (LeBlanc et al. 2013; Zhang & Davies 2016). Studies with germ-free or antibiotic treated mice have highlighted that the host-microbiota can play an essential role in preventing colonisation by, and overgrowth of, pathogens (Bäumler & Sperandio 2016; Kamada et al. 2013). It is also important to note that whilst the microbiota play key roles in supporting host-health, they can also significantly impair it when disrupted, and are in dysbiosis. Dysbiosis can be defined as an imbalance in the composition of the intestinal microbiota communities that leads to disruption of host-microbe homeostasis (Kinross et al. 2011; Sommer & Bäckhed 2013). Gut microbiota dysbiosis has been associated with several diseases, ranging from obesity (Ley et al. 2005; Turnbaugh et al. 2006), inflammatory bowel disease (Lavelle & Sokol 2018; Schirmer et al. 2019) and diabetes (Giongo et al. 2011), to atopic asthma and allergy (Durack et al. 2018; Fujimura & Lynch 2015), cardiovascular disease (Wang et al. 2011) and autoimmune conditions (Cekanaviciute et al. 2017; Wells et al. 2020). Therefore, the relationship between the gut microbiota and the host gut immune system is complex, and what denotes a ‘healthy’ microbiota appears highly context dependent. This theme of the complex interplay between gut microbiota and host health will be expanded upon throughout this thesis.

1.3. An overview of the gut

Within mammals, the GI tract is the largest mucosal site and due to its continuous contact with internal and external foreign antigens, it is a crucial site for host innate and adaptive immunity (Artis 2008; Mason et al. 2008; Mowat & Agace 2014; Soderholm & Pedicord 2019; Turner 2009). However, the GI immune system must differentiate between self and non-self, commensal bacteria, and pathogenic bacteria,

and thus strike a balance between immune tolerance and activation, which the gut microbiota can also directly influence (Artis 2008; Mowat & Agace 2014; Sommer & Bäckhed 2013).

The cellular structure and morphology of the GI tract is uniquely adapted to its function as an interface for the cross-communication of the gut microbiota and host immune system (Goto & Kiyono 2012; Mason et al. 2008; Soderholm & Pedicord 2019). It is lined, on the luminal surface, by a single layer of Intestinal Epithelial Cells (IECs) called the epithelium. This acts as a physical barrier to separate host connective tissues and access to the body's microvasculature from the external environment (Goto & Kiyono 2012; Soderholm & Pedicord 2019). Throughout the small intestine, the epithelium forms structures known as crypts and villi, increasing the mucosal surface area for more efficient nutrient absorption (Clevers 2013; Mowat & Agace 2014; Sumigray et al. 2018). Villi are finger-like protrusions that begin at the gut wall and project into the lumen of the gut, composed mostly of absorptive IEC's called enterocytes, under which lie a complex network of lymph vessels and capillaries which meditate nutrient absorption (Ensari & Marsh 2018; Mowat & Agace 2014). Enterocytes constitute approximately 80% of all epithelial cells and are also responsible for secreting the predominant antibody found within the gut, immunoglobulin A (IgA) (de Santa Barbara et al. 2003; Hand & Reboldi 2021), which plays a crucial role in preventing pathogen invasion and shaping the composition of the gut microbiota, through several mechanisms. For instance, IgA has been shown to be able to neutralise pathogens and toxins, such as those produced by *Vibrio cholerae* and *Escherichia coli* (Clements & Norton 2018; Hand & Reboldi 2021). Secretory IgA can also bind to and modulate bacterial surface proteins, disrupting their function and expression (Hand & Reboldi 2021; Pabst & Slack 2020; Peterson et al. 2007), and can aid in the production of agglutinated clumps of proliferating bacteria which are then more easily removed from the gut through wave-like muscle contractions known as peristalsis (Moor et al. 2017). In the small intestine, each villus is flanked by smaller invaginations of the epithelium, which form structures called the crypts of Lieberkühn, the large intestine is made up of these crypt structures only, with no villi (Mowat & Agace 2014). The base of these crypts is home to pluripotent stem cells, which can differentiate into specialised IECs (Clevers 2013; Mowat & Agace 2014). For example, tuft cells, which promote type 2 immunity, are needed for cell repair and expulsion of extracellular parasites (Howitt et

al. 2016). Paneth cells secrete antimicrobial peptides which help keep the microbiota in check and prevent pathogenic bacteria from colonising, and microfold cells capture antigens entering the gut from the environment and present these to immune cells (Mowat & Agace 2014). Approximately 10% of the total IECs distributed throughout the GI tract are goblet cells, which are responsible for reinforcing the physical defence barrier within the gut, through production of the mucus layer (Kim & Ho 2010). The epithelium of the GI tract is coated in a viscous, sticky, mucus layer, made up of mucin proteins such as mucin 2 (MUC2), that are secreted by goblet cells (Kim & Ho 2010). This mucus layer is composed of an inner and outer layer, which range in thickness along the GI tract and provide the epithelium with vital protection against both physical and chemical injury from host-ingested food and microbes (Goto & Kiyono 2012; Paola & Patrice 2020). Alongside the epithelium, the intestinal environment is also populated by structures known as gut-associated lymphoid tissue (GALT) including Peyer's patches, mesenteric lymph nodes and lymphoid follicles, which are enriched with a suite of immune cells including various types of T cells and B cells as well dendritic cells, which act as the gut's armoury in case defence is needed (Artis 2008; Mowat & Agace 2014; Sommer & Bäckhed 2013). As alluded to, the gut microbiota are active participants in the gastrointestinal ecosystem and thus, play a key role in maintaining immune homeostasis here (Yoo et al. 2020).

1.4. Host-microbiota interactions within the gut

The relationship between the gut microbiota and host immune system of humans and other mammals, including rodents, has been studied thoroughly over recent years (Arrieta & Finlay 2012; Goto & Kiyono 2012; Kabat et al. 2014; Macpherson & Harris 2004; Smith et al. 2007; Soderholm & Pedicord 2019). In this section, I will highlight some key interactions that are relevant to the specific content of my thesis. To date, much of our understanding of microbiota-host interactions has arisen from studies using germ-free (axenic) and gnotobiotic (colonised with a few known bacterial species) mice within the laboratory (Arrieta & Finlay 2012; Smith et al. 2007). These studies have highlighted that in the absence of the gut resident microbes, the host immune system develops poorly. For instance, in conventional lab mice and humans,

IECs within the GI tract express pathogen recognition receptors (PRR) on their cell surface, which identify specific molecular patterns on both commensal and pathogenic bacteria, leading to production of antimicrobial peptides and mucus secretion to combat pathogenic invasion (Soderholm & Pedicord 2019). However, germ-free mice were found to have lower proliferation rates of IEC's and these IECs also expressed lower levels of PRRs compared to conventional mice (Arrieta & Finlay 2012; Willing & Van Kessel 2007). Moreover, colonisation of germ-free with segmented filamentous bacteria (SFB), that come into direct contact with the IEC's, has been shown to induce development of the mucosal immune system in these mice, mostly through T cell activation (Gaboriau-Routhiau et al. 2009; Ivanov et al. 2009a; Khoury et al. 1969), suggesting these bacteria may play a key role in inducing IEC's to activate an immune response to pathogens.

Whilst the majority of bacteria within the gut inhabit the gut lumen, some, such as those from the *Lactobacilli* genus and SFB have developed the ability to penetrate the outer mucus layer via adhesion molecules including lectins and glycosidases and mucus binding proteins (Derrien et al. 2010; Juge 2012). Further, several bacteria belonging to the *Streptococcus, Bifidobacterium, Helicobacter and Clostridium* genus, to name a few, have been shown to produce enzymes that can specifically degrade the mucin proteins that make up the outer mucus layer (Derrien et al. 2010). However, studies in conventional lab mice have shown that the inner mucus layer of the colon lack the presence of any bacteria, whereas mice deficient in the main mucin protein of the mucus layer, MUC2, had evidence of bacterial penetration beyond the mucus layer and into the intestinal crypts (Hansson & Johansson 2010; Johansson et al. 2011). Again, studies in germ-free mice demonstrated that without microbes, the number of mucus producing goblet cells was reduced and the mucus layer of the small intestine was anchored to the goblet cells (Johansson et al. 2015). The production of IgA has also been shown to be influenced by the presence of gut microbes. A study comparing two conventional lab mouse strains, found that an increase in IgA abundance and diversity was associated with increased microbiota diversity (Fransen et al. 2015; Soderholm & Pedicord 2019). A common commensal bacterium, *Bacteroides fragilis,* is able to bind to intestinal IgA, allowing it to colonise an intestinal niche close to the IECs (Donaldson et al. 2018) and mice with deficient IgA levels within the gut show persistent colonisation with SFB (Suzuki et al. 2004). Moreover, the GALT structures

within the gut of germ-free mice, including the mesenteric lymph nodes and Peyer's patches, have also been found to be underdeveloped, suggesting a key role of the commensal gut bacteria in the development of these lymphoid structures (Bouskra et al. 2008; Macpherson & Harris 2004; Mazmanian et al. 2005b). Our ability to define how bacteria within the GI tract interact with the host-immune system is not only thanks to the creation of germ-free animal models, but also due to advances in computational programs and tools that make analysis and interpretation of complex datasets achievable.

1.5. Studying the microbiota in the lab

There is a wealth of bioinformatic methods available for the study of microbiota data. However, the lack of a 'gold standard' approach to the analysis and the wealth of options available can make comparative analysis difficult. However, several studies have analysed and compared the differing approaches and have provided recommendations for analysis (Galloway-Peña & Hanson 2020; Knight et al. 2018; Lutz et al. 2022; Peeters et al. 2021). Currently, most studies characterising the gut microbiota use a technique of amplifying bacterial 16s ribosomal RNA (rRNA) genes through polymerase-chain-reaction (PCR) and then sequencing the resultant DNA. These rRNA genes are highly conserved among bacteria and contain hypervariable regions, generating species-specific sequences which allow for the identification of individual bacterial taxa (Woese & Fox 1977; Woo et al. 2008). Until recently, most studies organised bacterial sequences into operational taxonomic units (OTU) which usually contained a group of sequences with approximately 97% similarity (Knight et al. 2018). However, over the last 5-7 years, there has been a shift to instead use amplicon sequence variants (ASVs) whereby the exact sequence for each read is used, this allows for the detection of subtle biological variation, such as single-nucleotide polymorphisms (SNPs) (Knight et al. 2018). Taxonomy can then be assigned to ASVs using machine learning tools such as the naïve Bayesian classifier, the Ribosomal Database Project (RDP) (Wang et al. 2007), to predict the alignment of sequences against taxonomic databases including Greengenes or Silva (McDonald et al. 2012; Yilmaz et al. 2013). This process typically results in a large data matrix whereby individual ASV abundances are assigned to their respective samples and can then undergo statistical analyses (Knight et al. 2018; Peeters et al. 2021). Here, we

describe some of the most common methodologies for extracting meaningful information from microbiota sequence data and highlight those that are used throughout this thesis.

To gain an initial overview of which taxa are most or least prevalent among samples, studies often convert sequence count data into a relative abundance value. This provides the abundance, as a percentage, of a single bacterium within a sample, relative to the total number of bacteria within the sample, as such the relative abundance for an entire sample is equal to 1. Data are then typically visualised in this format through bar charts or heat maps (Peeters et al. 2021). For a more detailed insight into the within-sample microbiota community structure, alpha diversity measurements are employed. These approaches measure the number of bacterial taxonomic groups present within a sample (referred to as taxonomic richness) and the distribution of the abundance of each taxonomic group within a sample (referred to as taxonomic evenness) (Whittaker 1960; Willis 2019). Usually, the output of these approaches generates a mean value for a sample, which can then be compared to another sample, and scaled up so that means between two groups of samples can be statistically compared (Knight et al. 2018). Many methods for estimating alpha diversity exist, for the analysis of data throughout this thesis, I selected four commonly used metrics to cover both breadth and depth, with each metric considering distinct properties within the data. All samples were analysed at the ASV level here.

As the most basic way of comparing diversity between samples, I used observed richness, which provides a count of the number of individual bacterial species identified within a sample (Fisher et al. 1943). Next, the second metric of alpha diversity that I used, Faith's phylogenetic diversity, considered how closely related bacterial taxa within a sample are by estimating their phylogeny and is calculated as the sum of the branch lengths of a phylogenetic tree connecting all species within a sample (Faith 1992). Here, the longer the branch lengths, the higher the phylogenetic diversity value, the more distinctly related taxa are from each other. The third metric I used was the Shannon diversity index, which calculates a value between 0-5 that represents the uncertainty in predicting a single species identity when taken at random within a community and considers both species richness and evenness within a sample (Shannon 1948). The more species within a sample, the greater the

uncertainty, as such the Shannon index has a species richness bias. Finally, the Simpson index, also considers both species richness and evenness, by estimating the probability that two taxa selected at random from a sample will belong to the same taxa (Simpson 1949). As such, the Simpson index has a bias towards the most abundant species within a sample. Traditionally, a higher Simpson index value, usually between 0-1, indicates a lower diversity, therefore, we use the inverse Simpson index, so that with each of our metrics, a higher value equates to a higher within sample diversity. Alpha diversity metrics are sensitive to the sequencing depth of a sample, for instance, greater numbers of unique taxa are more likely to be detected in samples with the highest number of sequencing reads. Rarefaction is a method that adjusts for differences in sequencing depth between samples. Here, the number of reads for the sample with the lowest sequencing depth within a dataset, is selected, known as the minimum library size (Hughes & Hellmann 2005; Willis 2019). All subsequent samples are then subsampled, without replacement at the depth of the minimum library size, which means each sample is subsampled equally, at a constant depth, allowing samples to be compared, independently of their library size (McMurdie & Holmes 2014; Willis 2019). Although commonly used, there has been some dispute among the research community as to whether rarefaction should be employed, as this process leads to the omission of sequence data in samples with library sizes larger than the minimum library size, which can lead to the loss of valid biological information about such samples (McMurdie & Holmes 2014; Schloss 2023; Willis 2019). However, to date, a consensus has not been conclusively reached about what alternative method to rarefaction is most appropriate.

To quantify taxonomic diversity between samples at the ASV level, I used three metrics of beta diversity, which measures how different each sample is compared to every other sample within the population, as such more than one value is generated per sample and these values are usually reported in a distance matrix with pairwise sample comparisons (IMPACTT investigators 2022; Knight et al. 2018). Again, each metric considers different sample properties, so the metric used can influence the outcome (Kuczynski et al. 2010). Therefore, I used three common approaches throughout this thesis to account for this. The Bray-Curtis dissimilarity metric accounts for both the taxa abundances shared between two samples and the number of taxa detected in each sample (Bray & Curtis 1957). A distance value between 0-1 is

generated, whereby two samples sharing the same number of taxa in the same abundance, would have a dissimilarity distance of 0, thus the lower the Bray-Curtis distance, the more similar the samples compositions (Bray & Curtis 1957). The UniFrac distance metrics assess the phylogenetic distances between taxa across samples. The Weighted UniFrac also considers the abundance of each taxa present, whilst the Unweighted Unifrac accounts for difference in presence and absence of taxa between two samples (Lozupone et al. 2007).

Whilst beta diversity analysis provides intel on how (dis)similar the composition of microbiota samples are when compared to each other, it does not provide specific information about what taxa may be driving these differences, for this, I employed differential abundance analysis. Several methods have been developed for this; however, results have been shown to vary between techniques (Nearing et al. 2022). I chose to use two popular approaches, based on the structure of my datasets and the research questions I aimed to answer. DESeq2 utilises sequencing read counts via a matrix of integer values and fits a Generalised Linear Model (GLM) with a negative binomial distribution to this count data. Differences between biological replicates are modelled by a dispersion parameter using empirical Bayes shrinkage, to estimate priors for log fold change and then calculate posterior estimates for these values (Love et al. 2014). Differences in bacterial taxon abundance are reported as log2 fold-change (L2FC) and a Wald test used to test for significance. As such, a negative L2FC indicates significant depletion and a positive L2FC indicates a significant enrichment with a bacterial taxon (Love et al. 2014).

The ANOVA-like differential expression tool estimates per taxon technical variation within each sample using Monte-Carlo instances drawn from the Dirichlet distribution and applying centred-log-ratio transformation to data to account for its compositional nature (Fernandes et al. 2014). Here, I compared samples among groups in a pairwise manner, and the difference in abundance of taxa is reported as an ALDEx2 effect size. This provides an estimate of the median standardised difference between two groups where the size of the effect is proportional to the difference in abundance of taxa between samples, thus, the greater the effect size the greater the difference between two taxa (Fernandes et al. 2014; Gloor 2023). The direction of the effect (positive or negative) is dependent upon the order that two groups were compared;

thus, a negative effect does not necessarily signify a significant depletion. Here p-values are adjusted for multiple comparisons using the Benjamini-Hochberg (BH) correction (Fernandes et al. 2014; Gloor 2023). Further analysis can be undertaken, to understand functional differences between microbiota communities, however, this analysis was beyond the scope of the research questions in this thesis.

Section 2

2.1. Gut interactions: Hosts, helminths, and bacteria

Evidence suggests that bacteria and helminths have co-evolved with their hosts over millions of years, during which time the mammalian immune system has also evolved (Araujo et al. 2008; Clark 1994; Fumagalli et al. 2009; Jackson et al. 2009). A well described feature of helminth infections is their ability to modulate host immunity to establish chronic infections (Maizels et al. 2004; McSorley et al. 2013) and we have previously outlined examples of how the gut microbiota can interact with the host immune system within the gut. Chronic helminth infection has previously been associated with an increased susceptibility to co-infection (Liu et al. 2010; Maizels 2005) whilst the presence of pathogens has also been shown to regulate the gut microbiota (Spor et al. 2011), thus, it is highly plausible that as these two communities occupy the same niche within the host, they may interact here and influence each other pathogenicity. As such, studies are beginning to explore how these macro-parasites and microbes interact with each other in the gut and what impact this relationship may have on host health and disease.

The gastrointestinal tract of many free-living mammals is occupied by soil transmitted helminths (STH) alongside their commensal bacterial communities. STH's are parasitic worms that are ingested by the host though contaminated soil or food sources, more than 1.5 billion humans are estimated to be infected with these helminths globally (World Health Organisation 2023). Once in the intestinal environment, the worms mature into adults, which reproduce, and shed up to thousands of infective eggs into the environment via host faeces every day (Bethony et al. 2006; World Health Organisation 2023). Furthermore, helminth infections in

livestock are ubiquitous (Bethony et al. 2006; Morgan et al. 2013; Wolstenholme et al. 2004), resulting in dramatic losses in productivity (meat and milk yields) and significant economic costs (Bethony et al. 2006; Charlier et al. 2014; Morgan et al. 2013). The lifespan of helminth infections vary, with the shortest infections lasting 2-3 weeks, but more often infections are chronic, existing within hosts for years with significant negative health consequences (Grencis 2015b; McKay et al. 2017).

Observational studies in humans have provided evidence that helminths and bacteria may interact within a host. Faecal samples collected from helminth infected and non-helminth infected individuals from two Malaysian villages showed that infection was associated with a significant increase in bacterial diversity. More specifically, infections with *Trichuris trichiura* (whipworm) were associated with an increased prevalence of bacteria belonging to the *Paraprevotellaceae* family (Lee et al. 2014). Another study of school children in Ecuador, found that the microbiota composition of children with *T. trichiura* infection was not significantly different to uninfected children, however, children that were co-infected with *T. trichiura* and *Ascaris lumbricoides* (roundworm), had lower abundance *Firmicutes* bacteria, in particular those from the *Clostridia* genus (Cooper et al. 2013). Whilst demonstrating that parasite infections may influence microbiota diversity, these studies do not provide mechanistic insight into what drives these possible bacteria-helminth interactions.

In vitro experiments have explored possible mechanisms, for example mouse and swine specific whipworm strains (*T. muris* and *T. suis* respectively) have demonstrated that the helminth eggs are unable to hatch into their infective larvae stage without the presence of specific gut bacteria, *Escherichia coli* and *Staphylococcus aureus* in the case of *T. muris* and Gram-positive bacteria for *T. suis* (Hayes et al. 2010; Vejzagić et al. 2015). Early studies with germ-free mice also indicated that intestinal bacteria may be required to promote helminth infection. Low-dose infection with the murine gastrointestinal nematode *H. bakeri*, resulted in lower worm burdens among both germ-free and gnotobiotic mice (colonised with *Lactobacillus spp.* only) compared to conventional lab mice (Chang & Wescott 1972). Similar findings were also observed with experimental infection of conventional and germ-free mice with the roundworm *Nippostrongylus brasiliensis* (Wescott & Todd 1964). More recently, the gut microbiota of two conventional lab mouse strains with differing degrees of susceptibility to *H.*

bakeri infection were compared. C57BL/6 mice are highly susceptible to *H. bakeri* infection and upon infection were found to have significantly increased abundance of the bacterium *Lactobacillus taiwanensis*, compared to BALB/c mice which are relatively resistant to *H.bakeri* infection. BALB/c mice were then exposed to *L. taiwanensis* through their drinking water, and challenged with *H. bakeri,* which led to an increased level of infection compared to BALB/c mice who were provided with normal drinking water (Reynolds et al. 2014). Conversely, another study administered the bacterium *L. casei* to lab mice infected with the roundworm *Trichinella spiralis* and found that this was associated with lower worm burdens (Bautista-Garfias et al. 2001) and mice exposed to the bacterium *Bifidobacterium animalis* were resistant to infection with the helminth *Strongyloides venezuelensis* (Oliveira-Sequeira et al. 2014). These studies provide evidence of a complex interplay between helminth infection and microbiota diversity, demonstrating that helminth infection can influence the specific composition of bacteria, which in turn can impact the success of helminth infection. However, it appears that outcome of bacteria-helminth interactions may also be context dependent and host-specific and helminth-specific.

2.2. A closer look at gastrointestinal helminths

Infection with gastrointestinal helminths can cause significant damage and restructuring of host-tissues (Colombo & Grencis 2020; King & Li 2018; Sorobetea et al. 2018), this is due to their often-substantial size and invasive nature. Upon infection, the intestinal epithelial cells (IECs) are exposed to parasites and contact with antigenic parasite proteins , lead's to host-immune cell activation and release of antimicrobial peptides, cytokines, and chemokines (Artis & Grencis 2008; Li et al. 1998). Proliferation and apoptosis of enterocytes leads to increased intestinal crypt and villi length, and this accelerated epithelial cell turnover has been associated with infection clearance (Allaire et al. 2018b; Cliffe & Grencis 2004; Cliffe et al. 2005). Upregulation of IgA secretion (Entwistle & Wilson 2017; Ramos et al. 2022) and goblet cell proliferation (Grencis 2015b; McKay et al. 2017), have also been observed during GI helminth infection, increasing production of intestinal mucins, and reinforcing the mucus barrier which can impede parasite motility and aid expulsion (Grencis 2015b;

McKay et al. 2017). Furthermore, Induction of Paneth cell hyperplasia also occurs; residing at the base of crypts (Clevers & Bevins 2013) these cells synthesise and secrete antimicrobial peptides and proteins (Allaire et al. 2018a), whilst the chemosensory tuft cells play a key role in promoting type 2 cell-mediated immunity (Gerbe et al. 2016).

Gastrointestinal worms have co-evolved with their hosts (Blaxter & Koutsovoulos 2015; Stear et al. 2011), their ability to survive for long periods within the GI tract demonstrates that worms are adapted to the physio-chemical barriers encountered here and can obtain sufficient nutrition within their intestinal niche (Coop & Kyriazakis 2001; McKay et al. 2017; Read 1968; Shea-Donohue et al. 2017a). Establishment of helminth infections is mediated by host immune responses (Bundy & Golden 2009; Koski & Scott 2001), an adaptive type-2 immune response is critical for control and expulsion of worms (Ben-Smith et al. 2003; Hotez et al. 2008; Reynolds et al. 2012b), whereas an inappropriate type-1 immune response exacerbates infection via pro-inflammatory mechanisms (Allen & Maizels 2011; Ben-Smith et al. 2003; Cliffe & Grencis 2004). Helminth parasites have been shown to avoid and/or counteract host immune responses through potent immunomodulatory mechanisms (Maizels et al. 2018). This is no mean feat; the parasites must strike a balance between promoting an immune response that supports their survival whilst also preventing extreme disease in the host which could lead to host-mortality, and secondary infections (Colombo & Grencis 2020). Meanwhile, the host immune response plays a vital role in determining the outcome of the host-parasite relationship; ideally the host immune system will provide an efficient response that is detrimental to the parasite but minimises host immunopathology without consuming vital resources (Graham et al. 2005). Hence, it is plausible that anti-helminth immune responses have evolved to reduce worm burden and promote wound and tissue repair, rather than causing complete parasite expulsion (Colombo & Grencis 2020).

Given the ubiquity and importance of helminth infections in humans and livestock, control efforts at present rely on periodic mass administration of anthelmintic drugs to all at-risk individuals in a population (World Health Organisation 2001). However, due to frequent widespread use and misuse, drug-resistance is rising rapidly (Bethony et al. 2006; Morgan et al. 2013; Wolstenholme et al. 2004). In addition, anthelmintics are

unable to confer protective immunity and given the longevity of infective stages within the environment, both humans and animals can be continuously exposed and rapidly re-infected post-treatment (Bethony et al. 2006; Speich et al. 2016). Without the availability of an effective vaccine, programmes to control helminth infections in endemic regions may lead to short-term improvements in individual health but have been largely ineffective for population-level control (World Health Organization 2005). As such, a more holistic approach to curb infections and reduce the burden of disease may be needed, however, this requires a greater understanding of both the broader environmental conditions and the specific host-helminth interactions that drive infection, immunity, and transmission.

2.3. You are what you eat: the role of host diet in shaping helminth and microbiota interactions.

Whilst the mammalian gastrointestinal tract is home to the gut microbiota communities and a preferential niche for intestinal helminths, it also plays an essential role in acquiring the nutrition needed for growth and maintenance. The GI tract digests food consumed by the host, during which nutrients crucial for supplying the body with energy to carry out essential functions are extracted and absorbed (McKay et al. 2017; Zhang & Davies 2016). Notably, the gut microbiota and helminth parasites also come into direct contact with the ingested nutrients which they can utilise to aid their survival (Coss-Bu & Mehta 2016; Liao et al. 2009). Moreover, the ability of the gut microbiota to digest components of the diet which are otherwise indigestible undoubtedly helps to aid fitness and survival of the host (Coss-Bu & Mehta 2016; Liao et al. 2009). However, in most free-living populations, availability of optimum host nutrition is not consistent or stable, for instance more than 700 million humans are currently estimated to be undernourished (Food and Agriculture Organization of the United Nations 2021), and wild animal populations experience seasonal fluctuations in their food availability (Altizer et al. 2006; Nelson & Demas 1996; Sheldon & Verhulst 1996).

Intestinal helminth parasites can cause malnutrition via their pathophysiology within the gut, leading to nutrient malabsorption and in some cases iron-deficient anaemia,

thus, negatively altering the nutritional status of their host (Koski & Scott 2001; Shea-Donohue et al. 2017b). Indeed, the association between GI helminth infections and malnutrition is highlighted by their shared geographical distribution among humans (Strunz et al. 2014; Yap et al. 2014). Nutrition plays a fundamental role in the development of an effective and protective immune response and malnutrition is a major cause of immunodeficiency globally, altering immunocompetence and increasing susceptibility to infection (Chandra 1972; Chandra 1997; Coop & Holmes 1996). Therefore, it is plausible that changes in host diet and nutrition may not only have consequences for the host itself, but may also affect members of the gut ecosystem, with possible feedback and consequences on host health and susceptibility to disease.

Deficiencies in macro-nutrients such as protein and energy have been shown to significantly impair host immunity to GI helminths (Chandra 1972; Chandra 1997; Coop & Holmes 1996). Studies where lambs were given either low protein or a high protein diet, and experimentally infected with the roundworm, *Oesophagostomum columbianum,* revealed that although parasite establishment was not different among the diet groups, the number of parasites detected at termination of the lambs was significantly lower in those on high protein diets (Bawden 1969; Coop & Holmes 1996; Dobson & Bawden 1974). Similar findings were observed in rats infected with the hookworm, *N. brasiliensis,* whereby animals fed diets deficient in both protein and the micronutrient, iron, displayed chronic infection, with delayed worm expulsion compared to rats given high protein diets and iron supplemented diets (Bolin et al. 1977; Cummins et al. 1978). Further studies in mice have shown that low protein diets lead to delayed expulsion of *T.muris, T. spiralis* and *H. bakeri* infections (Boulay et al. 1998b; Bundy & Golden 2009; Gbakima 1993; Slater 1988). Additionally, trace elements and vitamins are essential for several metabolic pathways and immune cell function (Chandra 1997). The interrelationship between micronutrient deficiencies and GI helminth infections is complex and most research to date has focused on a select few deficiencies (Koski & Scott 2001; Shea-Donohue et al. 2017b). For instance, zinc deficiency in mice infected with *H. bakeri*, has been associated with prolonged infection, increased worm burdens and egg shedding (Boulay et al. 1998b; Shi et al. 1997; Shi et al. 1994).

The diet an individual consumes has an influential role in driving the gut microbiota composition, as both a key source of microbes and by also providing these microbes with fuel in the form of micronutrients and metabolites (David et al. 2014; Muegge et al. 2011). Studies in humans have often focused on comparing the microbiotas of urban and rural communities to distinguish how differing diets impact composition and diversity. Studies on long-term diet patterns of humans found that diets high in protein and animal fat are associated with high levels of bacteria from the *Bacteroides* phylum, whilst high levels of carbohydrates are associated with elevated levels of *Prevotella* phylum (Wu et al. 2011). Moreover, experimental perturbation of 5 conventional lab mouse strains that were fed either a low fat, high-plant-polysaccharide diet (LFPP) or a high-fat, high-sugar diet (HFHS) for at least 15 weeks, revealed that the HFHS diet altered the gut microbiota of each mouse strain in a similar fashion, regardless of genotype (Carmody et al. 2015). Mice on the HFHS diet had significantly increased levels of bacteria belonging to the *Firmicutes* and *Verrucomicrobia* phyla and decreased levels of *Bacteroidetes* compared to those on the LFPP diet regimen (Carmody et al. 2015). Evidence from a wide range of wild animal populations, from primates to rodents, have also demonstrated that the gut microbiota composition shifts in response to seasonal changes in the diet (Amato et al. 2015; Carey et al. 2013; Li et al. 2023; Maurice et al. 2015b; Ren et al. 2017; Xue et al. 2015). Moreover, studies examining the microbiota composition of paired captive and free-living mammals, found that those in the wild tend to have a more diverse microbiota which may be a consequence of, or allow for greater adaptability to, a changing external environment and nutrient intake (Clayton et al. 2016a; Kohl & Dearing 2014; Kohl et al. 2014; Martínez-Mota et al. 2020; McKenzie et al. 2017b; Wang et al. 2023).

However much of the work today investigating the complex interactions between diet, helminth infections and the gut microbiota have either studied just two factors at a time and/or used experimental studies in controlled, laboratory conditions or observational studies of humans. To better understand how these three factors - helminths, diet and the gut microbiota interact and potentially impact host health, we need to move to a more holistic approach where all factors are considered and/or manipulated in a more real-world, ecologically relevant system. For example, a recent study, took this approach and provided novel mechanistic insights into the effect diet changes can have on modulating the gut microbiota and reducing damage caused by helminth

infection. Here, the investigators provided krill oil supplementation, a product rich in Omega-3 fatty acids and antioxidants, to pigs that were infected with the whipworm, *T. suis* (Liu et al. 2020). They found that supplementation reduced helminth-induced intestinal damage and led to a reduction in the abundance of bacteria from the *Rickettsiales* and *Lactobacillus* genera (Liu et al. 2020). These findings, suggest that changes within the host nutritional status, may have the ability to restore homeostasis within the gut, even when tissue damaging helminths are present, and may provide a novel resolution to sustain livestock productivity in an era of reduced efficacy of anthelmintics.

In addition, a second study, gave *T. suis* infected pigs supplementation with dietary inulin, a type of prebiotic fiber. Here, they found that the parasite infection induced a typical Type 2 immune response with increased goblet and tuft cell proliferation in the colon. However, the presence of inulin, suppressed key pro-inflammatory genes and reduced the levels of bacterial phyla associated with inflammation including *Proteobacteria* and *Firmicutes* (Myhill et al. 2018). Again, this study suggests that the natural Th2 polarisation was amplified by the changes in gut microbiota brought about by dietary intervention. Moreover, supplementation with dietary inulin has also been associated with accelerated expulsion of adult *T.suis* parasites, which could be a result of this amplified Th2 response (Thomsen et al. 2005). Interestingly, another study found an interaction between a high fat diet and *Trichenella spiralis* helminth infection, whereby mice parasitised mice had significantly reduced weight gain, fat mass and total cholesterol. Importantly the gut microbiota community also changed as *T. spiralis* infection decreased the ratio of *Firmicutes* to *Bacteriodetes*, thereby restoring the previously increased ratio of *Firmicutes* to *Bacteriodetes* in high fat diet fed mice (Kang et al. 2021). While these studies have started to dissect this complex tripartite relationship, most have been conducted in controlled settings, without incorporating the variation we see in hosts, the gut microbiota and environment of natural systems.

Section 3

3.1. The gut ecosystem in lab models

The house mouse, *Mus musculus*, has been a powerful tool for biomedical research for more than a century and have provided enormous insight into mammalian anatomy, and disease pathophysiology (Ericsson et al. 2013; Morse 2007; Perlman 2016). The development of genetic manipulation tools that led to the creation of transgenic, knock-in and knock-out mice strain within the laboratory greatly increased the potential of modern medical advancement (Ericsson et al. 2013; Morse 2007; Waterston et al. 2002). To date, these 'lab mice' have increased understanding of diseases that affect humans and facilitated the development of treatments for such diseases, including diabetes, cardiovascular disease, and cancer (King 2012; Zaragoza et al. 2011; Zhang et al. 2011). Moreover, studies using lab mice have enabled the characterisation of the mammalian immune response to serval infectious agents such as malaria, human immunodeficiency virus (HIV), tuberculosis, and helminth parasites (Grencis 2015a; Grencis et al. 2014; Minkah et al. 2018; Singh & Gupta 2018; Victor Garcia 2016), and aided in the development of effective vaccines and antibiotic treatments (Gaynes 2017; Kiros et al. ; Lobanovska & Pilla 2017; Racaniello 2006).

However, the translation from pre-clinical mouse trials to human clinical trials has a low success rate (Hackam & Redelmeier 2006; Hay et al. 2014; Pound & Ritskes-Hoitinga 2018; Robinson et al. 2019). For example, several promising anti-cancer drugs that have successfully treated the disease in mice, but when trialled as a human treatment, have shown limited efficacy (Anisimov et al. 2005; Mak et al. 2014). There are a number of reasons why translation from animal models to other animal systems fail to succeed (Mestas & Hughes 2004; Springer & Murphy 2007). One important factor, that cannot be overlooked is the vast differences between the environments that lab mice and natural populations, including humans, inhabit. For instance, to reduce variation within and between experiments, lab mice are housed under standardised, specific-pathogen free (SPF) conditions, with ambient temperature and 12-hour day/night cycles, and provided with a constant supply of food and water

(Home Office 2014). These envrioments of course, do not truly reflect the realities of most free-living mammals, whereby populations are genetically heterogeneous, individuals are adapted to living in vastly different habitats, from deserts to rainforests and are therefore, exposed to varying ecological conditions including nutrient sources and infectious agents within the environment such as microbes, parasites and fungi (Graham 2021; Maizels & Nussey 2013; Pedersen & Babayan 2011). Consequently, humans and animals face a diverse range of antigenic exposure within nature, and this can directly impact their susceptibility to infections (Abolins et al. 2017; Bradley 2015; Graham 2021; Maizels & Nussey 2013; Pedersen & Babayan 2011).

This difference in antigenic exposure between lab mice and wild animals, leads to remarkable differences in the gut microbiota (Lozupone et al. 2012; McFall-Ngai et al. 2013; Sommer & Bäckhed 2013) and is vulnerable to shifts caused by environmental changes (Ericsson et al. 2015; Ericsson et al. 2018; Goertz et al. 2019; Linnenbrink et al. 2013; Maurice et al. 2015b). For instance, genetically identical lab mice reared in different research facilities have been shown to have notable differences in their gut microbiota communities, even when housed under, controlled, semi-sterile environments (Ericsson et al. 2015). Such differences have been attributed to unexpected variation in experimental findings (Bowerman et al. 2021; Kohl et al. 2014; Kreisinger et al. 2014; Rosshart et al. 2017; Wang et al. 2015). Recent studies, that have characterised the gut microbiota of conventional lab mice and compared these to wild caught mice, have unsurprisingly revealed that the gut microbiota compositions here differ significantly (Bowerman et al. 2021; Kohl et al. 2014; Kreisinger et al. 2014; Rosshart et al. 2017; Wang et al. 2015). On the whole, lab mice appear to have less diverse gut bacterial communities which leads to poor maturation of immune cells and immune response that resemble human new-borns, whereas both wild-caught and pet shop mice have been found to have immune responses much more similar to adult humans (Beura et al. 2016; Bowerman et al. 2021; Kohl et al. 2014; Thomson et al. 2022).

The research community has come to appreciate that these differences in gut microbiota and immune phenotypes, may be a strong contributing factor to the lack of success in translation of animal trials. As such, a number of approaches have been adopted in an effort to bridge the gap between the lab and wild by increasing the

microbial and pathogenic exposure of lab mice. Key examples of this include co-housing conventional lab mice with mice purchased from pet shops (Beura et al. 2016; Pierson et al. 2021), transfer of faecal material from wild-caught mice to conventional lab mice (Rosshart et al. 2017), and sequential administration of viruses and intestinal parasites to lab mice prior to vaccination (Reese et al. 2016). These studies have all provided evidence that increased microbial exposure of mice within the laboratory environment generates more mature immune phenotypes (more closely resembling those of adult humans) and increases resistance to subsequent infectious challenges (Beura et al. 2016; Reese et al. 2016; Rosshart et al. 2017). Another study, took this approach to the next level, by establishing a novel mouse cohort known as “wildlings”, which are created by transferring embryos of a commonly used inbred lab mouse strain into pseudo-pregnant wild mice, that gave birth naturally and reared the wildling pups within a controlled lab environment (Rosshart et al. 2019). Upon characterisation of the different mucosal surfaces, including the gastrointestinal tract, they found that the wildlings gut microbiota differed considerably from the lab mice. Moreover, upon exposing wildlings to two treatments (anti-tumour necrosis factor (Fisher et al. 1996) and CD28 super-agonist (Suntharalingam et al. 2006)) that had shown promise in lab mouse studies, but caused severe pathology in human clinical trials, they found that wildlings in similar manner to humans and thus had greater translational potential (Rosshart et al. 2019).

A further, more complex approach to creating more natural reflecting lab mouse model, is to take the lab into the wild. For example, notable study Leung and colleagues (2018) exposed inbred lab mice to outdoor enclosures for several months, thereby not only increasing their microbial exposure, but also adding variation to ecological factors such as the weather/temperature, diet, and movement behaviour (Yeung et al. 2020). Here, investigators found that these ‘rewilded’ mice had increases susceptibility to helminth infection compared to their lab-reared littermates (Yeung et al. 2020), but also greater immune system maturation and a more diverse gut microbiota (Bär et al. 2020; Lin et al. 2020; Yeung et al. 2020). These studies, all offer exciting possibilities for studying lab mice under more natural, ‘real-world’ conditions, however, they do not allow us to directly compare animals in both the lab and wild environments. Here, in this thesis, I take a different, but complementary approach, by adapting a wild mouse model to more conventional laboratory conditions.

Section 4

4.1. The lab-to-wild wood mouse - helminth system

Wood mice (*Apodemus sylvaticus*) are a common small rodent among European woodlands and their parasitology and ecology has been studied for more than 50 years (Behnke et al. 1999; Flowerdew 1972; Miller 1954; Pedersen & Greives 2008). Notably, wood mice can be readily captured using standard mark and recapture methods, are amenable to experimental perturbations in the wild, and are home to a diverse community of parasites and pathogens, including *Heligmosomoides polygyrus*, a gastrointestinal nematode used as a model for human infection (Behnke et al. 1999; Díaz & Alonso 2003; Gregory et al. 1990), as well as a suite of other zoonotic pathogens. Importantly, over the last 10 years the University of Edinburgh has maintained a colony of formerly wild, now laboratory reared wood mice. These mice, originally collected from UK woodlands, are intentionally outbred, genetically similar to wild wood mice, and breed well in captivity. Here, I describe the establishment of a second, distinct wood mouse colony, whereby the only difference involves their gut microbiota exposure during birth and rearing (see Chapter 2). By creating these two wild-derived wood mouse colonies, I have mitigated the impact of genetic differences through the use of the same outbred population of wood mice, but also facilitated differences in gut microbiota by manipulating environmental factors, through caesarean re-derivation and cross-fostering.

A particularly well-studied model for chronic gastrointestinal helminth infection is the worm *Heligmosomoides bakeri,* which has been used extensively in mouse models of human hookworm infections such as *Ancylostoma duodenale* (Behnke & Harris 2010b; Behnke et al. 2009). Importantly, *H. bakeri* is a sister taxon to *H. polygyrus* which naturally infect wood mice in the wild (Behnke et al. 1999; Cable et al. 2006). *Heligmosomoides* infections follow similar lifecycles of other soil-transmitted nematode species, whereby infective larval stages are ingested by the host through environmental contact or a contaminated food or water source (Brooker et al. 2006). Once in the small intestine of the host, the larvae penetrate the muscular layer of the gut, undergo a final developmental stage and moult into male and female adult *H.*

polygyrus in the lumen of the gut, and subsequent eggs are shed into the environment through host faeces (Brooker et al. 2006).

As such, these worms have ample opportunity to interact with the host gastrointestinal ecosystem, including the resident microbial community and the vast mucosal immune system residing within the gut. Moreover, with humans and livestock, *H. polygyrus* infections of wood mice are usually chronic (Knowles et al. 2013) and whilst anthelmintic treatment effectively reduces worm burdens, re-infection is rapid with mice returning to their pre-treatment worm burdens within 3 weeks (Clerc et al. 2019a; Knowles et al. 2013). Likewise, the epidemiology of *H. polygyrus* infection in wood mice is typical of other mammals, whereby there is high prevalence, but significant variation in infection intensity (worm burden) across individuals (Gregory et al. 1992; Woolhouse et al. 1997). This wood mouse – *H. polygyrus* system offers unique opportunities because we can study this interaction in the wild and in controlled laboratory settings. There have also been some recent studies examining the gut microbiota of wood mice in wild setting, for instance, a two-year study of wood mice in two UK woodlands, found that like other mammals, the wood mouse gut microbiota was dominated by bacterial belonging to the phyla, *Bacteroidetes, Firmicutes* and *Proteobacteria* (Maurice et al. 2015a). Interestingly, this study also revealed that the gut microbiota of wood mice was sensitive to environmental changes, with a strong shift in bacterial community structure between the spring/early summer and late summer/early fall, which was attributed to the switch from an insect to seed-based diet during this period (Maurice et al. 2015a). Another study, monitored two wood mouse populations in South-East England, and found that although these populations were only approximately 50 km apart, the mice did not share a single bacterial amplicon sequence variant (ASV), however, at higher taxonomic resolution, they did have 8 bacterial taxa at the phylum level in common (Marsh et al. 2022). However, upon comparison of the gut microbiota composition of these wild wood mice populations with our lab-reared wood mouse colony, the investigators revealed that up to 95% of the ASVs detected in lab-reared wood mouse samples were also found in animals from Whytham Woods in Oxfordshire (Marsh et al. 2022). Further, a social network analysis study of world wood mice, provided evidence that the gut microbiota of these mice is influences by their social interactions, in particular among male-male and male-female contacts (Raulo et al. 2021).

Using this paired wild and lab-reared wood mouse - helminth system, my research group has experimentally manipulated both nutrition (with a high-quality whole-diet supplementation) and helminth infection in either wild or a controlled laboratory experiment with the gastrointestinal nematode, *H. polygyrus.* Across both environments, they found that mice given access to the supplemented high-quality diet were more resistant to *H. polygyrus* infection, shed fewer eggs in faeces, had higher parasite specific and nonspecific immunological responses, and had higher efficacy when given anthelmintic drug treatment (Sweeny et al. 2021). Here, I investigated the interaction between diet-helminths-microbiota in wood mice in this paired lab and wild system, specifically testing if diet supplementation impacts the diversity and composition of the gut microbiota, whether it impacts the response of the gut microbiota to helminth infection, and whether the gut microbiota may be involved in the diet-induced resistance documented in both wild and laboratory wood mice (Sweeny et al. 2021). To this end, I quantified the relative abundance, alpha and beta diversity, and differential abundance of the gut microbiota of wood mice from both a wild population in a UK woodland and our wild-derived colony following experimental manipulation of diet and *H. polygyrus* infection during the described study (Sweeny et al. 2021). Specifically, I aimed to determine how diet and helminth infection shape the diversity and composition of the gut microbiota within a natural host-parasite system.

Section 5

5.1. Thesis aims and objectives

The overall aim of this work was to explore the interaction between the gut microbiota, nutrition, helminth infection and immunity, by establishing a novel lab-to-wild system using wood mice as the model species. Here, the lab-to-wild system refers to a spectrum incorporating several mouse cohorts spanning conventional *Mus musculus,* lab-mouse strains (lab) to free living wild-caught wood mice (wild) and two unique cohorts in-between including wild-derived, lab-reared mice (wild-like) and a cohort of wild-derived wood mice that were caesarean re-derived and fostered by conventional lab mice (lab-like). The term lab-to-wild system will be used throughout this thesis to refer to these mouse cohorts.

Chapter 2. To investigate how laboratory housing and/or rearing affects the gut microbiota composition

Objectives for Chapter 2:

- To create and maintain two wood mouse colonies, one derived directly from the wild and one re-derived with laboratory CD1 foster mothers
- To characterise the gut microbiota composition of these two wood mice colonies using 16S rRNA sequencing and compare them to wood mice in the wild and conventional lab mice

Chapter 3. To investigate the role of the microbiota in shaping the host response to helminth infection

Objectives for Chapter 3:

- To assess the stability and resilience of microbiota composition in the two wood mouse colonies created in Aim 1, compared to laboratory mice, in response to experimental infection with *H.polygyrus*
- To quantify the difference in the immune response, gut pathology and infection outcome in the two wood mouse colonies, compared to laboratory mice, in response to experimental infection with *H.polygyrus*

Chapter 4. To determine how diet and helminth infection shape the diversity and composition of the gut microbiota within a natural host-parasite system in controlled and natural environments

Objectives for chapter 4:

- To test if diet supplementation impacts the diversity and composition of the gut microbiota in both wild and laboratory-housed wood mice
- To assess the impact of diet supplementation on the shift of the gut microbiota in response to *H. polygyrus* infection
- To investigate whether the gut microbiota may be involved in the diet-induced resistance to *H. polygyrus* infection documented in both wild and laboratory wood mice

Overall, I hope to use this novel lab-to-wild mouse system to better understand the possible interactions between nutrition, helminth infections and the gut microbiota, and importantly, the consequences of these interactions for host health and infection dynamics.

Chapter 2

2. Wild wood mouse gut microbiota are influenced by captivity and cross-fostering

2.1. Abstract

The contributions of host genetics and rearing environment to the gut microbiota are not well understood. To address this, we established Lab-like:As and Wild-like:As genetic lines by maintaining *Apodemus sylvaticus* (As) and *Mus musculus* (Mm) wood mice colonies at the University of Edinburgh. Our analysis of the gut microbiota using four diversity metrics revealed significant differences in intra-individual diversity among lab-to-wild cohorts. Lab:Mm mice had high within-sample diversity, while Wild:As mice had the highest mean observed richness and phylogenetic diversity among the *A. sylvaticus* cohorts. Lab-like:As mice displayed intermediate gut microbiota diversity between Lab:Mm and Wild:As. Microbiota composition analysis showed distinct differences in bacterial taxa among the cohorts. Six phyla were exclusive to Wild:As mice, and the relative abundance of *Firmicutes* and *Bacteroidota* differed significantly between Lab:Mm and Wild:As. We identified 3691 unique amplicon-sequence-variants (ASVs), with 60.7% found only in the Wild:As cohort. Lab-like:As mice had a distinct microbial composition that positioned them between the other *A. sylvaticus* cohorts and Lab:Mm mice in principal coordinate analysis (PCoA) based on multiple beta diversity metrics.

2.2. Introduction

The house mouse, *Mus musculus*, has been the powerhouse of biomedical research since the mid 20th century (Ericsson et al. 2013). Due to their ease in breeding, maintenance in large numbers, and their physiological and genetic similarities to other mammals, including humans, they have been fundamental to our understanding of mammalian anatomy, and disease pathophysiology (Ericsson et al. 2013; Morse 2007; Perlman 2016). The development of transgenic, knock-in and knock-out mice within the laboratory setting have greatly increased the potential of modern medical advancement (Ericsson et al. 2013; Morse 2007; Waterston et al. 2002). As such,

these ‘lab mice’ have aided both our understanding of diseases that affect humans and the development of treatments for such diseases, including diabetes, cardiovascular disease, and cancer (King 2012; Zaragoza et al. 2011; Zhang et al. 2011). Moreover, lab mice have provided extraordinary insight into the functioning of the immune system, defining how the mammalian-host responds to various infection challenges including malaria, human immunodeficiency virus (HIV), tuberculosis, and parasitic worm infections (Grencis 2015a; Grencis et al. 2014; Minkah et al. 2018; Singh & Gupta 2018; Victor Garcia 2016) and aided in the development of effective vaccines and antimicrobial drugs (Gaynes 2017; Kiros et al. ; Lobanovska & Pilla 2017; Racaniello 2006).

Despite this, lab mice lack essential aspects of human physiology, and the translation from pre-clinical mouse trials to human clinical trials has a low success rate (Hackam & Redelmeier 2006; Hay et al. 2014; Pound & Ritskes-Hoitinga 2018; Robinson et al. 2019). For instance, several anti-cancer drugs that have shown great promise in lab mouse studies, have gone on to show limited effectiveness in humans, with the average rate of successful translation to humans at an estimated 8% (Anisimov et al. 2005; Mak et al. 2014). The reasons behind the lack of success in translation are multifaceted, but considering that modern rodents and primates are believed to have diverged from their last common ancestor more than 65 million years ago, the fact that mice and humans often respond differently to external stimuli is not surprising (Mestas & Hughes 2004; Springer & Murphy 2007).

Moreover, we must also consider the huge differences between the environments that lab mice and natural populations inhabit. For instance, to increase reproducibility and limit variation within experiments, lab mice are maintained under standardised, specific-pathogen free (SPF) conditions, with ambient temperature and 12-hour day/night cycles and provided with constant access to nutrition (in the form of standardised mouse chow pellets) and water (Home Office 2014). However, this does not reflect the realities of life for most wild mammals, including humans, whereby populations are genetically heterogeneous, thrive in diverse habitats and are exposed to differing ecological conditions such as food and water sources, plus potential infectious agents (microbes, parasites, and fungi) and allergens within the environment (Graham 2021; Maizels & Nussey 2013; Pedersen & Babayan 2011).

Thus, humans and animals in the wild face a diverse range of antigenic exposure, which can directly impact their immune system and susceptibility to infection (Abolins et al. 2017; Bradley 2015; Graham 2021; Maizels & Nussey 2013; Pedersen & Babayan 2011).

Another crucial difference between lab mice and wild animals lies within the gut microbiota, which plays an essential role in mammalian health and fitness (Lozupone et al. 2012; McFall-Ngai et al. 2013; Sommer & Bäckhed 2013) and has been shown to be strongly influenced by environmental changes (Ericsson et al. 2015; Ericsson et al. 2018; Goertz et al. 2019; Linnenbrink et al. 2013; Maurice et al. 2015b). Indeed, recent studies have shown that, genetically identical lab mice reared in different research facilities can lead to marked differences in their gut microbiota, even when housed under standard conditions (Ericsson et al. 2015). These differences in the gut microbiota have been shown to contribute to variable experimental results (Flannigan & Denning 2018; Ivanov et al. 2009b; Rosshart et al. 2019; Thomson et al. 2022). Unsurprisingly, studies have also recently highlighted marked differences in the microbiota composition between lab mice and their wild counterparts (Bowerman et al. 2021; Kohl et al. 2014; Kreisinger et al. 2014; Rosshart et al. 2017; Wang et al. 2015). Lab mice tend to have much less diverse microbiotas that cannot drive immune maturation, leading to immune responses that resemble those of a human neonate, whereas wild and pet shop mice have an immune system that is more similar to human adults (Beura et al. 2016; Bowerman et al. 2021; Kohl et al. 2014; Thomson et al. 2022).

In an attempt to bridge the disparity between lab mice and wild animals, two different approaches have been adopted. The first involves increasing lab mice exposure to microbes and potential pathogens that are usually excluded from their SPF environments, and thus creating “dirty” mice. Key examples of this include co-housing conventional lab mice with mice purchased from pet shops (Beura et al. 2016; Pierson et al. 2021), collecting faecal material from wild-caught mice and transferring it directly to the gut of closely related lab mice (Rosshart et al. 2017), and sequentially infecting lab mice with viruses and intestinal parasites prior to vaccination (Reese et al. 2016). Although differing in their exact methods, each of these studies highlighted that microbial exposure of mice within the laboratory environment generated more mature

immune phenotypes (more closely resembling those of adult humans) and increased resistance to subsequent microbial challenges (Beura et al. 2016; Reese et al. 2016; Rosshart et al. 2017). One recent study took this approach further, by creating a mouse cohort known as “wildlings”, created by transferring embryos of a commonly used inbred lab mouse strain into pseudo-pregnant wild mice, that birthed and reared the wildling pups within the controlled lab environment (Rosshart et al. 2019). Upon characterisation of the skin, gastrointestinal and vaginal microbiota, they found that the wildlings microbiota differed considerably from the lab mice. Moreover, upon exposing wildlings to two treatments (anti-tumour necrosis factor (Fisher et al. 1996) and CD28 super-agonist (Suntharalingam et al. 2006)) that had failed to transition from rodent models to human clinical trials, they found that wildlings responses better resembled those of humans and thus had greater translational potential (Rosshart et al. 2019).

The second approach adds further complexity, by attempting to take the lab into the wild, and thus, alter microbial as well as other environmental and ecological influences such as the weather/temperature, diet, and differences in pathogen exposure. A notable study exposed inbred lab mice to outdoor enclosures for several months. These “rewilded” mice exhibited higher susceptibility to helminth infection compared to their littermates housed in laboratory environments (Leung et al. 2018a). Additionally, these mice showed enhanced immune system maturation and a more diverse gut microbiota (Bär et al. 2020; Lin et al. 2020; Yeung et al. 2020). Both approaches provide exciting new avenues for studying lab mice under more naturalised conditions, but they do not allow us to directly compare mice in both the lab and wild environments to elucidate unique microbial and immune influences. Therefore, we provide a potential third approach, with the creation of a lab-to-wild model, with a focus on the gut microbiota, that bridges both the lab and wild systems and adds a real-world, ecologically relevant model for future research.

Wood mice (*Apodemus sylvaticus*) are common small rodent found in British and European woodlands, which have been extensively studied in terms of ecology and parasitology (Behnke et al. 1999; Flowerdew 1972; Miller 1954; Pedersen & Greives 2008). Notably, wood mice can be readily captured using standard mark and recapture methods, are amenable to experimental perturbations in the wild, and are home to a

diverse community of parasites and pathogens, including *Heligmosomoides polygyrus*, a gastrointestinal nematode used as a model for human infection (Behnke et al. 1999; Díaz & Alonso 2003; Gregory et al. 1990) as well as a suite of other zoonotic pathogens. Importantly, over the last 10 years the University of Edinburgh has maintained a colony of formerly wild, now laboratory reared wood mice. These mice, originally collected from UK woodlands, are intentionally outbred, genetically similar to wild wood mice, and breed and survive well in captivity. Here, we describe the establishment of a second, distinct wood mouse colony, whereby the only difference involves their gut microbiota exposure during birth and rearing. By creating these two wild-derived wood mouse colonies, we have mitigated the impact of genetic differences through the use of the same outbred population of wood mice, but also facilitated differences in gut microbiota by manipulating environmental factors, through caesarean re-derivation and cross-fostering.

In our study here, we describe the establishment and maintenance of our new real-world lab-to-wild mouse model. We first describe how the gut microbiota wild-like and lab-like colonies were created, then we characterise the diversity and composition of gut microbiota of each colony in relation to wild wood mice and laboratory house mice (*Mus musculus*). Lastly, we determine how the composition of the gut microbiota differs from each other with particular interest in the cohorts that share the same genotype but have had differing microbiota exposures.

To the best of our knowledge, the establishment of a wild-derived, laboratory-reared wood mouse colony, that has been caesarean re-derived to a conventional lab-mouse strain, is a first of its kind. Alongside our colony of formerly wild, now laboratory reared wood mice, this allows for direct comparisons of wild, genetically outbred mice, under controlled laboratory conditions. In contrast to field studies within wild populations, this allows for the control of various environmental and ecological variables that can influence findings and make causal inferences difficult. As such, these two wild-derived wood mouse colonies provide not only an additional approach to the creation of a more natural, lab-to-wild mouse model, but also provide a new resource for the research community allowing for the controlled study of immunity and disease in a more natural and contextually relevant setting.

2.3. Materials & Methods

2.3.1. Wild-like wood mouse colony (Wild-like:As)

Since 2011, The University of Edinburgh has maintained a breeding colony of originally wild-caught, but now lab-reared wood mice (*Apodemus sylvaticus*). Currently, there are 12 male lines (originally there were >16 lines) which have been purposely outbred for many generations, allowing the wood mice to retain their genetic diversity. The colony is housed under standard specific-pathogen free (SPF) laboratory conditions in individually ventilated cages, with a 12-hour light cycle, ambient temperature, and the provision of food (Rat mouse 3, SDS, UK) and water *ad libitum*. Mice utilised in experiments from this colony will be referred to as Wild-like:As (referring to wild-like gut microbiota; *Apodemus sylvaticus*) henceforth (Table 1).

As these Wild-like:As mice originated from woodlands within the UK, they are screened annually for murine pathogens following the Federation of European Laboratory Animal Science Associations (FELASA) guidelines (Mähler Convenor et al. 2014). Upon routine screening in 2017, two protozoan parasites, *Tritrichomonas muris* and *Spironucleus muris*, were detected by PCR diagnostics (IDEXX Bioresearch, Germany) within samples collected from the Wild-like:As colony, but not in any of the conventional lab mouse (*Mus musculus*) colonies housed at the animal facility within Edinburgh. As such, our Wild-like:As colony is housed in a separate facility that is not shared by any other laboratory rodents.

2.3.2. Generation of lab-like wood mouse colony (Lab-like:As)

In an attempt to eliminate the two parasitic protozoa from our Wild-like:As colony and thus create a second breeding colony that was truly specific pathogen free, caesarean re-derivation with an outbred lab mouse strain (CD1, *M. musculus*) as foster-mothers was conducted. CD1 mice were chosen as they were suggested to be the best mouse strain for cross-fostering success (Charles River Laboratories 2023; Martín-Sánchez et al. 2015). Briefly, 18 unrelated mating pairs were selected at random from the Wild-like:As wood mouse colony and matched with CD1 breeding pairs, which at the time

were also bred in-house at University of Edinburgh animal facilities. Synchronised mating's were conducted between the Wild-like:As and CD1 breeding pairs, with all female mice checked daily for a vaginal plug.

Exactly 19 days after the vaginal plug was identified for female Wild-like:As mice, caesarean sections were performed, under aseptic surgery conditions by a Named Veterinary surgeons (NVS) resident at the Bioresearch and Veterinary Sevices, University of Edinburgh. Upon caesarean section, pregnant wood mice were terminated via cervical dislocation, the uterine horns were detached from the cervix with metal clamps and placed into a sterile container on a heat pad containing 1% Virkon (Dupont, UK) to remove any microbial contamination. Pups were then retrieved from the uterine horns in a sterile B60 Bell Isolator unit (thus, preventing any contamination with bacterial areseols), washed in 1% Virkon and stimulated on a heat pad. A CD1 foster mother who had recently given birth, was encouraged to urinate and this was smeared onto the wood mice pups with an otherwise sterile gauze pad to reduce the chance of rejection. Wood mouse pups were then placed into individually ventilated cages (IVC) with their respective CD1-foster mother within the B60 Bell Isolator unit, and then moved to an IVC rack in a room housing only CD1 foster mothers and pups, with no other mice, including wood mice present and housed under standard specific-pathogen free (SPF) laboratory conditions.

An average of 4 wood mouse pups per CD1 mother were added to CD1 litters, replacing the same number of CD1 pups. The CD1 mother and her litter of CD1 and cross-fostered wood mouse pups were left undisturbed for 24 hours to allow for bonding and then subsequently checked at 12-hour intervals with minimal disturbance. Successfully fostered litters were weaned after 25 days, wood mice pups were easily distinguished from their CD1 foster siblings due to differences in coat colour, wood mice are brown with white stomachs and CD1's are albino white. Once sexually mature, the fostered wood mice pups, henceforth known as Lab-like:As mice (Table 1), were allocated to unrelated breeding pairs with other Lab-like:As mice. For instance, a female cross-fostered wood mouse pup was placed with a male cross-fostered wood mouse pup that different biological wood mouse parents and from a different CD1-fostered litter, thus, unrelated. Through this process 6 successful breeding pairs, producing F1 offspring, with 6 unique genetic lines were established

for the Lab-like:As mouse colony. Breeding of these genetic lines was continued, ensuring that mice were purposely outbred so that no two genetically related mice were allowed to brred. This Lab-like:As colony was maintained under specific pathogen-free laboratory conditions in a room within the BVS facility that was shared with other conventional lab mouse strains (*Mus musculus*). The Wild-like:As wood mouse colony is housed within the same building, but in a different animal facility separated by 5 floors, movement between these floors is strictly limited, whereby staff members are only able to travel from the Lab-like:As colony to the Wild-like:As colony. Movement from the Wild-like:As colony to the Lab-like:As colony is prohibited, in order to maintain specific pathogen free conditions and limit cross-contamination between facilities.

Caesarean sections and cross-fostering of wood mouse pups was performed from February 2019 until March 2020. Samples for the study described (see section 2.3.3.) were collected between June and October 2021, allowing for several generations of Lab-like:As mice to be bred prior to sample collection.

2.3.3. Sampling for gut microbiota characterisation

Lab-reared mice

Upon commencement of this study, CD1 mice were no longer bred in-house, as such 36 CD1 mice were purchased from Charles River Laboratories (Edinburgh, UK). Immediately upon arrival at the BVS facility, University of Manchester, each CD1 mouse received a unique small ear biopsy so that individuals could be distinguished from each other, and faecal samples collected. This sample collection was performed under sterile conditions, in a class 2 laminar flow hood with rodent handling conducted in the previous 48 hours. CD1 mice were then placed in individual IVC cages with the same animals that they travelled with, in groups of 2-6 individuals. These mice were then housed in the same room as our Wild-like:As colony, on the same IVC rack, albeit on a separate higher row. Within one-hour of CD1 mouse sample collection, 36 mice from the Lab-like:As colony were transferred to the Wild-like:As mouse facility. These Lab-like:As mice were then given a small ear biopsy to allow individual identofcation

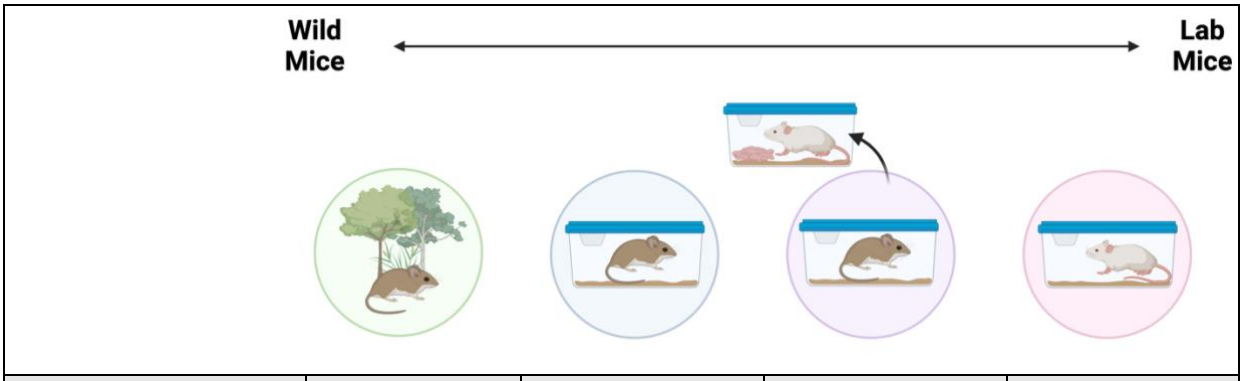
and faecal samples collected as described for the CD1 mice. Again, Lab-like:As mice, were then placed into IVC cages with the same mice that they were transported with, in groups of between 2-6 individuals. They were placed on the same IVC rack, directly below the CD1 mice and directly above the Wild-like:As colony mice. Finally, faecal samples were collected from 36 individual Wild-like:As mice, housed under the standard conditions described above. Faecal samples were then collected from each mouse cohort at weekly intervals over a 2-3 week period. Sample collection was always conducted under sterile laboratory conditions in a class 2 laminar flow hood. To prevent cross-contamination between cohorts, mice were always sampled in the following order, CD1 mice first, followed by Lab-like:As mice and then Wild-like:As mice. Mice were provided with fresh, autoclaved IVC cages on a weekly basis, cage changing was performed by one member of BVS staff and again followerd the order of CD1 mice first, then Lab-like:As mice and finally Wild-like:As mice to prevent cross-contamination between cohorts. All mice received *ad libitum* access to the same diet of Rat Mouse 1 (RM1^{TM}) chow pellets, a standard maintenance diet (SDS, UK) which was autoclaved before being put into cages.

The gut microbiota of lab mice has been shown to differ between vendors and housing facilities (Ericsson et al. 2015; Ericsson et al. 2018). To account for the fact that the CD1 mouse used to cross foster were reared in Edinburgh, but that this colony is no longer maintained, we also collected faecal samples from other lab mice strains which were bred in-house and maintained in the same facility as the Lab-like:As colony. At the time of the experiment, MF1 strain mice (an outbred strain, closely related to CD1 mice) were available to fit these criteria; thus, we collected faeces from 10 cages of MF1 mice. Each cage of MF1 mice contained between 2-4 individual mice and these faecal samples were included as part of the Lab:Mm mice cohort for downstream analysis (Table 1; N=82). For each mouse cohort, we collected samples from mice of both sexes in equal proportions that were aged between 5-13 weeks at the first sample collection, and age-matched within the cohort where possible. Faecal samples were collected fresh, straight from the mouse, and stored within 4 hours of collection at -80^{o}C until downstream processing.

Wild mice

To establish how the gut microbiotas of the Wild-like:As, Lab-like:As and Lab:Mm mouse cohorts compared to natural, free living wood mice, we collected faecal samples from three different populations of wild wood mice in Scottish woodlands. In 2017, faecal samples from 16 individual adult wood mice (5 females and 10 males) were collected, during a previously described field study in Callender Wood (Falkirk, Scotland) (Sweeny et al. 2021). In 2020, as part of a longitudinal NERC-funded field study (data not yet published) we collected faecal samples from 27 adult mice at Hewan wood (Polton, Scotland) and 14 adult mice at Penicuik House Estate woodlands (Penicuik, Scotland). All wild mouse faecal samples were collected from mice at first capture, before any experimental intervention, and stored at -80^{o}C within 4-6 hours of collection. The faecal samples from mice at all three woodlands were analysed as one cohort, henceforth referred to as Wild:As (wild *Apodemus sylvaticus*; Table 1).

Table 1: Establishment of the real-world lab-to-wild mouse model, specifically noting the four mouse cohorts analysed here.



	Wild:As	Wild-like:As	Lab-like:As	Lab:Mm
Mouse species	*A. sylvaticus*	*A. sylvaticus*	*A. sylvaticus,* cross fostered by *M. musculus* (CD1 strain)	*M. musculus*
Mouse strain	NA	NA	NA	CD1 strain & MF1 strain
Rearing/sampling environment	Wild environment	Lab environment	Lab environment	Lab environment
Specific sampling location	Falkirk 2017 (n=16) Penicuik 2020 (n=14) Hewan 2020 (n =27)	University of Edinburgh (n=36)	University of Edinburgh (n=36)	University of Edinburgh (n=36 CD1, n= 10 MF1 cages)
Sex Ratio	34 M / 23 F	18 M /18 F	18 M / 18 F	23 M / 23 F

2.3.4. Ethics Statement

All animal work was conducted under the UK Home Office Project Licence 70/8543 and health monitoring and handling conducted following the guidelines of the Scot PIL and the Home Office Scientific Procedures Act (1986).

2.3.5. DNA extraction and sequencing

QIAamp PowerFecal Pro DNA Kits (Qiagen Ltd, UK) were used for the manual extraction of DNA from faecal samples, following manufactures instructions. Briefly, in

batches of 24 including at least one dH_2O-only sample to act as a negative control, samples were homogenised at a 1:2 ratio with dH_2O. Next, 0.03g homogenate was added to a PowerBead Pro Tube with lysis buffer. Samples were vortexed using a Vortex Genie 2 at maximum speed for 20 minutes to ensure thorough cell-lysis, then centrifuged at 16,100 RCF for 1 min 30 seconds. The supernatant for each sample was then transferred to a clean 2ml microcentrifuge tube and inhibitor removal buffer added, vortexed briefly and centrifuged at 16,100 RCF for 1 min. The supernatant was transferred to a clean 2ml microcentrifuge tube, DNA binding buffer added and then vortexed briefly. The lysate was loaded onto an MB Spin column and centrifuged at 16,100 RCF for 1 min and the flow through discarded. This was repeated until all the lysate had been filtered, then washed twice with ethanol buffer. The MB Spin Column was then placed into a 1.5ml Elution Tube and 50µl was eluted into elution buffer. An estimate of DNA quantity of each sample and confirmation that DNA was not detected in dH_2O-only (negative control) samples was obtained through NanoDrop™ spectrophotometry. DNA extracts were stored at -20°C until required.

The V4 region of the bacterial 16S rRNA gene was amplified using a barcoded adaptor-based polymerase chain reaction (PCR) method, with the 515F forward primer and 806R reverse primer series (Caporaso et al. 2012). Each PCR reaction was set up under contaminant-free conditions using an ultra-violet (UV) sterilisation cabinet (SCIE-PLAS Ltd, Cambridge, UK) whereby all plasticware and reagents (excluding those containing nucleotides) were UV treated for 20 minutes. Individual PCR reactions were then set up at a final volume of 50µl using Roche reagents as follows: 37µl Nuclease-free PCR-grade H_2O, 5µl 10x PCR Buffer, 2µl $MgCl_2$ (25 mM), 1µl dNTP mix (10 mM), 0.5µl Taq DNA polymerase (5 U/µl), 1.25µl Forward barcode primer (10µM), 1.25 µl Reverse barcode primer (10 µM) and 1 µl DNA sample. For each DNA sample, a unique combination of 515F and 806R primers were added to the amplification reaction, allowing multiple samples to be pooled together for sequencing.

PCR reactions were performed using 96-well plates, whereby 91 DNA samples were randomly assigned to each plate, as well as 5 control samples including; 1x 2µl nuclease-free PCR grade water-only sample (negative control), 1x 2µl nuclease-free

PCR grade water plus Forward and Reverse barcode primers (negative control), 1 x 2µl Mock Community (20 strain staggered mix genomic material, ATCC MSA-1003; positive control), 1 x 2µl Golden Colony DNA sample (positive control) and 1 x 2µl Golden Wild DNA sample (positive control). The Golden Colony sample consisted of DNA that had been extracted from faecal samples collected from 6 individual wood mice from the lab-reared colony and pooled together after extraction. Similarly, the Golden Wild sample consisted of DNA that had been extracted from faecal samples collected from 6 individual wild wood mice during fieldwork conducted at Callendar Wood, Scotland (see Chapter 4 for further details) and the DNA pooled together after extraction.

The PCR cycling protocol was as follows: initial denaturation at 94°C for 3 minutes, followed by 25 cycles of, 94°C for 45 sec (denaturation), 50°C for 1 min (annealing) and 72°C for 1.5 min (extension) and a final extension step at 72°C for 10 mins. PCR amplicon size was verified using gel electrophoresis and to confirm that there was no visible DNA amplification of the two negative control samples and that there was visible DNA amplification of the three positive controls used. DNA of all samples and controls was then purified using AMPure XP Beads (Beckman Coulter, UK), then quantified with a Quant-iT PicoGreen dsDNA Assay Kit (Thermo-Fisher, UK) and pooled at equal final concentrations. For all negative control samples DNA levels were undetectable using the PicoGreen assay, thus, a volume of 2µl of each was added to the DNA pool for downstream sequencing. Next-generation DNA sequencing was conducted by Edinburgh Genomics, with the addition of custom primers (Caporaso et al. 2012) using an Illumina MiSeq v2 platform to generate 250 base pair (bp) paired-end reads and ~11 million raw reads. A total of 8 sequencing runs were performed, with samples randomly assigned to each run to limit bias.

2.3.6. Processing of sequence data

Raw Illumina sequences were processed following the DADA2 Pipeline Tutorial (version 1.16; (Callahan et al. 2016b)) using RStudio (2023.03.1+446) with identical parameters used for each of the 8 sequencing runs. Sequences were examined for quality to determine appropriate trimming parameters, forward sequences were trimmed between 220-240 bp and reverse sequences trimmed between 170-190 bp.

Default filtering parameters were used; ambiguous nucleotides removed, the maximum number of “expected errors” was set to 2 for forward reads and 5 for reverse reads and reads of a quality score $\leq$2 were truncated. During denoising, amplicon sequence variants (ASVs) were inferred for each sample, paired reads were merged, and putative chimeras removed. Taxonomy was assigned to each ASV using the naive Bayesian classifier of DADA2 and the Silva Project v132 database (version 138.1; (Yilmaz et al. 2013)) and tabulated. The average proportion of reads retained per sample at the end of the bioinformatics processing was ~ 0.80.

ASV's with non-bacterial taxa including; ASV’s with no taxonomic assignment, Eukaryota, Mitochondria and Chloroplasts, were removed, resulting in a total of 3,691 unique ASV’s. Samples with <10,000 reads were excluded, including 4 samples (mean reads = 141.5 ,range 98-222) and all negative control samples including water-only (mean reads = 152.3, range 95-207) and water plus primers (mean reads = 186.8, range 59-409) were removed from downstream analysis. Details of the bacteria identified within the negative control samples at the Genus taxonomic level can be observed in Table S1. Further, the Mock Community (mean reads = 58,399.3, range 33,731-71,636) for each sequencing run was explored at the Genus taxonomic level to ensure that the 20 bacterial strains present were detected (Supp Fig 1) and then excluded from further analyses. The microbial profile of the Golden colony and Golden wild samples was investigated through principal coordinate analyses (PCoA) with Bray-Curtis, Weighted and Unweighted UniFrac distances to ensure these clustered with our lab-reared colony and wild wood mouse samples as expected (Supp Fig 2) and then dropped from further analyses. Thus, a total of 20,360,304 reads (mean 73,238 per sample; range 16,648 – 737,454), from 278 faecal samples, 55 Wild:As, 70 Wild-like:As, 71 Lab-like:As and 82 Lab:Mm, were analysed.

2.3.7. Statistical analysis

All statistical analyses were conducted in R version 4.2.1 (R Core Team 2022). The Phyloseq package (version 1.40.0) was used to integrate taxonomy tables, ASV abundance tables and sample metadata into a phyloseq object for downstream analysis (McMurdie & Holmes 2013).

Alpha diversity

To evaluate how the diversity of the gut microbiota of our four mouse cohorts differed, we assessed four common alpha diversity metrics: observed richness, Faith's phylogenetic diversity, the Shannon diversity index, and the inverse Simpson index, using the estimate_richness function in the phyloseq package (version 1.40.0; (McMurdie & Holmes 2013)).

Alpha diversity metrics provide a summary of the within sample microbiota community structure, by measuring the number of taxonomic groups present (richness) and/or the distribution of the abundances of the taxonomic groups present (evenness) (Whittaker 1960; Willis 2019). The simplest measure is observed richness, which is a count of the number of individual species detected within a sample (Fisher et al. 1943). Faiths phylogenetic diversity is defined as the sum of the branch lengths of a phylogenetic tree connecting all species within a sample, thus, the higher value (the longer the branch lengths), the more taxa present that are distantly related to each other (Faith 1992). The Shannon index considers both species richness and evenness within a sample, it is a calculation that represents the uncertainty in predicting a single species identity when taken at random within a community, so the higher the value, the higher the diversity (Shannon 1948). The Simpson index also considers the species richness and evenness, by measuring the probability that two taxa randomly selected from a sample will belong to the same taxa. A higher value for the Simpson index equates to a lower diversity, as such, for simplicity, we report the inverse Simpson index, so that for all four metrics reported a higher value indicates higher diversity (Simpson 1949).

Although samples were randomly allocated to sequence runs, this was not balanced with a different number of samples on each sequencing run, plus, there were large differences in sequencing depth between sequencing runs. As such, we performed alpha diversity analysis on rarefied data. Rarefaction is a process that adjusts for differences in sequencing depth between samples, whereby the number of reads for the sample with the lowest sequencing depth is selected and all subsequent samples are then subsampled at this depth (Hughes & Hellmann 2005; Willis 2019). As such, reads from each sample were subsampled without replacement to a constant depth of 16,648, which removed a further 367 ASV's from our dataset, leaving a total of 3324 unique ASVs analysed for alpha diversity metrics.

To determine if differences in alpha diversity between cohorts were statistically robust, we used Generalised Linear Mixed Models (GLMMs) with a Gaussian distribution for each environment. Where necessary, data were log-transformed to enforce a normal distribution. The alpha metric of interest was set as the response variable for each model and the fixed effects in all models included: cohort (factor; Lab:Mm, Lab-like:As, Wild-like:As and Wild:As) and sex (factor; male or female). Individual mouse Id and sequencing run were included as a random effect for all models to account for multiple faecal samples for some individual mice and unequal distribution of samples across sequence run.

Beta diversity

To determine how the gut microbiota composition of our four mouse cohorts differed, we estimated beta diversity using three common ordination metrics. First, we filtered out rare taxa by removing ASV's with a count (abundance) of <5 and had <10% prevalence within the entire dataset, leaving a total of 563 ASVs analysed here. We then normalised read abundances to compositional proportion data and calculated pairwise dissimilarities among samples using; Bray-Curtis dissimilarity matrix, Unweighted UniFrac and Weighted UniFrac, in the Phyloseq package of R (version 1.40.0; (McMurdie & Holmes 2013)) and visualised via principle coordinates analysis (PCoA). Bray-Curtis dissimilarity examines both the abundance of taxa shared between two samples and the number of taxa detected in each sample, a value between 0-1 is calculated, where two samples sharing the same number of taxa in the same abundance would have a dissimilarity distance of 0 (Bray & Curtis 1957). UniFrac distance metrics consider the phylogenetic distances between taxa across two samples. The Weighted UniFrac is quantitative, accounting for the abundance of each taxa present, whilst the Unweighted UniFrac in qualitative and only considers the difference in presence and absence of taxa between two samples (Lozupone et al. 2007).

To test for statistically robust differences between cohorts, we used a permutational multivariate analysis of variance (PERMANOVA, (Anderson 2001)) for each beta diversity metric, using the Vegan package (version 2.6.4) with the *adonis2* function and 999 permutations (Oksanen 2012). For each PERMANOVA, the respective Beta

diversity metric set as the response variable and the explanatory variables included cohort, sex, and sequence plate. As PERMANOVA's may be sensitive to data dispersion differences, we performed an analysis of multivariate homogeneity (PERMDISP) between each cohort with the *betadisper* function in the Vegan package (version 2.6.4; (Oksanen 2012)) and 1000 permutations, with any significant results reported.

Differential abundance

To further understand how specific bacterial taxa differed between our four mouse cohorts, we performed differential abundance analysis at the phylum and genus taxonomic level, using differential gene expression analysis based on a negative binomial distribution, with the DESeq2 package (version 1.36.0; (Love et al. 2014)) on our phyloseq objects. A 10% threshold for prevalence of the taxa was applied as recommended (Nearing et al. 2022), leaving a total of 574 ASVs analysed here.

DESeq2 uses counts of sequencing reads in the form of a matrix of integer values, then fits a Generalised Linear Model (GLM) with a negative binomial distribution to read counts. The variability between replicates is modelled by a dispersion parameter, using empirical Bayes shrinkage, to estimate priors for log fold change and then calculates posterior estimates for these values (Love et al. 2014). Differential abundance is reported as log2 fold-change, thus, a positive value indicates significant enrichment and a negative value depletion. A Wald test is used for significance testing (Love et al. 2014)and to correct for multiple testing, we calculated an adjusted p-value by dividing 0.05 by the number of tests performed. Therefore, at the phylum level, we only report data with an adjusted p value of <0.005 as significant and at the genus level this threshold is <0.0007 for significance.

2.4. Results

2.4.1. Development of the Lab-like:As outbred colony using caesarean rederivation and cross-fostering

We successfully cross fostered and now maintain 6 genetic lines of Lab-like:As wood mice in our University of Edinburgh in addition to the 12 genetic lines of Wild-like:As. This Lab-like:As colony is housed in a facility alongside conventional lab mouse strains, near to, but separate from the Wild-like:As colony.

2.4.2. Intra-individual gut microbiota diversity varies among mouse cohorts.

Firstly, using the four alpha diversity metrics outlined above, we characterised the intra-individual diversity of the gut microbiota of our four mouse cohorts. Unsurprisingly, we found that alpha diversity differed considerably between the lab-to-wild cohorts (Fig. 1A-D). Interestingly, across all four metrics measured, the Lab:Mm mice had the greatest mean within sample diversity, significantly higher than each of the other mouse groups (GLMM, observed richness mean = 237 ± 5.71 SE, SD = 51.7, phylogenetic diversity mean = 674 ± 11.0 SE, SD = 99.6, Shannon diversity mean = 3.92 ± 0.06 SE, SD = 0.50, inverse Simpson mean = 25.0 ± 1.54 SE, SD = 13.9, p = <0.01 all, Fig. 1A-D, Table S2). Although significantly lower than the Lab:Mm cohort at each metric (p = <0.01 all, Table S2), the Wild:As cohort had the highest mean observed richness and phylogenetic diversity, among the three *A. sylvaticus* cohorts and the greatest variance across these two metrics across all four mouse groups (GLMM, observed richness mean = 215 ± 11.1 SE, SD = 82.1, phylogenetic diversity mean = 623 ± 22.8 SE, SD = 169.0, Fig. 1A-B, Table S2), indicating that the taxonomic richness (number of species) of the microbiota was higher in the wild wood mouse population than in the two lab-reared wood mice cohorts and that the spread in the number of species in the microbiota was greater here than in the Lab:Mm mice.

Interestingly, the Lab-like:As cohort appeared to have intermediate levels of intra-individual diversity within the gut microbiota, falling between the Lab:Mm and Wild:As

cohorts in both the Shannon and inverse Simpson metrics (GLMM, Shannon mean 3.58 ± 0.06 SE, SD = 0.5 and inverse Simpson mean = 25.0 ± 1.54 SE, SD = 13.9, Fig. 1C-D), indicating that although they had lower mean taxonomic evenness (relative abundance of species) per sample than the Lab:Mm (GLMM, Shannon, Est = -0.33 ± 0.10 SE, p = 0.001, and inverse Simpson, Table S2), this was higher than Wild:As on average (GLMM, p= <0.01 both, Table S2). In addition, the mean and variance in observed richness and phylogenetic diversity for Lab-like:As was lower than both Lab:Mm and Wild:As (GLMM, observed richness mean = 182 ± 4.95 SE, SD = 41.7 and phylogenetic diversity mean = 568 ± 10.8 SE, SD = 90.9, Fig. 1A-B) suggesting that they had a lower number of species per sample than these cohorts. Overall, the Wild-like:As wood mice had the lowest intra-individual diversity at all 4 metrics measured (GLMM, observed richness mean = 135 ± 6.16 SE, SD = 43.2, phylogenetic diversity mean = 460 ± 12.3 SE, SD = 103.0, Shannon diversity mean = 2.66 ± 0.09 SE, SD = 0.73, inverse Simpson mean = 8.19 ± 0.92 SE, SD = 8.58, p = <0.001 all, Fig. 1A-D, Table S2), indicating that they had lower taxonomic richness and evenness compared to the other three mouse cohorts.

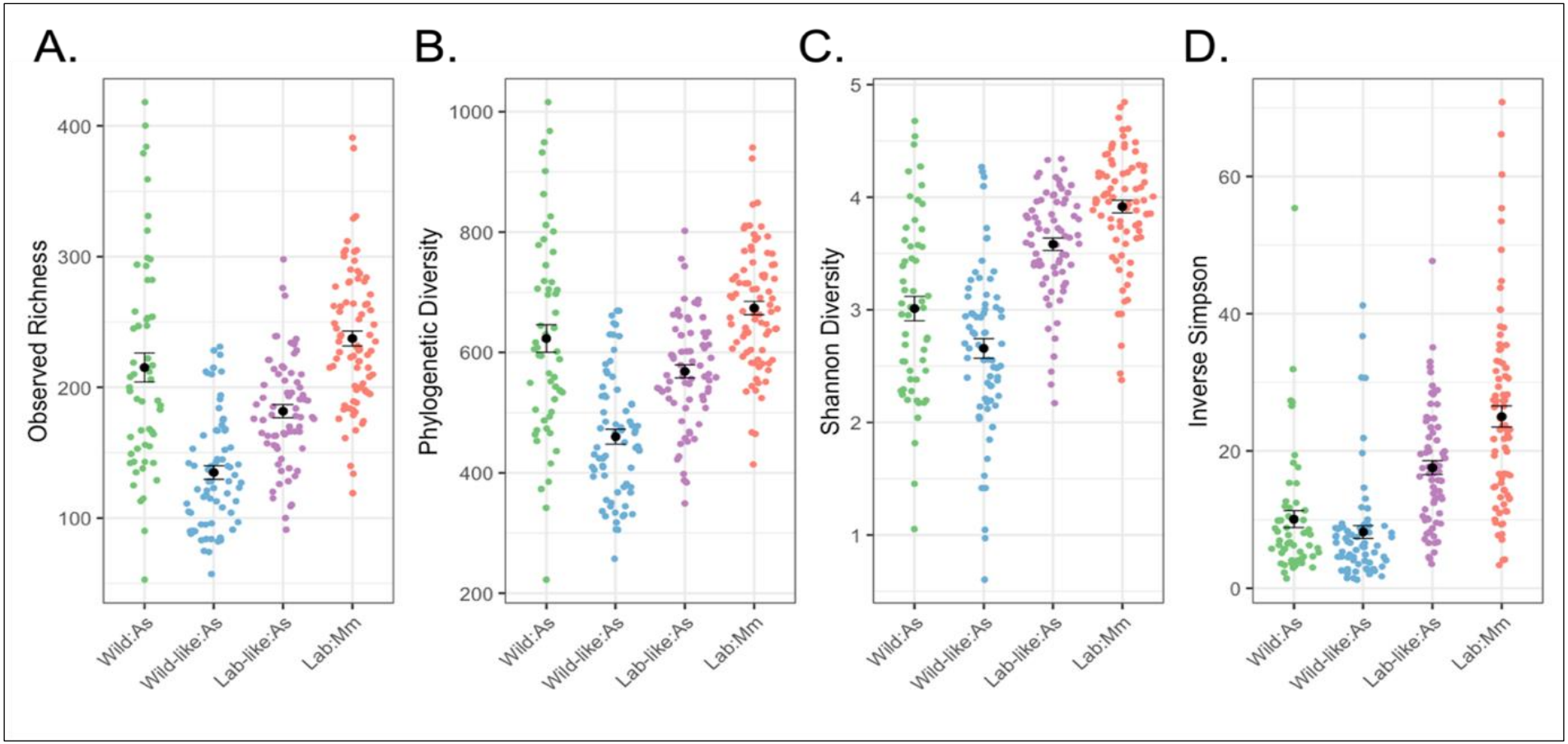


Figure 1. Intra-individual gut microbiota diversity varies among mouse cohorts. Alpha diversity measurements of 16s rRNA sequenced faecal samples collected from four mouse cohorts; wild caught wood mice (Wild: *Apodemus sylvaticus;* Green), wild-derived, lab-reared wood mice, referred to as wild-like wood mice (Wild-like:As; Blue), wild-like wood mice that were caesarean re-derived and fostered by conventional lab mice, thus, referred to as lab-like wood mice (Lab-like:As; Purple) and conventional lab mice (Lab: *Mus musculus;* Pink). **(A)** Observed species richness, **(B)** Faiths phylogenetic diversity, **(C)** Shannon diversity index, **(D)** inverse Simpson index. Each coloured dot represents the alpha diversity value for an individual mouse, error bars represent the standard error (SE) of the mean alpha diversity value of all samples within the cohort. For each metric, a higher value is indicative of higher within sample diversity.

2.4.3. Some similarities observed between Wild-like and wild wood mouse microbiota composition.

Next, we sought to characterise the composition of the gut microbiota of our four mouse cohorts. Upon examining the relative abundance of bacterial taxa at the lowest taxonomic resolution of phylum, for each mouse cohort, some key differences emerge. For instance, bacteria were distributed across a total of 17 phyla, of these six were unique to the Wild:As samples alone (*Acidobacteriota, Bdellovibrionota, Chloroflexi, Elusimicrobiota, Fusobacteriota* and *Myxococcota*) and one was attributed to the Wild-like:As samples only (*Spirochaetota*), the remaining 11 phyla were common to all four mouse cohorts (Fig. 3A). Moreover, with the two most abundant phyla, there is consistent trend of a reduction in *Firmicutes* and increase in *Bacteriodata* along the wild to lab spectrum, whereby Wild:As mice microbiota has the highest proportion of *Firmicutes* (72.1%) and lowest proportion of *Bacteroidota* (11.2%) of the four cohorts. This contrasts with Lab:Mm mice microbiota, which had the highest proportion of *Bacteroidota* (23.1%) and lowest proportion of *Firmicutes* (70.1%) across the four cohorts (Fig. 2A).

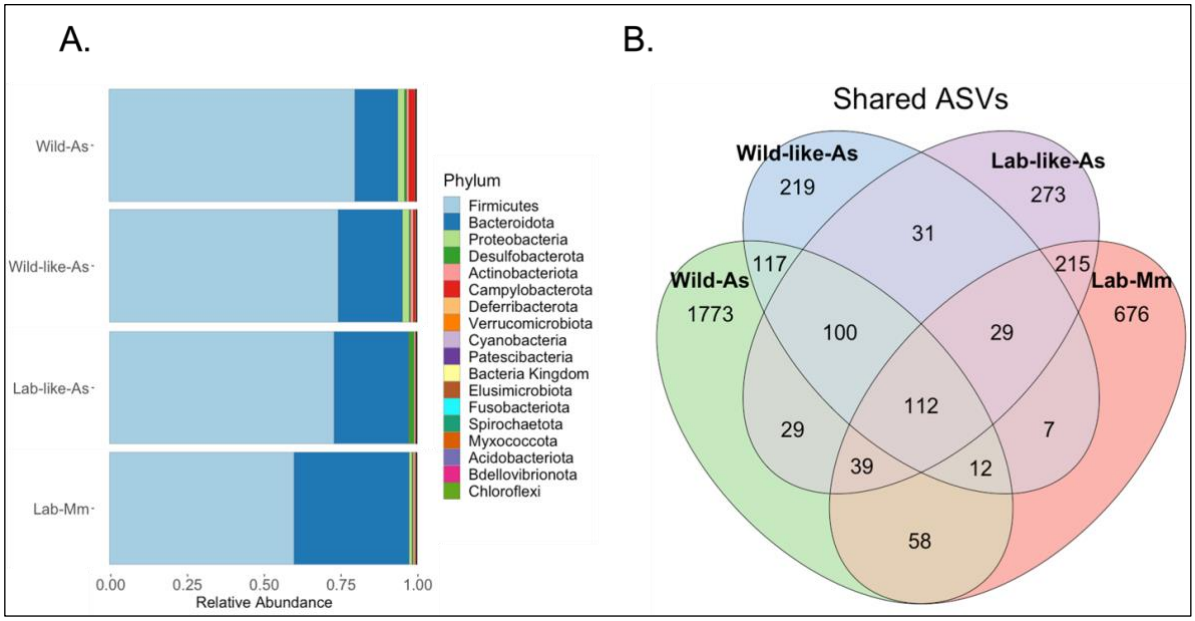


Figure 2. Some similarities observed between Wild-like and wild wood mouse microbiota composition. (A) The relative abundance of bacterial phyla among faecal samples collected from four mouse cohorts; wild caught wood mice (Wild: *Apodemus sylvaticus*), wild-derived, lab-reared wood mice referred to as wild-like wood mice (Wild-like:As), wild-like wood mice that were caesarean re-derived and fostered by conventional lab mice, referred to as lab-like wood mice (Lab-like:As) and conventional lab mice (Lab: *Mus musculus*). **(B)** The number of unique and shared amplicon sequence variants between the distinct mouse cohorts, circles overlapping indicate commonality.

At a higher taxonomic resolution, a total of 3691 unique ASV's were identified, however only 112 of these were shared across all four mouse cohorts (Fig. 2B). Indeed, the vast majority of the total ASV's detected were attributed to the Wild:As mice (60.7%; 2240) and the Lab:Mm mice (31.1%;1148) cohorts alone. We identified 1773 (79.2%) ASV's unique to the Wild:As mice, however, they also shared a large number with the other *A. sylvaticus* cohorts. The Wild-like:As mice shared nearly one-fifth (117; 18.7%) of their total ASVs with the Wild:As mice and similarly the Lab-like:As cohort shared nearly one-eighth of their ASV's (100; 12.1%) with the Wild:As mice. Over half of the Lab:Mm mice microbiota composition was made up of ASVs unique to their samples (676; 58.9%), but they also shared the largest proportion of ASV's with the Lab-like:As mice (215; 18.7%) than any other cohort (Fig. 2B).

2.4.4. The gut microbiota composition of Lab-like wood mice is distinct from lab and wood mouse cohorts.

To further characterise the variation in microbiota composition across the four mouse cohorts, we calculated pairwise dissimilarity among samples for three beta diversity metrics: Bray-Curtis dissimilarity, plus, Weighted and Unweighted Unifrac. We visualised these distance matrices via principal coordinate analysis (PCoA) and found similar clustering patterns of each cohort across all three ordination analyses (Fig. 3A-C). In general, the Wild:As and Wild-like:As mice samples clustered closely and often overlapped (Bray-Curtis and Unweighted Unifrac), indicating similar microbiota compositions within these two cohorts. Interestingly, Lab-like:As samples appeared to form a distinct cluster in all ordinations, falling directly between the other two *A. sylvaticus* cohorts and the Lab:Mm mice samples, on at least one of the PCoA axes (Fig. 3A-C). The Bray-Curtis and Weighted Unifrac highlight a small amount of overlap of samples across the three *A.sylvaticus* cohorts here (Fig 4A-B), which may indicate that there are similar levels of abundance and close phylogenetic relatedness in some taxa across the three mouse cohorts, which supports our earlier finding that there are some ASVs shared among all the mouse cohorts (see Fig. 3B). Further, the Lab:Mm mice samples clustered separately from all other cohorts at each ordination, suggesting they also have distinct microbiota compositions. PERMDISP analysis of each ordination metric revealed that samples from each cohort were highly dispersed

and were not homogenous with each other, indicating that there were both dispersion and location effects within our data (Supp Fig. 3A-C).

Statistical analysis of the Bray-Curtis dissimilarity matrix by PERMANOVA supported PCoA's, whereby differences in the microbiota composition between the Lab:Mm and Lab-like:As cohorts explained 15.0% (R^2) of the variation within the data set (F = 66.44, p = 0.001, Table S3). Similarly, differences between the Lab:Mm and Wild:As and the Lab:Mm and Wild-like:As mice drove 15.0% and 9.0% (R^2) of the variation within the data respectively (F = 66.84, p = 0.001 and F= 43.26, p = 0.001, Table S3). Sequence run and sex also accounted for a significant, but small amount of variation with the microbiota composition of the mouse cohorts (R^2 = 0.01 , F = 5.19, p = 0.002 and R^2 = 0.01 , F = 2.31, p = 0.028 respectively). These results were robust and consistent across PERMANOVA analyses of both Weighted and Unweighted Unfriac distance matrices too, with the only difference of sex not having a significant effect here (Table S3).

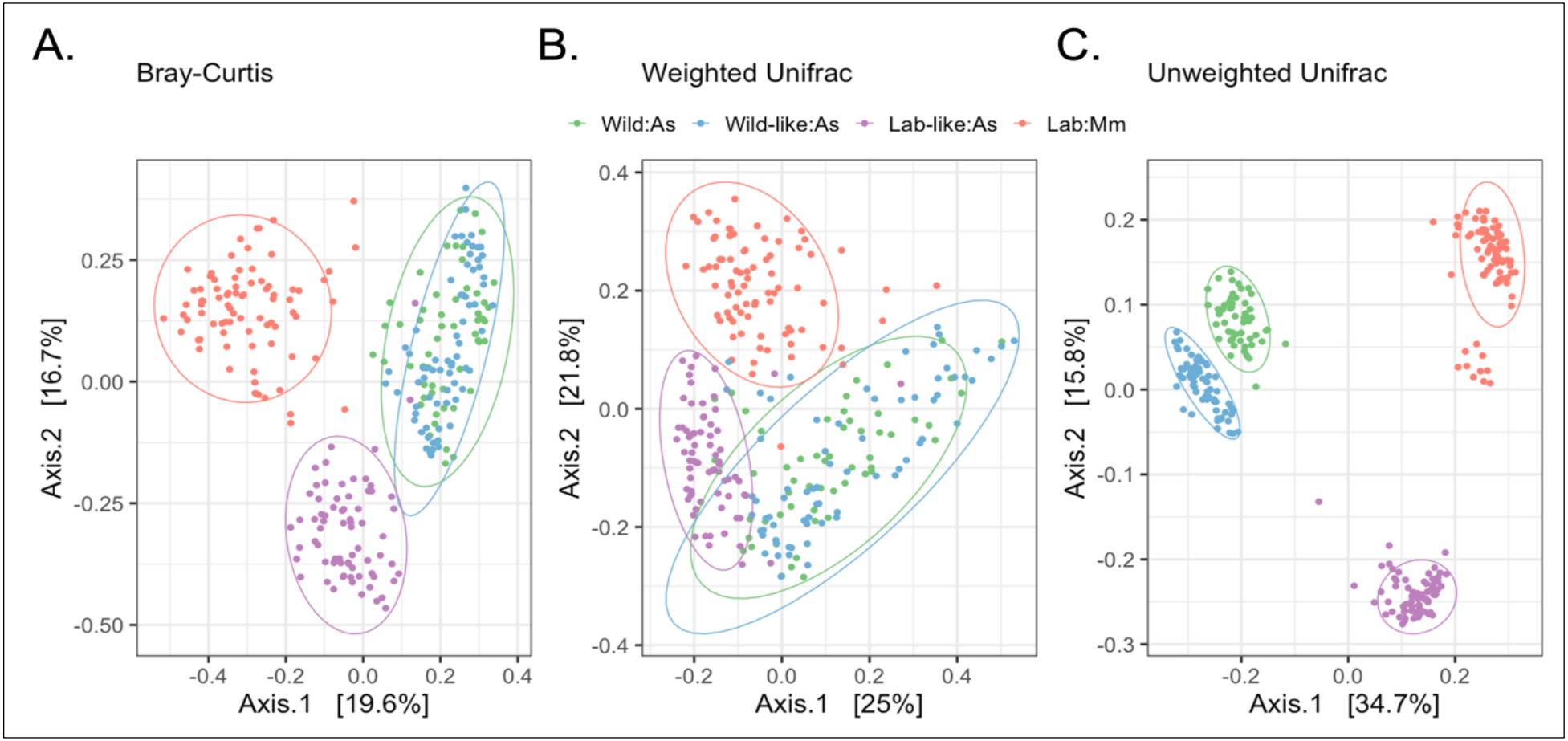


Figure 3. The gut microbiota composition of Lab-like wood mice is distinct. Principal coordinate analysis (PCoA) ordination of beta diversity dissimilarity matrices, for 16s rRNA sequenced faecal samples collected from four mouse cohorts; wild caught wood mice (Wild: *Apodemus sylvaticus;* Green), wild-derived, lab-reared wood mice referred to as wild-like wood mice (Wild-like:As; Blue), wild-like wood mice that were caesarean re-derived and fostered by conventional lab mice, referred to as a lab-like wood mice (Lab-like:As; Purple) and conventional lab mice (Lab: *Mus musculus;* Pink).**(A)** Bray Curtis dissimilarity **(B)** Weighted Unifrac distance **C)** Unweighted Unifrac distance. Each coloured dot represents data for an individual samples, whilst ellipses represent 95% confidence intervals for each mouse cohort. Each axis has an eigenvalue in parenthesis indicating the percentage of variation within the dataset captured at that axis.

2.4.5. Wood mice cohorts show similar patterns of enrichment and depletion of taxa.

To examine which bacterial taxa were driving differences in the microbiota composition of our mouse cohorts, we conducted differential abundance analysis using DESeq2, data is only reported as significant if adjusted p values were below the threshold of <0.005 for Phylum analysis and <0.0007 for Genus analysis. As the microbiota composition of the Lab:Mm cohort appeared to be the most distinct, we set this as our 'baseline' and compared each of the three wood mouse cohorts (Lab-like:As, Wild-like:As, Wild:As) to these samples. First, we explored the lowest taxonomic resolution of Phylum to get a general picture of the differences. Here we found a total of 10 Phyla differentially expressed among the cohorts, of these *Bacteroidota* and *Proteobacteria* were significantly decreased in all three wood mouse cohorts compared to the Lab:Mm mice, whereas *Patescibacteria* was significantly enriched (Fig. 4). Interestingly, *Deferribacterota* was depleted*, Campylobacterota* was enriched in the Wild:As and Wild-like:As groups only compared to Lab:Mm, whilst *Actinobacteriota* was only enriched in Lab-like:As compared to Lab:Mm.

Next, we investigated the Genus level where we found a total of 65 genus differentially expressed among the wood mice cohorts compared to Lab:Mm (Fig. 5). Of these 13 were enriched across all three wood mouse groups compared to Lab:Mm mice (including, *Bifidobacterium, Lactobacillus, Rikenella, Streptococcus*), whilst a further 11 significantly were depleted across all cohorts compared to Lab:Mm mice (including, *Bacteroides, Faecalibaculum, Monoglobus and Ruminococcus*).

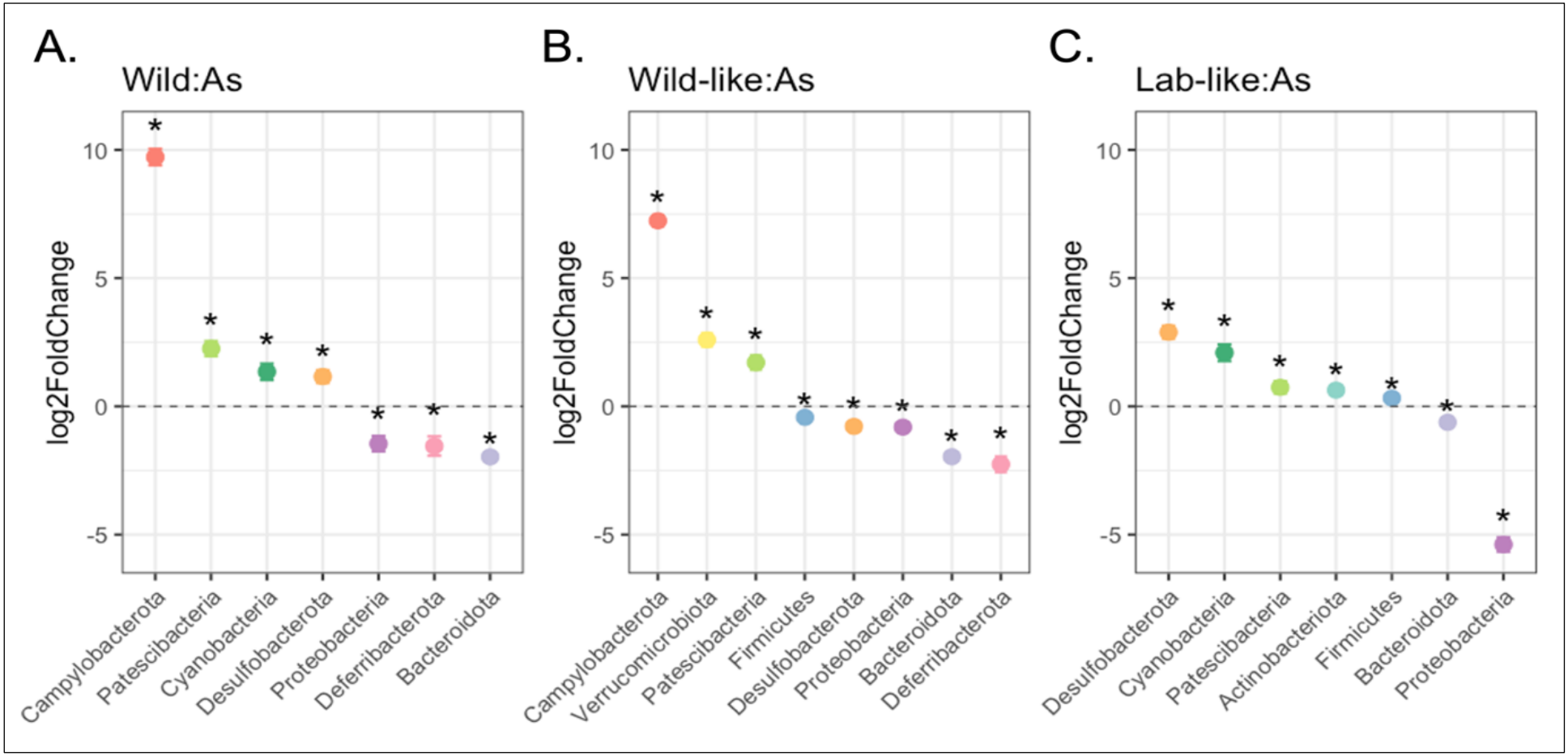


Figure 4. At the phylum taxonomic order wood mice cohorts show similar patterns of enrichment and depletion of taxa. Differential abundance analysis of 16s rRNA sequenced faecal samples collected from three mouse cohorts compared to a conventional lab mouse strain (*Mus musculus*). **(A)** Wild caught wood mice (Wild: *Apodemus sylvaticus*), **(B)** Wild-derived, lab-reared wood mice referred to as wild-like wood mice (Wild-like:As) and **(C)** Wild-like wood mice that were caesarean re-derived and fostered by conventional lab mice, thus referred to as lab-like wood mice (Lab-like:As). Analysis was conducted through the DESeq2 package in R software, using sequence read counts of individual phyla and Generalised Linear Models with a negative binomial distribution. Each coloured dot represents the differential abundance of a specific bacterial Phylum for each mouse cohort. This is reported as log2 fold-change whereby a positive value indicates significant enrichment and a negative value depletion. P-values were adjusted for multiple testing and only differences with a p-value of <0.005 were treated as significant and reported.

Among the Wild:As and Wild-like:As samples, the most significantly enriched taxa compared to the Lab:Mm mice was *Helicobacter* (DESeq2; L2FC = 11.86 ± 0.32 SE and L2FC = 9.93 ± 0.23 SE respectively), whereas there was no significant difference in abundance of this bacteria between the Lab-like:As and Lab:Mm cohorts (DESeq2; LC2F = 0.60 ± 0.23 SE, adj p = 0.015, Fig. 6). The most enriched taxa in Lab-like:As samples compared to Lab:Mm mice was *Gemella* (DESeq2; LC2F = 7.32 ± 0.31 SE, adj p = <0.0001), which was also significantly enriched in Wild-Like:As mice and Wild:As but to lesser degree (DESeq2; L2FC = 7.43 ± 0.27 SE and L2FC = 4.49 ± 0.28 SE) in comparison to other bacterial taxa. Among Lab-like:As and Wild-like:As samples, the most significantly depleted taxa compared to Lab:Mm was *Prevotellaceae* (UCG-001), this was also the second most depleted in Wild:As samples (DESeq2; Lab-like:As, L2FC = -9.14 ± 0.33 SE, Wild-like:As, L2FC = -8.20 ± 0.33 SE and Wild:As, L2FC = -7.90 ± 0.37 SE). However, the most significantly depleted bacterial taxa among Wild:As mice compared to Lab:Mm mice was *Rikenellaceae* (RC9 gut group), again this was also depleted in Wild-like:As and Lab-like:As samples, but to lesser degree (DESeq2; Wild:As, L2FC = -8.61 ± 0.28 SE, Wild-like:As, L2FC = -8.14 ± 0.24 SE and Lab-like:As, L2FC = -1.02 ± 0.27 SE, Fig. 6).

Finally, there were four bacterial taxa, *Anaeroplasma, Eubacterium* (siraeum group)*, Lachnospiraceae (*NK4B4 group*), Lachnospiraceae* (UCG-004), that were significantly enriched in Lab-like:As mice compared to Lab:Mm samples, but these were significantly less abundant in the Wild-like:As and Wild:As compared to Lab:Mm mice. On the other hand, *Ligilactobacillus* was the only taxa found to be increased in both Wild:As and Wild-like:As cohorts but depleted in the Lab-like:As samples compared to Lab:Mm mice.

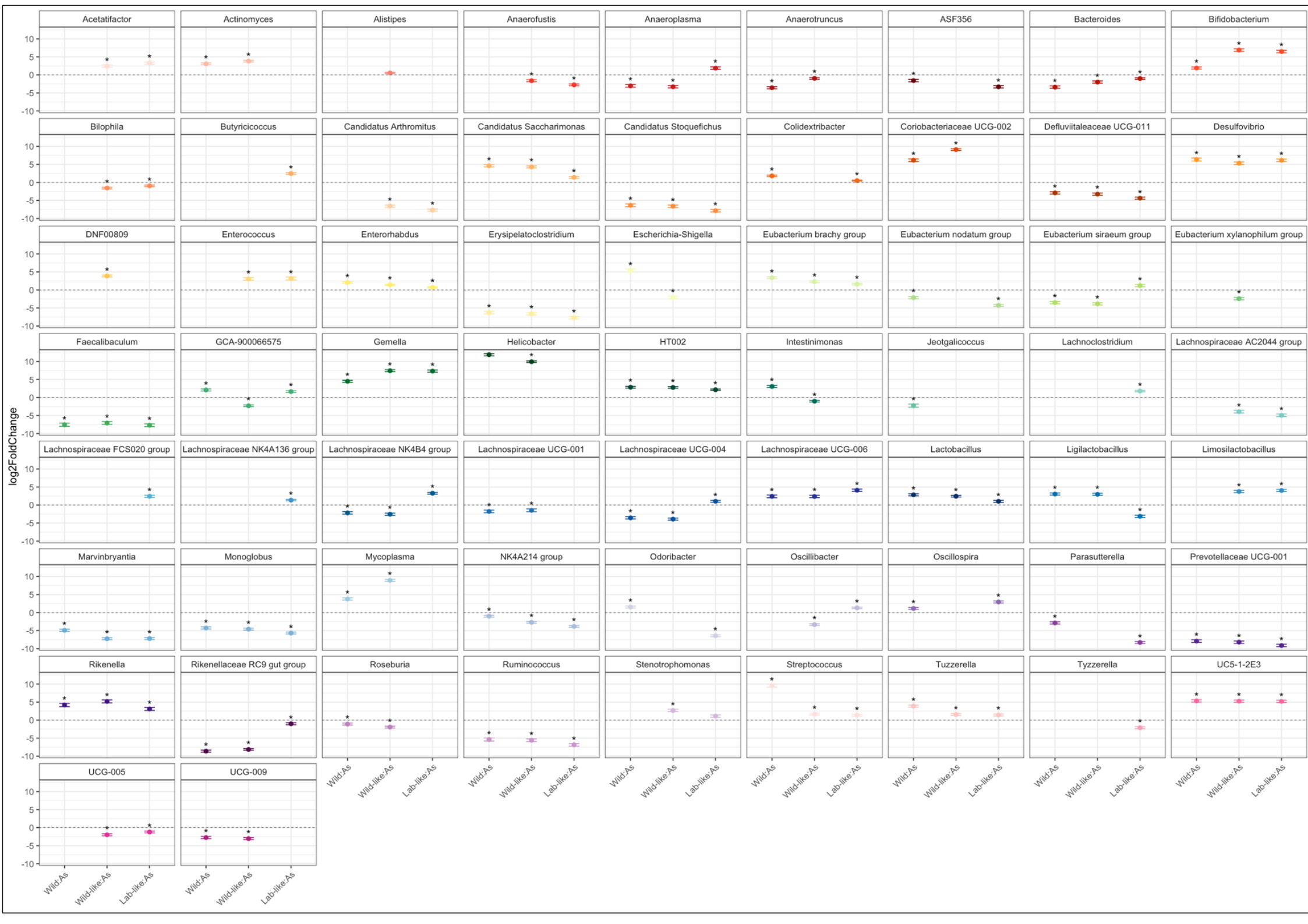

Acetatifactor
Actinomyces
Alistipes
Anaerofustis
Anaeroplasma
Anaerotruncus
ASF356
Bacteroides
Bifidobacterium
Bilophila
Butyricicoccus
Candidatus Arthromitus
Candidatus Saccharimonas
Candidatus Stoquefichus
Colidextribacter
Coriobacteriaceae UCG-002
Defluviitaleaceae UCG-011
Desulfovibrio
DNF00809
Enterococcus
Enterorhabdus
Erysipelatoclostridium
Escherichia-Shigella
Eubacterium brachy group
Eubacterium nodatum group
Eubacterium siraeum group
Eubacterium xylanophilum group
Faecalibaculum
GCA-900066575
Gemella
Helicobacter
HT002
Intestinimonas
Jeotgalicoccus
Lachnoclostridium
Lachnospiraceae AC2044 group
Lachnospiraceae FCS020 group
Lachnospiraceae NK4A136 group
Lachnospiraceae NK4B4 group
Lachnospiraceae UCG-001
Lachnospiraceae UCG-004
Lachnospiraceae UCG-006
Lactobacillus
Ligilactobacillus
Limosilactobacillus
Marvinbryantia
Monoglobus
Mycoplasma
NK4A214 group
Odoribacter
Oscillibacter
Oscillospira
Parasutterella
Prevotellaceae UCG-001
Rikenella
Rikenellaceae RC9 gut group
Roseburia
Ruminococcus
Stenotrophomonas
Streptococcus
Tuzzerella
Tyzzerella
UC5-1-2E3
UCG-005
UCG-009
log2FoldChange
10
5
0
-5
-10
Wild:As
Wild-like:As
Lab-like:As

Figure 5. A total of 65 bacterial genus differed in abundance between wood mice cohorts and a conventional lab-mouse strain. Differential abundance analysis between 16s rRNA sequenced faecal samples collected from three mouse cohorts; Wild caught wood mice (Wild: *Apodemus sylvaticus*), Wild-derived, lab-reared wood mice referred to as wild-like wood mice (Wild-like:As) and Wild-like wood mice that were caesarean re-derived and fostered by conventional lab mice, thus referred to as lab-like wood mice (Lab-like:As) and compared to conventional lab mouse samples (Lab:Mm). Analysis was conducted through the DESeq2 package in R software, using sequence read counts of individual genera and Generalised Linear Models with a negative binomial distribution. Each coloured dot represents the differential abundance of a specific bacterial Genera for each mouse cohort. This is reported as log2 fold-change whereby a positive value indicates significant enrichment and a negative value depletion. P-values were adjusted for multiple testing and only differences with a p-value of <0.0007 were treated as significant and reported.

2.5. Discussion

Here, we created a novel, low maintenance, lab-to-wild mouse model of wild-derived, but now laboratory-reared wood mice (*A. sylvaticus*) that vary in the diversity and composition of their gut microbiota. Through paired pregnancies, caesarean rederivation, and cross-fostering with standard laboratory mice (CD1; *M. musculus*), we were able to create two distinct colonies of wood mice: 'Wild-like:As' which is our wood mouse colony that has been reared in captivity under standard conditions for many generations, and 'Lab-like:As' which are from the same wood mouse colony, but after caesarean rederivation and cross fostering with CD1 mice (Lab:Mm) have a more 'lab-like' gut microbiota. Through the collection of faecal samples and 16s rRNA sequencing, we then characterised the gut microbiota of these mouse cohorts. Our results suggest that our Wild-like:As retain much of the diversity and composition of the gut microbiota of wild wood mice (Wild:As), even though they have been reared under SPF settings and fed standard mouse chow for more than a decade. Interestingly, the gut microbiota diversity and composition of our Lab-like:As mice were in between the Wild-like:As mice and the lab mice (Lab:Mm) cohorts, which suggests that although they would only have had environmental exposure of the gut microbiota of Lab:Mm, their gut microbiota shares many characteristics and taxa with both Wild-like:As and Lab:Mm mice cohorts. Our findings align with existing research indicating the microbiota remains remarkably stable in wood mouse over long periods, even when exposed to antiparasitic treatment, and across multiple generations (Marsh et al. 2022; Maurice et al. 2015a; Rocca et al. 2019).

In contrast, the unique microbiota profile exhibited by the Lab-like:As which shares only partial similarities with both its wild-like progenitors and lab-maintained *M. musculus* samples, warrants deeper inquiry into the interactions between genetic and external microbial exposures. Recent studies have begun to unravel the interplay between host genetics and microbiota, highlighting the role of specific genetic factors in microbiota composition and diversity (Goodrich et al. 2016; Goodrich et al. 2014; Wu et al. 2022). Here, we observe that while Lab-like:As only ever came into contact with Lab:Mm foster mother‘s microbiota, they did not share the exact same microbiota composition and diversity, and indeed had some commonality with their biological Wild-like:As mothers, indicating that genetics, and potentially the “*in-utero*” environment, play a strong role in determining wood mouse microbiota (Chen et al. 2021; Miko et al. 2022; Walker et al. 2017; Younge et al. 2019).

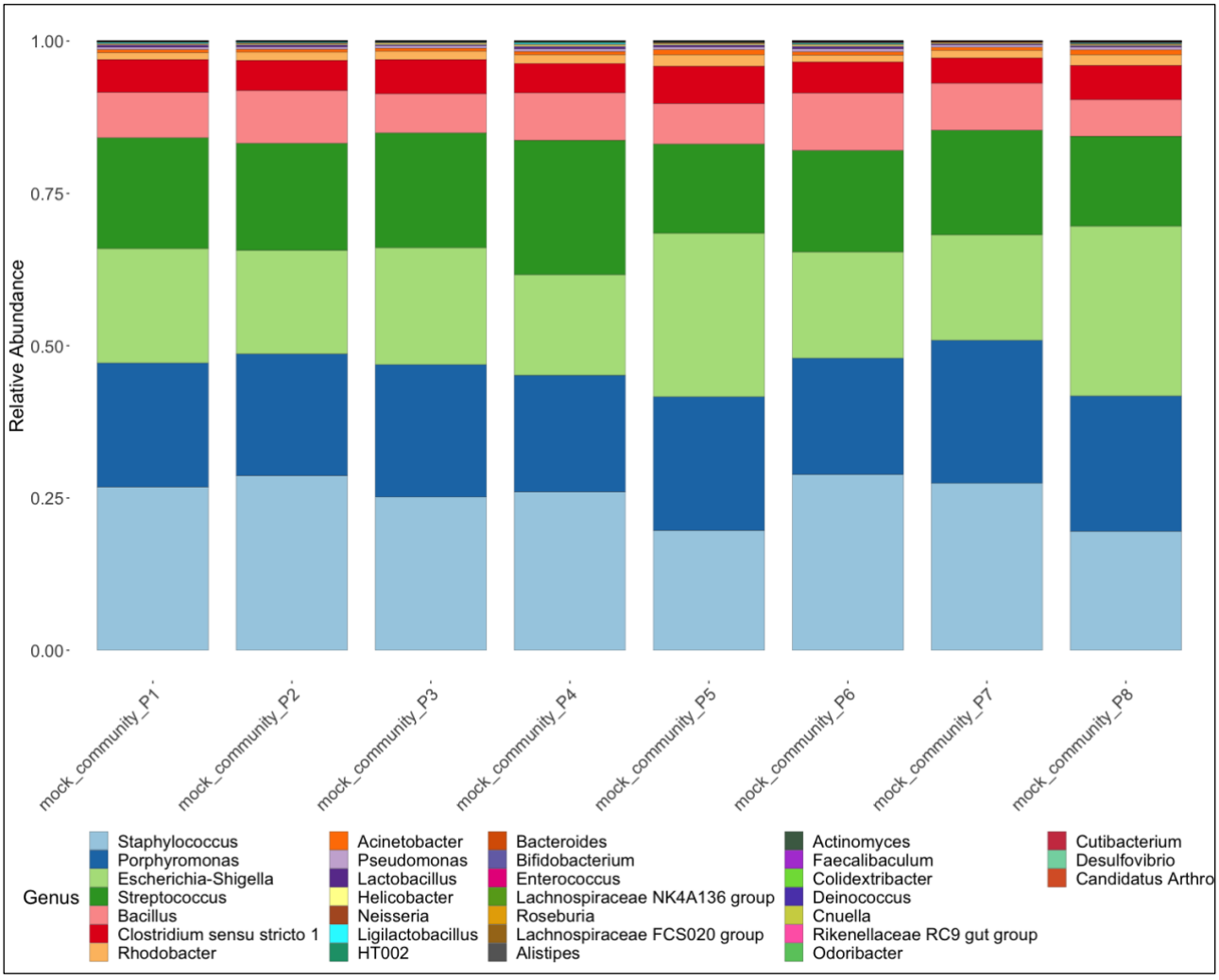
We found strong evidence that all four mouse cohorts had significantly different gut microbiota diversity. However, in contrast to our expectations, we found that, on average, the Lab:Mm mice had the greatest within sample diversity (alpha diversity) compared to the Wild:As, Wild-like:As and Lab-like:As cohorts. This Lab:Mm cohort included samples collected from both CD1 strain and MF1 strain mice, both of which are maintained as outbred strains, and studies have provided evidence that these mice to have more diversity than the inbred mouse strains such as C57BL/6 or BALB/c (Churchill et al. 2012; Hufeldt et al. 2010; Tuttle et al. 2018; Whary et al. 2015). Further, because we included both strains in the Lab:Mm group, we anticipated more diversity than had we used only one outbred strain. Indeed, previous research has shown that the diversity of lab mice’ gut microbiota can vary significantly across strains, and even facilities (Ericsson et al. 2015; Ericsson et al. 2018; Viney 2019).

Among the three wood mouse cohorts, Wild:As had the highest mean species richness and phylogenetic diversity, these wild mice had significantly more variation in diversity compared to all three other mouse cohorts. In terms of alpha diversity, the Lab-like:As mice had intermediate levels of diversity, sitting between Wild-like:As and Lab:Mm mice. Moreover, on average, Lab:Mm had more unique bacterial sequences as measured by the number of Amplicon Sequence Variants (ASVs) detected within a sample. However, Wild:As mice exhibited a greater variation in the number of ASVs

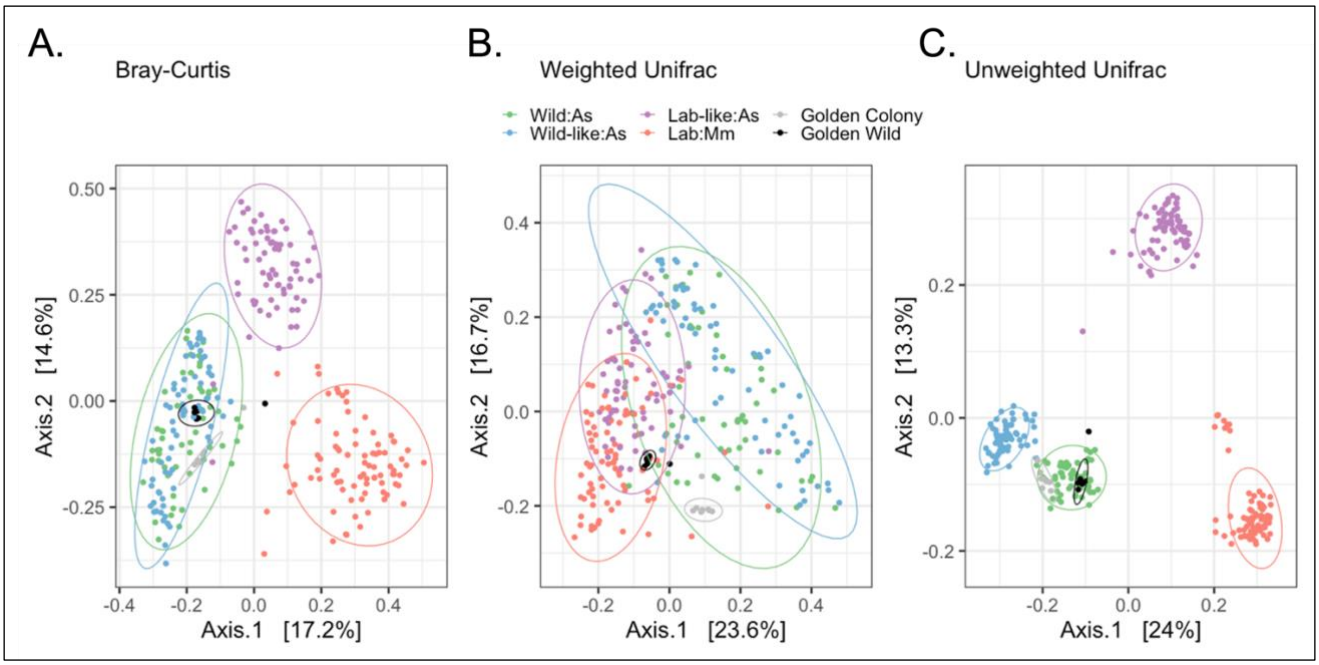
among samples, making them comparatively more diverse than other cohorts. At present, it is unclear why the Wild-like:As had the lowest alpha diversity across the various metrics, although it could be due to more than 10 years of breeding in captivity with very few new, wild animals added to the gene pool. It is possible that the gut microbiota diversity was reduced due to taxa being lost by chance or over time (Kohl & Dearing 2014; Kohl et al. 2014; McKenzie et al. 2017b; van Leeuwen et al. 2020). Here, we also decided to rarefy data (Hughes & Hellmann 2005), due to differences in sequencing depth between sequencing run, however, this process has been known to lead to loss of data on more rare ASV's (McMurdie & Holmes 2014; Willis 2019). Additionally, wild wood mice and mice with wild-like characteristics are more prone to having a higher number of rare ASVs. Therefore, rarefaction could introduce a bias in the data towards the laboratory mice (McMurdie & Holmes 2014; Willis 2019).

2.6. Chapter 2: Supplementary Material

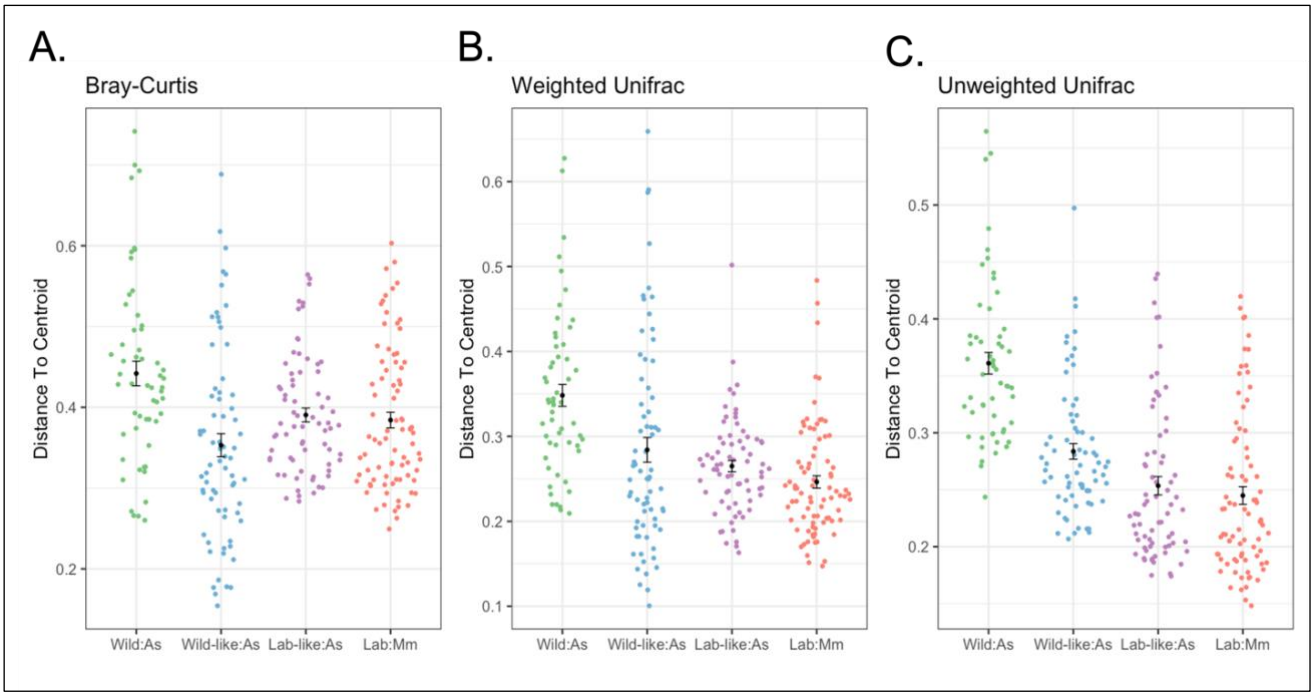
2.6.1. Supplementary Figures



Supplementary Figure 1. The relative abundance of bacteria, at the Genus taxonomic level, detected within each Mock Community on each of the 8 sequencing runs is presented here. During preparation for sequencing, the V4 region of the bacterial 16S rRNA gene of each sample was amplified using a barcoded adaptor-based polymerase chain reaction (PCR). Among the controls for each PCR plate and subsequent sequencing run 1 x 2µl Mock Community (20 strain staggered mix genomic material, ATCC MSA-1003; positive control) was included. Here, we ensured that the 20 bacterial strains expected to be detected were indeed identified using the Silva Project v132 database (version 138.1; (Yilmaz et al. 2013)) for taxonomic assignment.



Supplementary Figure 2. Principal coordinate analysis (PCoA) ordination of beta diversity dissimilarity matrices, for 16s rRNA sequenced faecal samples. Along with the four mouse cohorts wild caught wood mice (Wild: *Apodemus sylvaticus;* Green), wild-like wood mice (Wild-like:As; Blue), lab-like wood mice (Lab-like:As; Purple) and conventional lab mice (Lab: *Mus musculus;* Pink), two positive control samples were examined to ensure these clustered with samples as expected. Here the Golden Colony (Gray) sample consisted of DNA that had been extracted from faecal samples collected from 6 individual wood mice from the lab-reared colony and pooled together after extraction. Similarly, the Golden Wild (Black) sample consisted of DNA that had been extracted from faecal samples collected from 6 individual wild wood mice during fieldwork conducted at Callendar Wood, Scotland and the DNA pooled together after extraction. **(A)** Bray Curtis dissimilarity **(B)** Weighted Unifrac distance **C)** Unweighted Unifrac distance. Each coloured dot represents data for an individual sample, ellipses represent 95% confidence intervals for each sample group. Each axis has an eigenvalue in parenthesis indicating the percentage of variation within the dataset captured at that axis.



Supplementary Figure 3. Distance to centroids of four mouse cohorts; Wild:As (green), Wild-like:As (blue), Lab-like:As (purple), Lab:Mm (pink) for different beta-diversity ordination metrics: (**A**) Bray Curtis dissimilarity, (**B**) Weighted Unifrac distance, (**C**) Unweighted Unifrac distance. Each coloured dot represents the beta diversity distance at the PC1 axis for an individual sample to the centroid of the cohort and the error bars represent the standard error (SE) of the mean beta diversity distance to centroid value for each mouse cohort.

2.6.2. Supplementary Tables

Table S1. Bacterial species identified within negative control samples of water-only and water plus Forward and Reverse primers of the 8 sequencing runs. Taxonomic assignment is at the genus level.

	Water-only samples	**Water plus primers samples**
Bacteria Detected (Genus taxonomic level)	**Frequency observed (N)**	**Frequency observed (N)**
Aerococcus	NA	1
Alistipes	1	4
Bacteroides	1	NA
Clostridium (sensu stricto 1)	NA	1
Desulfovibrio	1	NA
Escherichia-Shigella	1	1
Faecalibaculum	1	NA
Helicobacter	1	NA
HT002	1	1
Lachnoclostridium	NA	1
Lachnospiraceae (FCS020 group)	1	1
Lachnospiraceae (NK4A136 group)	2	2
Lactobacillus	2	3
Ligilactobacillus	1	1
Oscillibacter	NA	1
Porphyromonas	NA	1
Rikenellaceae (RC9 gut group)	NA	1
Roseburia	NA	1
Staphylococcus	NA	2
Stenotrophomonas	1	1
Streptococcus	NA	1

Table S2. Model outputs for fixed effects are shown from Generalised Linear Mixed Models on observed richness, phylogenetic diversity, Shannon diversity and Inverse Simpson alpha diversity metrics in the lab and wild environments. Data that were log transformed to correct for non-normal distribution are indicated in parentheses. All p-values <0.05 are in bold.

	Observed richness (Log)			**Phylogenetic diversity (Log)**			**Shannon Diversity**			**Inverse Simpson (Log)**		
Model covariates	Est	SE	p value	Est	SE	p value	Est	SE	p value	Est	SE	p value
Intercept	5.42	0.04	**<0.001**	6.49	0.03	**<0.001**	3.91	0.08	**<0.001**	3.04	0.10	**<0.001**
Cohort; Lab-like:As	-0.26	0.05	**<0.001**	-0.17	0.03	**<0.001**	-0.33	0.10	**0.001**	-0.31	0.11	**0.003**
Cohort, Wild-like:As	-0.59	0.05	**<0.001**	-0.39	0.03	**<0.001**	-1.26	0.10	**<0.001**	-1.23	0.11	**<0.001**
Cohort, Wild:As	-0.20	0.07	**0.005**	-0.14	0.05	**0.005**	-0.93	0.13	**<0.001**	-1.10	0.15	**<0.001**
Sex, Male	0.02	0.04	0.491	0.02	0.03	0.506	0.02	0.08	0.81	0.04	0.08	0.64

Table S3. Model outputs are shown from a PERMANOVA on Bray-Curtis, Weighted Unifrac and Unweighted Unifrac dissimilarity values in the lab and wild environments (999 permutations). Multivariate homogeneity of group dispersions between environments were carried out for each beta diversity metric (1000 permutations) and the PERMDISP results also shown. All p-values <0.05 are in bold.

Response Variable: Bray-Curtis Ordination				
Model covariates	Sum Sq	R^2	F	p value
Cohort, Lab-like:As	10.66	0.15	66.44	**0.001**
Cohort, Wild-like:As	6.94	0.09	43.26	**0.001**
Cohort, Wild:As	10.72	0.15	66.84	**0.001**
Sex, Male	0.37	0.01	2.31	**0.028**
Sequence Run	0.83	0.01	5.19	**0.002**
Residual	43.64	0.60	-	-
Total	73.17	1.00	-	-
PERMDISP				
Groups	0.25	-	8.43	**<0.001**
Residuals	2.67	-	-	-
Response Variable: Weighted Unifrac Ordination				
Model covariates	Sum Sq	R^2	F	p value
Cohort, Lab-like:As	4.51	0.13	55.23	**0.001**
Cohort, Wild-like:As	2.88	0.08	35.27	**0.001**
Cohort, Wild:As	4.48	0.13	54.88	**0.001**
Sex, Male	0.16	0.01	2.01	0.066
Sequence Run	0.36	0.01	4.39	**0.003**
Residual	22.2	0.64	-	-
Total	34.59	1.000	-	-
PERMDISP				
Groups	0.33	-	18.77	**<0.001**
Residuals	1.60	-	-	-
Response Variable: Unweighted Unifrac Ordination				
Model covariates	Sum Sq	R^2	F	p value
Cohort, Lab-like:As	7.02	0.17	104.40	**0.001**
Cohort, Wild-like:As	7.58	0.18	112.65	**0.001**
Cohort, Wild:As	8.63	0.21	128.25	**0.001**
Sex, Male	0.11	0.00	1.62	**0.129**
Sequence Plate	0.36	0.01	5.28	**0.003**
Residual	18.30	0.44	-	-
Total	42.00	1.000	-	-
PERMDISP				
Groups	0.58	-	46.70	**<0.001**
Residuals	1.13	-	-	-

Chapter 3

3. The impact of helminth infection on gut microbiota diversity and composition

3.1. Abstract

The gut microbiota and intestinal helminths are both key players in the mammalian gut ecosystem and have the ability to modulate the localised immune system within the gastrointestinal tract. Studies are beginning to explore how these two communities interact and what implications this could have for host health and disease. However, to date, many of these studies have used laboratory mouse models, often using strains of helminths that have also been maintained in a laboratory environment for a number of years. Whilst these studies help us gain some mechanistic insights, they do not truly reflect infections of wild populations. Here, we investigate how the gut microbiota diversity and composition is altered during infection in a natural host-helminth model, using wood mice (*Apodemus sylvaticus*) and wild derived *Heligmosomoides polygyrus* infective larvae, a natural parasite of wood mice. In addition, with the establishment of two wild-derived, but laboratory reared wood mice colonies, that have distinct gut microbiota phenotypes, we can directly compare how differing microbial communities interact with helminth infection. We found that *H.polygyrus* was able to establish patent infection in both colonies and levels of pathology were similar. However, mice with a wild-like gut microbiota, had significantly higher faecal IgA concentrations and a decrease in microbiota composition diversity, that appeared to be driven by infection.

3.2. Introduction

Gastrointestinal helminths are some of the most prevalent parasites within wildlife, livestock, and humans (Bethony et al. 2006; Hotez et al. 2008), with one quarter of the world's population estimated to be infected (World Health Organisation 2023). These infections are usually chronic in nature and are associated with significant morbidities and pathology within the gastrointestinal tract of their host (Finkelman et al. 2004; Gentile & King 2018; Grencis 2015a). The enclosed environment, extensive vascular system, and access to host dietary nutrients, makes the gut not only a preferential

niche for helminths, but home to the dense community of bacterial microbes known as the gut microbiota (Lozupone et al. 2012). Consequently, a growing body of research has begun to investigate how these two communities, helminths, and microbiota, interact with each other and the host immune system within the gastrointestinal environment and to explore what consequences this may have on infection dynamics and host health (Leung et al. 2018b; Zaiss & Harris 2016). One study provided strong evidence that some helminth species may even rely on specific intestinal bacteria within the host to establish infection. The mouse specific whipworm, *Trichuris muris*, a close relative of the human parasite *T. trichiura*, is unable to hatch from its egg stage to the infective larvae stage without the presence of certain gut bacteria, including *Escherichia coli* and *Staphylococcus aureus* (Hayes et al. 2010). A similar finding was observed with whipworm of pigs, *T. suis*, whereby eggs are unable to hatch *in vitro* without the presence of Gram-positive bacteria (Vejzagić et al. 2015). However, other studies have shown that these helminth-microbiota interactions can have positive outcomes for the host too, for instance, *T. trichiura* in macaque monkeys was associated with reductions in *Cyanobacteria* and increases in *Mycoplasmatota* (formerly known as *Tenericutes*) within the gut, which are thought to have helped improve clinical symptoms of idiopathic chronic diarrhoea (Broadhurst et al. 2012). Thus, it appears that interactions within the gut ecosystem are complex, and host-outcomes may be context dependent.

A particularly well-studied model for chronic gastrointestinal helminth infection is the worm *Heligmosomoides bakeri,* which has been used extensively in mouse models of human hookworm infections such as *Ancylostoma duodenale* (Behnke & Harris 2010b; Behnke et al. 2009). Additionally, *H. bakeri* is a sister taxon to *H. polygyrus* which has been shown to naturally infect wood mice (*Apodemus sylvaticus*) in the wild (Behnke et al. 1999; Cable et al. 2006). *Heligmosomoides* infections follow similar life cycles to other soil-transmitted nematode species, whereby infective larval stages are ingested by the host through environmental contact or a contaminated food or water source (Brooker et al. 2006; Reynolds et al. 2012a). Once in the small intestine of the host, the larvae penetrate and encyst the muscular layer of the gut, undergo final developmental stages, moult into adults, and emerge into the lumen of the gut where they feed on host tissue (Bansemir & Sukhdeo 1994; Sukhdeo et al. 1984). Adults

worms then secure themselves in the gut by coiling around the intestinal villi, here they mate, and subsequent eggs are shed into the environment through host faeces (Brooker et al. 2006; Reynolds et al. 2012a). As such, these worms have ample opportunity to interact with the host gastrointestinal ecosystem during both larval and adult stages, including the resident microbial community and the vast mucosal immune system residing within the gut.

Indeed, much of the success of *H. bakeri* as a parasite is attributed to its ability to modulate the host immune response, which leads to a reduction or blocking of the Type-2 immune response needed to expel worms (McMurdie & Holmes 2013; Reynolds et al. 2012a).. It now appears that some host immune modulation and disruption in gut physiology may be beneficial to the gut bacterial community too. For example, *H. bakeri* has been shown to alter intestinal barrier function, allowing bacteria and bacterial proteins to cross the epithelial membrane of the gut and enter host circulation (Chen et al. 2005; McDermott et al. 2003; Shea-Donohue et al. 2001). Infection with *H. bakeri* has also been associated with an impairment of the Type-1 immune response needed to control bacterial growth, leading to an inability to control bacterial replication (Chen et al. 2005; Su et al. 2018). Plus, the increased mucus production in the gut caused by *H. bakeri* has been linked to increased growth of mucin utilising *Clostridiales* bacteria (Ramanan et al. 2016) and several studies have also found an association with *H. bakeri* and the *Lactobacillaceae* bacterial family, whereby they appear to have a symbiotic relationship and promote each other's survival within the gut (Reynolds et al. 2014; Walk et al. 2010a).

Whilst these studies have undoubtedly provided insight into helminth-microbiota interactions and their influence on host health, they have all been conducted in laboratory mouse models of infection. Not only do these studies use laboratory mice, but they also utilise the lab strain *H. bakeri*, which has been shown to be a different species than the wild wood mouse species, *H. polygyrus* (Behnke & Harris 2010a; Cable et al. 2006; Lewis et al. 2023). These controlled lab experiments therefore do not fully reflect real-world dynamics. In the wild, hosts experience much more genetic, ecological, and environmental variation, leading to much more varied microbial and

parasite exposure and consequently, more diverse within-host communities and variation in immunological responses (Babayan et al. 2011; Bradley 2015). For example, the immune system of the wild house mouse *Mus musculus domesticus* has been found to be profoundly different from the lab house mice. Studies have shown that although these mice are genetically identical, their immune systems have marked differences. Wild mice are more immunologically responsive and maintain a highly activated state (Abolins et al. 2017), whilst those of lab mice are naïve and resemble those of new-born humans (Beura et al. 2016). There are also significant differences in the gut microbiota of wild *M. musculus* compared with inbred lab strains of *Mus musculus,* with approximately 16% of the bacterial taxa differing between these mice (Kreisinger et al. 2014).

However, such studies that make direct comparisons between the lab and wild are rare (Abolins et al. 2017; Kreisinger et al. 2014; Sweeny et al. 2021) and studies within wild populations that look at helminth-microbiota interactions and link this with immune responses are even less well understood (Kreisinger et al. 2015). This is likely due to the difficulty in studying parasite interactions in the wild, which often require experimental perturbation in order to disentangle direct and indirect relationships due to the heterogeneity in individuals behaviour, exposure and immunity (Pedersen & Babayan 2011; Pedersen & Fenton 2015). Instead, research has shifted to approaches that attempt to naturalise conditions of the lab mouse to better reflect the wild. For instance, the creation of “wildling” mice, whereby lab-mouse embryos were implanted into wild-mice, the resultant pups had immune and microbiota phenotypes much closer to those of wild mice and even recapitulated human-like responses to failed clinical trials (Rosshart et al. 2019). Another such study moved inbred laboratory mice into wild enclosures for long periods of time and found that this caused shifts in the microbiota composition and increased their susceptibility to helminth infection (Leung et al. 2018a).

In our study, we aim to expand on these current approaches, with the creation of a new lab-to-wild model of helminth infection, using wild-derived wood mice *(Apodemus sylvaticus),* which are the natural host of *H. polygyrus*. We have established two wild-

derived, out-bred wood mouse colonies, that have differing gut microbiota compositions due to caesarean rederivation and cross-fostering by lab mice (*Mus musculus*). We exposed both wood mice colonies and a third cohort of lab mice to *H. polygyrus* infection, measured their physiological responses to infection through gut health metrics and also sequenced their gut microbiota at key time points throughout the course of infection. As such, here we aim to determine if the cohorts differ in their response to infection at both the immune and gut microbiota level and determine if differences in their microbiota lead to differing infection outcomes.

3.3. Materials and Methods

3.3.1. Study Design

As discussed previously, we have created a lab-to-wild mouse system that incorporates two distinct colonies of wood mice (*Apodemus sylvaticus)* maintained at the University of Edinburgh (see Chapter 2 for further details). To investigate how infection with *H. polygyrus* impacts the gut microbiota and health in this model, we selected a total of 36 mice from three mouse cohorts (Fig. 1). This included 12 mice from our originally wild-caught, but now lab-reared wood mice colony, henceforth referred to as the Wild-like:As cohort, another 12 mice from our colony of wood mice that were caesarean re-derived and fostered by CD1 lab mice, known as the Lab-like:As cohort from this point onwards, and finally 12 CD1 strain (*Mus musculus*) mice were purchased from Charles River Laboratories (Edinburgh, UK), referred to as the Lab:Mm cohort from here onwards (Fig. 1).

The mice were all housed in the same room, under standard specific-pathogen free laboratory conditions in individually ventilated cages, with a 12-hour light cycle, ambient temperature, and the provision of food (Rat mouse 3, SDS, UK) and water *ad libitum*. We allowed two-weeks for the Lab-like:As and Lab:Mm cohorts to acclimatise to the new environment prior to any experimental perturbation. Mice were co-housed in cages of between 2-6 animals within their cohort, and each cohort of mice included 6 males and 6 females. We matched mouse ages between cohorts where possible, and all mice were aged between 7-13 weeks at the commencement of the experiment.

On Day 0, all mice were administered 150 L3 infective stage *H.polygyrus* larvae via oral gavage. Faecal samples were collected for microbiota analysis at Day 0 (pre-infection), Day 7 post-infection (p.i), Day 14 p.i and Day 21 p.i and stored at -80ºC within 4-6 hours of collection. Half of the mice from each cohort were sacrificed at Day 14 p.i, when we expect to see peak infection levels based on previous work (Clerc et al. 2019c), via cervical dislocation and exsanguination. The second half of the mice were culled, post-peak at Day 21 p.i, which is when we have previously shown that wood mice start to naturally clear their *H. polygyrus* infections (Clerc et al. 2019c). Mice were weighed weekly throughout the duration of the experiment to monitor for weight loss associated with infection and assessed for any signs of distress. Due to time constraints, we conducted this experiment in two parallel experimental blocks with a two-day difference in timing between the blocks. Half the mice from each cohort were randomly allocated to each block. The same culture of *H. polygyrus* larvae was used for each experimental block with thorough mixing between animals and larvae were administered within cages among cohorts at random, to limit any cohort biases.

3.3.2. *H.polygyrus* parasites

H. polygyrus L3 larvae were isolated from faecal samples collected from wild wood mice as part of a longitudinal NERC-funded field study at Hewan and Penicuik House Estate woodlands (Scotland, UK), via previously described methods (Johnston et al. 2015). Briefly, faecal pellets were homogenised with distilled H2O at a 1:1 ratio, then mixed with an equal amount of deactivated granulated charcoal (Darco®, 20-40 mesh particle size). An approximate 10-pence piece size of faecal-charcoal mix was then smeared on dampened filter paper (Whatman No. 40 Filter Paper circles) in a petri-dish and placed in a humid container in the dark at room temperature. After 7 days, petri-dishes were checked to ensure larvae had migrated away from the charcoal mix to the edge of the filter paper and were then harvested by flushing the petri-dish with 5 ml distilled water. Larvae were washed three times with distilled water and stored at 4°C until required. A batch of these larvae were screened by an independent laboratory via PCR diagnostics for contamination with any known mouse-infective pathogens (IDEXX Bioresearch, Germany) and then passaged several times in our wood mouse colony to maintain stocks for future experiments.

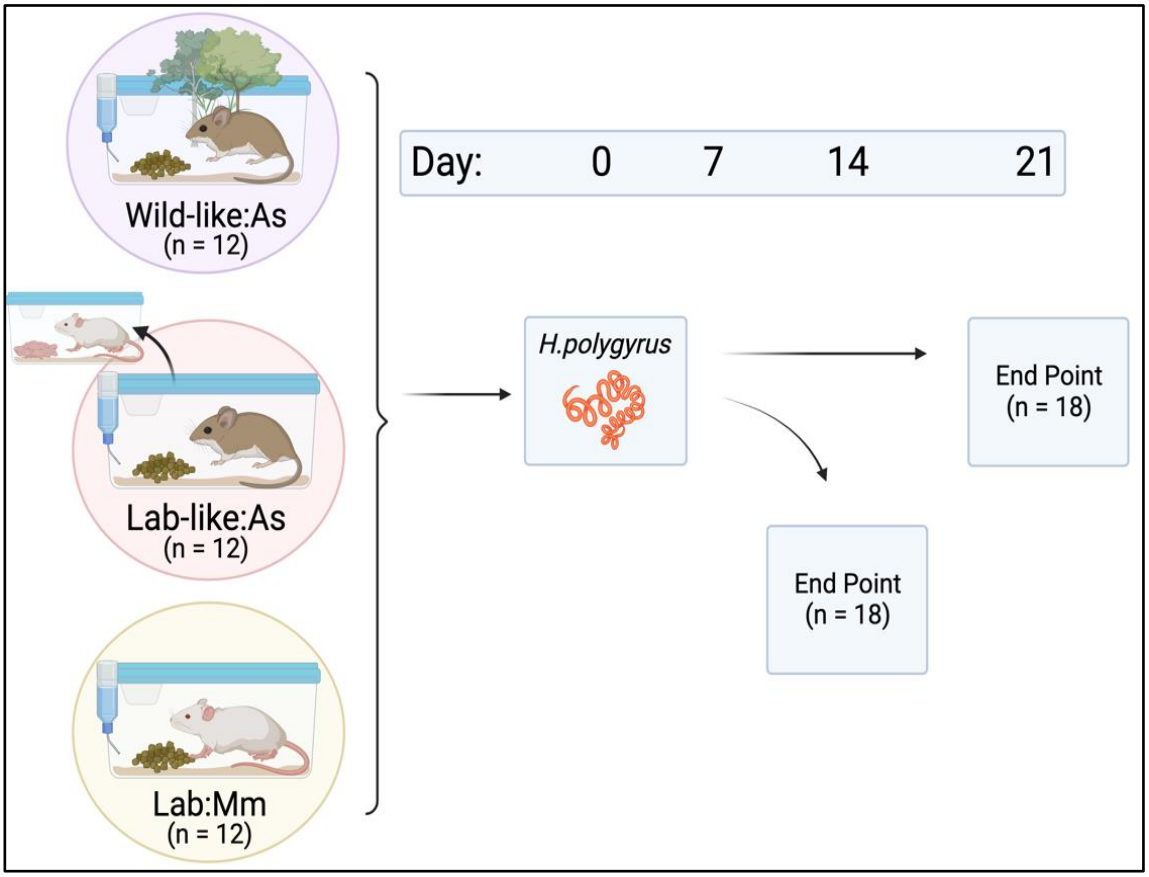


Figure 1. Experimental design of lab-to-wild mouse model infected with *H.polygyrus*. A total of 36 mice from three mouse cohorts were used; 12 wild-derived, lab-reared wood mice, referred to as wild-like (Wild-like: *Apodemus sylvaticus*; Purple), 12 wild-like wood mice that were caesarean re-derived and fostered by conventional lab-mice, referred to as lab-like wood mice (Lab-like:As; Pink) and 12 conventional lab mice (Lab: *Mus musculus;* Yellow). On Day 0, all mice were administered 150 L3 infective stage *H.polygyrus* larvae via oral gavage. Faecal samples were collected for microbiota analysis at Day 0 (pre-infection), Day 7 post-infection (p.i), Day 14 p.i and Day 21 p.i. Half of the mice from each cohort were sacrificed at peak infection (Day 14 p.i) and the second half at post-peak infection (Day 21 p.i) to examine the small intestine for the presence of adult *H.polygyrus*.

3.3.3. Total faecal IgA ELISA assay

Total faecal IgA antibody concentration was measured using an enzyme-linked immunoassay (ELISA), as previously described (Clerc et al. 2019b). Faecal IgA is an important defence for gastrointestinal nematodes and may be considered an indicator of general gut health (Macpherson & Harris 2004; Watt et al. 2016). Briefly, faecal samples were weighed, and a standard volume of protease inhibitor solution was

added (PIS, Complete Mini Protease Inhibitor Tablets, Roche), before thorough homogenisation. Samples were left to stand for 1 hour at room temperature, then centrifuged at 12,000 rpm for 5 mins and the resultant supernatant containing IgA was used. Next, 96-well microplates (NuncMicroWell, ThermoScientifc) were coated with 2 µg/ml of unlabelled goat anti-mouse IgA antibody (Southern Biotech) diluted in 0.5M carbonate buffer and incubated overnight at 4 °C. Plates were then flicked to remove capture antibody then non-specific binding was blocked with 4% BSA-TBS for 2 hours at 37 °C. Faecal extracts were diluted 1:50 in 1% BSA-TBS and added to plates in triplicate. Positive controls of two serial dilutions (250 ng/mL to 0.24 ng/mL) of purified mouse IgA standard antibody (BD Pharmingen) were added to each plate and incubated overnight at 4 °C. Plates were washed three times in TBS-Tween, then goat anti-mouse Horseradish peroxidase (HRP) conjugated detection antibody (Southern Biotech) added at a 1:4000 dilution and incubated at 37 °C for 1 h in the dark. Plates were then washed four times with TBS-Tween and two times with dH2O, then 3,3',5,5'-tetramethylbenzidine (TMB) solution was added, and plates were immediately covered with tinfoil. This enzymatic reaction was allowed to develop for 7 mins, then the reaction stopped with 0.18 M Sulphuric acid. Plates were read at 450 nm using a FLUOstar® Omega plate reader and concentrations of total faecal IgA were obtained by fitting 4-parameter logistic regression to standard curves.

3.3.4. Histopathology

At autopsy of mice at day 14 or 21 p.i, the entire small intestine of each mouse was removed, assessed under light microscope for the presence of adult *H.polygyrus* and then flushed with methacarn fixative (60% methanol, 30% chloroform and 10% acetic acid), and rolled following the Swiss roll technique, as previously described (Bialkowska et al. 2016). Samples were then fixed for 24 hours in methacarn, transferred to 100% methanol for 1 hour and then stored in 70% ethanol. Samples were then sent to the Histology Research Service (University of Edinburgh, UK) where they were processed (Thermo Excelsio AS processor), embedded in paraffin wax (Leica EG1160), and sectioned (4-5 µm thick) with a microtome, mounted to microscope slides and stained with Periodic acid-Schiff (PAS). Slides were then scanned using Nanozoomer slide scanner (Hamamatsu Photonics UK Limited) and visualised using the NDP viewer 2 program.

PAS stain was chosen to highlight the small intestine cellular architecture, allowing for the visualisation of the characteristic crypt and villi structures here. For each mouse, approximately 30 full-length longitudinal crypts of Lieberkühn (base of the crypt at the gut wall to the gut lumen) were measured. An elongation of intestinal crypts is indicative of increased epithelial cell proliferation and apoptosis, which is a type-2 immune mediated response to intestinal nematode infection, to aid parasite expulsion (Cliffe et al. 2005; Cliffe et al. 2007; Erben et al. 2014). In addition, due to the specific nature of *H. polygyrus* adult worms coiling around the intestinal villi, they can cause direct damage to these structures, leading to villous blunting or atrophy (Erben et al. 2014; Grencis et al. 2014). As such, we measured approximately 30 intestinal villi (base at the muscularis mucosa to the tip in the gut lumen) for each animal. Finally, PAS stain, dyes the mucus producing, goblet cells found within the crypt and villi, a bright magenta colour. Therefore, we counted the number of goblet cells present within the crypts and villi measured, as a proxy for mucus production. Increased mucus production within the gut is another type-2 immune mechanism which reduces nematodes motility and inhibits their ability to feed on host tissues, leading to expulsion (Artis 2008; Khan & Collins 2004; Sharpe et al. 2018).

3.3.5. Ethics Statement

All animal work was conducted under the UK Home Office Project Licence 70/8543 and health monitoring and handling conducted following the guidelines of the Scot PIL and the Home Office Scientific Procedures Act (1986).

3.3.6. DNA extraction

DNA was extracted from faecal samples in randomised batches of 24 samples, using QIAamp PowerFecal Pro DNA Kits (Qiagen Ltd, UK) following manufacturer's instructions (see Chapter 2 for further details). Each batch of samples contained at least one dH_2O-only sample to act as a negative control. In summary, samples were homogenised at a 1:2 ratio with dH2O, then 0.03g homogenate was added to PowerBead Pro tubes and vortexed at top speed for 20 minutes to ensure thorough homogenisation. Faecal samples then underwent cell lysis, inhibitor removal, DNA binding, washing and finally 50ul DNA was eluted into elution buffer. An estimate of DNA quantity of each sample and confirmation that DNA was not detected in dH_2O-

only (negative control) samples was obtained through NanoDrop™ spectrophotometry and DNA extracts were stored at -20ºC until required.

3.3.7. Amplification of 16S rRNA & sequencing

The V4 region of the bacterial 16S rRNA gene was amplified using a barcoded adaptor-based polymerase chain reaction (PCR) method, with the 515F forward primer and 806R reverse primer series (Caporaso et al. 2012)(see Chapter 2 for further details). PCR's were conducted under contaminant-free conditions using an ultra-violet (UV) sterilisation cabinet (SCIE-PLAS Ltd, Cambridge, UK) whereby all plasticware and reagents (excluding those containing nucleotides) were UV treated for 20 minutes. Individual PCR reactions were then set up at a final volume of 50µl using Roche reagents, as follows: 37µl Nuclease-free PCR-grade H_2O, 5µl 10x PCR Buffer, 2µl $MgCl_2$ (25 mM), 1µl dNTP mix (10 mM), 0.5µl Taq DNA polymerase (5 U/µl), 1.25µl Forward barcode primer (10µM), 1.25 µl Reverse barcode primer (10 µM) and 1 µl DNA sample. For each DNA sample, a unique combination of 515F and 806R primers were added to the amplification reaction, allowing multiple samples to be pooled together for sequencing.

PCR reactions were performed using 96-well plates, whereby 91 DNA samples were randomly assigned to each plate, as well as 5 control samples including; 1x 2µl nuclease-free PCR grade water-only sample (negative control), 1x 2µl nuclease-free PCR grade water plus Forward and Reverse barcode primers (negative control), 1 x 2µl Mock Community (20 strain staggered mix genomic material, ATCC MSA-1003; positive control), 1 x 2µl Golden Colony DNA sample (positive control) and 1 x 2µl Golden Wild DNA sample (positive control). The Golden Colony sample consisted of DNA that had been extracted from faecal samples collected from 6 individual wood mice from the lab-reared colony and pooled together after extraction. Similarly, the Golden Wild sample consisted of DNA that had been extracted from faecal samples collected from 6 individual wild wood mice during fieldwork conducted at Callendar Wood, Scotland and the DNA pooled together after extraction.

The PCR cycling protocol was as follows: initial denaturation at 94ºC for 3 minutes, followed by 25 cycles of, 94ºC for 45 sec (denaturation), 50ºC for 1 min (annealing) and 72ºC for 1.5 min (extension) and a final extension step at 72ºC for 10 mins. PCR

amplicon size was verified using gel electrophoresis and to confirm that there was no visible DNA amplification of both negative control samples and that there was visible DNA amplification of the three positive controls used. DNA of all samples and controls was then purified using AMPure XP Beads (Beckman Coulter, UK), then quantified with a Quant-iT PicoGreen dsDNA Assay Kit (Thermo-Fisher, UK) and pooled at equal final concentrations. For all negative control samples DNA levels were undetectable using the PicoGreen assay, thus, a volume of 2µl of each was added to the DNA pool for downstream sequencing. Next-generation DNA sequencing was conducted by Edinburgh Genomics, with the addition of custom primers (Caporaso et al. 2012), using an Illumina MiSeq v2 platform to generate 250 base pair (bp) paired-end reads and ~11 million raw reads. In total, 8 sequencing runs were performed, however, for this experiment a subset of samples was analysed which included samples from 3 of the 8 sequencing runs.

Processing of sequence data

Raw Illumina sequences were processed following the DADA2 Pipeline Tutorial (version 1.16; (Callahan et al. 2016a)) using RStudio (2023.03.1+446) with identical parameters used for each of the 3 sequencing runs. Sequences were examined for quality to determine appropriate trimming parameters, forward sequences were trimmed between 220-240 bp and reverse sequences trimmed between 170-190 bp. Default filtering parameters were used; ambiguous nucleotides removed, the maximum number of “expected errors" was set to 2 for forward reads and 5 for reverse reads and reads of a quality score $\leq$2 were truncated. During denoising, amplicon sequence variants (ASVs) were inferred for each sample, paired reads were merged, and putative chimaeras removed. Taxonomy was assigned to each ASV using the naive Bayesian classifier of DADA2 and the Silva Project v132 database (version 138.1; (Yilmaz et al. 2013)) and tabulated. The average proportion of reads retained per sample at the end of the bioinformatics processing was ~0.81.

Non-bacterial taxa including; ASV’s with no taxonomic assignment, Eukaryota, Mitochondria and Chloroplast ASV’s, resulting in a total of 1,341 unique ASV’s. Samples with <10,000 reads were excluded, which included all negative control samples including water-only and water plus primers were removed from downstream

analysis. Details of the bacteria identified within the negative control samples at the Genus taxonomic level can be observed in Table S1 (Chapter 2, page 61). Further, the Mock Community for each sequencing run was explored at the Genus taxonomic level to ensure that the 20 bacterial strains present were detected (Supp Fig. 1, Chapter 2, page 58) and then excluded from further analyses. The microbial profile of the Golden colony and Golden wild samples was investigated through principal coordinate analyses (PCoA) with Bray-Curtis, Weighted and Unweighted UniFrac distances to ensure these clustered with our lab-reared colony and wild-like wood mouse samples as expected (Supp Fig. 2) and then dropped from further analyses. A total of 10,490,228 reads (mean 65, 157 per sample; 27, 164 - 239, 060 range). Thus, a final total of 161 faecal samples, 42 Wild-like:As, 42 Lab-like:As and 41 Lab:Mm, were analysed.

3.3.8. Statistical analysis

Due to the absence of adult *H. polygyrus* worms in the small intestine of all the Lab:Mm mice at both peak (Day 14 p.i) and post-peak (Day 21 p.i) timepoints, they were excluded from all infection dynamics analyses, including worm burden, total faecal IgA and histopathology. However, as the Lab:Mm had still been exposed to *H.polygyrus*, we still wanted to determine if and how their microbiota was impacted by this and make comparisons between the other two mouse cohorts, thus, we included Lab:Mm in our microbiota analyses, unless otherwise stated. All statistical analyses were conducted in R version 4.2.1 (R Core Team 2022).The Phyloseq package (version 1.40.0) was used to integrate taxonomy tables, ASV abundance tables and sample metadata into a phyloseq object for downstream analysis (McMurdie & Holmes 2013).

Infection dynamics and gut health

To determine if *H.polygyrus* infection and subsequent gut health and pathology differed between the Lab-like:As and Wild-like:As cohorts, we used Generalised Linear Model (GLM) and Generalised Linear Mixed Models (GLMMs). The following response variables were tested, (i) total number of adult worms counted in the small intestine (*H.polygyrus* worm burden), (ii) total faecal IgA concentration for experiment duration (Day 0 to Day 21 p.i), (iii) total faecal IgA concentration at endpoints, (vi) mean goblet cells per villi at endpoints, (v) mean goblet cells per crypt at endpoints (vi) mean villi

length at endpoints and (vii) mean crypt length at endpoints. Worm burden data was zero-inflated and over-dispersed, as such we fit a GLMM with a Poisson distribution and individual mouse ID was included as a random effect, to account for this. Unless otherwise stated, fixed effects in all models included; cohort (factor; Lab:Mm, Lab-like:As and Wild-like:As), experiment time point (factor; Day 14 p.i and Day 21 p.i), sex (factor; male or female) and experimental block (factor; block 1 or block 2).

To examine faecal IgA concentration over the duration of the experiment, timepoint was set as a continuous explanatory variable and individual mouse ID was included as a random effect to account for repeated measures for individuals. ELISA plate was also included in all IgA models to account for plate bias and IgA data was log transformed to enforce normal distribution of residuals. The data for the histology metrics of intestinal crypt and villi length and goblet cell counts were also log transformed and to determine if total faecal IgA concentration and worm burden impacted gut pathology, they were included as fixed effects in these models. All model outputs and covariates are provided in the supplementary tables (Table S1-S3).

Alpha diversity

To evaluate how the intra-individual diversity of the gut microbiota differed among our three mouse cohorts, we assessed four common alpha diversity metrics; observed richness, Faith's phylogenetic diversity, the Shannon diversity index, and the inverse Simpson index, using the estimate_richness function in the phyloseq package (version 1.40.0; (McMurdie & Holmes 2013)).

Alpha diversity metrics provide a summary of the within-sample microbiota community structure, by measuring the number of taxonomic groups present (richness) and/or the distribution of the abundances of the taxonomic groups present (evenness) (Whittaker 1960; Willis 2019). Observed richness provides a count of the number of individual species detected within a sample (Fisher et al. 1943). Faith's phylogenetic diversity is the sum of the branch lengths of a phylogenetic tree connecting all species within a sample, thus, the higher the value, the more taxa present that are distantly related to each other (Faith 1992). The Shannon index considers both species richness and evenness within a sample, it is a calculation that represents the uncertainty in predicting a single species identity when taken at random within a community, so the

higher the value, the higher the diversity (Shannon 1948). The Simpson index also considers the species richness and evenness, by measuring the probability that two taxa randomly selected from a sample will belong to the same taxa. A higher value for the Simpson index equates to a lower diversity, as such, thus, we report the inverse Simpson index, so that for all four metrics reported a higher value indicates higher diversity (Simpson 1949).

Although samples were randomly allocated to sequence runs, this was not balanced with a different number of samples on each sequencing run, plus, there were differences in sequencing depth between sequencing runs. As such, we accounted for this using rarefaction, whereby the number of reads for the sample with the lowest sequencing depth is selected and all subsequent samples are then subsampled at this depth (Hughes & Hellmann 2005; Willis 2019). Therefore, reads from each sample were subsampled without replacement to a constant depth of 27,164, which removed a further 310 ASV's, leaving a total of 1,229 unique ASVs in this analysis.

To determine if differences in alpha diversity between cohorts were statistically robust, we used GLMMs with a Gaussian distribution. Where necessary, data were log-transformed to enforce a normal distribution. The alpha metric of interest was set as the response variable for each model and the fixed effects in all models included; cohort (factor; Lab:Mm, Lab-like:As and Wild-like:As), experimental time point (continuous), sex (factor; male or female), experimental block (factor; block 1 or block 2), and sequence run (continuous). Individual mouse ID was included as a random effect to account for multiple faecal samples per mouse (see Table S4 for model outputs).

To determine if *H. polygyrus* burden was associated with alpha diversity among our wood mouse cohorts, we fit GLMs with Gaussian distribution with each alpha metric, log transformed where necessary, as the response variable. Here, worm burden was included as an additional fixed effect, and time point was set to factor level (Day 14 p.i and Day 21 p.i). To assess if total faecal IgA concentration was associated with alpha diversity among the Wild-like:As and Lab-like:As samples, we fit GLMMs with each alpha diversity metric set as the response variable and individual mouse Id was set as a random effect to account for multiple samples per individual. Here, total faecal IgA concentration was set as an additional fixed effect, and time point was set as a

continuous variable so that we could look at faecal IgA levels over the full course of the experiment, not just the two endpoints.

Beta diversity

To determine how the gut microbiota composition of our lab-to-wild mouse cohorts differed we estimated beta diversity using three common metrics. First, we filtered out rare taxa by removing ASV's with a count (abundance) of <5 and had <10% prevalence within the entire dataset, leaving a total of 588 ASVs analysed here. We then normalised read abundances to compositional proportion data and calculated pairwise dissimilarities among samples using; Bray-Curtis dissimilarity matrix, Unweighted UniFrac and Weighted UniFrac, in the Phyloseq package of R (version 1.40.0; (McMurdie & Holmes 2013)) and visualised via principal coordinates analysis (PCoA). Bray-Curtis dissimilarity considers the abundance of taxa shared between two samples and the number of taxa detected in each sample (Bray & Curtis 1957). UniFrac distance metrics examine the phylogenetic distances between taxa across samples. The Weighted UniFrac is quantitative, accounting for the abundance of each taxa present, whilst the Unweighted UniFrac is qualitative and considers the difference in presence and absence of taxa between two samples (Lozupone et al. 2007).

To test for statistically robust differences between cohorts, we used a permutational multivariate analysis of variance (PERMANOVA, (Anderson 2001)) for each beta diversity metric, using the Vegan package (version 2.6.4) with the *adonis2* function and 999 permutations (Oksanen 2012). For each PERMANOVA model, the beta diversity metric was set as the response variable and the explanatory variables included cohort, timepoint (continuous), sex, sequence plate and experimental block. PERMANOVA currently does not allow for random effects, and we were unable to use an alternative restricted PERMANOVA here due to unequal sample sizes in each cohort. As PERMANOVA's may be sensitive to data dispersion differences, we performed an analysis of multivariate homogeneity (PERMDISP) between each cohort with the *betadisper* function in the Vegan package (version 2.6.4; (Oksanen 2012)) and 1000 permutations, with any significant results reported.

Further, among the two wood mouse cohorts, we investigated if *H. polygyrus* burden and total faecal IgA concentration were associated with the first principal component

(PC1) of each beta diversity ordination metric, as this explained the largest amount of variation in the gut community structure within our dataset. To determine if *H.polygyrus* burden was associated with differences in distribution between cohorts along the PC1 axes, we used GLMs with each beta diversity PC1 axis set as the response variable. Fixed effects were the same as the PERMANOVA's, however, timepoint was set at the factor level, as we were only assessing samples at Day 14 p.i and Day 21 p.i. To examine if faecal IgA antibody levels were associated with differences at the PC1 axes, we performed GLMMs, with each beta diversity PC1 axis as the response variable and faecal IgA concentration included as a fixed effect. As we were examining antibody levels across the entire duration of the experiment, timepoint was included as a continuous explanatory variable and individual mouse Id was set as a random effect to account for repeated measures. Again, all model outputs and covariates can be observed in the supplementary tables (Table S5-S7).

Differential abundance

To further understand how specific bacterial taxa differed between our four mouse cohorts we performed differential abundance analysis at the genus taxonomic level, using differential gene expression analysis based on a negative binomial distribution, with the DESeq2 package ((Love et al. 2014); version 1.36.0) on our phyloseq objects. A 10% threshold for prevalence of the taxa was applied as recommended, leaving a total of 602 ASVs analysed here (Nearing et al. 2022).

DESeq2 uses a matrix of sequencing read counts and fits a Generalised Linear Model (GLM) with a negative binomial distribution to this data (Love et al. 2014). Differential abundance is reported as log2 fold-change, thus, a positive value indicates significant enrichment and a negative value depletion. A Wald test is used for significance testing (Love et al. 2014) and to correct for multiple testing, we calculated an adjusted p-value by dividing 0.05 by the number of tests performed. Therefore, we only report data with an adjusted p value of <0.0009 as significant.

3.4. Results

3.4.1. Susceptibility to H.polygyrus infection and subsequent immune response

To determine if our three mouse cohorts differed in their response to *H. polygyrus* infection, we selected two key time points to quantify worm burdens. Day 14 post infection (peak) from our previous work has been shown to be the peak levels of infection and day 21 post infection (post-peak), is when we have shown that wood mice typically start clearing their worms and we see worm burdens starting to decline. First, we found that the Lab:Mm mice had no adult worms recovered from their intestines at either peak or post-peak timepoints, thus, they were excluded from all infection dynamics analyses.

However, both the Wild-like:As and Lab-like:As cohorts developed patent infection to a similar degree, as the overall mean for Wild-like:As mice was 42.5 worms and for Lab-like:As mice was 40.4 worms (GLMM, Est = -0.10 ± 0.70 SE, p = 0.882, Table S1, Fig. 2). Worm burdens were significantly lower across each cohort by day 21, mean Wild-like:As at day 14 p.i was 71.3 ± 10.0 and day 21 p.i was 13.7 ± 9.70, whilst mean Lab-like:As at day 14 p.i was 54.0 ± 9.48 and day 21 p.i was 26.8 ± 16.0 (GLMM, Est -2.80 ± 0.81, p = <0.001, Table S1, Fig.2), but, we found no significant cohort and timepoint interaction (GLMM, Est = -0.43 ±1.07, p =0.692, Table S1). Thus, suggesting that both groups of mice were beginning to clear their infections by day 21 p.i. at a similar rate. Indeed, at this point exactly 50% of the mice in each cohort had eliminated their *H.polygyrus* infection entirely. Surprisingly, we also found that mice in experiment block 2 also had significantly higher worm burdens than those in block 1, as such we included experiment block as a covariate in all subsequent analyses, to account for this bias (GLMM, Est 2.07 ± 0.59, p = <0.001, Table S1).

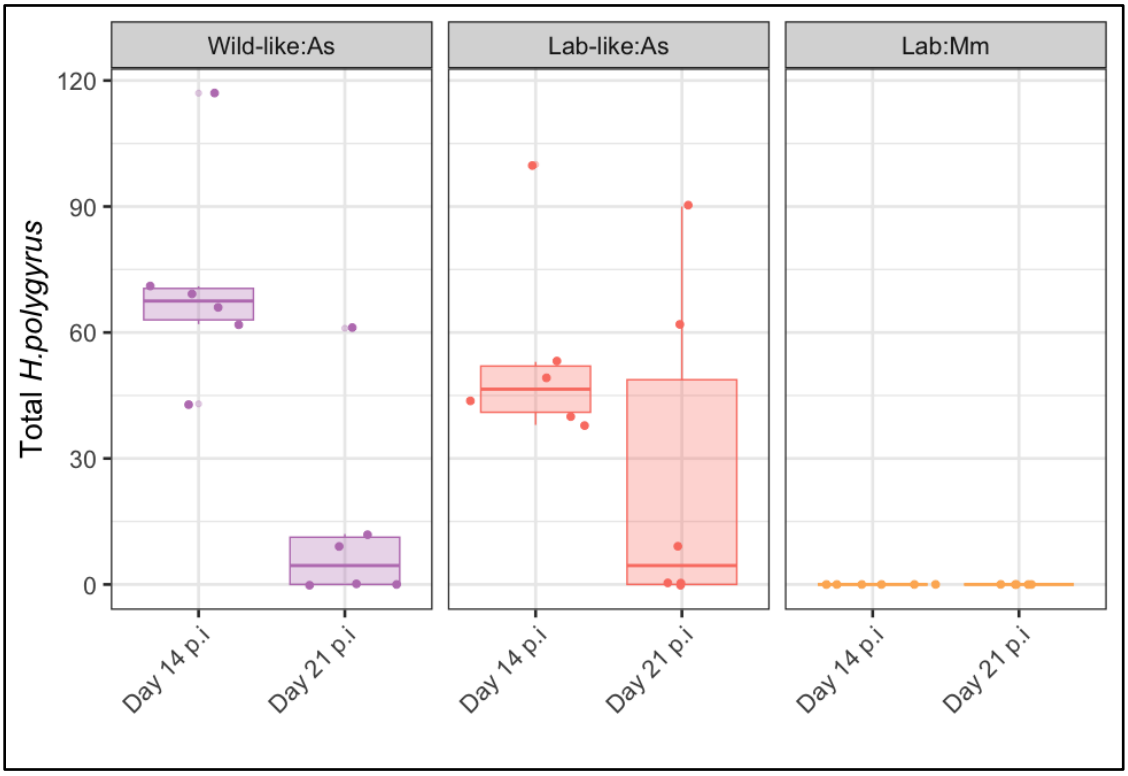


Figure 2. Susceptibility to *H.polygyrus* infection. The total number of adult *H. polygyrus* adult parasites (worm burden) detected in the small intestine, 14- and 21-days post infection of three mouse cohorts; wild-derived, lab-reared wood mice referred to as wild-like wood mice (Wild-like: *Apodemus sylvaticus;* Purple), wild-like wood mice that were caesarean re-derived and fostered by conventional lab mice, referred to as lab-like wood mice (Lab-like:As; Pink) and conventional lab mice (Lab: *Mus musculus;* Yellow). Box indicates interquartile range (IQR), with vertical lines representing median worm burdens and horizontal lines indicating ± 1.5 IQR. Points represent individual worm burdens.

To determine if the gut health of the two wood mouse cohorts differed during the course of *H.polygyrus* infection, we assessed total faecal IgA antibody levels at weekly intervals from day 0 p.i to day 21 p.i (Fig. 3). Here, we found that levels of faecal IgA were relatively stable during infection for both groups of mice, with no significant cohort and time point interaction, when time point was treated as a continuous variable (GLMM, Est = -0.03 ± 0.02 SE, p = 0.065, Table S1). However, Wild-like:As mice, did have significantly higher levels of faecal IgA in general compared to Lab-like:As mice (GLMM, Est = 0.45 ± 0.20 SE, p= 0.022, Wild-like:As mean = 19.7 ng ml^{-1} and Lab-like:As mean = 13.6 ng ml^{-1}, Table S1, Fig. 3).

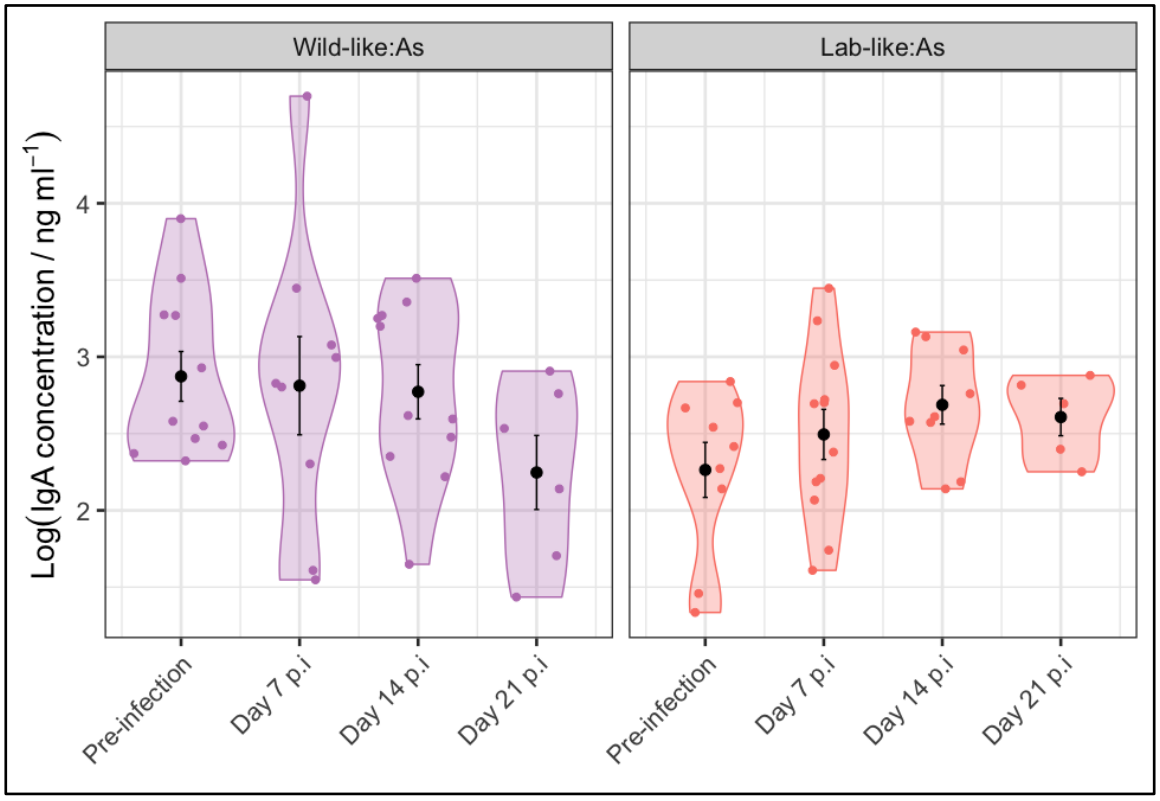


Figure 3. Faecal antibody levels are relatively stable among wood mice during *H.polygyrus* infection. Log transformed total faecal IgA concentrations (ng ml-1) of two wood mouse (*Apodemus sylvaticus*) cohorts over the course of *H.polygyrus* helminth infection. Wild-like:As indicate wild-derived lab-reared wood mice and Lab-like:As indicate wood mice that were caesarean re-derived and fostered by conventional lab mice. Error bars indicate standard error of the mean log IgA concentration at each time point for each cohort. Coloured points represent individual worm burdens.

We next assessed if there was an association between *H. polygyrus* worm burden and total faecal IgA levels at our experiment end points only, with time point treated as a factor variable (levels; Day 14 p.i and Day 21 p.i, Fig. 4). Here, we found a significant difference in faecal IgA antibody concentration between cohorts (Wild-like:As mean = 15.7 ± 2.17 ngml^{-1} and Lab-like:As mean = 15.0 ± 1.32 ngml^{-1}, Est = -0.58 ± 0.26 SE, p = 0.028, Table S2), but this was significantly affected by worm burden, whereby Wild-like:As mice with a higher total number of *H. polygyrus* had higher IgA antibody concentrations (GLMM, Est = 0.01 ± 0.01 SE, p = 0.018, Table S2, Fig.4). Male mice also had significantly lower faecal IgA antibody concentrations at the experimental endpoints compared to female mice (GLMM, Est = -0.28 ± 0.11 SE, p = 0.013, Table

S2), whilst mice in experimental block 2 had on average higher IgA concentrations than those in block 1 (GLMM, Est = 0.31 ± 013 SE, p = 0.015, Table S2).

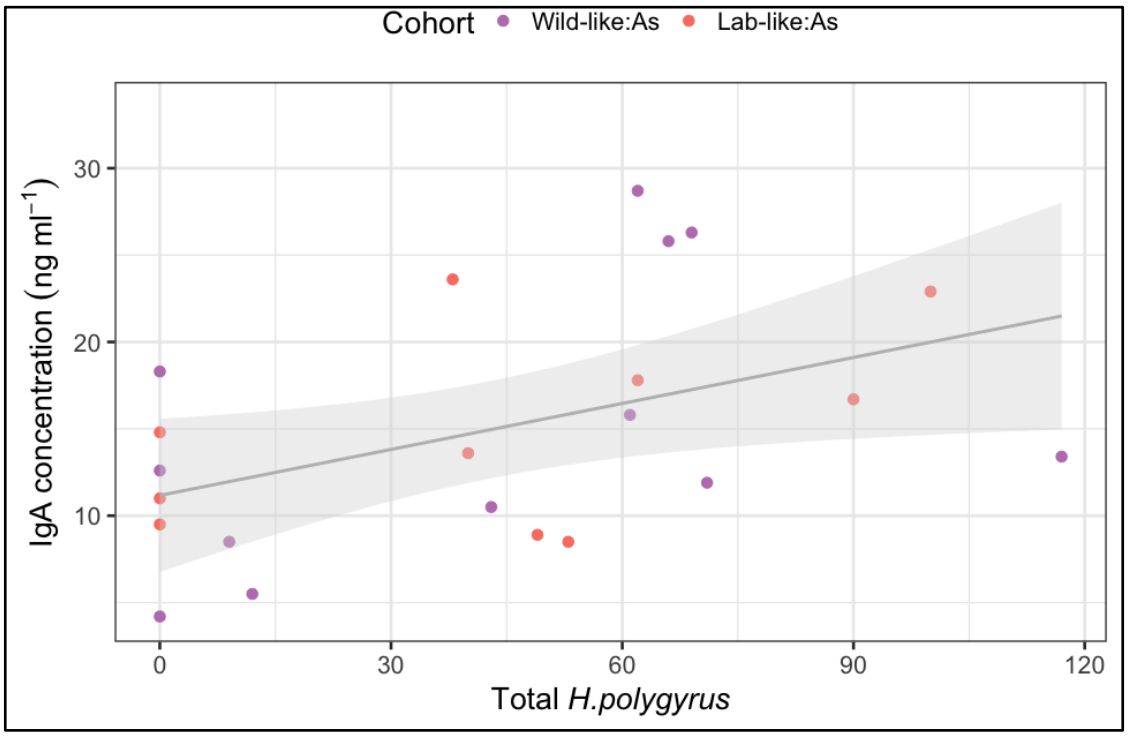


Figure 4. Faecal antibody levels are positively associated with *H.polygyrus* burden in Wild-like wood mice. The association between Log transformed total faecal IgA concentrations (ng ml-1) and number of *H.polygyrus* worms detected 14- and 21-days post-infection, of two wood mouse (*Apodemus sylvaticus*) cohorts. Wild-like:As (Purple) indicate wild-derived, lab-reared wood mice and Lab-like:As (Pink) indicate wood mice that were caesarean re-derived and fostered by conventional lab mice. Line represents the mean with the shaded area around the line representing 95% confidence intervals. Points represent individual samples.

Finally, we assessed the gut pathology of our two wood mice cohorts at the two infection endpoints, by measuring intestinal crypt and villi length as a proxy for cell damage and goblet cell counts per crypt and villi as proxy for mucus production (Fig. 5A-D). Here there were similar findings across both mouse cohorts, except with goblet cell counts per villi, whereby, the Wild-like:As mice had significantly lower goblet cell counts, compared to the Lab-like:As group (Wild-like:As mean = 12.4 ± 1.30 SE, Lab-like:As mean = 16.2 ± 2.20 SE, GLM, Est = -0.56 ± 0.23 SE, p= 0.031, Table S3, Fig. 5A). We found no significant association between *H. polygyrus* burden or total faecal

IgA concentration and any of the four-histology metrics measured across either Wild-like:As or Lab-like:As cohorts (Supplementary Fig. 2 and 3, Table S3).

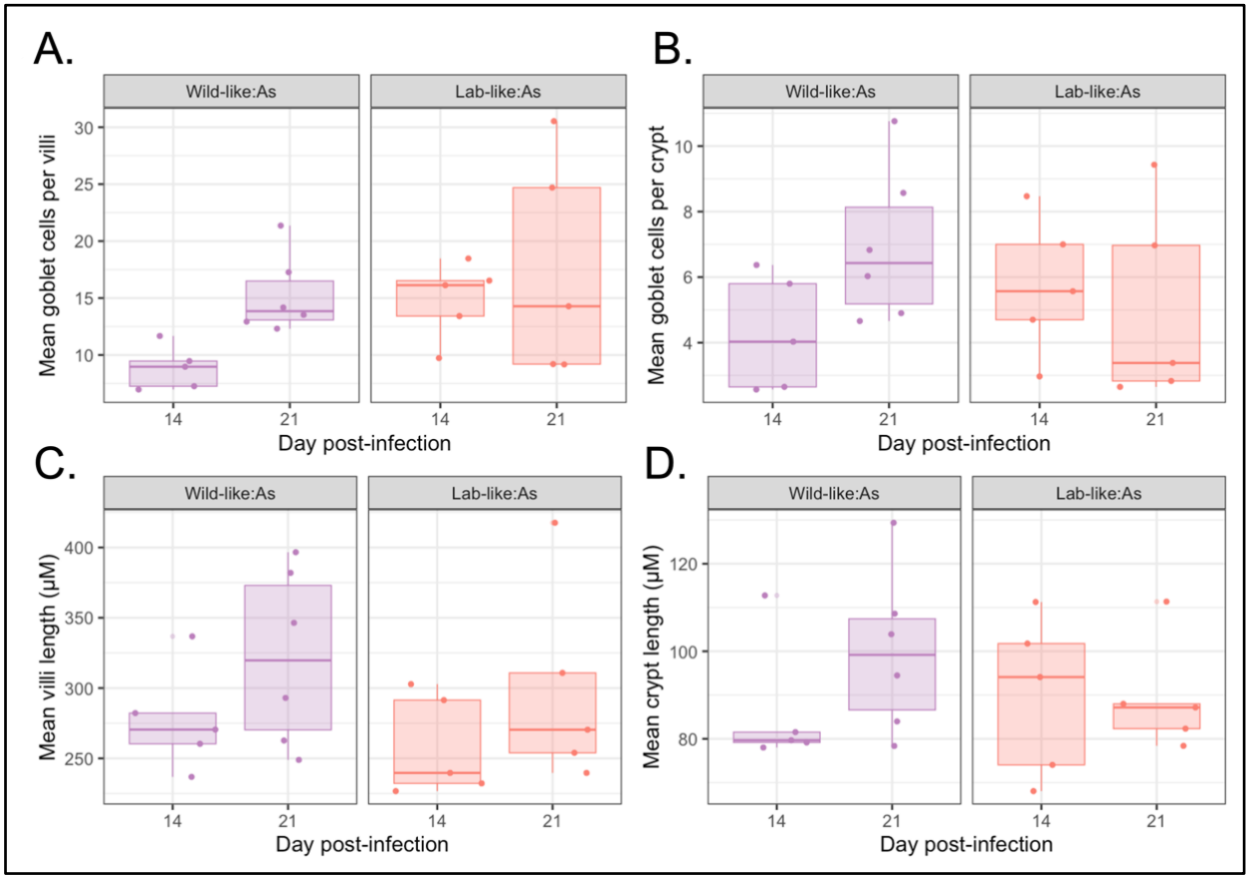


Figure 5. Gut pathology associated with *H. polygyrus* infection is similar among wood mouse cohorts. Pathology within the small intestine of two wood mice (*Apodemus sylvaticus*) cohorts 14- and 21-days post-infection with *H. polygyrus.* (**A**) mean goblet cells per villi, (**B**) mean goblet cells per crypt, (**C**) mean villi length (µM) and (**D**) mean crypt length (µM). Wild-like:As (Purple) indicate wild-derived, lab-reared wood mice and Lab-like:As (Pink) indicate wood mice that were caesarean re-derived and fostered by conventional lab mice. Box indicates interquartile range (IQR), with vertical lines representing medians and horizontal lines indicating ± 1.5 IQR. Points represent individual samples.

3.4.2. Gut microbiota diversity increased during infection in Wild-like:As mice.

Although the Lab:Mm mice did not have any adult worms in their guts by the experimental end points, we were still interested to establish if exposure to *H.polygyrus* had any effect on their microbiota in comparison to mice that were patently infected. As such, we included the Lab:Mm cohort in our analysis of the gut microbiota diversity and composition. To examine how the within-sample microbiota diversity differed between our three cohorts and how infection may have influenced this, we assessed four commonly used alpha diversity metrics at weekly intervals from pre-infection (day 0) until day 21 p.i (Fig. 6A-D). This revealed that the microbiota diversity of Wild-like:As mice significantly increased over the course of infection in comparison to Lab:Mm mice and this was consistent across all four metrics measured (GLMM, $p = <0.01$ for all except inverse Simpson where $p = 0.013$, Table S4, Fig. 6A-D).

Overall, Lab:Mm samples had significantly higher observed richness and phylogenetic diversity compared to Lab-like:As mice (GLMM, observed richness mean = 238 ± 7.16 SE, Est = -0.23 ± 0.01 SE, $p = 0.005$ and phylogenetic diversity mean = 485 ± 9.04 SE, $p = 0.01$, Fig. 6A-B). However, there was no significant difference in mean Shannon and inverse Simpson diversity measurements between these two groups (GLMM, Est = -0.30 ± 0.21 SE, $p= 0.160$ and Est = -0.27 ± 0.23 SE, $p = 0.238$ respectively, Fig. 6C-D). Surprisingly, the Wild-like:As samples had significantly lower mean alpha diversity at all four metrics when compared to the Lab:Mm cohort (GLMM, $p= <0.001$ all, Table S4), however, Wild-like:As mice had the greatest variance in observed richness and phylogenetic diversity values across all three mouse cohorts (observed richness variance = 2341, SD = 48.4 and phylogenetic diversity variance = 5312, SD =72.9), indicating that the spread in the species within Wild-like:As microbiota samples was greater than in the Lab:Mm mice.

We also investigated if there was an association between each of the alpha diversity metrics and *H. polygyrus* burden or total faecal IgA antibodies, in the two wood mouse cohorts at the experimental endpoints of day 14 p.i and day 21 p.i. Here we found no

significant association between either worm burden or gut health and the intra-individual microbiota diversity in either Wild-like:As or Lab-like:As cohorts (Supp fig. 4A-D and 5A.D)

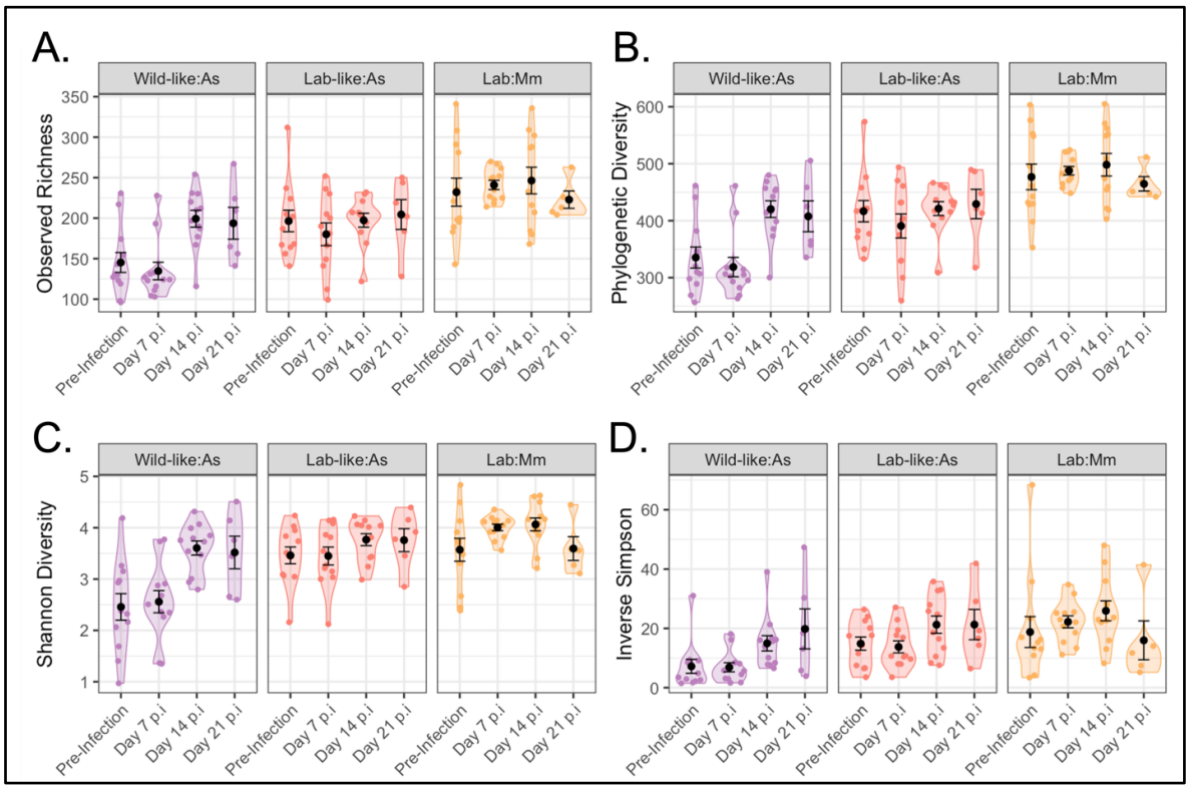


Figure 6. Gut microbiota diversity increased during infection in Wild-like:As mice. Alpha diversity measurements of 16s rRNA sequenced faecal samples collected from three distinct mouse cohorts, wild-derived, lab-reared wood mice referred to as wild-like wood mice (Wild-like: *Apodemus sylvaticus*; Purple), wild-like wood mice that were caesarean re-derived and fostered by conventional lab mice, referred to as lab-like wood mice (Lab-like:As; Pink) and conventional lab mice (Lab: *Mus musculus;* Yellow). (**A**) Observed species richness, (**B**) Faith's phylogenetic diversity, (**C**) Shannon diversity index, (**D**) inverse Simpson index. Each coloured dot represents the alpha diversity value for an individual sample, error bars represent the standard error (SE) of the mean alpha diversity value of all samples within the cohort. For each metric, a higher value is indicative of higher within sample diversity.

3.4.3. Infection has subtle influences on observed differences in the microbiota composition of Wood mouse cohorts.

Next, we explored how the gut microbiota composition varied between the three mouse groups. First, we examined the relative abundance of taxa across the 13 Phyla detected, which highlighted that although each cohort shared the same four most prevalent Phyla, this was to varying degrees depending on cohort (Fig. 7A). For example, we see a general trend of increasing proportion of *Bacteriodota* from Wild-like:As (14.6%) to Lab:Mm (19.1%) but increasing levels of *Actinobacteriota* and *Proteobacteria* from Lab:Mm (2.3% and 2.0% respectively) to Wild-like:As (5.4% and 3.7% respectively, Fig.7A). We found a total of 1341 unique bacterial sequences within the data set, with more than one third of these associated with the Lab:Mm samples only (31.2%; 418, Fig. 7B). Indeed, there were a marked number of unique ASV's associated with both the Lab-like:As (15.6%; 209) and Wild-like:As (16.9%; 227) cohorts as well. However, there was also a noteworthy core microbiota of 123 ASV's shared among all three mouse cohorts, and both wood mouse cohorts had 161 ASV's in common, whilst the Lab:Mm and Lab-like:As mice shared 193 ASVs.

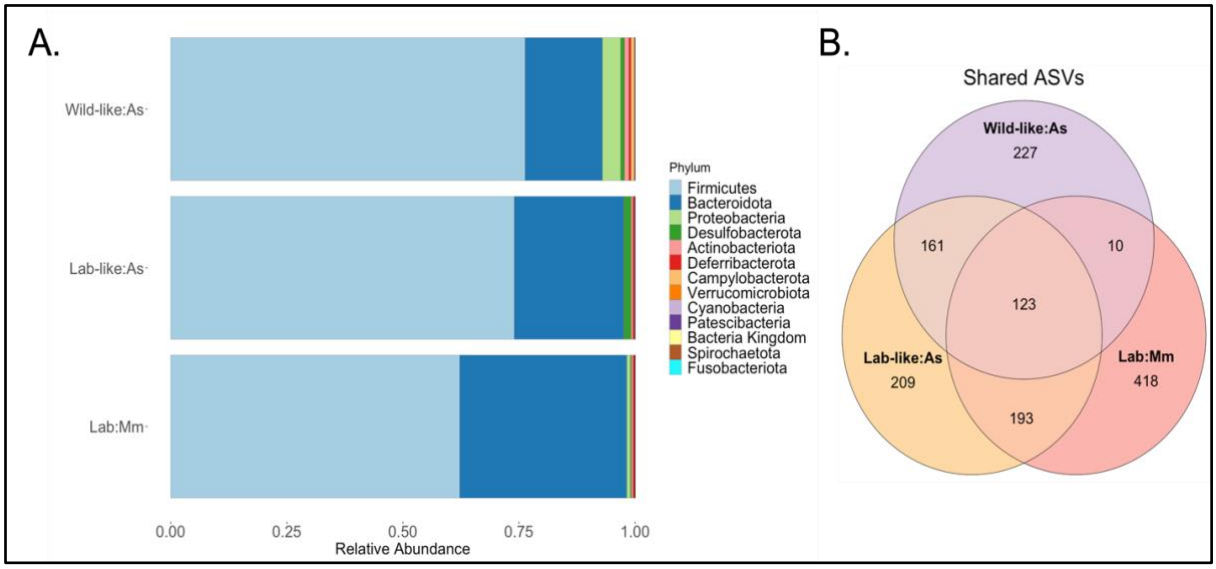


Figure 7. Wood mouse cohorts have similar microbiota composition even when infected. (**A**) The relative abundance of bacterial phyla among faecal samples collected from three mouse cohorts; wild-derived lab-reared wood mice referred to as wild-like wood mice (Wild-like: *Apodemus sylvaticus*), wild-like wood mice that were caesarean re-derived and fostered by conventional lab mice, thus referred to as lab-like wood mice (Lab-like:As) and conventional lab mice (Lab: *Mus musculus*). (**B**) The number of unique and shared amplicon sequence variants (ASV's) between the distinct mouse cohorts, circles overlapping indicate commonality.

To examine the microbiota composition differences in further detail, we went on to calculate pairwise dissimilarity between samples in each mouse cohort, using three beta diversity metrics: Bray-Curtis dissimilarity, plus, Weighted and Unweighted Unifrac. We visualised these distance matrices via principal coordinate analysis (PCoA) and found distinct clustering patterns of each cohort across all three ordination analyses (Fig. 8A-C). At both Bray-Curtis and Weighted Unifrac distances, Wild-like:As and Lab-like:As samples clustered closely and often overlapped, indicating similar microbiota compositions within these two cohorts, in terms of taxa abundance (Fig. 8A) and phylogenetic relatedness (Fig. 8B). Moreover, the Lab:Mm cohort also clustered closely to the two wood mice cohorts, in particular the Lab-like:As samples at Weighted Unifrac distances, where the two cohorts a difficult to distinguish, indicating a strong overlap in phylogenetic relatedness of bacterial taxa here (Fig.8B).

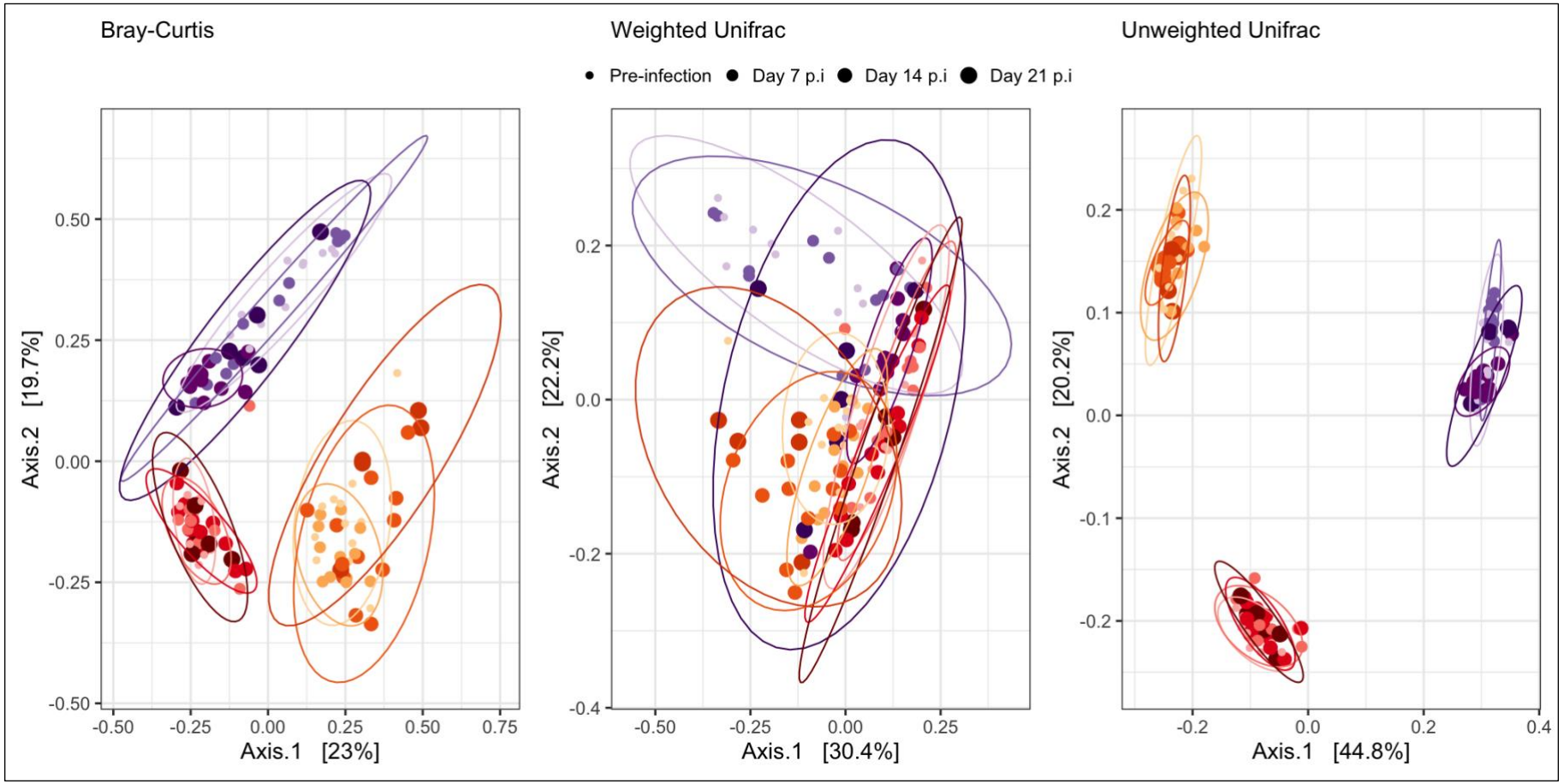


Figure 8. The gut microbiota composition of wood mice are similar regardless of *H.polygyrus* infection. Principal coordinate analysis (PCoA) ordination of beta diversity dissimilarity matrices, for 16s rRNA sequenced faecal samples collected from three mouse cohorts; wild-derived, lab-reared wood mice referred to as wild-like wood mice (Wild-like: *Apodemus sylvaticus*; Purple), wild-like wood mice that were caesarean re-derived and fostered by conventional lab mice, thus referred to as lab-like microbiota (Lab-like:As; Pink) and conventional lab mice (Lab: *Mus musculus;* Yellow). **(A)** Bray Curtis dissimilarity **(B)** Weighted Unifrac distance **C)** Unweighted Unifrac distance. Each coloured dot represents data for an individual samples, whilst ellipses represent 95% confidence intervals for each mouse cohort at each timepoint. Size of points represents sampling point. The closer the dots (samples) are ordinated together, the more similar their microbiota compositions are. Each axis has an eigenvalue in parenthesis indicating the percentage of variation within the dataset captured at that axis.

As such, we also partitioned the PCoA plots into a matrix of panels for each cohort at each beta diversity metric, to visualise the within cohort variation across time more clearly (SuppFig. 5A-C). Overall, we can see that there is a large amount of within cohort variation across timepoints for all three mouse cohorts, indicated by 95% confidence interval ellipses and their varying sizes at Bray-Curtis and Weighted Unifrac distances (Fig. 8A-B & Supp Fig. 6A-B). At Unweighted Unifrac distances, the three mouse cohorts form distinct clusters with no overlap between cohorts, suggesting that the presence and absence of different bacterial taxa is unique to each cohort (Fig. 8C). PERMDISP analysis of each ordination metric support this finding, as here we found that dispersion of samples was homogenous between cohorts at Bray-Curtis and Weighted Unifrac, whereas Wild-like:As mice were significantly more dispersed at Unweighted Unifrac distances (Supp Fig. 7A-C, Table S5).

Statistical analyses of each distance matrix via PERMANOVA also supported the ordination findings (Table S5). For instance, at Bray-Curtis, differences in the microbiota composition between Lab:Mm mice and Wild-like:As mice accounted for 21% (R^2) of the variation within the data set ($F = 45.09$, $p = 0.001$), whilst differences between the Lab:Mm and Lab-like:As also accounted for 17% (R^2) of the variation observed here ($F = 36.65$, $p = 0.001$). Both Wild-like:As and Lab-like:As cohorts had significantly increased microbiota composition diversity over time ($F = 6.90$, $R^2=0.03$ $p= 0.001$ and $F = 1.68$ $R^2 = 0.01$, $p = 0.020$ respectively). These findings were all robust and consistent across both Weighted and Unweighted unifrac measures too (Table S5).

3.4.4. *H. polygyrus* infection does not appear to alter overall gut microbiota composition of wood mice.

The significant variation in microbiota composition over time for both the Lab-like:As and Wild-like:As cohorts could suggest that *H.polygyrus* infection influences the composition. To test this, we again calculated dissimilarity matrices using the three beta diversity metrics (Bray Curtis, Weighted and Unweighted Unifrac), for Wild-like:As and Lab-like:As at the experimental timepoints of Day 14 and Day 21 post-infection, where the total *H.polygyrus* burden for each mouse is known. We visualised these

distance matrices via principal coordinate analysis (PCoA). Here, we observed distinct clustering patterns of each cohort across all three ordinations, with some overlap between the two cohorts at Weighted Unifrac distance (Fig. 9A-9C). Interestingly, within the Wild-like:As samples, Day 14 p.i samples appear to cluster more closely with other Day 14 p.i samples than Day 21 p.i. samples, at both Bray-Curtis and Weighted Unifrac distances in particular (Fig. 9A-B). However, this does not appear to be replicated within the Lab-like:As cohort, where samples cluster closely together regardless of post-infection timepoint.

We performed statistical analyses of each distance matrix via PERMANOVA, whereby *H.polygyrus* burden was fit as a covariate. Here, we found that at Bray-Curtis, the only significant explanatory variable tested was differences between the mouse cohorts, accounting for 31% (R^2) of the variation within the data set ($F = 10.05$, $p = 0.001$, Table S6). This finding was replicated with both the Weighted and Unweighted Unifrac distance metrics too (0.22 R^2 , $F = 5.92$, $p = 0.001$ and 0.55 R^2 , $F = 26.64$, $p = 0.001$, Table S6). Here, we found no statistical evidence that the overall gut microbiota composition varied with *H.polygyrus* worm burden among either Wild-like:As mice or Lab-like:As mice at any of the beta diversity metrics we used ($p > 0.05$ for all, Table S6). Similarly, we found no significant interaction between the mouse cohort and total number of *H.polygyrus* worms present, in driving differences between the gut microbiota composition ($p > 0.05$ for all metrics, Table S6).

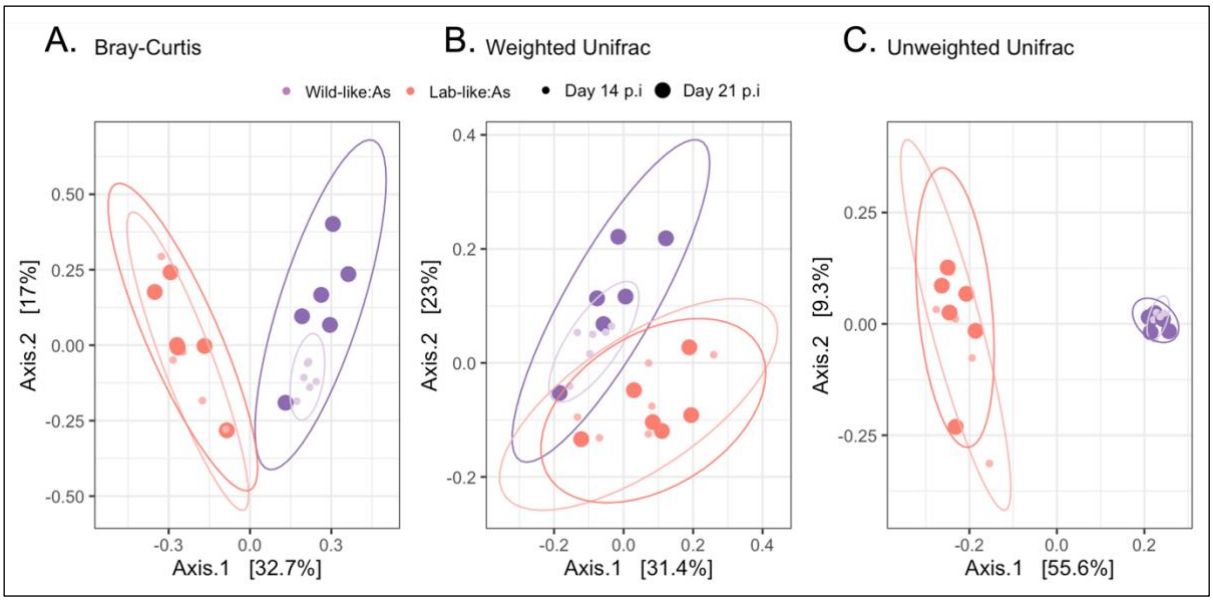


Figure 9. *H. polygyrus* infection does not appear to alter overall gut microbiota composition of wood mice. Principal coordinate analysis (PCoA) ordination of beta diversity dissimilarity matrices, for 16s rRNA sequenced faecal samples collected from two mouse cohorts; wild-derived, lab-reared wood mice referred to as wild-like wood mice (Wild-like: *Apodemus sylvaticus*; Purple) and wild-like wood mice that were caesarean re-derived and fostered by conventional lab mice, thus referred to as lab-like microbiota (Lab-like:As; Pink) at both 14 and 21 days posi-infection with *H.polygyrus* **(A)** Bray Curtis dissimilarity **(B)** Weighted Unifrac distance **C)** Unweighted Unifrac distance. Each coloured dot represents data for an individual samples, whilst ellipses represent 95% confidence intervals for each mouse cohort at each timepoint. Size of points represents sampling point. The closer the dots (samples) are ordinated together, the more similar their microbiota compositions are. Each axis has an eigenvalue in parenthesis indicating the percentage of variation within the dataset captured at that axis.

Further, we assessed if there was an association between gut health of the wood mice cohorts, as measured by total faecal IgA antibody levels, and difference in microbiota composition (Fig. 10A-C). At each ordination metric, there is distinct clustering within mouse cohort and specific timepoint clustering too, particularly at both Bray-Curtis and Unweighted Unifrac metrics (Fig. 10A and 10C). We then performed statistical analyses of each distance matrix via PERMANOVA, whereby total faecal IgA antibody concentration was fit as a covariate. Here, we found that at Bray-Curtis, that IgA concentration, mouse cohort and experimental timepoint were all significant explanatory variables, accounting for 3.0%, 27.0% and 5.0% (R^2) of the variation within the data set respectively ($p = 0.001$ for all three covariates, Table S7). This result was also observed with Weighted and Unweighted Unifrac measures too (Table S7).

However, we found no significant interaction between mouse cohort and total faecal IgA concentration at any of the metrics tested (Bray Curtis; 0.01 R^2 , F = 1.54, p = >0.05, Weighted Unifrac; 0.05 R^2 , F = 1.24, p = >0.05 and Unweighted Unifrac; 0.01 R^2 , F = 1.31, p = >0.05 Table S7).

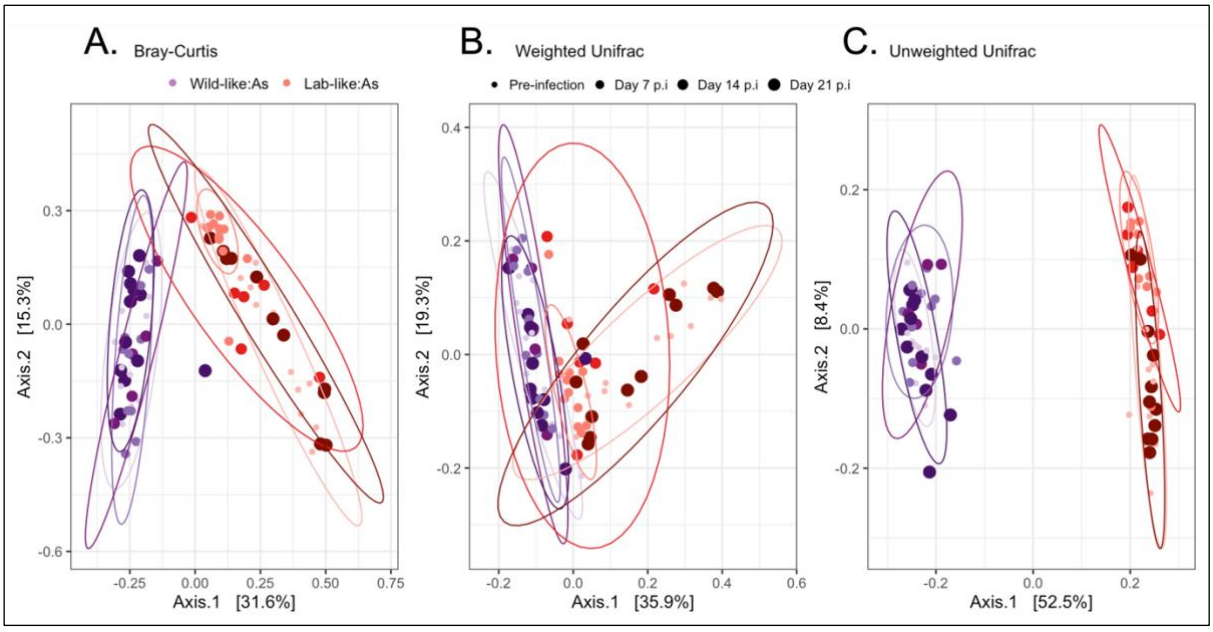


Figure 10. IgA concentration, mouse cohort and experimental timepoint affect microbiota composition independently. Principal coordinate analysis (PCoA) ordination of beta diversity dissimilarity matrices, for 16s rRNA sequenced faecal samples collected from two mouse cohorts; wild-derived, lab-reared wood mice referred to as wild-like wood mice (Wild-like: *Apodemus sylvaticus*; Purple) and wild-like wood mice that were caesarean re-derived and fostered by conventional lab mice, thus referred to as lab-like microbiota (Lab-like:As; Pink), whereby total faecal IgA concentration was recorded at several timepoints. **(A)** Bray Curtis dissimilarity **(B)** Weighted Unifrac distance **C)** Unweighted Unifrac distance. Each coloured dot represents data for an individual samples, whilst ellipses represent 95% confidence intervals for each mouse cohort at each timepoint. Size of points represents sampling point. The closer the dots (samples) are ordinated together, the more similar their microbiota compositions are. Each axis has an eigenvalue in parenthesis indicating the percentage of variation within the dataset captured at that axis.

3.4.5. *H.polygyrus* infection is associated with depletion in specific bacterial taxa in wood mice.

Finally, we examined how specific bacterial taxa, at the genus level, were shifting over the course of infection through differential abundance analysis using DESeq2. To determine how *H. polygyrus* infection in the Wild-like:As and Lab-like:As cohorts, and exposure in the Lab:Mm cohort, influenced the microbiota, we compared the composition of samples from day 7, 14 and 21 post-infection to pre-infection (day 0) samples (Fig. 11A-C). Here, data is only reported as significant if adjusted p values were below the threshold of 0.0009 to account for multiple testing.

In total, we found 15 taxa that were differentially abundant among the three mouse cohorts at post-infection timepoints compared to pre-infection. Of these, most were significantly depleted during infection rather than enriched. For instance, *Staphylococcus* was significantly depleted in samples from all three mouse cohorts at day 14 and day 21 post-infection, but not at day 7 (DESeq2; Wild-like:As, L2FC = -5.67 ± 0.66 SE and L2FC = -6.46 ± 0.70 SE, Lab-like:As, L2FC = -6.04 ± 1.03 SE, and L2FC = -7.52 ± 0.88 SE, Lab:Mm, L2FC = -5.88 ± 0.86 SE and L2FC = -10.36 ± 1.14 SE respectively, Fig. 11A-C). *Jeotgalicoccus* was also significantly depleted in Wild-like:As and Lab:Mm at day 21 p.i (DESeq2; L2FC = -4.12 ± 0.96 SE and L2FC = -6.33 ± 1.44 SE respectively) and in Wild:like:As and in Lab-like:As at day 14 p.i (DESeq2, L2FC = -4.35 ± 0.72 SE and L2FC = -6.43 ± 0.74 SE respectively Fig. 11A-C).

Interestingly, we found no significant differences in taxa abundance of Wild-like:As mice at day 7 compared to pre-infection samples (Fig. 11C) and in Lab-like:As samples at this time point, only *Tyzzerella* differed, with a significant enrichment (DESeq2, L2FC = 3.15 ± 5.21 SE, Fig 11B). In contrast, the Lab:Mm samples had significant enrichment of *Clostridium* (ASF356) and depletion of Corynebacterium and *Candidatus Arthromitus* (DESeq2, L2FC = 2.65 ± 0.50, L2FC = -4.80 ± 0.95 SE and L2FC = -6.31 ± 0.87 SE respectively, Fig 11C). The only other taxa we found enriched in samples was *Bilophila* in Wild-like:As and *Eubacterium* (*xylanophilum* group) in Lab-like:As, at day 14 and *Roseburia* in Lab:Mm at day 21 p.i DESeq2; Wild-like:As,

L2FC = 1.88 ± 0.39 SE, Lab-like:As, L2FC = 2.63 ± 0.62 SE, and Lab:Mm L2FC = 2.77 ± 0.62 SE, Fig. 11A-C).

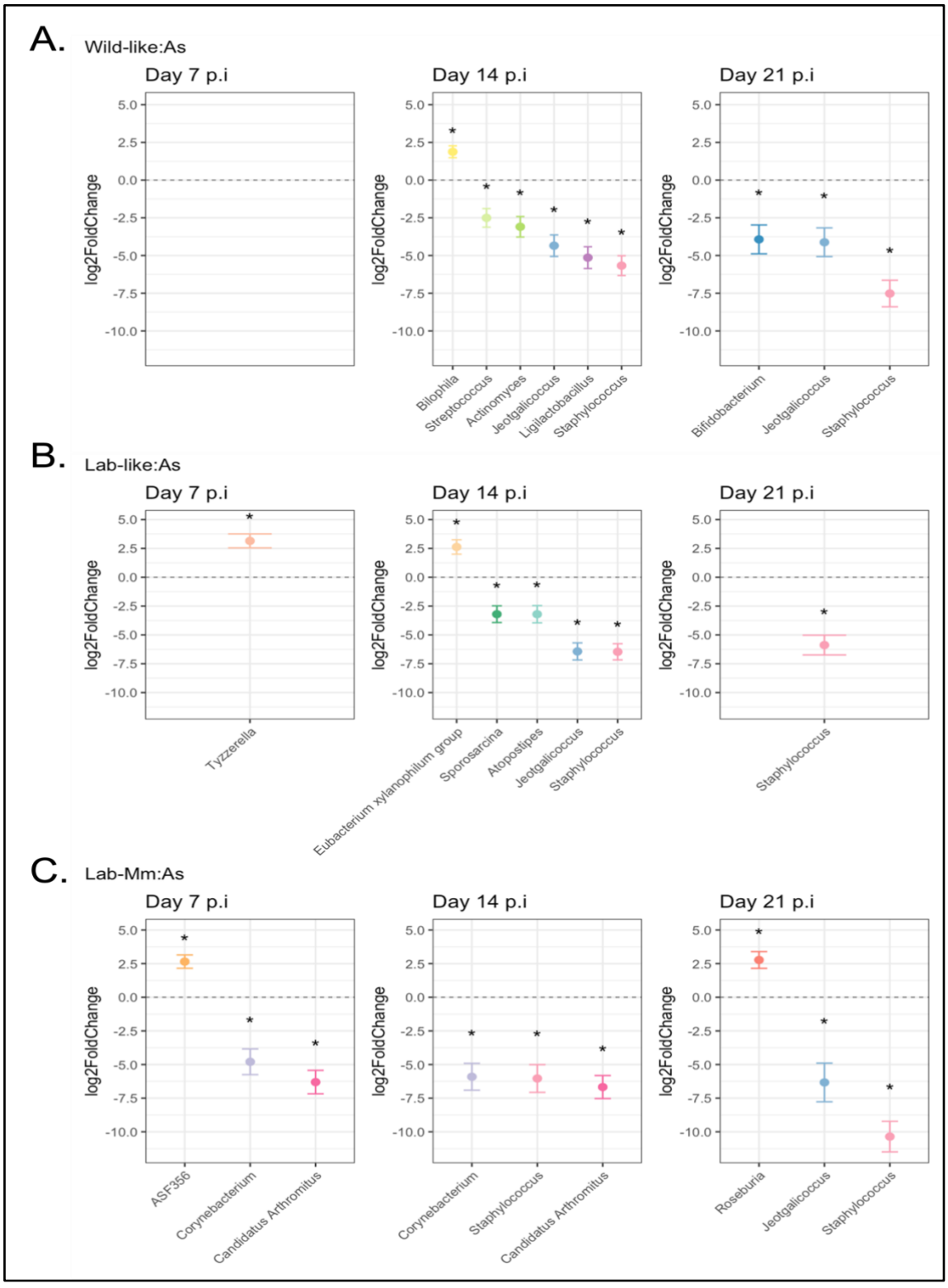


Figure 11. *H.polygyrus* infection is associated with depletion in specific bacterial taxa in wood mice. Differential abundance analysis of 16s rRNA sequenced faecal samples pre-infection with *H.polygyrus* compared to day 7, 14 and 21 post-infection samples. Samples collected from three mouse (**A**) Wild-derived, lab-reared wood mice, referred to as wild-like wood mice (Wild-like: *Apodemus sylvaticus*), (**B**) Wild-like wood mice that were caesarean re-derived and fostered by conventional lab mice, referred to as lab-like wood mice (Lab-like:As) and (**C**) conventional lab mice (Lab: *Mus musculus*). Differential abundance is reported as log2 fold-change whereby a positive value indicates significant enrichment and a negative value depletion. P-values were adjusted for multiple testing and only differences with a p-value of <0.0009 were treated as significant and reported.

3.5. Discussion

In this study, I find that the gut microbiota within sample diversity increases, and the composition is altered after *H. polygyrus* infection in our Wild-like:As mice cohort, but that there is very little change after infection in our Lab-like:As mice. By using a unique system with the same outbred species held in captivity, but with differing gut microbiota diversity and composition, we have been able to test what contribution is made by the gut microbiota during helminth infection.

Interestingly, we found, among the two wood mouse cohorts, very few changes in bacterial composition between pre-infection (Day 0) and Day 7 post infection, for instance among Wild-like:As samples, there were no significant differences in taxa abundance during this period and for the Lab-like:As, we observed a significant enrichment in bacterium from the *Tyzzerella* genus, with a 3.15 log2 fold change. Within the first 24 hours if infection, *H.polygyrus* larvae have been shown to have penetrated into the submucosa of the small intestine and here they encyst the muscle layer of the intestine for a number of days to undergo two developmental moults (Maizels et al. 2012; Reynolds et al. 2012a). Consequently, the infective larvae stage are most likely to come into direct contact with the host gut microbiota during the first 24-48 hours, whereby the larvae are establishing themselves in the gut niche. As such, collecting faecal samples from mice during that initial 48-hour window, would provide greater insight into how the microbiota respond to the establishment and invasive nature of the parasite larvae. Our samples at day 7 post-infection, unfortunately appear to have missed this window, and here we provide support that whilst the larvae

are encysted in the gut wall and undergoing moults, they may have less direct interaction with the bacteria dwelling in the gut lumen, thus leading to fewer microbiota shifts. However, previous studies with patients suffering from Crohn's disease, a chronic inflammatory bowel disease, found a remarkable (27-fold, log2 change) increase in the abundance of *Tyzzerrella* bacteria among patients with active Crohn's disease, and associated this genera with an increase in levels of inflammation in the gut (Lewis et al. 2023; Walker et al. 2017). Therefore, it is possible that the significant increase of this same bacterial genera among our Lab-like:As cohort may indicate that *H.polygyrus* infection did cause an inflammatory response here.

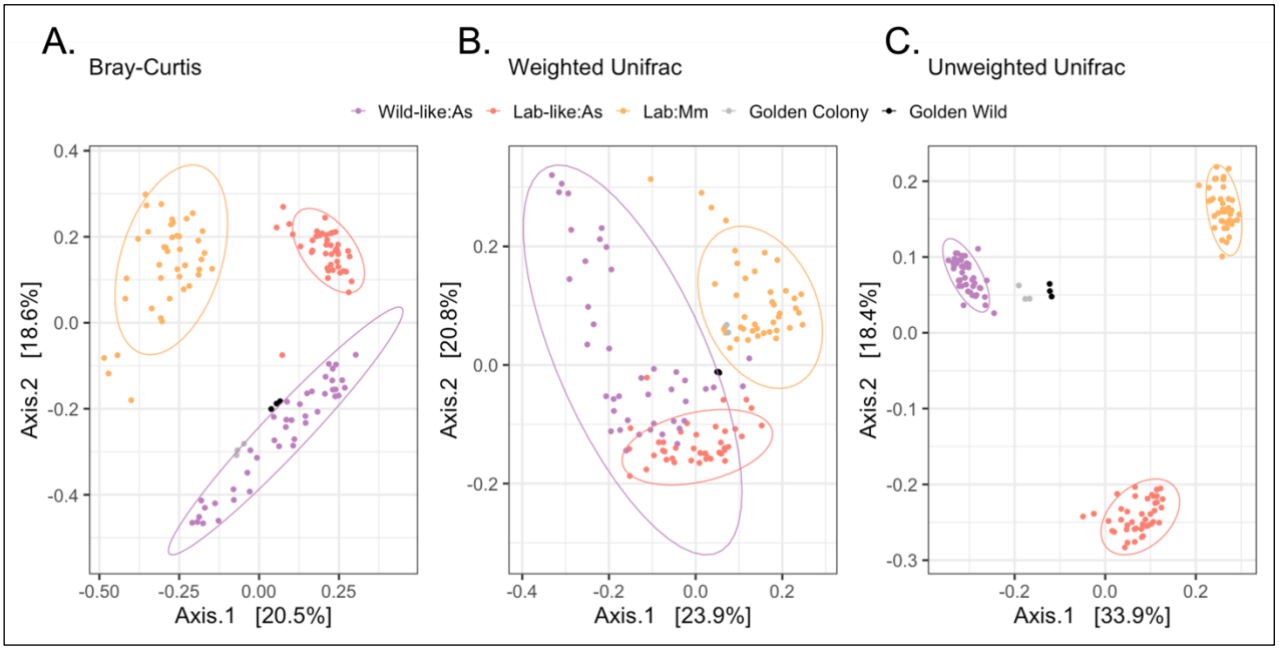
Moreover, the IgA immune response within the gut and the levels of pathology observed upon *H.polygyrus* infection were similar among the two wood mouse cohorts, this aligns with the fact that infection intensity as measured by worm burdens in the gut was also similar across the two cohorts, highlighting that their immune mediated response to infection was similar. However, within the Wild-like:As mice, we did find significantly higher levels of IgA at Day 14 and Day 21 p.i when compared to the Lab-like:As mice which appeared to be driven by infection intensity, whereby Wild-like:As mice with a higher total number of *H. polygyrus* had higher IgA antibody concentrations. This weak positive correlation between IgA and *H.polygyrus* worm burden suggests that IgA does not play a dominant role in protection. This finding aligns with a previous study, that demonstrated that only the IgG antibody concentrations within the gut were correlated with resistance to *H.polygyrus* infection, whereas IgA and IgM levels were not associated with resistance (Zhong & Dobson 1996). Furthermore, there was no clear interaction observed with the microbiota and IgA either.

Finally, a surprising finding during this study, was that *H.polygyrus* was unable to establish patent infection among the Lab:Mm cohort, as demonstrated by absences of the worms in the small intestine at Day 14 and 21 post infection, among every mouse in the Lab:Mm cohort. Previous studies with CD1 strain mice have shown that *H.polygyrus* can cause infection among these mice (Minkus et al. 1992; Valanparambil et al. 2017). However, upon closer inspection of the methods of these studies, it appears they have all used parasite strains that have been passaged and maintained within the laboratory for a number of years, and not wild-derived, as such these

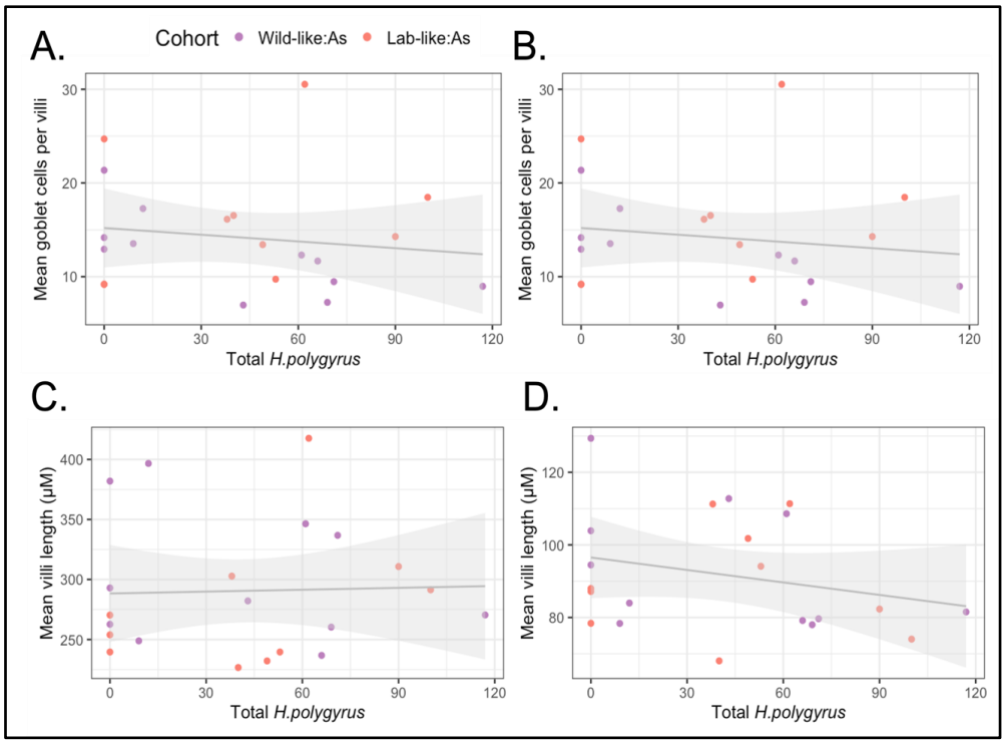
parasites are actually more likely to be *H.polygyrus* sister taxon of *H.bakeri* (Minkus et al. 1992; Valanparambil et al. 2017). There has been some confusion and controversy over the years within the helminth research community about whether *H.bakeri* and *H.polygyrus* are truly two different worms and as such, the name are often used interchangeably (Behnke & Harris 2010a). There have now been a number of studies providing evidence that these are two distinct species of worm (Behnke & Harris 2010a; Cable et al. 2006; Lewis et al. 2023) and we believe our findings support that notion. In addition, a recent study in collaboration with the Buck and Pedersen research groups at the University of Edinburgh, we performed a cross-species experiment with *H.polygyrus* larvae, derived from faeces collected from wild wood mice and *H.bakeri* larvae that have been maintained by the Buck group for a number of years via passage experiments in conventional lab mice. Here we found that both *H.polygyrus* and *H.bakeri* larvae were able to establish chronic infection in our Wild-like:As wood mouse colony, but that only *H.bakeri* larvae was able to establish patent infection in the inbred, commonly used conventional lab mouse (C57BL/6, data unpublished 2021). Further, a previous study also had similar findings whereby investigators also found that *H.polygyrus* showed host-specificity towards wood mice only and was unable to establish chronic infection in lab mice (Quinnell et al. 1991). Thus, we believe, that much of our current understand of helminth infection using *Heligmosomoides*, especially studies that have used *H. bakeri,* may not be fully applicable to more natural settings and more work needs to be done using natural host-helminth models to better understand how infections and response occur in 'real-world' contexts.

3.6. Chapter 3: Supplementary Material

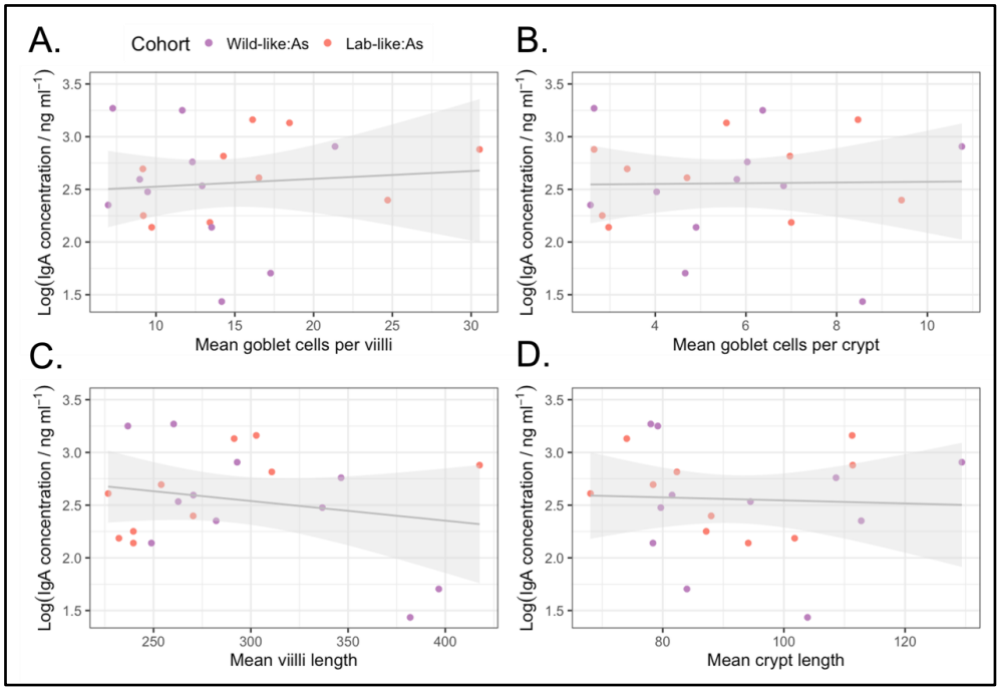
3.6.1. Supplementary Figures



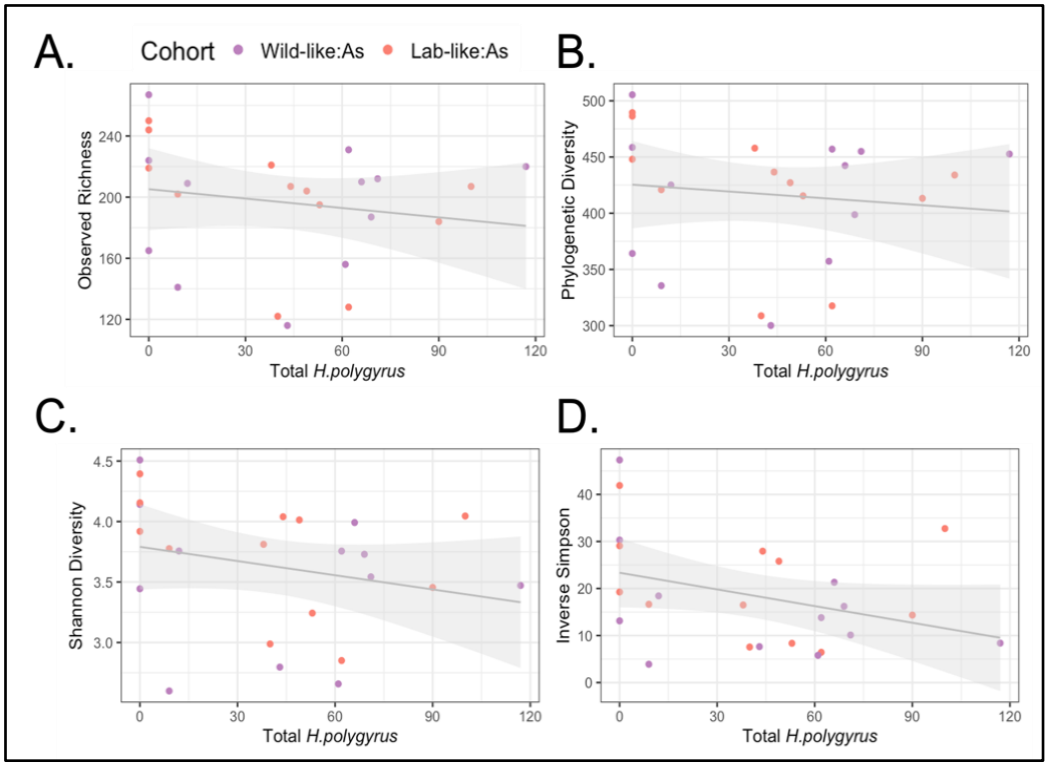
Supplementary Figure 1. Principal coordinate analysis (PCoA) ordination of beta diversity dissimilarity matrices, for 16s rRNA sequenced faecal samples. Along with the three mouse cohorts, wild-like wood mice (Wild-like:As; Purple), lab-like wood mice (Lab-like:As; Pink) and conventional lab mice (Lab: *Mus musculus;* Pink), two positive control samples were examined to ensure these clustered with samples as expected. Here the Golden Colony (Gray) sample consisted of DNA that had been extracted from faecal samples collected from 6 individual wood mice from the lab-reared colony and pooled together after extraction. Similarly, the Golden Wild (Black) sample consisted of DNA that had been extracted from faecal samples collected from 6 individual wild wood mice during fieldwork conducted at Callendar Wood, Scotland and the DNA pooled together after extraction. **(A)** Bray Curtis dissimilarity **(B)** Weighted Unifrac distance **C)** Unweighted Unifrac distance. Each coloured dot represents data from individual sample, ellipses represent 95% confidence intervals for each mouse cohort. Each axis has an eigenvalue in parenthesis indicating the percentage of variation within the dataset captured at that axis.



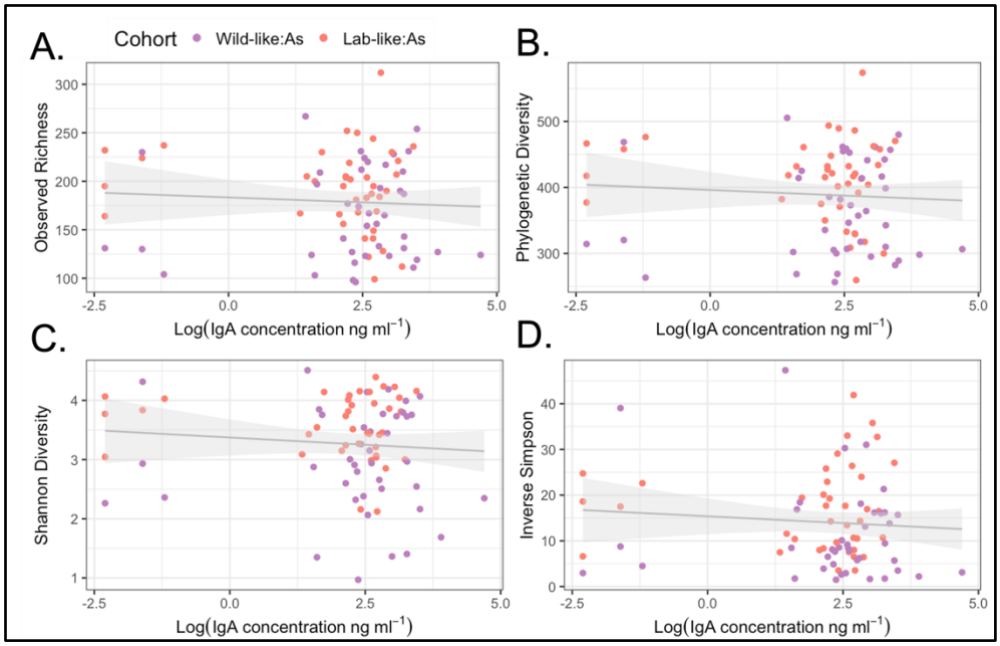
Supplementary Figure 2. Associations between gut pathology measures and Total *H.polygyrus* worm burdens, for Wild-like:As (purple) and Lab-like:As (pink) cohorts. (**A**) mean goblet cells per villi, (**B**) mean goblet cells per crypt, (**C**) mean villi length (µM) and (**D**) mean crypt length (µM). Line represents the mean with the shaded area around the line representing 95% confidence intervals. Points represent individual samples.



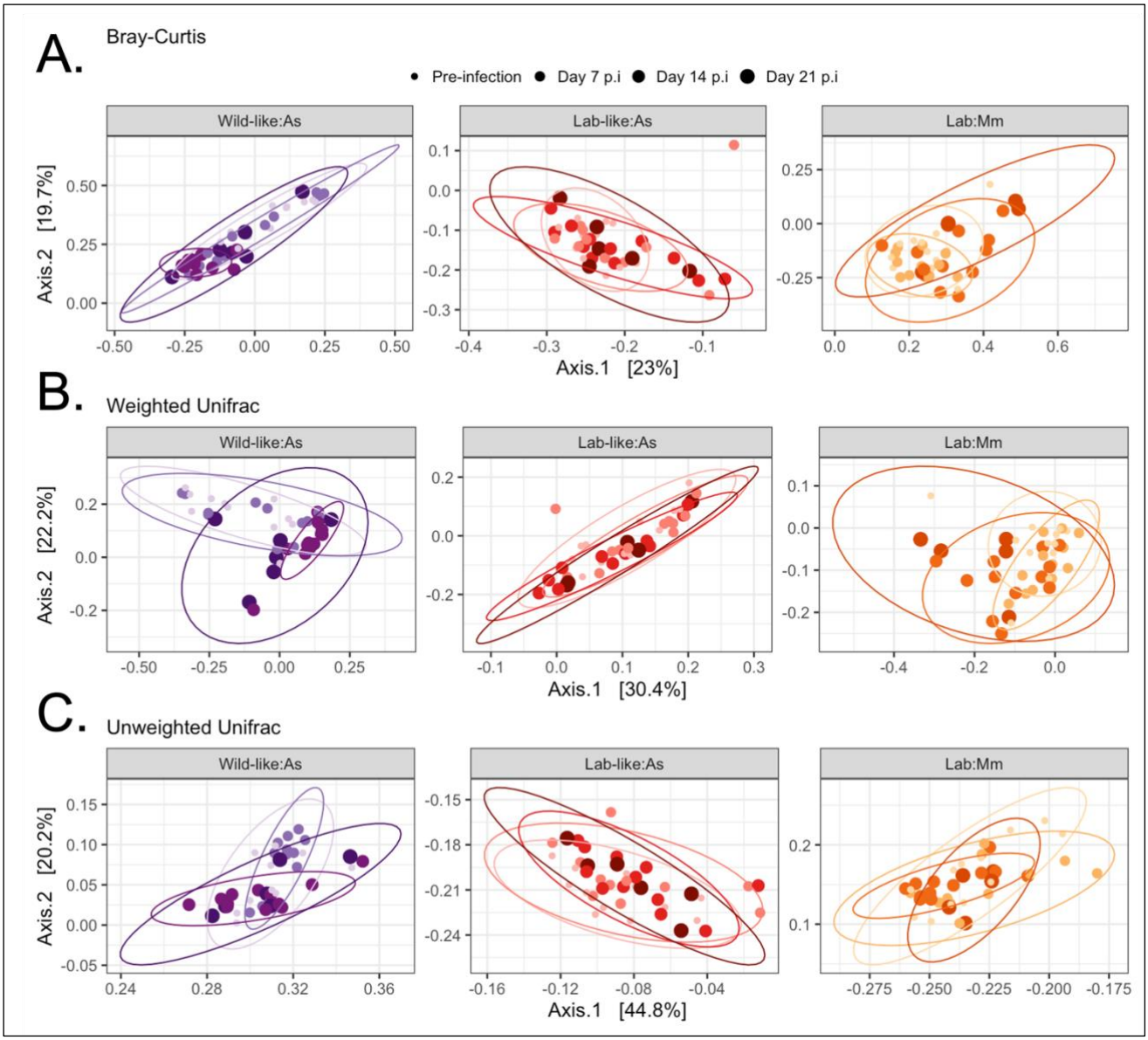
Supplementary Figure 3. Associations between Log transformed total faecal IgA concentrations (ng ml-1) and gut pathology metrics for Wild-like:As (purple) and Lab-like:As (pink) cohorts. (**A**) mean goblet cells per villi, (**B**) mean goblet cells per crypt, (**C**) mean villi length (μM) and (**D**) mean crypt length (μM). Line represents the mean with the shaded area around the line representing 95% confidence intervals. Points represent individual samples.



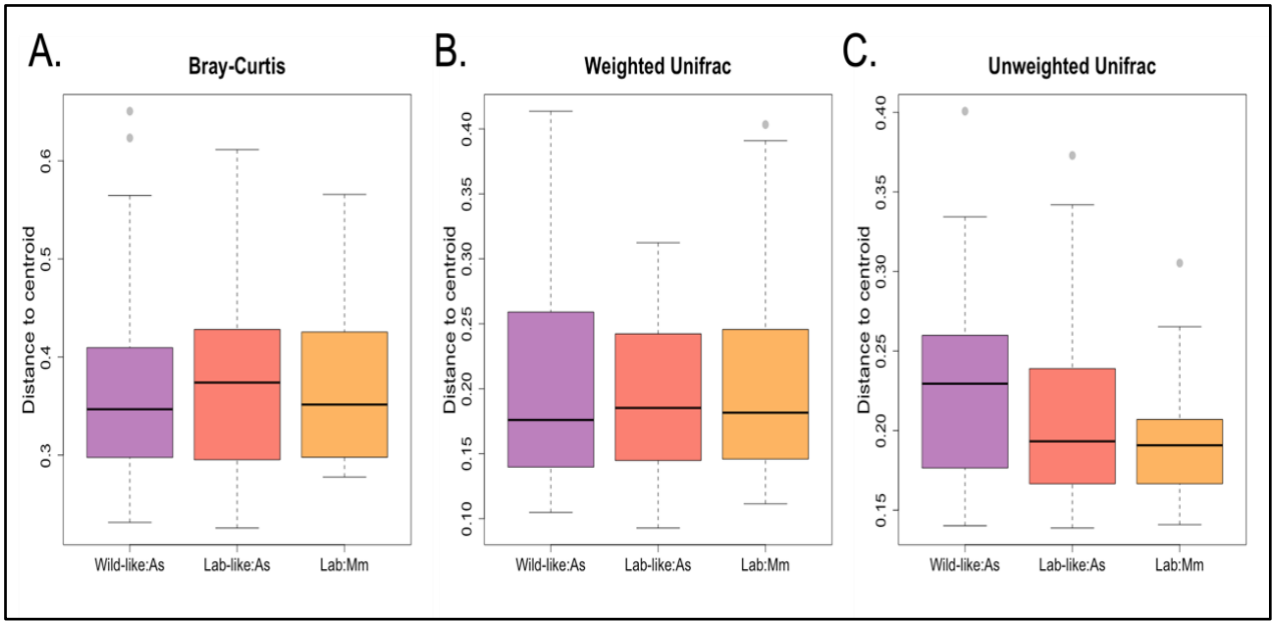
Supplementary Figure 4. Associations between alpha diversity metrics and Total *H.polygyrus* worm burdens for Wild-like:As (purple) and Lab-like:As (pink) cohorts. **A**) Observed species richness, (**B**) Faith's phylogenetic diversity, (**C**) Shannon diversity index, (**D**) inverse Simpson index. Line represents the mean with the shaded area around the line representing 95% confidence intervals. Points represent individual samples.



Supplementary Figure 5. Associations between alpha diversity metrics and Log transformed total faecal IgA concentrations (ng ml-1) for Wild-like:As (purple) and Lab-like:As (pink) cohorts. **A**) Observed species richness, (**B**) Faith's phylogenetic diversity, (**C**) Shannon diversity index, (**D**) inverse Simpson index. Line represents the mean with the shaded area around the line representing 95% confidence intervals. Points represent individual samples.



Supplementary Figure 6. Represents the same data shown in Figure 8 but faceted by mouse cohort to distinguish within cohort differences at each beta-diversity metric and mouse cohort. Principal coordinate analysis (PCoA) ordination of beta diversity dissimilarity matrices, for 16s rRNA sequenced faecal samples collected from three mouse cohorts; Wild-like:As (Purple), Lab-like:As (Pink) and Lab: Mm (Yellow). Each coloured dot represents data for an individual samples, whilst ellipses represent 95% confidence intervals for each mouse cohort at each timepoint. Size of points represents sampling point.



Supplementary Figure 7. Distance to centroids of mouse cohorts Wild-like:As, (Purple), Lab-like:As (Pink) and Lab:Mm (Yellow) for different beta-diversity dissimilarity matrices: (**A**) Bray Curtis dissimilarity, (**B**) Weighted Unifrac distance, (**C**) Unweighted Unifrac distance. Box indicates interquartile range (IQR), with vertical lines representing medians and horizontal lines indicating ± 1.5 IQR.

3.6.2. Supplementary Tables

Table S1. Model outputs for fixed effects on *H.polygyrus* infection and faecal IgA concentration (ng ml^{-1}) where Generalised Linear Mixed Models with a Poisson and Gaussian distribution were used respectively. The Lab:Mm cohort was excluded from this analysis due to absence of *H.polygyrus* in the small intestine. All p-values <0.05 and considered significant are highlighted in bold.

	***H.polygyrus* burden**			**Log faecal IgA concentration (ng ml^{-1})**		
Model covariates	Est	SE	p value	Est	SE	p value
Intercept	3.05	0.59	**<0.001**	2.37	0.20	**<0.001**
Cohort, Wild-like:As	-0.10	0.70	0.882	0.45	0.20	**0.022**
Timepoint, Day 21 p.i	-2.80	0.82	**<0.001**	-	-	-
Timepoint (continuous)	-	-	-	0.02	0.01	0.084
Sex, Male	0.35	0.54	0.516	-0.04	0.13	0.770
Experiment Block, Block 2	2.07	0.59	**<0.001**	0.02	0.12	0.883
Cohort, Wild-like:As: Timepoint, Day 21 p.i	-0.43	1.07	0.692	-	-	-
Cohort, Wild-like:As: Timepoint (continuous)	-	-	-	-0.03	0.02	0.065

Table S2. Model outputs for fixed effects on faecal IgA concentration (ng ml^{-1}) at experimental endpoints (Day 14 and Day 21 post infection). A Generalised Linear Mixed Model with a Gaussian distribution was used, and data were log transformed to correct for non-normal distribution. The Lab:Mm cohort was excluded from this analysis due to absence of *H.polygyrus* in the small intestine. All p-values <0.05 and considered significant are highlighted in bold.

	Log faecal IgA concentration (ng ml^{-1})		
Model covariates	Est	SE	p value
Intercept	3.72	0.48	**<0.001**
Total *H.polygyrus*	-0.01	0.00	**0.029**
Cohort: Wild-like:As	-0.58	0.26	**0.028**
Timepoint: Day 21 p.i	-0.03	0.18	0.085
Sex: Male	-0.28	0.11	**0.013**
Experiment Block: Block 2	0.31	0.13	**0.015**
Total *H.polygyrus*: Cohort: Wild-like:As	0.01	0.01	**0.018**

Table S3. Model outputs for fixed effects on gut pathology metrics of *H.polygyrus* infected mice, at experimental endpoints (Day 14 and Day 21 post infection). Generalised Linear Models with a Gaussian distribution were used, and data were log transformed to correct for non-normal distribution. The Lab:Mm cohort was excluded from this analysis due to absence of *H.polygyrus* in the small intestine. All p-values <0.05 and considered significant are highlighted in bold.

	Mean goblet cells per villi (Log)			**Mean goblet cells per crypt (Log)**			**Mean villi length, µM (Log)**			**Mean crypt length, µM (Log)**		
Model covariates	Est	SE	p value	Est	SE	p value	Est	SE	p value	Est	SE	p value
Intercept	2.30	0.30	**<0.001**	1.41	0.26	**<0.001**	5.47	0.14	**<0.001**	4.46	0.17	**<0.001**
Cohort, Wild-like:As	-0.57	0.23	**0.031**	-0.15	0.20	0.461	-0.00	0.11	0.997	-0.02	0.13	0.895
Timepoint, Day 21 p.i	0.14	0.25	0.590	0.07	0.21	0.755	0.14	0.12	0.266	0.01	0.14	0.965
Total *H.polygyrus*	0.00	0.00	0.812	0.00	0.00	0.404	0.00	0.00	0.428	-0.00	0.00	0.779
IgA concentration	0.02	0.01	0.265	0.02	0.01	0.204	-0.00	0.01	0.781	0.00	0.01	0.734
Sex, Male	0.12	0.17	0.531	0.24	0.14	0.118	0.01	0.08	0.911	0.02	0.09	0.812
Experiment Block, Block 2	0.01	0.19	0.973	-0.43	0.16	**0.019**	0.13	0.09	0.184	-0.03	0.11	0.764
Cohort, Wild-like:As: Timepoint, Day 21 p.i	0.56	0.33	0.113	0.56	0.28	0.071	0.08	0.16	0.619	0.11	0.19	0.538

Table S4. Model outputs for fixed effects are shown from Generalised Linear Mixed Models on observed richness, phylogenetic diversity, Shannon diversity and Inverse Simpson alpha diversity metrics in all cohorts. Data that were log transformed to correct for non-normal distribution are indicated in parentheses. All p-values <0.05 are in bold.

	Observed richness (Log)			**Phylogenetic diversity (Log)**			**Shannon Diversity**			**Inverse Simpson (Log)**		
Model covariates	Est	SE	p value	Est	SE	p value	Est	SE	p value	Est	SE	p value
Intercept	5.44	0.21	**<0.001**	6.17	0.14	**<0.001**	3.52	0.56	**<0.001**	2.49	0.62	**<0.001**
Cohort; Lab-like:As	-0.23	0.08	**0.005**	-0.18	0.05	**0.001**	-0.30	0.21	0.160	-0.27	0.23	0.238
Cohort, Wild-like:As	-0.57	0.08	**<0.001**	-0.41	0.05	**<0.001**	-1.37	0.21	**<0.001**	-1.31	0.23	**<0.001**
Timepoint (continuous)	0.00	0.00	0.847	0.00	0.00	0.819	0.01	0.01	0.350	0.01	0.01	0.427
Sex, Male	0.02	0.05	0.751	0.00	0.03	0.966	0.04	0.11	0.737	0.07	0.12	0.579
Experiment Block, Block 2	0.02	0.05	0.631	0.02	0.03	0.582	0.17	0.11	0.123	0.20	0.12	0.086
Sequence Run	-0.00	0.03	0.921	-0.00	0.02	0.959	0.02	0.09	0.820	0.03	0.10	0.797
Cohort, Lab-like:As: Timepoint, (continuous)	0.00	0.01	0.769	0.00	0.00	0.724	0.01	0.02	0.779	0.01	0.02	0.595
Cohort, Wild-like:As: Timepoint, (continuous)	0.02	0.01	**0.003**	0.01	0.00	**0.002**	0.05	0.02	**0.004**	0.05	0.02	**0.013**

Table S5. Model outputs are shown from a PERMANOVA on Bray-Curtis, Weighted Unifrac and Unweighted Unifrac dissimilarity values in the lab and wild environments (999 permutations). Multivariate homogeneity of group dispersions between mouse cohorts were carried out for each beta diversity metric (1000 permutations) and the PERMDISP results also shown. All p-values <0.05 are highlighted in bold.

	Bray-Curtis PERMANOVA				**Weighted Unifrac PERMANOVA**				**Unweighted Unifrac PERMANOVA**			
Model covariates	Sum sq	R2	F	P value	Sum sq	R2	F	P value	Sum sq	R2	F	P value
Cohort; Lab-like:As	5.02	0.17	36.65	**0.001**	1.01	0.13	24.07	**0.001**	3.24	0.21	72.62	**0.001**
Cohort, Wild-like:As	6.18	0.21	45.09	**0.001**	1.25	0.16	29.65	**0.001**	6.07	0.40	135.95	**0.001**
Timepoint (continuous)	0.56	0.02	4.06	**0.001**	0.21	0.03	4.89	**0.001**	0.17	0.01	3.87	**0.001**
Sex: Male	0.18	0.01	1.33	**0.001**	0.06	0.01	1.38	**0.001**	0.07	0.01	1.59	**0.001**
Sequence Run	0.18	0.01	1.34	0.190	0.04	0.00	0.92	0.414	0.05	0.00	1.03	0.617
Experiment Block, Block 2	0.34	0.01	2.50	**0.001**	0.08	0.01	1.99	**0.001**	0.16	0.01	3.62	**0.001**
Cohort, Lab-like:As: Timepoint, (continuous)	0.23	0.01	1.68	**0.020**	0.07	0.01	1.77	**0.036**	0.10	0.01	2.18	**0.001**
Cohort, Wild-like:As: Timepoint, (continuous)	0.94	0.03	6.90	**0.001**	0.31	0.04	7.34	**0.001**	0.10	0.01	2.35	**0.002**
Residual	15.89	0.54	-	-	4.88	0.62	-	-	5.18	0.34	-	-
	Bray-Curtis PERMDISP				Weighted Unifrac PERMDISP				Unweighted Unifrac PERMDISP			
Groups	0.00	-	0.08	0.922	0.01	-	0.47	0.628	0.02	-	4.09	**0.024**

Table S6. Model outputs are shown from a PERMANOVA on Bray-Curtis, Weighted Unifrac and Unweighted Unifrac dissimilarity values (999 permutations), whereby only *H.polygyrus* infected mice from the Wild-like:As and Lab-like:As cohorts were included in the analyses.

	Bray-Curtis PERMANOVA				**Weighted Unifrac PERMANOVA**				**Unweighted Unifrac PERMANOVA**			
Model covariates	Sum sq	R2	F	P value	Sum sq	R2	F	P value	Sum sq	R2	F	P value
Total *H.polygyrus*	0.23	0.05	1.70	0.099	0.06	0.06	1.49	0.167	0.03	0.01	0.60	0.649
Cohort: Wild-like:As	1.37	0.31	10.05	**0.001**	0.24	0.22	5.92	**0.001**	1.26	0.55	26.64	**0.001**
Timepoint: Day 21 p.i	0.10	0.02	0.77	0.609	0.03	0.03	0.68	0.703	0.06	0.03	1.27	0.235
Sex: Male	0.08	0.02	0.62	0.796	0.02	0.02	0.57	0.834	0.04	0.02	0.74	0.502
Sequence Run	0.11	0.03	0.55	0.550	0.02	0.02	0.54	0.858	0.04	0.02	0.86	0.412
Experiment Block, Block 2	0.16	0.04	1.18	0.268	0.03	0.03	0.81	0.557	0.09	0.04	1.82	0.122
Total *H.polygyrus*: Cohort: Wild-like:As:	0.15	0.03	1.09	0.335	0.04	0.03	0.88	0.507	0.03	0.01	0.57	0.661
Residual	2.18	0.50	-	-	0.64	0.60	-	-	0.76	0.33	-	-

Table S7. Model outputs are shown from a PERMANOVA on Bray-Curtis, Weighted Unifrac and Unweighted Unifrac dissimilarity values (999 permutations), whereby total faecal IgA concentrations in *H.polygyrus* infected mice in with two wood mouse cohorts over the course of infection were assessed. All p-values <0.05 and considered significant are highlighted in bold.

	Bray-Curtis PERMANOVA				**Weighted Unifrac PERMANOVA**				**Unweighted Unifrac PERMANOVA**			
Model covariates	Sum sq	R2	F	P value	Sum sq	R2	F	P value	Sum sq	R2	F	P value
IgA concentration	0.45	0.03	3.34	**0.001**	0.13	0.03	2.98	**0.011**	0.25	0.03	5.51	**0.001**
Cohort: Wild-like:As	4.46	0.27	33.00	**0.001**	0.95	0.20	22.07	**0.001**	4.15	0.50	89.93	**0.001**
Timepoint (continuous)	0.76	0.05	5.63	**0.001**	0.27	0.06	6.18	**0.001**	0.20	0.02	4.34	**0.003**
Sex: Male	0.18	0.01	1.30	0.233	0.06	0.01	1.40	0.210	0.08	0.01	1.81	0.106
Sequence Run	0.14	0.01	1.07	0.357	0.03	0.01	0.71	0.630	0.06	0.01	1.21	0.266
Experiment Block, Block 2	0.47	0.03	3.48	**0.003**	0.12	0.02	2.84	**0.015**	0.18	0.02	3.94	**0.007**
IgA concentration Cohort: Wild-like:As:	0.21	0.01	1.54	0.141	0.05	0.01	1.24	0.258	0.06	0.01	1.31	0.211
Residual	9.86	0.60	-	-	3.15	0.66	-	-	3.37	0.40	-	-

Chapter 4

4. Diet and helminth infection shape the gut microbiota differently in wild and laboratory populations of wood mice.

4.1. Abstract

The mammalian gastrointestinal tract is a rich ecosystem and focal-point for a wealth of interactions; it is the largest site of the host immune system and diet-derived nutrient absorption, whilst also a preferential niche for helminth parasites and host to the community of microorganisms known as the gut microbiota. Whilst host-nutrition is a key source of intestinal microbiota it also plays a fundamental role in the development of an effective immune response, highlighted by immunodeficiency and increased susceptibility to infection in the malnourished. Moreover, intestinal helminth infections can impair nutrient absorption and compromised immunity can lead to reduced anthelmintic efficacy. To date, the relationship between nutrition, helminth immunity and the gut microbiota has been studied in controlled laboratory models focusing on alterations of specific macro- or micro-nutrients. However, these are unlikely to truly represent findings in natural populations where there is more genetic, ecological, and behavioural variation that can determine exposure and susceptibility to infection, as well as the occurrence of co-infections alongside fluctuations in resource availability due to seasonal shifts which can impact the gut microbiota composition and diversity. We used a high-quality, nutrient-rich diet to experimentally supplement both wild and wild-derived, lab-reared wood mice (*Apodemus sylvaticus)* and measured anthelmintic treatment efficacy and resistance to infection with the gastrointestinal nematode *Heligmosomoides polygyrus.* Previously, we have shown that in both settings, wood mice given this supplemented diet, were more resistant to *H. polygyrus* infection, cleared adult worms more efficiently after treatment and had higher general and parasite-specific immune responses. Here, we expand upon these findings with gut microbiota data from the same study, where we highlight key differences in the diversity and composition of the microbiota between the lab and wild, during infection

and determine how supplemented nutrition impacts this – beginning to unravel the mechanisms driving nutrition-induced *H. polygyrus* resistance.

4.2. Introduction

The mammalian gastrointestinal tract is a complex and diverse ecosystem home to trillions of bacterial microorganisms known as the gut microbiota (Lozupone et al. 2012). These diverse microbial communities are responsible for a wide range of biological processes which are vital for host health, including, but not limited to, aiding digestion to facilitate nutrient, energy, and drug metabolism (Nicholson et al. 2012; Turnbaugh et al. 2006; Wilson & Nicholson 2009), development and maturation of the host immune system (Fulde & Hornef 2014; Mazmanian et al. 2005a; Olszak et al. 2012), preventing colonisation by pathogenic bacteria (Kamada et al. 2013) and overall intestinal barrier integrity and homeostasis (Kinross et al. 2011; Lynch & Pedersen 2016; Rakoff-Nahoum et al. 2004; Sommer & Bäckhed 2013). To date, much of our understanding of the relationships between the microbiota composition and its impacts on host health has derived from laboratory based, animal models, in controlled environments (Johansson & Sarles 1949; Nguyen et al. 2015). However, host-associated microbiota are highly sensitive to the environments of their host, (Lozupone et al. 2012; Sommer & Bäckhed 2013), for instance, the gut microbiota of identical twins have been shown to differ substantially (Turnbaugh et al. 2010; Vilchez-Vargas et al. 2022). As such it is increasingly important to understand how the gut microbiota may be shaped by complex variable conditions experienced in real-world settings, such as in wild populations.

The diet an individual consumes plays a fundamental role in shaping the gut microbiota, as both a major source of microbes and concurrently a fuel source for these microbes, which metabolise host micronutrients and aid digestion (David et al. 2014; Muegge et al. 2011). For instance, in humans, long-term diet patterns have been associated with distinct gut microbiota signatures, whereby diets high in protein and animal fat are associated with high levels of bacteria from the *Bacteroides* phylum, whilst high levels of carbohydrates are associated with elevated levels of *Prevotella* phylum (Wu et al. 2011). Moreover, a shift in consumption to either a solely animal-based or plant-based diet significantly alters the gut microbial community within 24 hours (David et al. 2014). The profound effect of diet on the gut microbiota may, in

turn, affect host health in wild animal populations, where diet composition is often variable due to seasonal fluctuations in resource availability. Evidence from a range of wild animal populations have demonstrated that the gut microbiota can actively respond to these seasonal shifts in diet, as observed in gibbons (Li et al. 2023), howler monkeys (Amato et al. 2015), giant pandas (Xue et al. 2015), ground and red squirrels (Carey et al. 2013; Ren et al. 2017) and wood mice (Maurice et al. 2015b). Moreover, studies examining the gut microbiota of captive versus wild animals, have also shown that animals in the wild tend to have a more diverse gut microbiota which may be a consequence of, or allow for greater adaptability to, a changing external environment and nutrient intake (Clayton et al. 2016a; Kohl & Dearing 2014; Kohl et al. 2014; Martínez-Mota et al. 2020; McKenzie et al. 2017b; Wang et al. 2023).

In addition to a diverse range of commensals, wild animals are also continually exposed to a number of pathogens including, for example, protozoan or helminth parasites which commonly parasitize the gut (Jackson et al. 2009; Leung et al. 2018b). This provides the opportunity for direct parasite-microbiota interactions within the gut, which have the potential to affect both host health and infection outcomes. Although the effects of such relationships on their hosts are difficult to measure in the wild, a number of studies using laboratory mouse models have provided insight into helminth-gut microbiota interactions. For example, gut microbiota analysis of mice infected with the intestinal nematode *Trichuris muris,* revealed that between day 14 and 28 post-infection there was a reduction in the microbiota diversity, driven by a reduction in Bacteroidetes phylum. Interestingly, this effect was reversed when the parasite was removed via anthelmintic treatment (Holm et al. 2015; Houlden et al. 2015; White et al. 2018). It has also been shown that *T. muris* is reliant upon specific host gut microbiota to successfully establish infection, for instance without *Escherichia sp.* and *Staphylococcus sp.* the eggs fail to hatch and infection is not possible (Hayes et al. 2017). Further, expansion *Lactobacillus* bacteria is consistently found when mice are infected within either *T. muris* or another common gastrointestinal helminth*, Heligmosomoides polygyrus*, and correlates with susceptibility to infection (Holm et al. 2015; Rausch et al. 2013; Reynolds et al. 2015; Walk et al. 2010b).

The relationship between nutrition and helminth infection is also clearly important. Gastrointestinal nematodes, for example, can cause malnutrition through their

pathophysiology, impairing nutrient absorption and malnutrition, which can lead to increased vulnerability to further infection (Koski & Scott 2001; Scrimshaw & SanGiovanni 1997; Shea-Donohue et al. 2017a). Mouse models have also shown that low-protein diets can increase adult worm (*H.polygyrus* and *T.muris* helminths) survival and delay expulsion (Boulay et al. 1998a; Brailsford & Mapes 1987; MICHAEL & BUNDY 1992; SLATER & KEYMER 1988; Slater et al. 1986), while zinc, vitamin A and iron micronutrient deficiencies have been found to prolong infection, increased worm burdens, and egg shedding (Boulay et al. 1998a; Gagnon CMA et al. 1996; Shi et al. 1997; Shi et al. 1995). However, inferring causation these diet-infection-microbiota relationships within a wild setting is difficult and requires the consideration of several host variables including demography, behaviour, and immunity, plus, environmental factors including parasite prevalence, coinfection, seasonality, and resource availability - that all can impact this relationship.

Traditional laboratory-based studies have attempted to establish cause and effect of these complex interactions, but often consider variables independently of one another, which can oversimplify the real-world context of natural populations, where individuals compete for resources such as breeding partners and nesting sites. A recent pioneering study attempted to bridge the gap between the lab and the wild, by housing laboratory mice in controlled outdoor enclosures (a process known as “rewilding”). These re-wilded mice showed a marked increase in gut microbiota diversity and an increased susceptibility to *T. muris* infection compared to laboratory reared mice (Graham 2021; Leung et al. 2018a). Another recent study explicitly considered the effect of diet in wild and lab mice, by transferring C57BL/6 mouse (a traditional lab mouse strain) embryos into wild-caught house mice (also *Mus musculus domesticus*) and creating a colony of mice termed “wildlings” (genetic inbred mice with a wild-like gut microbiota). The authors demonstrated that the wildling bacterial microbiota resembled that of wild mice and significantly differed from conventional laboratory mice. Interestingly, through experimental perturbation of the gut microbiota with a 10-week high-fat-choline deficient diet regime, wildlings also displayed a more stable and resilient microbiota phenotype compared to their conventional laboratory counterparts which showed marked shifts in the composition of the gut microbiota (Rosshart et al. 2019).

We maintain a unique system of paired wild and lab-reared wood mouse (*Apodemus sylvaticus*) populations housed at the University of Edinburgh. Previous work in this system experimentally manipulated both nutrition (with a high-quality whole-diet supplementation) and infection in either wild or a controlled laboratory experiment with the gastrointestinal nematode, H.*polygyrus,* which naturally infects wild wood mice populations. In both lab and wild environments, they found that mice given access to the supplemented high-quality diet were more resistant to *H. polygyrus* infection, shed fewer eggs, had higher parasite specific and nonspecific immunological responses and had higher efficacy when given anthelmintic drug treatment (Sweeny et al. 2021).

Here, I investigated the interaction between diet-helminths-microbiota in wood mice in this paired lab-to-wild system, specifically testing if diet supplementation impacts the diversity and composition of the gut microbiota and whether it impacts the response of the gut microbiota to helminth infection in both wild and laboratory wood mice (Sweeny et al. 2021). To this end, we quantified the relative abundance, alpha and beta diversity, and differential abundance of the gut microbiota of wood mice from both a wild population in a UK woodland and our wild-derived colony following experimental manipulation of diet and *H. polygyrus* infection for Sweeny *et al.* 2021 (Sweeny et al. 2021). Specifically, I aimed to determine how diet and helminth infection shape the diversity and composition of the gut microbiota within a natural host-parasite system in controlled and natural environments.

4.3. Materials & Methods

4.3.1. Experimental design

Field experiment

The experimental design of both the field and laboratory experiment has been described in detail previously (Sweeny et al. 2021). Briefly, from June to August 2016, a nutritional perturbation experiment in a wood mouse population in Callendar Wood, Scotland, was conducted. Using mark and recapture methods, 180 sterilised-live traps (H.B. Sherman, Florida, USA), containing cotton bedding, seeds, mealworms, and fresh carrot were distributed evenly across 4 trapping grids (60x50m each) spaced

≥50m apart, to prevent mouse movement between grids. Trapping grids were equally and randomly assigned to one of two nutrition regimes: supplemented or control nutrition (no nutritional manipulation; mice only had access to their normal food sources). Two weeks before and throughout the duration of the experiment, twice per-week, 2kg/1000m^2 of sterilised, TransBreed^{TM} mouse chow pellets were evenly scattered across and added to traps on the supplemented nutrition grids. TransBreed^{TM} mouse chow pellets provide high-quality, whole-diet supplementation, they contain increased levels of key macro- and micro-nutrients compared to other standard rodent feed, such as 20% protein and 10% fat, alongside additional zinc, iron, selenium, vitamin A and vitamin E (SDS, UK).

At first capture, all wood mice weighing >10g received a subcutaneous microchip for individual identification (Friend Chip, AVID2028, California, USA) and were randomly assigned within sex to a control or treatment group (Fig. 1). Treated mice received a combinational anthelmintic previously shown to significantly reduce *H. polygyrus* burdens for 12-16 days (cite Clerc et al. 2017), which included 2ml/g Pyrantel pamoate and Ivermectin (Strongid-P, 100mg/kg and Eqvalan, 9.4mg/kg) via oral gavage, whilst the control group received an equivalent dose of dH_2O. At each capture, demographic details including sex, age, host condition (body mass, length, and fat scores) and reproductive status were recorded, and faecal samples collected from traps. A subset of the faecal pellets were later used to estimate *H. polygyrus* infection levels via faecal egg counts as previously described (Knowles et al. 2013) and for downstream microbiota analysis (stored at -80^{o}C). Mice caught between 12-16 days after first capture, when the anthelmintic treatment begins to lose efficacy (Clerc et al. 2019a; Knowles et al. 2013), were euthanised. The small intestine was removed from each individual and stored in 1xPBS until examination the same day for adult *H. polygyrus* presence.

In total, faecal samples and data collected from a subset of 29 individual wild wood mice was included in the analysis outlined here, with an average of 2.55 samples per individual (range 1-7). The average number of captures per individual was 5.08 (range 1-11) and there were 5 individuals were only captured once. Of our 29 study wood mice, a total of 11 were trapped on the control grids, with no dietary supplementation, within these 8 were treated with anthelmintic and a further 3 received the equivalent

dose of dH_2O as a control. The remaining 18 mice were captured on the supplemented nutrition grids, whereby 8 of these mice received anthelmintic treatment and 10 received an equivalent dose of dH_2O as a control. Of these 29 study mice, we were able to obtain an indication of *H.polygyrus* infection status at first capture, via faecal egg counts, for a total of 28 individuals. Here, we were able to detect the presence of *H.polygyrus* in 16 samples, therefore an overall prevalence of 69.6%. However, *H.polygyrus* prevalence differed between treatment groups, whereby at first capture (at the point of treatment) 7 wood receiving anthelmintic treatment had detectable *H.polygyrus* (prevalence = 63.6%) whereas of 12 mice that received a control dose of dH_2O 9 had *H.polygyrus* eggs detected in faeces (prevalence = 75%).

Laboratory experiment

A parallel nutrition and infection laboratory experiment was conducted using 24 wood mice from our lab-reared colony, aged 15-21 weeks and with an equal number of males and females within each group. Supplemented nutrition consisted of TransBreed™ chow pellets; a high-quality, nutrient-dense diet used to support breeding rodents that was the same diet supplemented to the wild wood mice. The control nutrition treatment consisted of adlib access to Rat Mouse 1 (RM1™) chow pellets, a standard maintenance diet (SDS, UK) where wood mice maintain weight and condition. Food and water were provided *ad libitum* and mice were given a significant diet acclimatisation period (Days -32 to 0).

A primary challenge of 200 wild-derived *H. polygyrus* larvae was administered by oral gavage to 16 mice (Day 0; Fig.1). At peak infection (Day 14), half of these mice were randomly assigned to treatment groups and administered either anthelmintics (same drugs and dose as in field) or a control of dH_2O via oral gavage . A secondary challenge of 200 *H. polygyrus* larvae was administered by oral gavage one-week post-treatment (Day 21) and mice were culled two-weeks after the secondary challenge (Day 35; but 14 days after secondary challenge infection). The remaining 8 mice were infection controls, as such were given a control of dH_2O via oral gavage on Day 0 and Day 14 and a primary challenge with 200 *H. polygyrus* on Day 21 and culled on Day 35. At cull, the small intestine of each animal was removed and examined for the presence

of adult *H. polygyrus.* Throughout the experiment, faecal samples were collected from each animal three times per week and used to estimate helminth infection levels via faecal egg counts and microbiota analysis. Unrelated to nutrition or infection status, 5 wood mice exhibited weight loss over the threshold of our experimental protocol, due to issues not associated with our experiment, and were subsequently culled and excluded from analysis.

Faecal samples collected from 19 individuyal lab-reared wood mice were included for the analysis outlined here, with 11 faecal samples collected for each individual throughout the duration of the study. In total, 9 mice were on a control diet of RM1™, of these 3 received anthelmintics at day 14 post-infection, whilst the remaining 6 mice recived a control dose of dH_2O. Further, 10 mice received the supplemented diet of TransBreed™ and among these, 4 individuals received anthelmintic treatment at day 14 post-infection, which the remaining 6 received a control dose of dH_2O.

Ethics statement

All animal work was conducted under the UK Home Office Project Licence 70/8543 and health monitoring and handling conducted following the guidelines of the Scot PIL and the Home Office Scientific Procedures Act (1986).

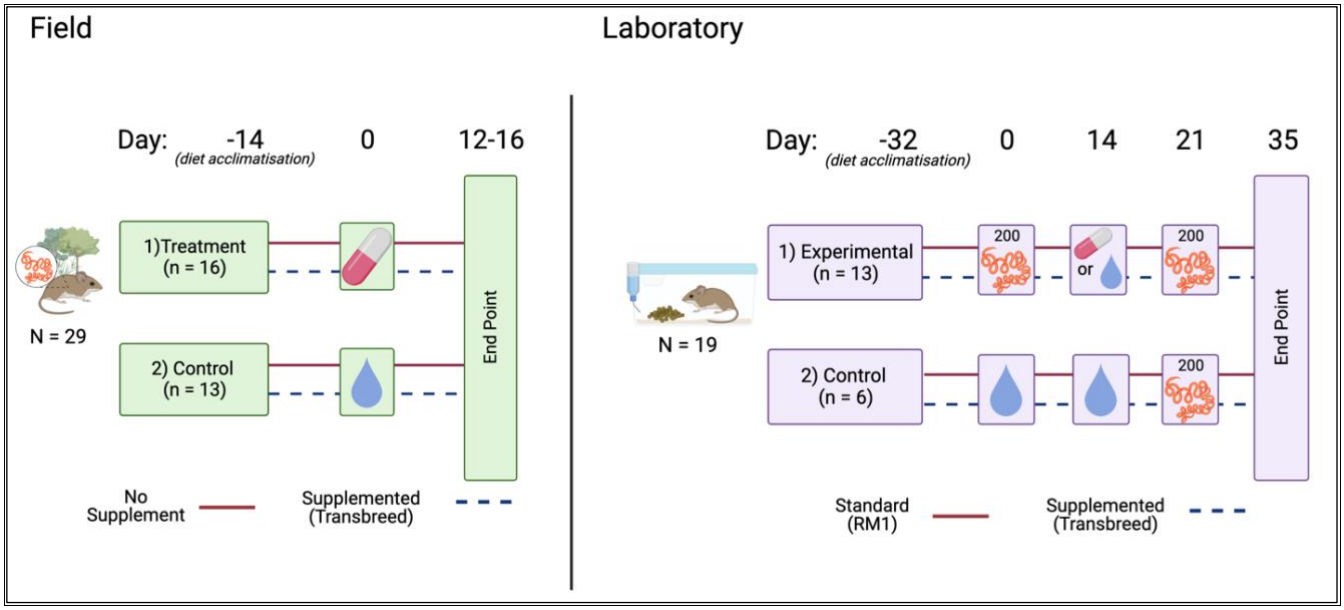


Figure 1. Experimental design of field and laboratory experiments of infection with *H.polygyrus* and anthelmintic treatment. In the field experiment (green), a total of 29 individual wood mice were split into Treatment or Control groups. Each of the Treatment and Control group mice were further split into 2 groups, which were either given No Supplement or were Supplemented (Transbreed), applied at the grid level. All mice were allowed 14 days of diet acclimatisation prior to any treatment. At day 0, Treatment group received an anthelmintic whilst the Control group received an equivalent dose of dH_2O. Mice caught between 12-16 days after first capture were euthanised and worm burdens of *H.polygyrus* assessed. In the Laboratory experiment (purple), a total of 19 Lab-reared wood mice were split into Experimental or Control groups, and each of these groups was further split into 2 diet groups - Supplemented (Transbreed), or Standard (RM1) to act as the control diet. All mice were allowed 32 days to acclimatise to the diet. At day 0, Experimental mice were given a dose of 200 *H.polygyrus* larvae, whilst control mice were given a dose of dH_2O. At day 14 (peak infection), experimental mice were either given a dose of anthelmintic or a control of dH_2O, whilst control mice were given a dose of dH_2O. At day At day 21, both Experimental and Control groups were given a dose of 200 *H.polygyrus* larvae, before being euthanised on Day 35 when worm burdens of *H.polygyrus* were assessed.

4.3.2. *H. polygyrus* parasites

Infective stage *H. polygyrus* L3 larvae were isolated from the Callendar Wood wild wood mouse population and were screened using PCR diagnostics to ensure the isolate was not contaminated with any other known mouse parasites or pathogens (IDEXX Bioresearch, Germany), and then passaged several times through the lab-reared wood mouse population at University of Edinburgh (Clerc et al. 2019c; Sweeny

et al. 2021). We measured *H. polygyrus* shedding as eggs per gram of faeces (EPG) using a salt flotation method as previously described (Knowles et al. 2013; Sweeny et al. 2021). In summary, *H. polygyrus* eggs were counted and standardized by the weight of the sample to estimate EPG, these values were then rounded to the nearest whole number (integer) for downstream analysis.

4.3.3. DNA extraction

DNA was extracted from faecal samples in randomised batches of between 12-24 samples using QIAamp PowerFecal DNA Kits (Qiagen Ltd, UK) and a QIAcube Connect instrument (Qiagen Ltd, UK). Each batch of samples contained at least one dH_2O-only sample to act as a negative control. Samples were homogenised at a 1:2 ratio with dH_2O, then 0.03g homogenate was added to a PowerBead Tube with 750µl PowerBead solution and 60µl cell lysis buffer. Samples were vortexed using a Vortex Genie 2 at maximum speed for 10 minutes and centrifuged at 16,100 RCF for 1 min 30 seconds. Next, 450µl supernatant for each sample was transferred to the QIAcube Connect whereby samples underwent further cell lysis, inhibitor removal, DNA binding, washing and finally 50 µl DNA was eluted into elution buffer. An estimate of DNA quantity of each sample and confirmation that DNA was not detected in dH_2O-only (negative control) samples was obtained through NanoDrop™ spectrophotometry and extracts were stored at -20°C until required.

4.3.4. Amplification of 16S rRNA & sequencing

The V4 region of the bacterial 16S rRNA gene was amplified using a barcoded adaptor-based polymerase chain reaction (PCR) approach, with the 515F forward primer and 806R reverse primer series (Caporaso et al. 2012). Each PCR reaction was set up under contaminant-free conditions using an ultra-violet (UV) sterilisation cabinet (SCIE-PLAS Ltd, Cambridge, UK) whereby all plasticware and reagents (excluding those containing nucleotides) were UV treated for 20 minutes. Individual PCR reactions were then set up at a final volume of 50µl using Roche reagents, as follows: 37µl Nuclease-free PCR-grade H_2O, 5µl 10x PCR Buffer, 2µl $MgCl_2$ (25 mM), 1µl dNTP mix (10 mM), 0.5µl Taq DNA polymerase (5 U/µl), 1.25µl Forward barcode primer (10µM), 1.25 µl Reverse barcode primer (10 µM) and 1 µl DNA sample. For each DNA sample, a unique combination of 515F and 806R primers were added to

the amplification reaction, allowing multiple samples to be pooled together for sequencing. PCR reactions were performed using 96-well plates, whereby 91 DNA samples were randomly assigned to each plate, as well as 5 control samples including; 1x 2µl nuclease-free PCR grade water-only sample (negative control), 1x 2µl nuclease-free PCR grade water plus Forward and Reverse barcode primers (negative control), 1 x 2µl Mock Community (20 strain staggered mix genomic material, ATCC MSA-1003; positive control), 1 x 2µl Golden Colony DNA sample (positive control) and 1 x 2µl Golden Wild DNA sample (positive control). The Golden Colony sample consisted of DNA that had been extracted from faecal samples collected from 6 individual wood mice from the lab-reared colony and pooled together after extraction. Similarly, the Golden Wild sample consisted of DNA that had been extracted from faecal samples collected from 6 individual wild wood mice during fieldwork conducted at Callendar Wood, Scotland and the DNA pooled together after extraction.

The PCR cycling protocol was as follows: initial denaturation at 94^{o}C for 3 minutes, followed by 25 cycles of, 94^{o}C for 45 sec (denaturation), 50^{o}C for 1 min (annealing) and 72^{o}C for 1.5 min (extension) and a final extension step at 72^{o}C for 10 mins.
PCR amplicon size was verified using gel electrophoresis and to confirm that there was no visible DNA amplification of both negative control samples and that there was visible DNA amplification of the three positive controls used. DNA of all samples and controls was then purified using AMPure XP Beads (Beckman Coulter, UK), and quantified with a Quant-iT PicoGreen dsDNA Assay Kit (Thermo-Fisher, UK) and pooled at equal final concentrations. For all negative control samples DNA levels were undetectable using the PicoGreen assay, thus, a volume of 2µl of each was added to the DNA pool for downstream sequencing. Next-generation DNA sequencing was conducted by Edinburgh Genomics, with the addition of custom primers (Caporaso et al. 2012), using an Illumina MiSeq v2 platform to generate 250 base pair (bp) paired-end reads and ~11 million raw reads. A total of 283 faecal samples were sequenced across 5 sequencing runs, with samples randomly assigned to each run to limit bias.

Processing of sequence data

Raw Illumina sequences were processed following the DADA2 Pipeline Tutorial (version 1.16; available at https://benjjneb.github.io/dada2/tutorial.html: (Callahan et

al. 2016a)) using RStudio (2023.03.1+446) with identical parameters used for each of the 5 sequencing runs. Sequences were examined for quality to determine appropriate trimming parameters, forward sequences were trimmed at 240 bp and reverse sequences trimmed at 160 bp. Default filtering parameters were used; ambiguous nucleotides removed, the maximum number of “expected errors” was set to 2 for forward reads and 5 for reverse reads and reads of a quality score $\leq$2 were truncated. During denoising, amplicon sequence variants (ASVs) were inferred for each sample, paired reads were merged, and putative chimeras removed. Taxonomy was assigned to each ASV using the naive Bayesian classifier of DADA2 and the Silva Project v132 database ((Yilmaz et al. 2013); version 138.1) and tabulated.

Non-bacterial taxa including; 3 ASV’s with no taxonomic assignment, 3 Eukaryota, 213 Mitochondria and 30 Chloroplast ASV’s were removed from analysis resulting in a total of 2,711 unique ASV’s. Samples with <10,000 reads were excluded, including 7 lab-reared colony samples (read range 37-3040) and all negative control samples including water-only (mean reads = 63.8, range 14-210) and water plus primers (mean reads = 62.7, range 3-202) were removed from downstream analysis. Details of the bacteria identified within the negative control samples at the Genus taxonomic level can be observed in Table S1. Further, the Mock Community (mean reads = 68,544.6, range 30,824-139,281) for each sequencing run was explored at the Genus taxonomic level to ensure that the 20 bacterial strains present were detected (Supp Fig. 1) and then excluded from further analyses. The microbial profile of the Golden colony and Golden wild samples was investigated through principal coordinate analyses (PCoA) with Bray-Curtis, Weighted and unweighted Unifrac distances to ensure these clustered with our lab-reared colony and wild wood mouse samples as expected (Supp Fig. 2) and then dropped from further analyses. Therefore, in total, 18,870,129 reads (mean per sample = 68,406.3, range 10,000-966,732) analysed from 275 faecal samples from a total of 48 individual wood mice, 19 lab-reared colony (N=209; 11 samples per individual) and 29 wild wood mice (N=74, mean 2.55, range 1-7 per individual) were analysed.

4.3.5. Statistical analysis

All statistical analyses were conducted in R version 4.2.1 (R Core Team 2022). The Phyloseq package (version 1.40.0) was used to integrate taxonomy tables, ASV abundance tables and sample metadata into a phyloseq object for downstream analysis (McMurdie & Holmes 2013).

Alpha diversity

To evaluate the diversity of the gut microbiota of wood mice in the lab and wild, we assessed four common alpha diversity metrics; observed richness, Faith's phylogenetic diversity, the Shannon diversity index, and the inverse Simpson index, using the estimate_richness function in the phyloseq package ((McMurdie & Holmes 2013); version 1.40.0).

Alpha diversity metrics provide a summary of the within-sample microbiota community structure, by measuring the number of taxonomic groups present (richness) and/or the distribution of the abundances of the taxonomic groups present (evenness) (Whittaker 1960; Willis 2019). Observed richness provides a count of the number of individual species detected within a sample (Fisher et al. 1943). Faith's phylogenetic diversity is the sum of the branch lengths of a phylogenetic tree connecting all species within a sample, thus, the higher the value, the more taxa present that are distantly related to each other (Faith 1992). The Shannon index considers both species richness and evenness within a sample, it is a calculation that represents the uncertainty in predicting a single species identity when taken at random within a community, so the higher the value, the higher the diversity (Shannon 1948). The Simpson index also considers the species richness and evenness, by measuring the probability that two taxa randomly selected from a sample will belong to the same taxa. A higher value for the Simpson index equates to a lower diversity, as such, thus, we report the inverse Simpson index, so that for all four metrics reported a higher value indicates higher diversity (Simpson 1949).

Moreover, rarefaction is a process that adjusts for differences in sequencing depth between samples, whereby the number of reads for the sample with the lowest

sequencing depth is selected and all subsequent samples are then subsampled at this depth (Hughes & Hellmann 2005; Willis 2019). However, this method has been shown to lead to loss of valid biological data, in particular that of rarer ASV's present within samples (McMurdie & Holmes 2014), which we expected to observe in our wild wood mouse faecal samples. As we randomised samples between the wild and lab environments in a balanced manner among sequence runs and found that the sequencing depth across sampling environments was also comparable (lab mean = 69,245 and wild mean = 67,514), we decided in this instance to perform analysis on raw, non-rarefied data.

To evaluate the impact of the environment on gut microbiota diversity, we compared the means of each alpha diversity metric between wild and lab mice, using a Wilcoxon Rank Sum Test. When examining the differences in microbiota diversity between diet groups we used Generalised Linear Mixed Models (GLMMs) with a Gaussian distribution for each environment. Where necessary, data were log-transformed to conform to a normal distribution. Each alpha metric was set as the response variable in a model and the fixed effects in all four alpha diversity models included; environment (factor: lab or wild), diet (factor: control or supplemented), sex (factor: male or female) and an environment and diet interaction. Individual mouse ID was included as a random effect for all models to account for multiple faecal samples per mouse.

To determine the impact of *H. polygyrus* infection in each diet regimen, we ran GLMMs for each environment with each of the four alpha metric as the response variable and the following fixed effects in all models; diet (factor: control or supplemented), infection status (factor: lab; pre-infection, primary infection, and secondary infection, wild; infected and uninfected), sex (factor: male or female) and a diet and infection status interaction. Individual mouse ID was included as a random effect for all models to account for multiple faecal samples per mouse.

Beta diversity

To determine how the gut microbiota composition differs between lab and wild wood mice, we estimated beta diversity using three common metrics. Beta diversity is a

measure of gut microbiota similarity or dissimilarity between groups. First, to ensure compositional differences were not driven by rare taxa, we filtered out rare taxa by removing ASV's with a count (abundance) of <5 and had <10% prevalence within the entire dataset. We then normalised read abundances to compositional proportion data and calculated pairwise dissimilarities among samples using; Bray-Curtis dissimilarity matrix, Unweighted UniFrac and Weighted UniFrac, in the Phyloseq package of R ((McMurdie & Holmes 2013); version 1.40.0). Bray-Curtis dissimilarity considers the abundance of taxa shared between two samples and the number of taxa detected in each sample (Bray & Curtis 1957). UniFrac distance metrics examine the phylogenetic distances between taxa across samples. The Weighted UniFrac is quantitative, accounting for the abundance of each taxa present, whilst the Unweighted UniFrac is qualitative and considers the difference in presence and absence of taxa between two samples (Lozupone et al. 2007).

To assess the extent to which the environment, diet, and infection status predicted the microbiota composition, these dissimilarities were used in principal coordinates analysis (PCoA) and permutational multivariate analysis of variance (PERMANOVA, (Anderson 2001)), using the Vegan package (version 2.6.4) with the *adonis2* function and 999 permutations (Oksanen 2012). To test for significant differences with the environment and diet, we used PERMANOVA analyses with the respective Beta diversity metric set as the response variable and the explanatory variables included environment, diet, and sex and an environment and diet interaction.

To evaluate the impact of *H. polygyrus* infection on the microbiota composition PERMANOVA's were run for each environment respectively, with the Beta diversity metric of interest set as the response variable and the explanatory variables included diet, infection status, and sex, and a diet and infection status interaction. As PERMANOVA's may be sensitive to data dispersion differences, we performed an analysis of multivariate homogeneity (PERMDISP) between environment, diet, and infection groups, with the *betadisper* function in the Vegan package ((Oksanen 2012);version 2.6.4) and 1000 permutations, with any significant results reported. Finally, we assessed the correlation between the PC1 axis of each beta diversity metric and number of *H. polygyrus* eggs per gram of faeces (EPG), log transformed, to determine if the variation in microbiota composition explained by worm burden

increased with the intensity of infection (number of adult worms). Here we ran GLMMs with a Gaussian distribution, at each environment respectively, with the respective Beta diversity PC1 axis set as the response variable and fixed effects in all models included; diet (factor: supplemented or control), infection status (factor: lab; pre-infection, primary infection, and secondary infection, wild; infected and uninfected) and sex (factor: male or female). Individual mouse ID was included as a random effect for all models to account for multiple faecal samples per mouse.

Differential abundance

To further understand the effects of environment, diet, and infection on the gut microbiota composition of wood mice, we performed differential abundance analysis at the taxonomic level of genus, using an ANOVA-like differential expression tool (ALDEx2; (Fernandes et al. 2014)) in the microbiomeMarker package ((Cao et al. 2022); version 1.2.2) on our feature tables. A 10% threshold for prevalence of the taxa was applied as recommended (Nearing et al. 2022). ALDEx2 estimates per taxon technical variation within each sample using Monte-Carlo instances drawn from the Dirichlet distribution and applying centred-log-ratio transformation to data to account for its compositional nature. Here, samples are compared among groups in a pairwise manner, and the difference in abundance of taxa is reported as an ALDEx2 effect size. This provides an estimate of the median standardised difference between two groups. Here, the size of the effect is proportional to the difference in abundance of taxa between samples, thus, the greater the effect size the greater the difference between two taxa (Fernandes et al. 2014; Gloor 2023). The direction of the effect (positive or negative) is dependent upon the order that two groups were compared, thus, a negative effect does not necessarily signify a significant depletion.

4.4. Results

4.4.1. Environment shapes the wood mouse gut microbiota composition.

We found that wood mice living in their wild, natural woodland environment had higher intra-individual gut microbiota diversity (alpha diversity) compared to originally wild-derived, but now laboratory reared wood mice. We found significant differences in

mean observed richness (Wilcoxon, Wild wood mice; mean = 239 SE ± 9.18, Lab wood mice; mean = 166 SE ± 3.06, W=2840, p = <0.001, Fig. 2A), Faith's phylogenetic diversity (Wilcoxon, Wild wood mice; mean = 616 SE ± 15.6, Lab wood mice; mean = 477 SE ± 6.33, W=2767, p = <0.001, Fig. 2B), and Shannon index (Wilcoxon, Wild wood mice; mean= 3.81 SE ± 0.08, Lab wood mice; mean = 3.82 SE ± 0.02, W=6179, p=<0.05, Fig. 2C); all demonstrating that wild mice had significantly higher alpha diversity, compared to lab mice. However, there was no significant difference between wild and lab wood mice gut microbiota diversity using the inverse Simpson diversity (Wilcoxon, Wild wood mice; mean = 24.0 SE ± 2.01, Lab wood mice; mean = 25.3 SE ± 0.67, W= 8550 p= 0.06 Fig. 2D), suggesting that the taxonomic evenness (relative abundance of species) of the microbiota was more similar across environments.

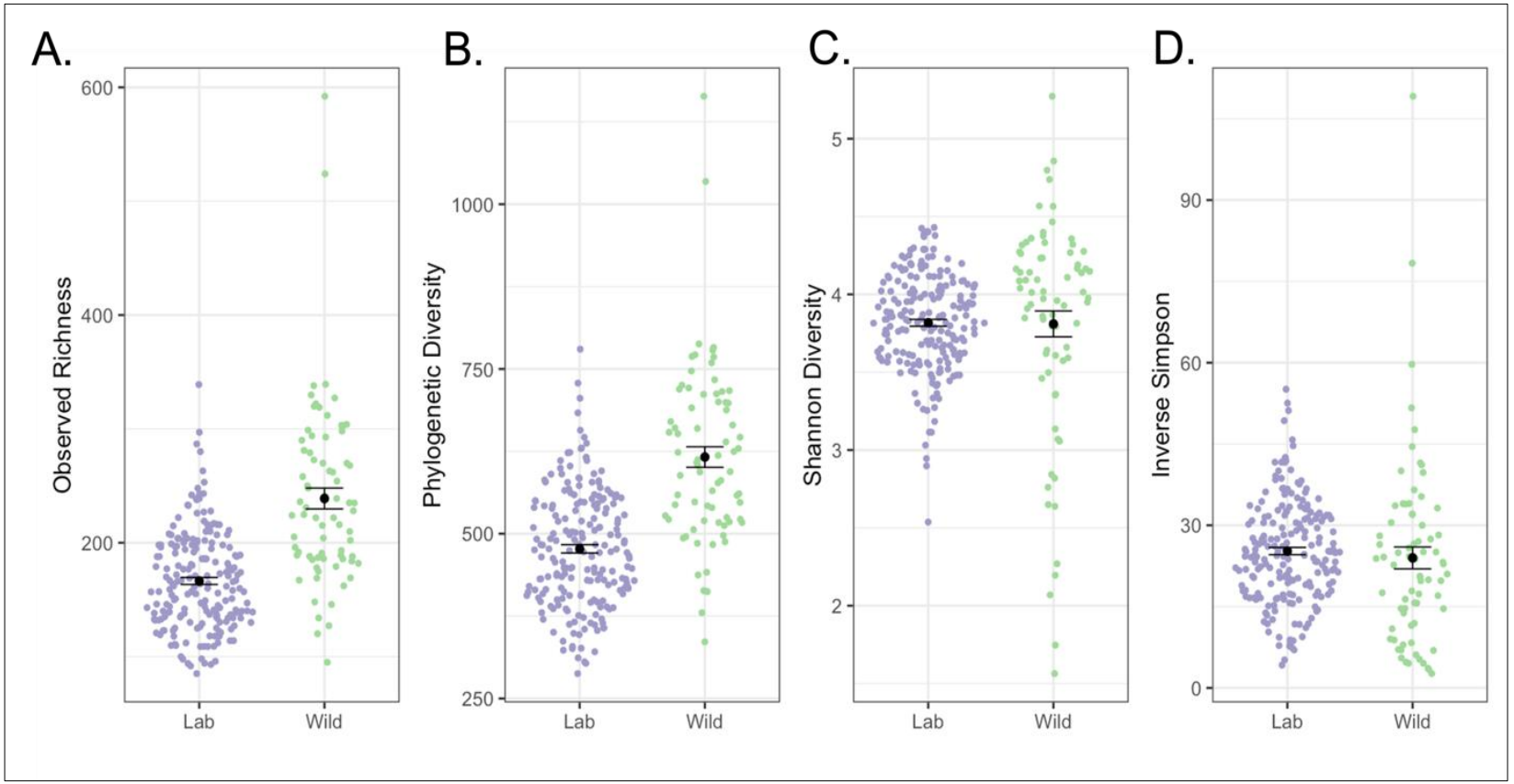


Figure 2. Gut microbiota alpha diversity of Lab-reared and Wild wood mice. Alpha diversity measurements of 16s rRNA sequenced faecal samples collected from Lab-reared wood mice (purple) and Wild wood mice (green). (**A**) Observed species richness, (**B**) Faith's phylogenetic diversity, (**C**) Shannon diversity index, (**D**) inverse Simpson index. Each coloured dot represents the alpha diversity value for an individual sample, error bars represent the standard error (SE) of the mean alpha diversity value of all samples within the group. For each metric, a higher value is indicative of higher within sample diversity.

Within laboratory wood mouse faecal samples, we identified 728 unique ASVs, distributed across 11 bacterial phyla which primarily comprise *Bacteroidota* (63.3%; 461/728) and Firmicutes (18.1%; 132/728, Supp Fig. 3). Interestingly, within wild wood mouse faecal samples, we identified over 3 times more ASVs, with 2350 ASVs distributed across 20 bacterial phyla, again, made up of mostly *Firmicutes* (62.9%; 1479/2350) and *Bacteroidota* (19.8%;466/2350 Supp Fig. 3). Furthermore, when examining core taxa shared between both laboratory and wild samples at the highest taxonomic resolution of unique sequences, we found that just 13.5% (367/2711) of the unique ASV's identified were shared between both laboratory and wild wood mice, with 1983 ASV's found only in wild mice and 361 ASV's unique laboratory mice (Fig. 3A-C).

We used three beta diversity ordination metrics to determine if the gut microbiota composition was significantly different between environments. To first assess how taxa abundances differed between samples, we used a Bray Curtis dissimilarity matrix and found that the sampling environment was a significant predictor, explaining 23.7% (R^2) of the total variation (PERMANOVA, F = 92.75, p = 0.001, Fig. 3D, Table S2). Diet (factor; control or supplemented) and sex (factor; male or female) also had significant main effects on the gut microbiota composition (PERMANOVA, 4.5 R^2, F = 17.63, p = 0.001 and 0.8 R^2, F = 3.18, p = <0.01 respectively, Table S2). We also found a significant environment and diet interaction, whereby wild wood mice on a supplemented diet explained 1.79% (R^2) of the variation in composition data (PERMANOVA, F = 6.99, p=0.001, Table S2). We used both Weighted and Unweighted Unifrac dissimilarities to evaluate the phylogenetic relatedness of taxa between samples, in addition to taxa abundances (Weighted Unifrac) and presence/absence (Unweighted Unifrac). Here, we again found that microbiota composition differences driven by environment type, diet and sex were robust and remained statistically significant (Fig. 3E-F; Table S2). PERMDISP analysis of each ordination metric revealed that samples from wild wood mice also had significantly higher dispersion than lab wood mice samples, indicating that there were both dispersion and location effects within our data (Supp Fig. 4A-C).

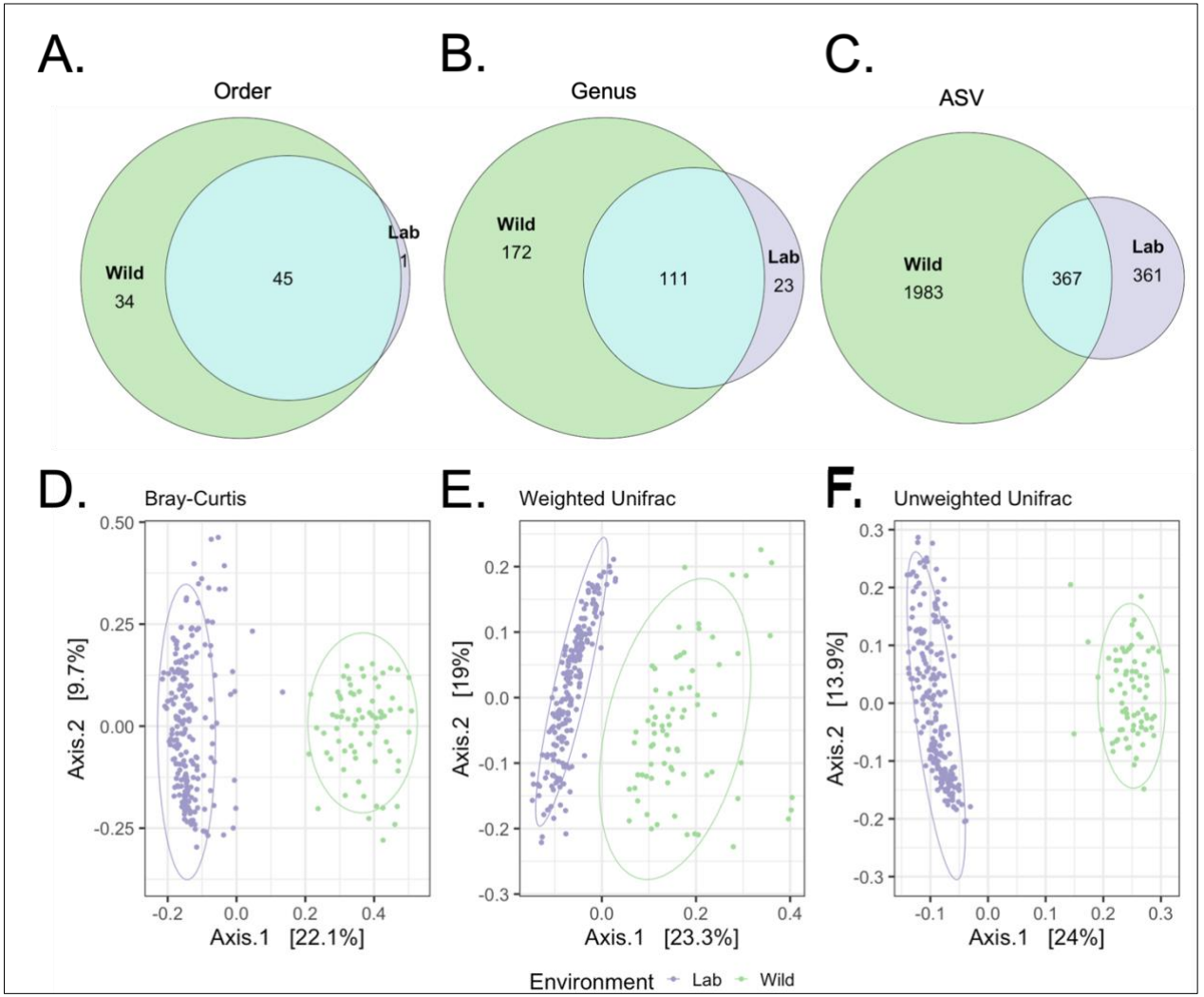


Figure 3. Lab-reared and Wild wood mice have distinct gut microbiota. The number of unique and shared taxa between the wild (green) and lab (purple) mice at the (**A**) Order, (**B**) Genus and (**C**) amplicon sequence variants (ASV) level, where circles overlapping indicate commonality. Principal coordinate analysis (PCoA) ordination of beta diversity dissimilarity matrices, for 16s rRNA sequenced faecal samples of wild (green) and lab (purple) mice. **(D)** Bray Curtis dissimilarity **(E)** Weighted Unifrac distance, **(F)** Unweighted Unifrac distance. Each coloured dot represents data for an individual samples, whilst ellipses represent 95% confidence intervals for each sample group. The closer the dots (samples) are ordinated together, the more similar their microbiota compositions are. Each axis has an eigenvalue in parenthesis indicating the percentage of variation within the dataset captured at that axis.

To further examine differences in the specific microbial community profiles between wild and lab wood mice, we used differential abundance analysis. At the genus level, there were 44 taxa found to be significantly differentially abundant between the two environments, 21 were enriched in wild mice, while and 23 were enriched in the lab mice (Fig. 4). The microbiota of wild mice was characterised by enrichment of bacteria from, for example, *Enterococcus and Mucispirillum* genera (ALDEx2, effect size = -1.17, p= <0.001 and effect size = -1.08, p=<0.001) and the lab mice microbiota was characterised by enrichment of *Lactobacillus* and HT002 (*Lactobacillus* oral clone) genera (ALDEx2, effect size 3.58, p= <0.001 and effect size 2.98, p= <0.001).

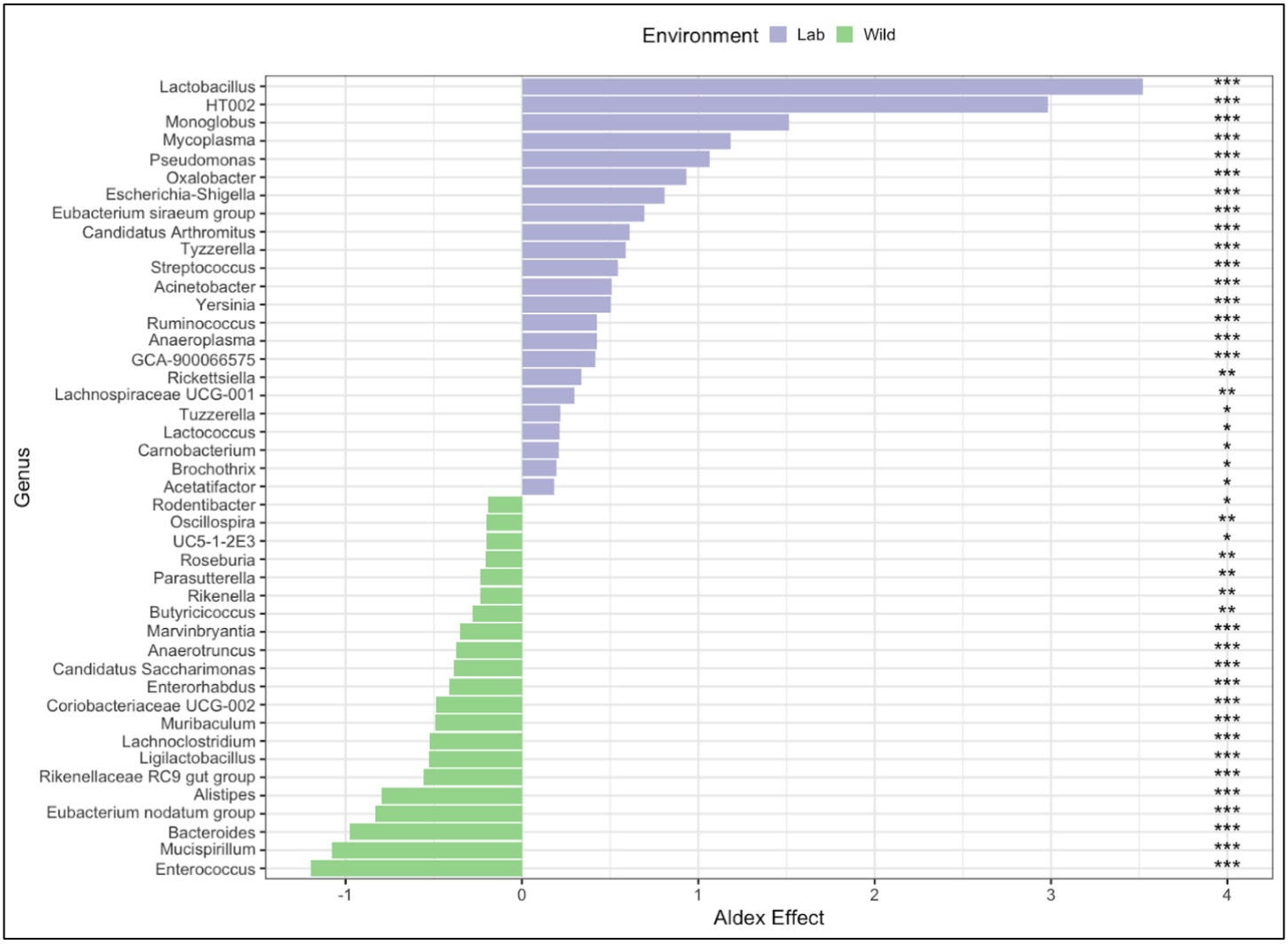


Figure 4.Differential abundance of microbial Genera in Lab-reared and Wild wood mice. Aldex index of differentially abundant genera (y axis) in faecal samples taken from Lab-reared wood mice (purple) and Wild wood mice (green). Aldex index >0 indicates genera more abundant in Lab mice than in Wild mice and Aldex index < 0 indicates genera more abundant in wild mice. Stars (*) indicate the level of statistical significance of difference between Lab and Wild wood mouse genera abundance.

4.4.2. Diet shapes wood mouse microbiota composition and diversity in both lab and wild environments

Next, we sought to determine whether higher quality, supplemented diet increased the individual diversity of the gut microbiota of wood mice in the lab and wild. We found that wood mice on a supplemented diet in both the lab and wild had significantly higher alpha diversity for 3 of metrics tested, compared to mice on control diets within the same environment (GLM, all p = <0.001 except inverse Simpson p = <0.05, Fig.5A-D, Table S3). Further, we found a significant environment and diet interaction when testing observed richness, phylogenetic diversity, and Shannon diversity, which revealed the increase in diversity driven by a supplemented diet was higher in the lab environment compared to the wild (GLM, all p = <0.05, Fig.5A-D, Table S3).

Diet supplementation also resulted in significant shifts in the microbiota composition within the lab environment, but this was more subtle in the wild (Fig. 6A-F). Bray-Curtis dissimilarity index revealed that a supplemented diet was responsible for a significant amount of variation (12.8% R^2) in the microbiota composition of wood mice within the laboratory setting (PERMANOVA, F = 29.42, p= <0.001, Fig. 6A). In addition, sex was also a significant explanatory variable (to a lesser degree), whereby male mice drove 1.23% (R^2) of the variation in microbiota composition within the laboratory (PERMANOVA, F= 2.91, p = 0.01, Table S4). These findings were robust across both Weighted and Unweighted Unifrac metrics (Fig. 6B-6C, Table S4). Using PERMDISP analysis, we found that at each beta diversity metric, wood mice in the lab on a control diet had significantly higher dispersion than those on the supplemented diet (Supp Fig. 5A-C). In the wild environment, supplemented diet only significantly affected the microbiota composition (3.48% R^2) under Unweighted Unifrac metrics (PERMANOVA, F = 2.47, p = 0.01, Fig. 6F, Table S4). However, sex had a similar effect on the microbiota of mice in the wild as the lab, with male mice driving significant variation within the microbiota composition at each metric measured (PERMANOVA, all <0.05, Table S4). Moreover, a significant difference in the dispersion of samples across diet groups was only found under Bray-Curtis dissimilarity, thus, dispersion effects were unlikely to have affected the PERMANOVA results (Supp Fig. 5D-F).

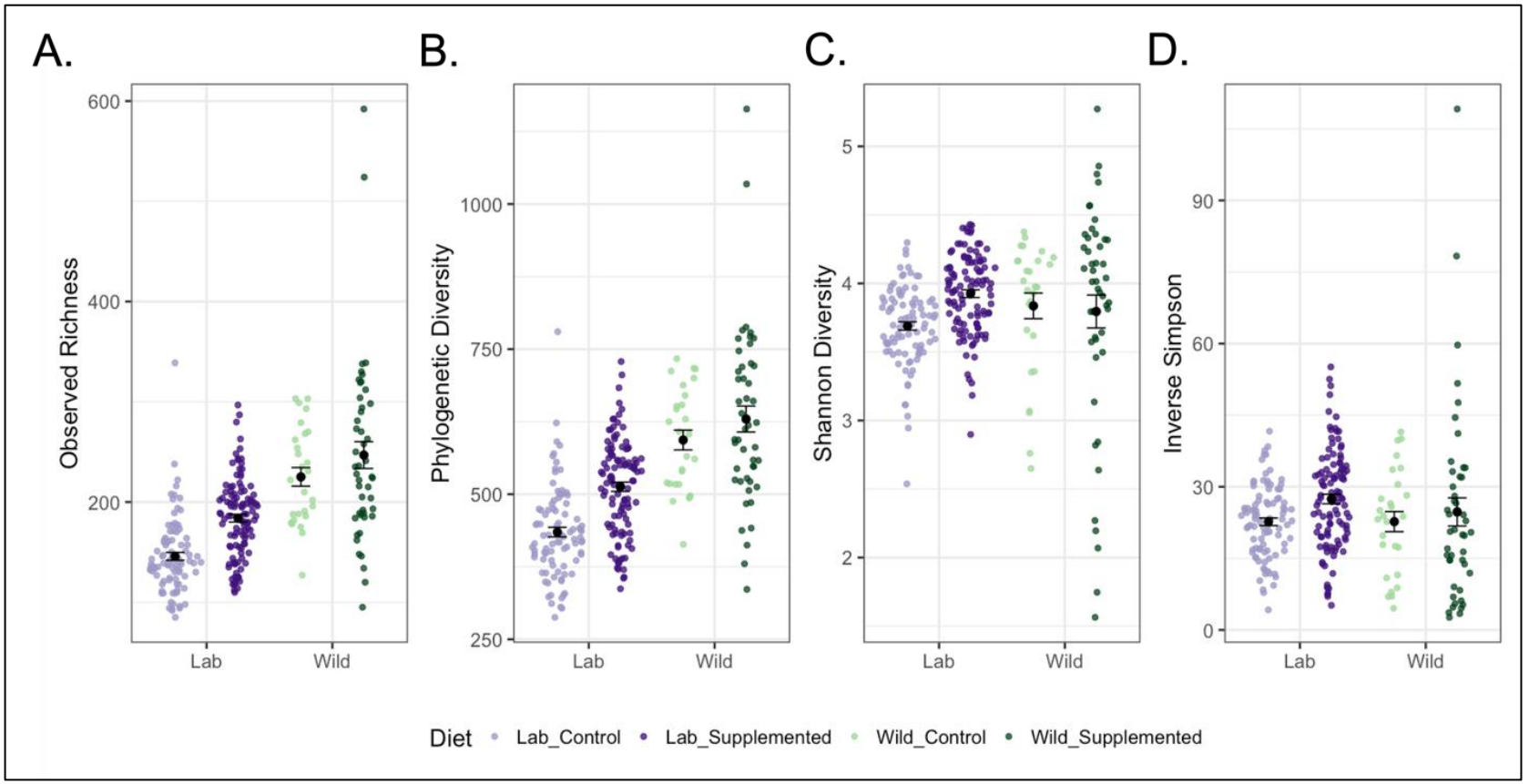


Figure 5. Gut microbiota alpha diversity is increased by supplemented diet in Lab-reared wood mice. Alpha diversity measurements of 16s rRNA sequenced faecal samples collected from Lab-reared wood mice on control diet (light purple), Lab-reared wood mice on supplemented diet (dark purple), Wild wood mice on control diet (light green), and Wild wood mice on supplemented diet (dark green). (**A**) Observed species richness, (**B**) Faith's phylogenetic diversity, (**C**) Shannon diversity index, (**D**) inverse Simpson index. Each coloured dot represents the alpha diversity value for an individual sample, error bars represent the standard error (SE) of the mean alpha diversity value of all samples within the group. For each metric, a higher value is indicative of higher within sample diversity.

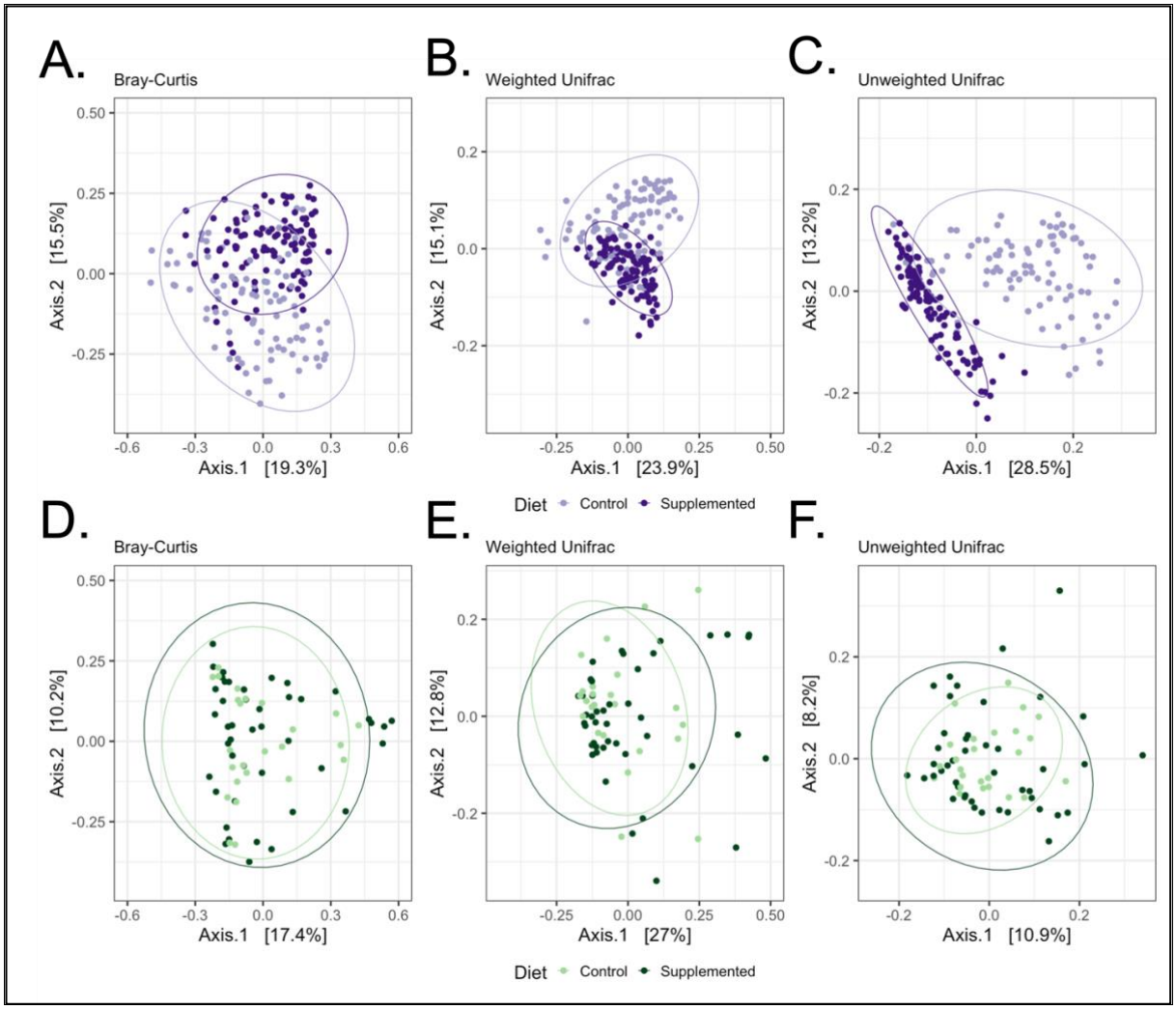


Figure 6. Diet shapes differences in gut microbiota composition among Lab-reared wood mice. Principal coordinate analysis (PCoA) ordination of beta diversity dissimilarity matrices, for 16s rRNA sequenced faecal samples of Lab-reared wood mice (purple) and Wild wood mice (green) on control (lightly shaded) and supplemented (darkly shaded) diets. Lab mice beta-diversity metrics: **(A)** Bray Curtis dissimilarity of **(B)** Weighted Unifrac distance, **(C)** Unweighted Unifrac distance. Wild mice beta-diversity metrics: **(D)** Bray Curtis dissimilarity **(E)** Weighted Unifrac distance, **(F)** Unweighted Unifrac distance. Each coloured dot represents data for an individual sample whilst ellipses represent 95% confidence intervals for the each sample group. The closer the dots are ordinated together, the more similar their microbiota compositions are. Each axis has an eigenvalue in parenthesis indicating the percentage of variation within the dataset captured at that axis.

Differential abundance analysis further highlighted that a supplemented diet had stronger effects on shaping the microbiota composition of wood mice in the lab than in the wild. In lab wood mouse samples, we found a total of 35 taxa at the genus level differentially expressed between the two diet regimens, with 19 genera significantly enriched in samples from mice on a control diet and 16 genera significantly enriched in the microbiotas of mice on a supplemented diet (Fig. 7A). In comparison, when investigating the microbiota profiles of wood mice from the wild, we found just 6 taxa were differentially expressed between the two diet treatments, 3 genera were significantly enriched in the microbiota of mice on a supplemented diet and 3 in mice on control diets (Fig. 7B). Interestingly, 4 of the taxa that were differentially expressed in wild faecal samples were also differentially expressed in the lab faecal samples too (Supp Fig. 6). Of these, both *Eubacterium xylanophilum* group and *Enterococcus* were enriched in mice given a control diet in both environments (ALDEx2, Lab, effect size 1.70, p= <0.001 and effect size 0.35, p= <0.001; Wild, effect size 0.55, p = <0.001 and effect size 0.48, p= <0.01 respectively, Supp Fig. 7). Mice given a supplemented diet across both environments had an enrichment of both *Helicobacter* and *Lachnospiraceae* FCS020 group (ALDEx2, lab, effect size -0.61, p = < 0.001 and effect size -1.09, p = <0.001; wild, effect size -0.22 <0.05 and effect size -0.19, p = <0.05 respectively, Supp Fig. 6).

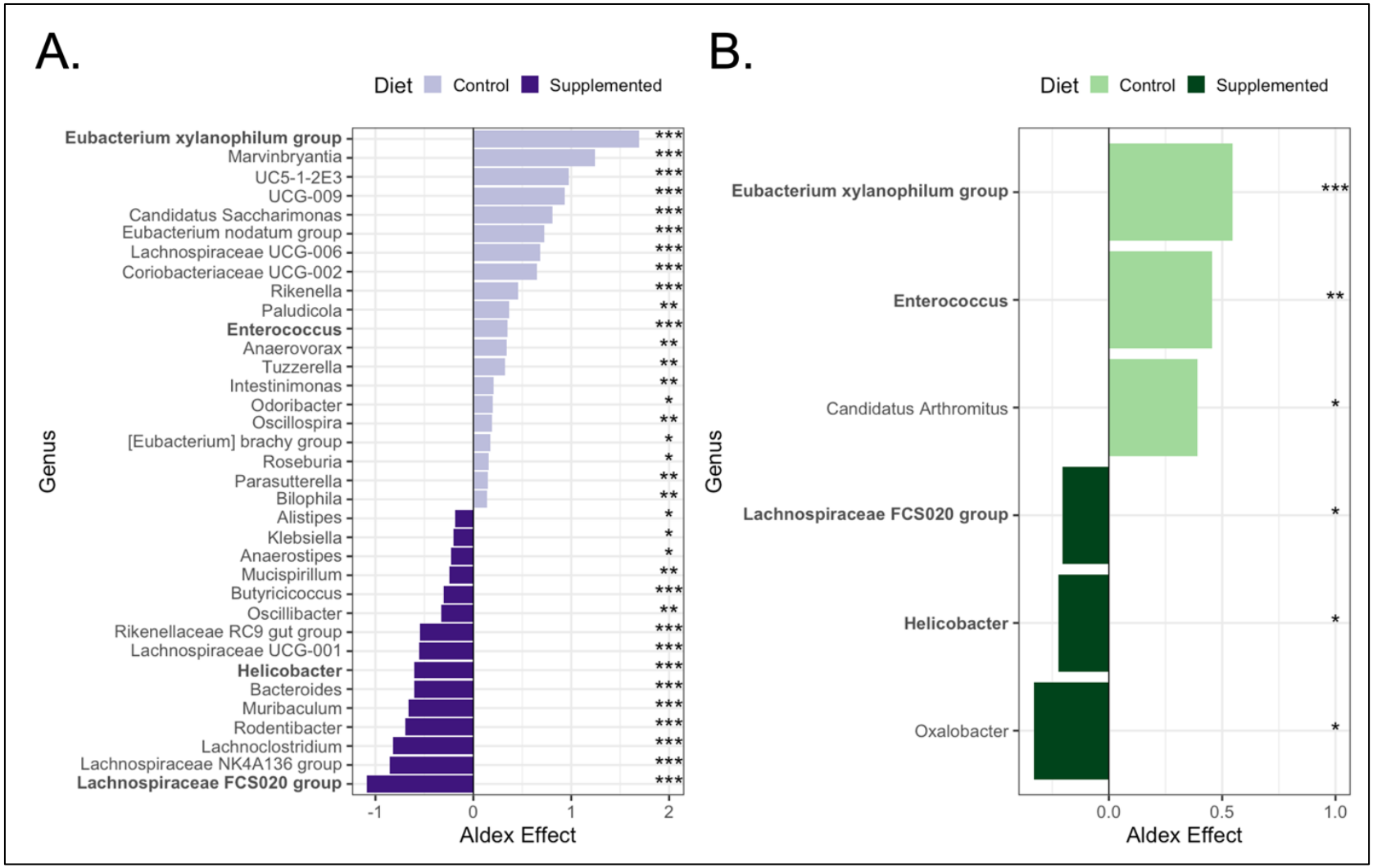


Figure 7. Differential abundance of microbial Genera in Lab-reared and Wild wood mice on different diets. Aldex index of differentially abundant genera (y axis) in faecal samples taken from (**A**) Lab-reared wood mice and (**B**) Wild wood mice on control (lightly shaded) and supplemented (darkly shaded) diets. Aldex index >0 indicates genera more abundant in mice on control diets than those on supplemented diets and Aldex index <0 indicates genera more abundant in mice on supplemented diet. Stars (*) indicate the level of statistical significance of difference between supplemented and control diet in genera

4.4.3. *H. polygyrus* infection has subtle effects on the gut microbiota diversity and composition in lab, but not wild, wood mice.

Finally, we tested if *H. polygyrus* impacted the alpha diversity of the gut microbiota of wood mice. We found that both the observed richness and phylogenetic diversity of lab-reared wood mice with a primary *H. polygyrus* infection was significantly lower than at pre-infection (GLM, p = <0.05 both, Fig. 8A-B, Table S5), indicating that infection may lead to a reduction in the number of bacterial species within the microbiota of lab wood mice. Observed richness, Shannon diversity and Inverse Simpson metrics here also revealed that lab-reared wood mice with a secondary *H. polygyrus* infection, had significantly lower microbiota diversity than lab-reared wood mice pre-infection (GLM, p = <0.05 all Fig. 8A-D, Table S5), suggesting that the infection may lead to a decrease in relative abundance of bacteria. However, this effect appears to be impacted by a supplemented diet, as a diet and infection status interaction revealed that at Shannon and Inverse Simpson metrics, lab-reared mice on a supplemented diet had significantly higher microbiota diversity at secondary infection than those on a control diet (GLM, Shannon, Est. = 0.39, SE ±0.11, p = <0.001; Inverse Simpson, Est. = 0.46, SE ±0.16, p = <0.001, Fig. 8C-D, Table S5). Conversely, in wild wood mice, we observed no statistically significant differences in alpha diversity between infection status and no significant infection status and diet interactions, across any of the 4 metrics measured (Fig. 8E-H, Table S5).

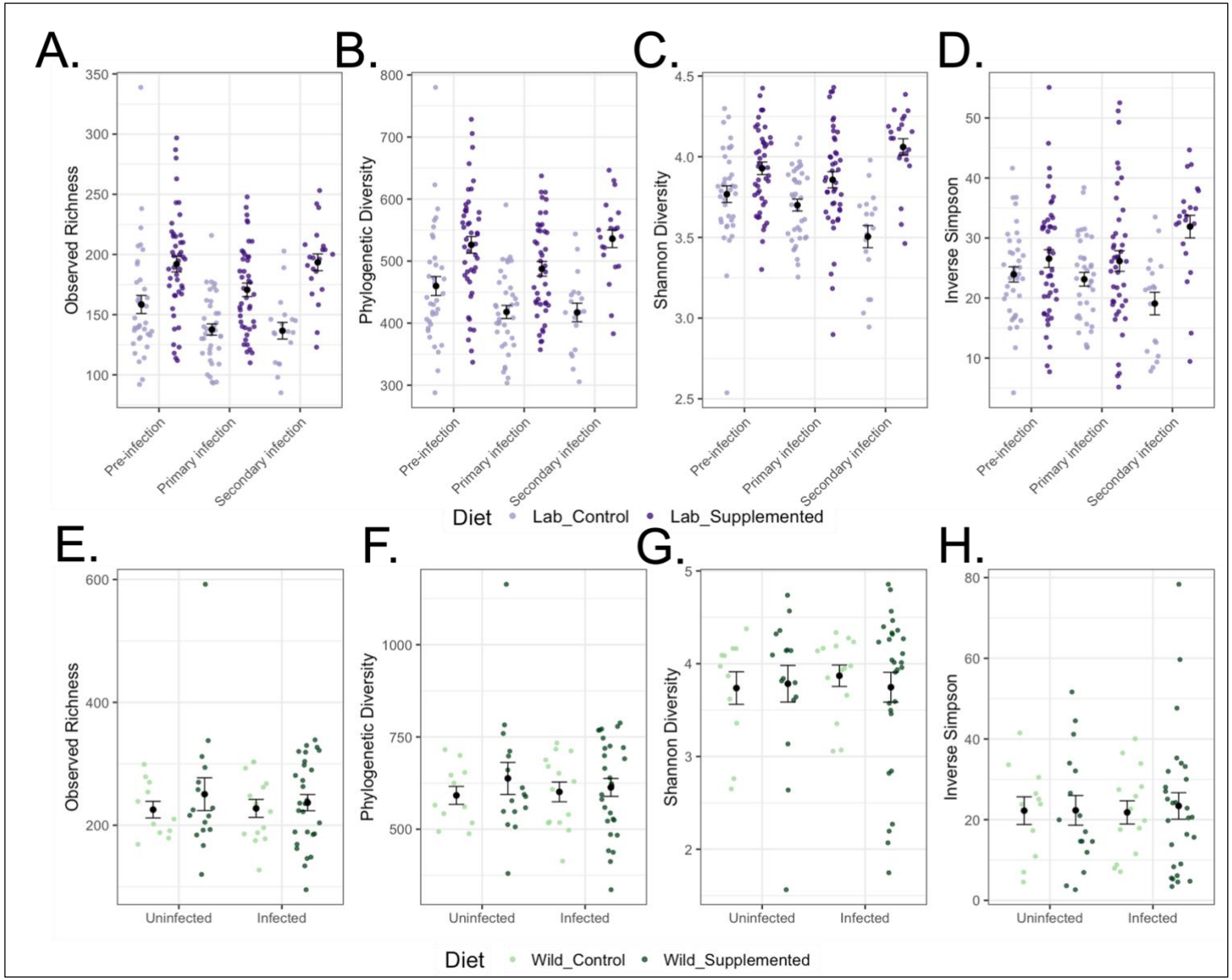

A.
B.
C.
D.
Observed Richness
Phylogenetic Diversity
Shannon Diversity
Inverse Simpson
Pre-infection
Primary infection
Secondary infection
Diet
Lab_Control
Lab_Supplemented
E.
F.
G.
H.
Uninfected
Infected
Wild_Control
Wild_Supplemented

Figure 8. *H. polygyrus* infection influences the alpha diversity of Lab-reared wood mice gut microbiota. Alpha diversity metrics for 16s rRNA sequenced faecal samples of Lab-reared wood mice (purple) and Wild wood mice (green) on control (lightly shaded) and supplemented (darkly shaded) diets (rows) across the duration of the experiment- pre-infection, primary infection with *H. polygyrus* and secondary infection with *H. polygyrus* (Lab mice) or uninfected and infected mice (Wild mice). Lab mice alpha diversity metrics: (**A**) Observed species richness, (**B**) Faith's phylogenetic diversity, (**C**) Shannon diversity index, (**D**) inverse Simpson index. Wild mice alpha diversity metrics: (**A**) Observed species richness, (**B**) Faith's phylogenetic diversity, (**C**) Shannon diversity index, (**D**) inverse Simpson index.

Infection with *H. polygyrus* also significantly impacted the composition of the gut microbiota of wood mice reared in the lab. Bray-Curtis dissimilarity ordination showed that both primary infection and secondary infection accounted for significant variation within the microbiota composition (0.91% and 2.25% R^2 respectively, Fig. 9A, Table S6). In addition, other explanatory factors including diet and sex were also significant and these findings were all replicated in both Weighted and Unweighted Unifrac ordination (Fig. 9B-C, Table S6). However, Unweighted Unifrac analyses also highlighted a significant infection status and diet interaction whereby mice on a supplemented diet with a secondary *H. polygyrus* infection drove 0.96% (R^2) variation within the data (PERMANOVA, F = 2.44, p = 0.014, Fig. 9C, Table S6).

PERMDISP analysis revealed that mice with a secondary infection had significantly higher dispersion under Weighted Unifrac ordination only, thus, was unlikely to have affected the PERMANOVA results (Supp Fig. 7A-C). *H. polygyrus* infection appeared to have minimal effects on driving the composition of the wild wood mice gut microbiota. With Bray-Curtis dissimilarity ordination, we found a significant diet and infection status interaction, whereby uninfected mice on a supplemented diet drove 2.30% R^2 variation in the microbiota composition (PERMANOVA, F = 1.64, p = <0.05). This finding was not replicated under Weighted or Unweighted Unifrac ordinations. Supplemented diet had a significant main effect in both Bray-Curtis and Unweighted dissimilarity analyses and sex was a significant explanatory variable across all 3 beta diversity metrics (PERMANOVA, p = <0.01 all, Table S6). There were no significant dispersion effects across any of the 3 measurements in wild wood mice, indicating that samples were homogenous regardless of infection status (Supp Fig. 7D-F).

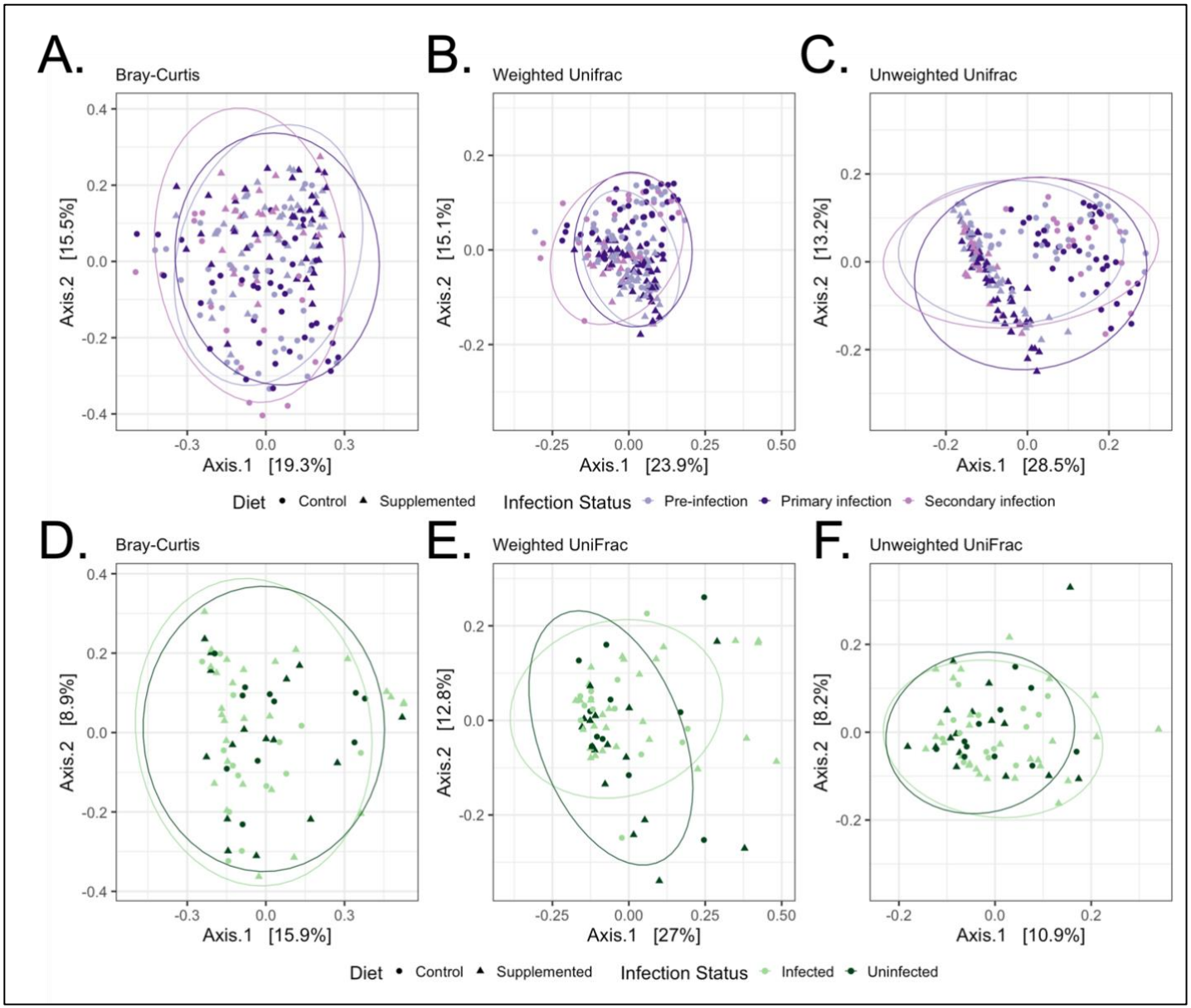

A.
Bray-Curtis
Axis.2 [15.5%]
Axis.1 [19.3%]
B.
Weighted Unifrac
Axis.2 [15.1%]
Axis.1 [23.9%]
C.
Unweighted Unifrac
Axis.2 [13.2%]
Axis.1 [28.5%]
Diet Control Supplemented
Infection Status Pre-infection Primary infection Secondary infection
D.
Bray-Curtis
Axis.2 [8.9%]
Axis.1 [15.9%]
E.
Weighted UniFrac
Axis.2 [12.8%]
Axis.1 [27%]
F.
Unweighted UniFrac
Axis.2 [8.2%]
Axis.1 [10.9%]
Diet Control Supplemented
Infection Status Infected Uninfected

Figure 9. **Diet has greater impact on gut microbiota composition than infection status in lab wood mice.** Principal coordinate analysis (PCoA) ordination of beta diversity dissimilarity matrices, for 16s rRNA sequenced faecal samples of Lab-reared wood mice (top row; purple) and Wild wood mice (bottom row; green) on control (circle) and supplemented (triangle) diets, and across different *H.polygyrus* infection status. Lab mice beta-diversity metrics: **(A)** Bray Curtis dissimilarity of **(B)** Weighted Unifrac distance, **(C)** Unweighted Unifrac distance. Wild mice beta-diversity metrics: **(D)** Bray Curtis dissimilarity **(E)** Weighted Unifrac distance, **(F)** Unweighted Unifrac distance. Each coloured dot represents data for an individual samples, whilst ellipses represent 95% confidence intervals for each sample group. The closer the dots are ordinated together, the more similar their microbiota compositions are. Each axis has an eigenvalue in parenthesis indicating the percentage of variation within the dataset captured at that axis.

Differential abundance analysis highlighted that the specific microbiota profile of lab wood mice at each infection status also varied by diet treatment (Fig. 10). In total, we found 30 taxa at the genus level that were differentially expressed by infection status, of these 18 genera were expressed in wood mice from all three infection groups, 9 enriched in mice on a supplemented diet (*Bacteroides, Helicobacter, Lachnoclostridium, Lachnospiraceae* FCS020 group, *Lachnospiraceae* NK4A136 group, *Lachnospiraceae* UCG-001, *Muribaculum, Rikenellaceae* RC9 gut group, *Rodentibacter)* and 9 enriched in mice on control diet (*Eubacterium nodatum* group, *Eubacterium xylanophilum* group, *Candidatus Saccharimonas*, *Coriobacteriaceae* UCG-002, *Lachnospiraceae* UCG-006, *Marvinbryantia, Rikenella,* UC5-1-2E3, UCG-009, Fig. 10). A further 4 genera were differentially expressed in two infection groups; *Butyricicoccus* was enriched in pre-infection and secondary infected mice on a supplemented diet (ALDEx2, effect size = -0.35, p = 0.01 and effect size = -0.50, p = 0.01, respectively), whereas *Oscillibacter* was enriched in pre-infection and primary infection mice on a supplemented diet (ALDEx2, effect size = -0.57, p = 0.01 and effect size = -0.29, p = 0.02 respectively). *Enterococcus* was enriched in pre-infection and primary infected mice on a control diet (ALDEx2, effect size = 0.38, p = <0.01 and effect size = 0.42, p = <0.001 respectively, Fig. 10) and *Oscillospira* was enriched in primary and secondary infected mice on a control diet (ALDEx2, effect size = 0.31, p = 0.04 and effect size = 0.55, p = 0.03, Fig. 10). We identified a final 8 genera that were differentially expressed at one infection status, 3 taxa were enriched only in pre-infection mice (*Parasutterella* and *Tuzzerella* with control diet and *Mucispirillum* with

supplemented diet) and a further 5 taxa were enriched only in secondary infected mice (*Bilophila,* GCA-900066575, *Paludicola* and *Roseburia* with control diet and *Alistipes* with supplemented diet; data not shown).

Across the wild wood mice faecal samples, we identified very few taxa at the genus level, that were differentially expressed by infection status. However, we found that *Helicobacter* (Genus), RF39 (Order) and *Peptococcaceae* (Family) were significantly enriched in uninfected wild mice on a control diet (ALDEx2, effect size = -0.57, p = 0.04, effect size = -0.60, p =0.02, effect size = -0.66, p =0.01 respectively, data not shown). Conversely, we found, Rs-E47 termite group (Family) and *Bacteroidia* (Class) significantly enriched in uninfected mice on a supplemented diet (ALDEx2, effect size = -0.85, p = 0.001 and effect size = -0.54, p = 0.03 respectively), whilst *Erysipelotrichaceae* (Family) and *Oscillospiraceae* (Family) were enriched in infected mice on a supplemented diet (ALDEx2, effect size = 0.35, p = 0.02 and effect size = 0.20 and p = 0.05 respectively, data not shown).

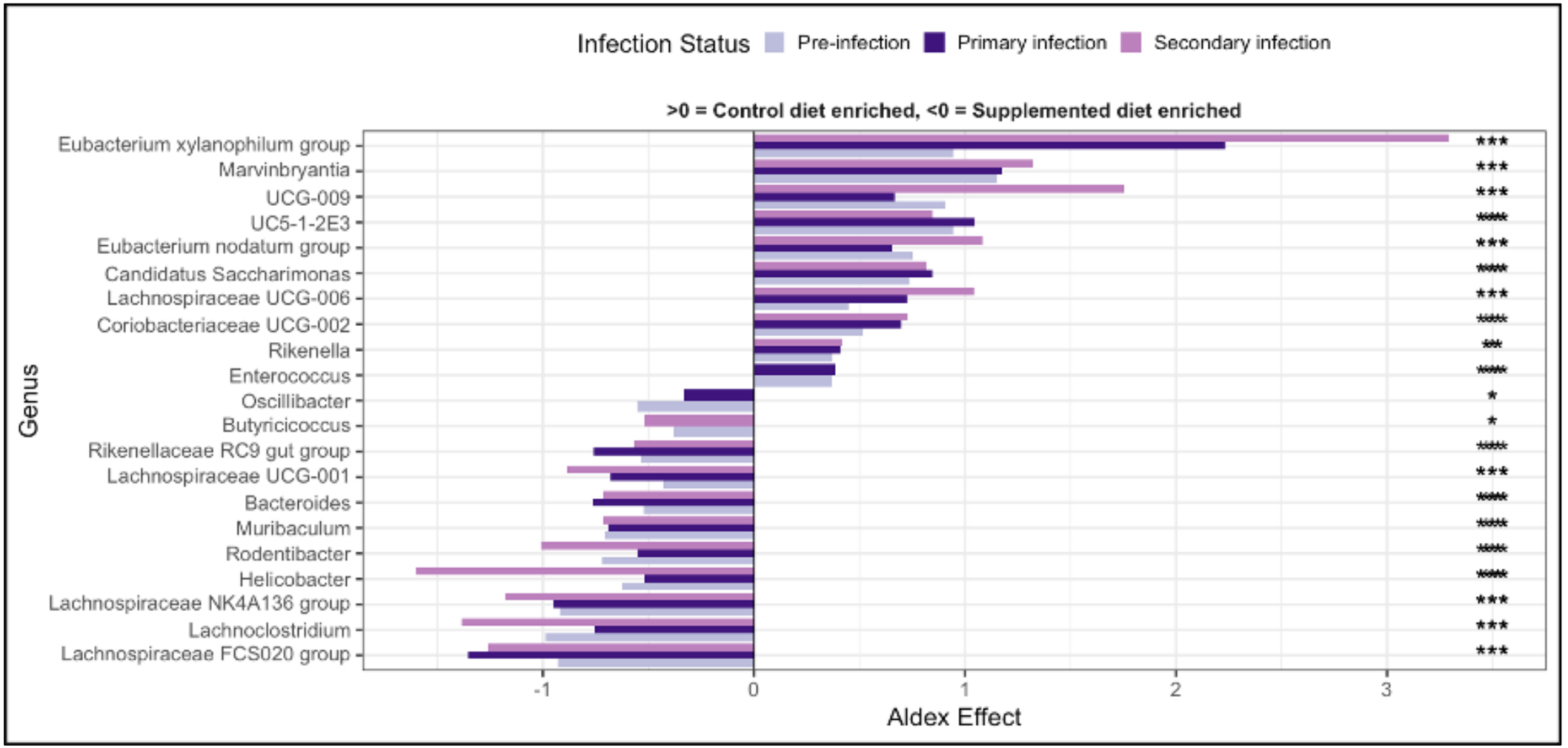


Figure 10. Differential abundance of microbial genera in Lab-reared wood mice on different diets and across *H.polygyrus* infection timepoints. Aldex index of differentially abundant genera (y axis) in faecal samples taken from Lab-reared wood mice at different time points across *H.polygyrus* infection. Aldex index >0 indicates genera more abundant in mice on control diets than those on supplemented diets and Aldex index < 0 indicates genera more abundant in mice on supplemented diet. Stars (*) indicate the level of statistical significance of difference between supplemented and control diet in genera abundance.

4.5. Discussion

In this study, I found that the environment plays a key role in shaping the gut microbiota of wood mice. Wood mice living in natural woodland environments had significantly greater alpha and beta diversity than laboratory wood mice from a colony derived originally from wild caught wood mice, but maintained under standard, controlled conditions. However, I also found evidence that nutrition and helminth infection can impact the diversity and composition of the gut microbiota, but that these effects are more pronounced in the controlled, lab environment. These results highlight the importance using a paired lab-to-wild system to understand the implications of interactions between diet, gut microbiota, and helminth infections.

My findings support previous research that has shown that diet and nutrition can have significant impacts on the diversity and composition of the gut microbiota in the lab (Boulay et al. 1998a; Gagnon CMA et al. 1996; Shi et al. 1997; Shi et al. 1995) and wild (Amato et al. 2015; Ren et al. 2017). However, importantly by testing the same diet supplementation in the same host species in both the controlled laboratory and natural wild environment, I was able to show that diet has a much larger impact in laboratory conditions. This result is not unexpected, as in the lab we are able to control 100% of the diet that mice have access to, however, in the wild, wood mice are omnivorous and have a diverse diet, and we are not able to measure what percent of an individual mouse's diet is composed of the high-quality supplemental mouse chow that was distributed in the woodland environment. From our previous results, we know that wood mice are consuming some of the diet provided, given the significant reductions found in H. polygyrus infection, egg shedding, and the similar increases we see in body condition and immune responses in mice given access to supplemental nutrition. Future work where we could measure the proportion of the supplemented diet consumed by each wild wood mouse wood allow us to better understand if the variation in diet between wild mice is why we find a smaller impact of diet on gut microbiota diversity and composition than in wild mice, Regardless of the variation in wild wood mice, I still find a significant higher gut microbiota diversity in wild wood

mice when given access to the supplemental high-quality diet, suggesting diet even has a detectable effect when wild mice are able to consume a diverse set of food.

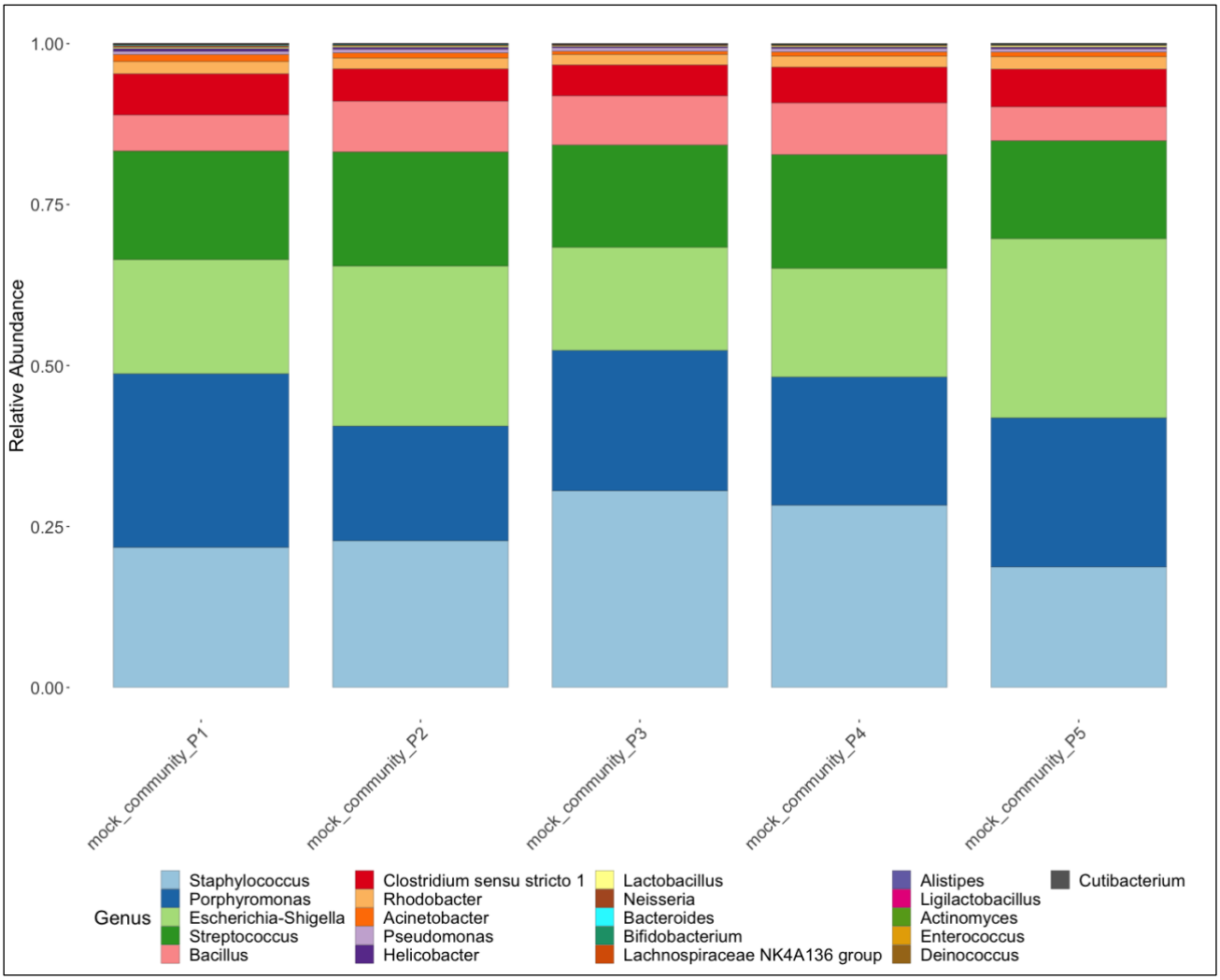
Diet supplementation resulted in some bacterial taxa being differentially expressed, with some genera enriched, while others were reduced in mice given access to high quality nutrition. Importantly, 4 genera were differential expressed in diet supplemented wood mice in both the laboratory and wild woodlands; with *Helicobacter* and *Lachnispiraceae* (FCS2020) significantly enriched, while *Eubacterium xylanophilum* and *Enterococcus* were significantly reduced in wood mice on the supplemented diet. Interestingly, bacteria from the *Lachnispiraceae* family are anaerobic bacteria that are able to ferment diverse polysaccharides into short-chain-fatty acids (Boutard et al. 2014), which may suggest upregulation of these bacteria is associated with some of the constituents of the supplemented diet and aid its digestion.

As has been demonstrated in controlled infection studies in lab mice, *H. polygyrus* infection can impact the gut microbiota diversity and composition (Reynolds et al. 2015; Reynolds et al. 2014). Here, I found the strongest effects of infection in the lab wood mice, specifically that gut microbiota diversity significantly decreased in both primary and secondary infection compared to before infection. In contrast, I found no clear impact of *H. polygyrus* infection on the gut microbiota in wild wood mice. This lack of a signature of infection is not too surprising, given that wild mice have lots of ecological and environmental sources of heterogeneity, such as differences in exposure and burdens of worms, coinfection, differences in diet, age and reproductive status (Babayan et al. 2018; Clerc et al. 2019a; Díaz & Alonso 2003; Marsh et al. 2022; Shaner et al. 2018; Sweeny et al. 2021). It is also possible that the more diverse wild mouse gut microbiota may be more stable to perturbations, as it is constantly being naturally 'perturbed' in the wild due to ever changing environment and resources. It is possible that the wild microbiota can essentially 'buffer huge microbiota shifts, because it is both more resistant to changes and more resilient after a perturbation (Maurice et al. 2015a; Viney 2019).

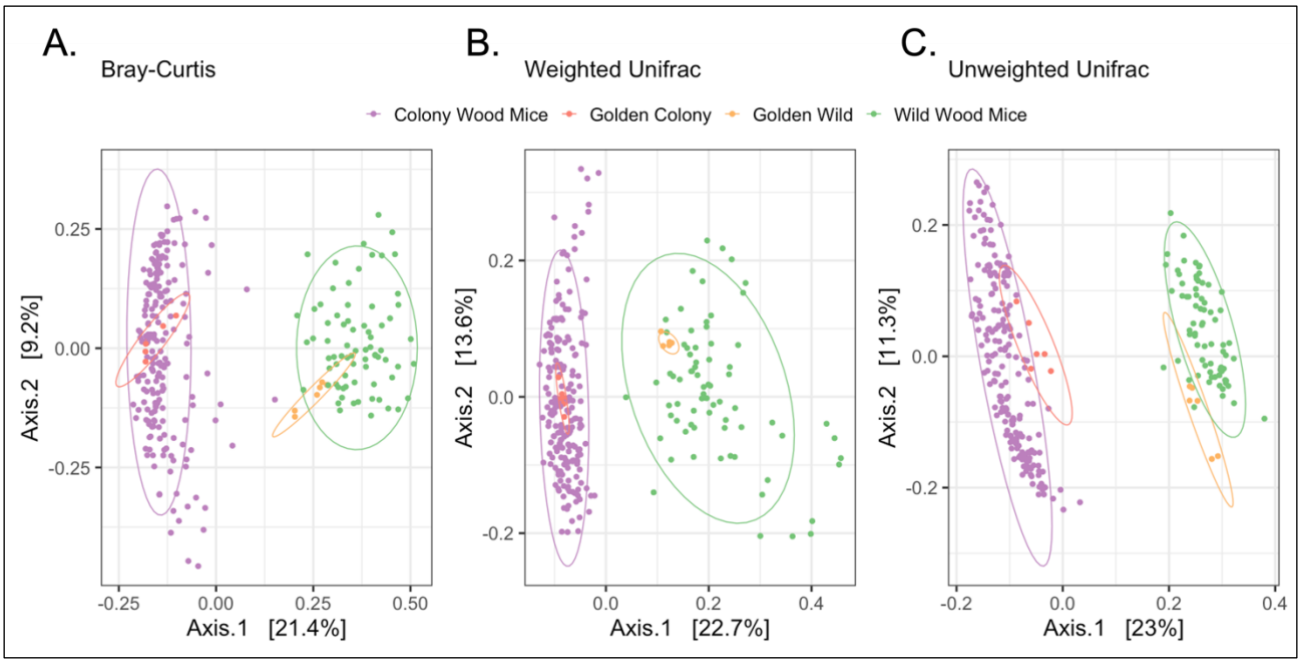
This study presents clear experimental results from a novel lab-to-wild system that nutrition and *H. polygyrus* infection can impact gut microbiota diversity and composition, although the diverse gut microbiota of wild wood mice may be more stable to perturbations than the lab mouse microbiota. We have previously shown that this high-quality diet supplementation significantly improves helminth resistance in both the lab and wild, here we show that this diet does lead to some changes in the abundance of specific bacterial taxa; several of which were consistently enriched across environments. Given the growing importance of understanding interactions between nutrition-gut microbiota-infections, my result show the need to go beyond the standard mouse model, as while we some shifts in the wild mouse microbiota, these were only demonstrated in a subset of taxa compared to the lab wood mice. Further experiments are needed to better understand the functional role of the bacterial that were enhanced/reduced after infection, especially those that were impacted by the interaction of diet and infection to see if the gut microbiota was impacting the impact of diet on helminth resistance.

4.6. Supplementary Material

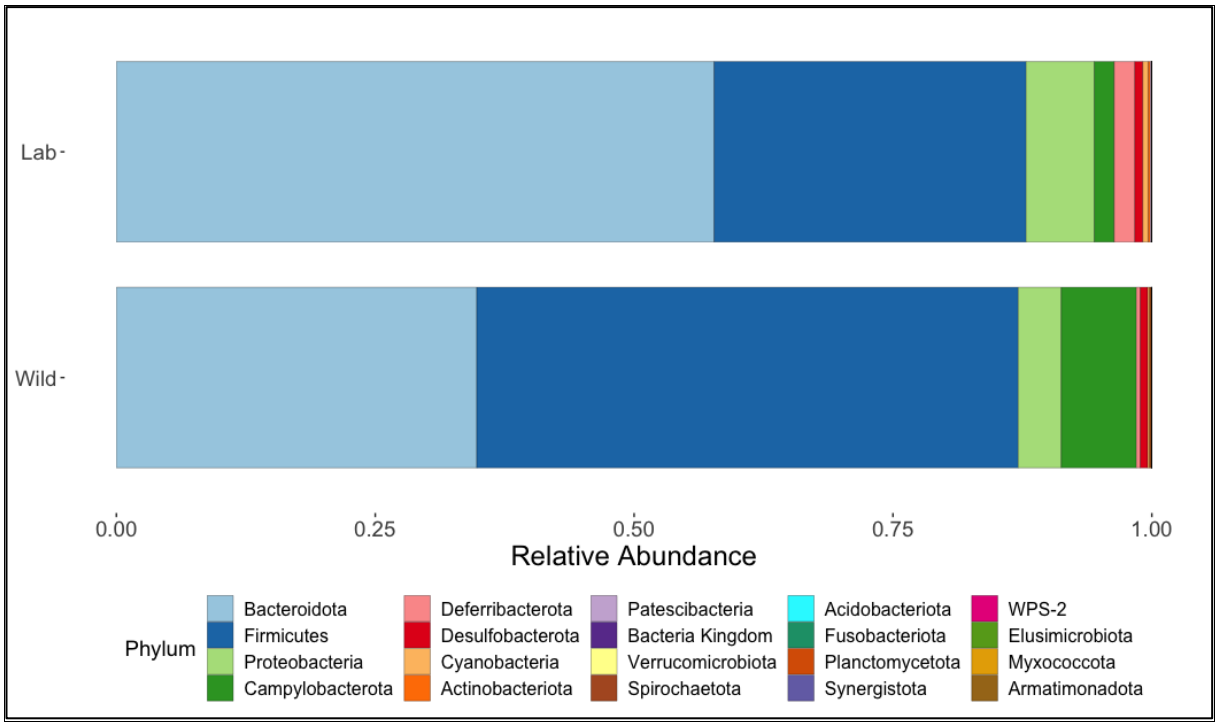
4.6.1. Supplementary Figures



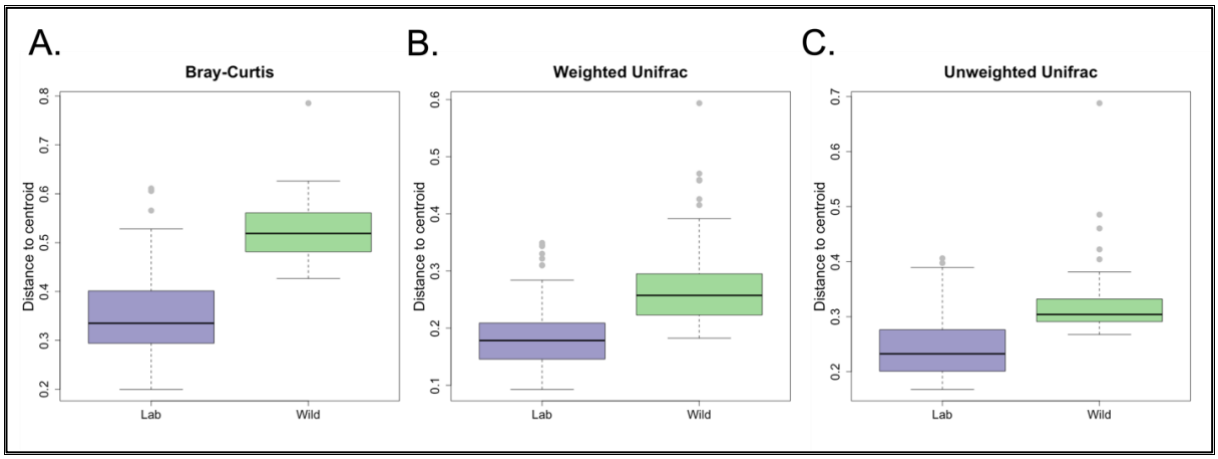
Supplementary Figure 1. The relative abundance of bacteria, at the Genus taxonomic level, detected within each Mock Community on each of the 5 sequencing runs is presented here. During preparation for sequencing, the V4 region of the bacterial 16S rRNA gene of each sample was amplified using a barcoded adaptor-based polymerase chain reaction (PCR). Among the controls for each PCR plate and subsequent sequencing run 1 x 2µl Mock Community (20 strain staggered mix genomic material, ATCC MSA-1003; positive control) was included. Here, we ensured that the 20 bacterial strains expected to be detected were indeed identified using the Silva Project v132 database (version 138.1; (Yilmaz et al. 2013)) for taxonomic assignment.



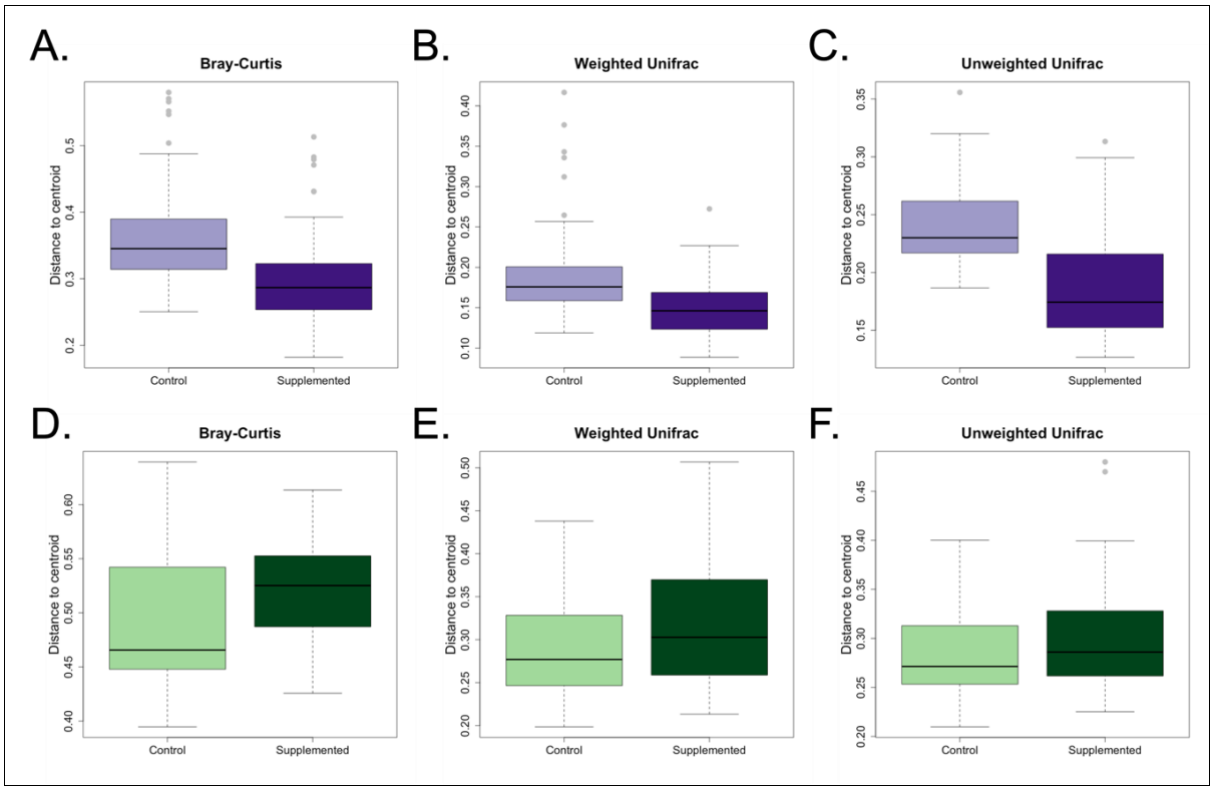
Supplementary Figure 2. Principal coordinate analysis (PCoA) ordination of beta diversity dissimilarity matrices, for 16s rRNA sequenced faecal samples. Along with the two mouse cohorts, Lab-reared (colony) wood mice (Purple) and Wild wood mice (green), two positive control samples were examined to ensure these clustered with samples as expected. Here the Golden Colony (Pink) sample consisted of DNA that had been extracted from faecal samples collected from 6 individual wood mice from the lab-reared colony and pooled together after extraction. Similarly, the Golden Wild (Yellow) sample consisted of DNA that had been extracted from faecal samples collected from 6 individual wild wood mice during fieldwork conducted at Callendar Wood, Scotland and the DNA pooled together after extraction. **(A)** Bray Curtis dissimilarity **(B)** Weighted Unifrac distance **C)** Unweighted Unifrac distance. Each coloured dot represents data for individual sample and ellipses represent 95% confidence intervals surrounding each sample group. The closer the dots are ordinated together, the more similar their microbiota compositions are. Each axis has an eigenvalue in parenthesis indicating the percentage of variation within the dataset captured at that axis.



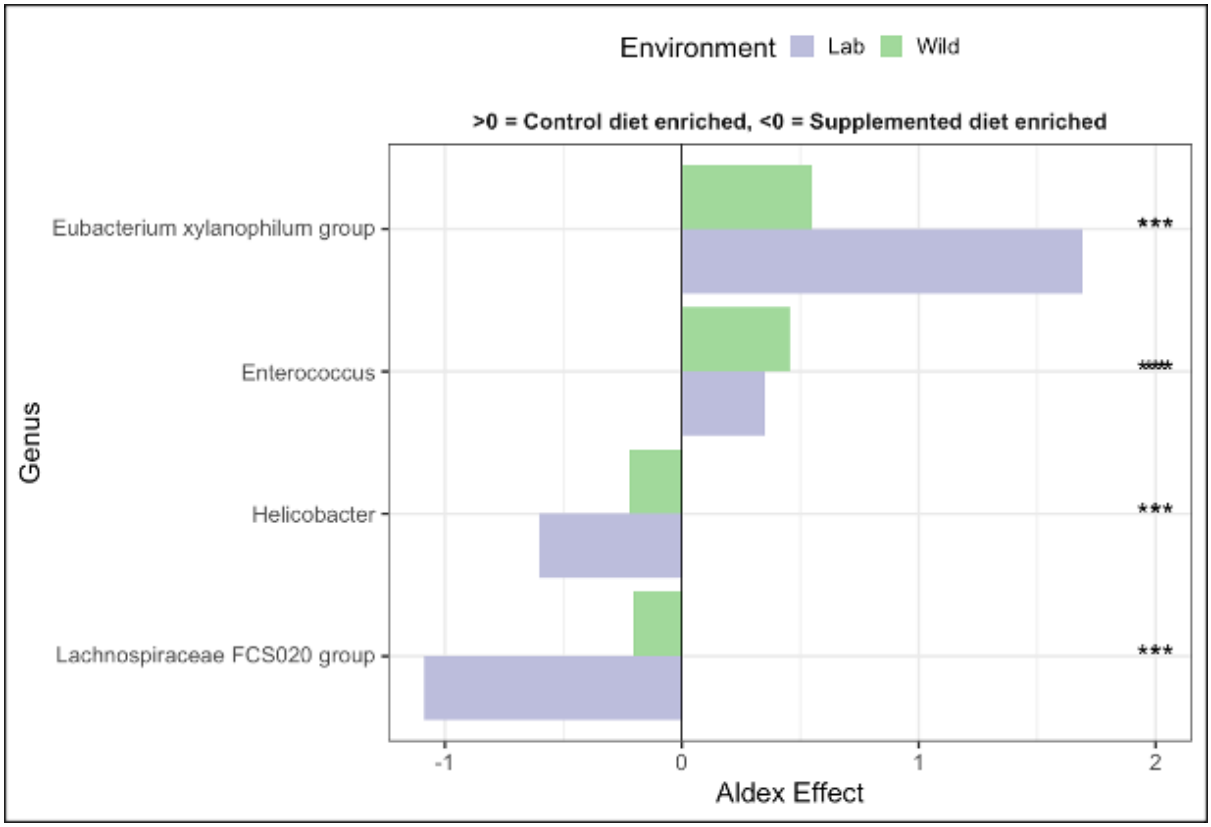
Supplementary Figure 3. The relative abundance of bacterial phyla within the gut microbiota of faecal samples collected from in Lab-reared wood mice (top bar) and Wild wood mice (bottom bar) faecal samples.



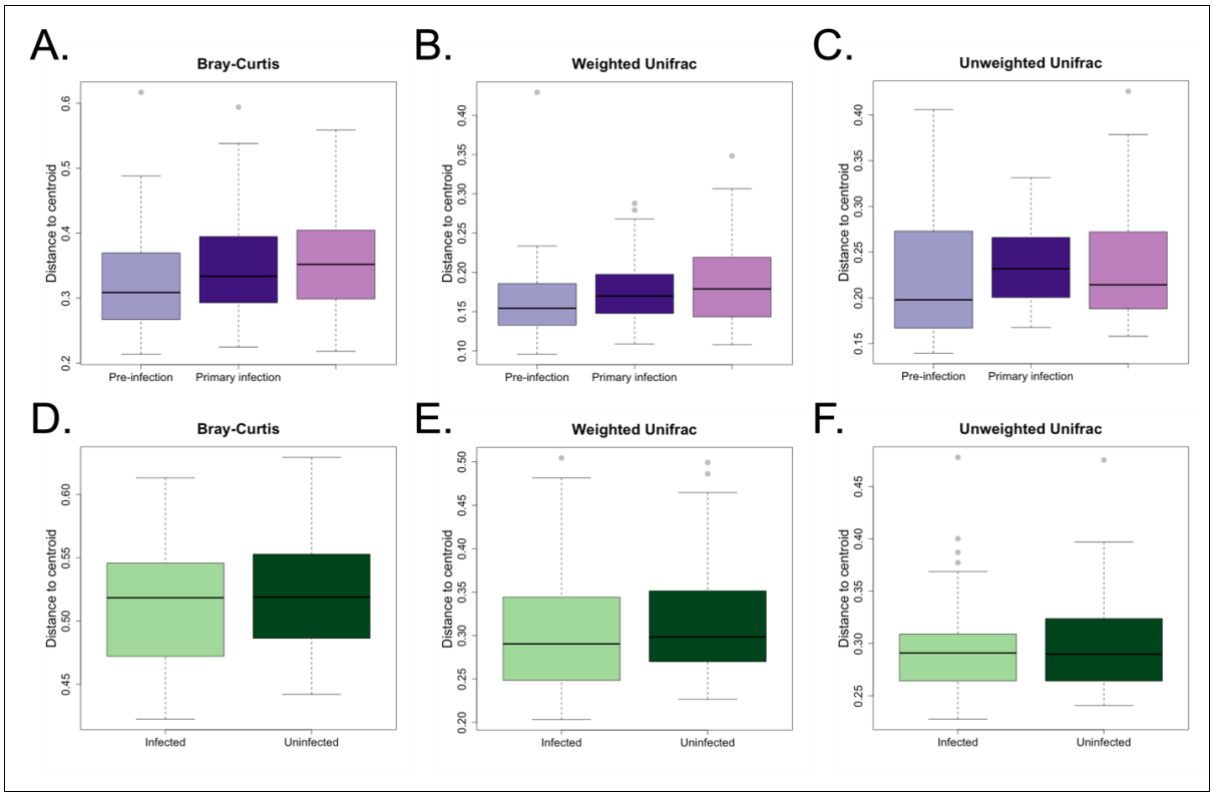
Supplementary Figure 4. Distance to centroids of Lab-reared wood mice (purple) and Wild wood mice (green) for different beta-diversity dissimilarity matrices: (**A**) Bray Curtis dissimilarity, (**B**) Weighted Unifrac distance, (**C**) Unweighted Unifrac distance. Box indicates interquartile range (IQR), with vertical lines representing medians and horizontal lines indicating ± 1.5 IQR.



Supplementary Figure 5. Distance to centroids of Lab-reared wood mice (top row; purple) and Wild wood mice (bottom row; green) on control (lightly shaded) and supplemented (darkly shaded) diets for different beta-diversity dissimilarity matrices. Lab wood mice: (**A**) Bray Curtis dissimilarity, (**B**) Weighted Unifrac distance, (**C**) Unweighted Unifrac distance. Wild wood mice: (**D**) Bray Curtis dissimilarity, (**E**) Weighted Unifrac distance, (**F**) Unweighted Unifrac distance. Box indicates interquartile range (IQR), with vertical lines representing medians and horizontal lines indicating ± 1.5 IQR.



Supplementary Figure 6. Differential abundance of microbial genera in Lab-reared wood mice (purple) and Wild wood mice (green) on different diets. Aldex index of differentially abundant genera (y axis) in faecal samples taken from mice on different diets. Aldex index >0 indicates genera more abundant in mice on control diets than those on supplemented diets and Aldex index < 0 indicates genera more abundant in mice on supplemented diet. Stars (*) indicate the level of statistical significance of difference between supplemented and control diet in genera abundance.



Supplementary Figure 7. Distance to centroids of Lab-reared wood mice (top row; purple) and Wild wood mice (bottom row; green) across *H.polygyrus* infection timepoints/status for different beta-diversity dissimilarity matrices. Lab wood mice: (**A**) Bray Curtis dissimilarity, (**B**) Weighted Unifrac distance, (**C**) Unweighted Unifrac distance. Wild wood mice: (**D**) Bray Curtis dissimilarity, (**E**) Weighted Unifrac distance, (**F**) Unweighted Unifrac distance. Box indicates interquartile range (IQR), with vertical lines representing medians and horizontal lines indicating ± 1.5 IQR.

4.6.2. Supplementary Tables

Table S1. Bacterial species identified within negative control samples of water-only and water plus Forward and Reverse primers of the 5 sequencing runs. Taxonomic assignment is at the genus level.

	Water-only samples	**Water plus primers samples**
Bacteria Detected (Genus taxonomic level)	**Frequency observed (N)**	**Frequency observed (N)**
Alistipes	NA	1
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	NA	1
Bacteroides	1	1
Bilophila	NA	1
Desulfovibrio	NA	1
Escherichia-Shigella	1	1
Helicobacter	1	2
Intestinimonas	NA	1
Lachnospiraceae (NK4A136 group)	1	2
Lactobacillus	NA	1
Ligilactobacillus	1	1
Micrococcus	NA	1
Mucilaginibacter	NA	1
Mucispirillum	1	NA
Mycoplasma	1	NA
Odoribacter	NA	1
Pseudomonas	NA	1
Rikenella	NA	3
Roseburia	NA	1
Staphylococcus	NA	1
Terriglobus	NA	1

Table S2. The environment shapes the microbiota composition of wood mice. Model outputs are shown from a PERMANOVA on Bray-Curtis, Weighted Unifrac and Unweighted Unifrac dissimilarity values (999 permutations). Multivariate homogeneity of group dispersions between environments were carried out for each beta diversity metric (1000 permutations) and the PERMDISP results also shown. All p-values <0.05 are in bold.

	PERMANOVA				**PERMDISP**		
Response Variable: Bray-Curtis Ordination							
Model covariates	Sum Sq	R^2	F	p value	Sum Sq	F	p value
Environment, Wild: Diet, Supplemented	1.084	0.018	6.99	**0.001**			
Environment, Wild	14.369	0.237	92.75	**0.001**	1.652	308.37	**<0.001**
Diet, Supplemented	2.732	0.045	17.63	**0.001**			
Sex, Male	0.493	0.008	3.18	**0.004**			
Residual	41.830	0.691					
Total	60.507	1.000					
Response Variable: Weighted Unifrac Ordination							
Model covariates	Sum Sq	R^2	F	p value	Sum Sq	F	p value
Environment, Wild: Diet, Supplemented	0.247	0.015	5.48	**0.001**			
Environment, Wild	3.389	0.204	75.10	**0.001**	0.458	135.1	**<0.001**
Diet, Supplemented	0.633	0.038	14.02	**0.001**			
Sex, Male	0.193	0.012	4.27	**0.001**			
Residual	12.184	0.732					
Total	16.645	1.000					
Response Variable: Unweighted Unifrac Ordination							
Model covariates	Sum Sq	R^2	F	p value	Sum Sq	F	p value
Environment, Wild: Diet, Supplemented	0.624	0.024	9.58	**0.001**			
Environment, Wild	6.199	0.235	95.20	**0.001**			
Diet, Supplemented	1.823	0.069	27.99	**0.001**	0.339	118.97	**<0.001**
Sex, Male	0.149	0.006	2.29	**0.022**			
Residual	17.582	0.667					
Total	26.376	1.000					

Table S3. Diet shapes the wood mouse microbiota diversity in the lab and wild. Model outputs for fixed effects are shown from Generalised Linear Mixed Models on observed richness, phylogenetic diversity, Shannon diversity and Inverse Simpson alpha diversity metrics in the lab and wild environments. Data that were log transformed to correct for non-normal distribution are indicated in parentheses. All p-values <0.05 are in bold.

	Observed richness (Log)			**Phylogenetic diversity (Log)**			**Shannon Diversity**			**Inverse Simpson (Log)**		
Model covariates	Est	SE	p value	Est	SE	p value	Est	SE	p value	Est	SE	p value
Intercept	4.95	0.03	**<0.001**	6.06	0.02	**<0.001**	3.69	0.05	**<0.001**	3.05	0.06	**<0.001**
Environment, Wild: Diet, Supplemented	-0.18	0.07	**0.011**	-0.12	0.05	**0.015**	-0.28	0.13	**0.028**	-0.25	0.15	0.104
Environment, Wild	0.44	0.06	**<0.001**	0.32	0.04	**<0.001**	0.15	0.10	0.134	-0.08	0.12	0.492
Diet, Supplemented	0.24	0.04	**<0.001**	0.17	0.03	**<0.001**	0.24	0.06	**<0.001**	0.17	0.08	**0.026**
Sex, Male	0.02	0.03	0.609	0.01	0.02	0.716	0.07	0.05	0.175	0.14	0.08	0.082

Table S4. Diet shapes the wood mouse microbiota composition in the lab and wild. Model outputs are shown from a PERMANOVA on Bray-Curtis, Weighted Unifrac and Unweighted Unifrac dissimilarity values in the lab and wild environments (999 permutations). Multivariate homogeneity of group dispersions between environments were carried out for each beta diversity metric (1000 permutations) and the PERMDISP results also shown. All p-values <0.05 are in bold.

	Laboratory Wood Mice				**Wild Wood Mice**			
PERMANOVA: Bray-Curtis Ordination								
Model covariates	Sum Sq	R^2	F	p value	Sum Sq	R^2	F	p value
Diet, Supplemented	3.257	0.128	29.42	**0.001**	0.437	0.023	1.64	0.055
Sex, Male	0.322	0.013	2.91	**0.009**	0.857	0.045	3.22	**0.001**
Residual	21.916	0.860	-	-	17.533	0.931	-	-
Total	25.495	1.00	-	-	18.826	1.000	-	-
PERMDISP: Bray-Curtis Ordination								
Groups	Sum sq		F	p value	Sum sq		F	p value
Diet	0.238		53.29	**<0.001**	0.013		4.81	**<0.05**
PERMANOVA: Weighted Unifrac Ordination								
Model covariates	Sum Sq	R^2	F	p value	Sum Sq	R^2	F	p value
Diet, Supplemented	0.615	0.093	20.66	**0.001**	0.145	0.020	1.39	0.162
Sex, Male	0.131	0.020	4.39	**0.002**	0.241	0.033	2.30	**0.023**
Residual	5.900	0.888	-	-	6.910	0.947	-	-
Total	6.643	1.000	-	-	7.300	1.000	-	-
PERMDISP: Weighted Unifrac Ordination								
Groups	Sum sq		F	p value	Sum sq		F	p value
Diet	0.078		41.56	**<0.001**	0.009		1.51	0.206
PERMANOVA: Unweighted Unifrac Ordination								
Model covariates	Sum Sq	R^2	F	p value	Sum Sq	R^2	F	p value
Diet, Supplemented	2.159	0.188	46.37	**0.001**	0.223	0.035	2.47	**0.001**
Sex, Male	0.107	0.009	2.29	**0.020**	0.220	0.034	2.43	**0.001**
Residual	9.216	0.803	-	-	5.980	0.931	-	-
Total	11.481	1.000	-	-	6.424	1.000	-	-
PERMDISP: Unweighted Unifrac Ordination								
Groups	Sum sq		F	p value	Sum sq		F	p value
Diet	0.143		94.84	**<0.001**	0.005		1.63	0.216

Table S5. ***H. polygyrus*** **infection has subtle effects on the gut microbiota diversity in lab, but not wild wood mice.** Model outputs for fixed effects are shown from Generalised Linear Mixed Models on observed richness, phylogenetic diversity, Shannon diversity and Inverse Simpson alpha diversity metrics in the lab and wild environments. Data that were log transformed to correct for non-normal distribution are indicated in parentheses. All p-values <0.05 are in bold.

Laboratory Wood Mice												
	Observed richness (Log)			**Phylogenetic diversity (Log)**			**Shannon Diversity**			**Inverse Simpson (Log)**		
Model covariates	Est	SE	p value	Est	SE	p value	Est	SE	p value	Est	SE	p value
Intercept	5.03	0.04	**<0.001**	6.11	0.03	**<0.001**	3.75	0.05	**<0.001**	3.01	0.09	**<0.001**
Inf Status, Primary infection: Diet, Supplemented:	0.01	0.07	0.841	0.02	0.05	0.773	-0.02	0.09	0.847	-0.05	0.13	0.703
Inf Status, Secondary infection: Diet, Supplemented:	0.16	0.09	0.068	0.12	0.06	0.070	0.37	0.11	**<0.001**	0.43	0.16	**0.008**
Inf Status, Primary infection	-0.13	0.05	**0.013**	-0.09	0.04	**0.020**	-0.06	0.06	0.317	-0.01	0.09	0.957
Inf Status, Secondary infection	-0.14	0.06	**0.031**	-0.09	0.05	0.056	-0.26	0.08	**0.002**	-0.24	0.12	**0.049**
Diet, Supplemented	0.20	0.05	**<0.001**	0.14	0.04	**<0.001**	0.16	0.06	**0.016**	0.10	0.11	0.344
Sex, Male	0.01	0.03	0.804	0.00	0.02	0.908	0.03	0.05	0.545	0.06	0.08	0.465
Wild wood mice												
	Observed richness (Log)			**Phylogenetic diversity (Log)**			**Shannon Diversity**			**Inverse Simpson (Log)**		
Model covariates	Est	SE	p value	Est	SE	p value	Est	SE	p value	Est	SE	p value
Intercept	5.38	0.08	**<0.001**	6.37	0.06	**<0.001**	3.77	0.19	**<0.001**	2.82	0.20	**<0.001**
Inf Status, Uninfected: Diet, Supplemented	0.07	0.15	0.639	0.07	0.11	0.530	0.34	0.36	0.353	0.20	0.38	0.600
Inf Status, Uninfected	-0.03	0.12	0.812	-0.03	0.08	0.706	-0.28	0.29	0.329	-0.21	0.30	0.495
Diet, Supplemented	0.01	0.10	0.907	0.00	0.07	0.956	-0.17	0.23	0.450	-0.14	0.24	0.547
Sex, Male	0.07	0.07	0.335	0.05	0.05	0.352	0.34	0.17	0.054	0.43	0.18	**0.019**

Table S6. *H. polygyrus* infection has subtle effects on the gut microbiota composition in lab, but not wild wood mice. Model outputs are shown from a PERMANOVA on Bray-Curtis, Weighted Unifrac and Unweighted Unifrac dissimilarity values in the lab and wild environments (999 permutations). Multivariate homogeneity of group dispersions between environments were carried out for each beta diversity metric (1000 permutations) and the PERMDISP results also shown. All p-values <0.05 are in bold.

Laboratory Wood Mice					**Wild Wood Mice**				
					PERMANOVA: Bray-Curtis Ordination				
Model covariates	Sum Sq	R^2	F	p value	*Model covariates*	Sum Sq	R^2	F	p value
Diet, Supplemented: Inf Status, Primary infection	0.103	0.004	0.96	0.436	Diet, Supplemented: Inf Status, Uninfected	0.432	0.023	1.64	**0.044**
Diet, Supplemented: InfStatus, Secondary infection	0.179	0.07	1.67	0.087	Diet, Supplemented	0.437	0.023	1.66	**0.044**
Diet, Supplemented	3.257	0.128	30.34	**0.001**	Inf Status, Uninfected	0.227	0.012	0.86	0.618
Inf Status, Primary infection	0.233	0.009	2.17	**0.027**	Sex, Male	0.881	0.047	3.35	**0.001**
Inf Status, Secondary infection	0.575	0.023	5.36	**0.001**	Residual	16.850	0.895	-	-
Sex, Male	0.323	0.013	3.01	**0.004**					
Residual	20.826	0.0817	-	-	Total	18.826	1.000	-	-
Total	25.495	1.000	-	-					
	PERMDISP: Bray-Curtis Ordination								
Groups	Sum sq		F	p value	Sum sq		F	p value	
Infection status	0.031		2.48	0.087	0.002		0.89	0.3467	
	PERMANOVA: Weighted Unifrac Ordination								
Model covariates	Sum Sq	R^2	F	p value	*Model covariates*	Sum Sq	R^2	F	p value
Diet, Supplemented: Inf Status, Primary infection	0.046	0.001	1.60	0.093	Diet, Supplemented: Inf Status, Uninfected	0.189	0.026	1.84	0.064
Diet, Supplemented: InfStatus, Secondary infection	0.054	0.001	1.90	0.061	Diet, Supplemented	0.145	0.020	1.41	0.154
Diet, Supplemented	0.615	0.093	21.46	**0.001**	Inf Status, Uninfected	0.128	0.017	1.24	0.245
Inf Status, Primary infection	0.062	0.001	2.15	**0.027**	Sex, Male	0.249	0.034	2.42	**0.012**
Inf Status, Secondary infection	0.175	0.026	6.09	**0.001**	Residual	6.586	0.903	-	-

Sex, Male	0.131	0.020	4.55	**0.002**					
Residual	5.562	0.837	-	-	Total	7.30	1.000	-	-
Total	6.643	1.000	-	-					

	PERMDISP: Weighted Unifrac Ordination					
Groups	Sum sq	F	p value	Sum sq	F	p value
Infection status	0.024	5.52	**0.005**	0.003	0.43	0.527

Total	**PERMANOVA:** Unweighted Unifrac Ordination								
Model covariates	Sum Sq	R^2	F	p value	*Model covariates*	Sum Sq	R^2	F	p value
Diet, Supplemented: Inf Status, Primary infection	0.045	0.004	1.00	0.401	Diet, Supplemented: Inf Status, Uninfected	0.081	0.013	0.90	0.680
Diet, Supplemented: InfStatus, Secondary infection	0.110	0.010	2.44	**0.014**	Diet, Supplemented	0.223	0.035	2.47	**0.001**
Diet, Supplemented	2.159	0.188	47.81	**0.001**	Inf Status, Uninfected	0.100	0.015	1.05	0.386
Inf Status, Primary infection	0.176	0.015	3.89	**0.002**	Sex, Male	0.226	0.035	2.49	**0.001**
Inf Status, Secondary infection	0.127	0.011	2.81	**0.008**	Residual	5.798	0.903	-	-
Sex, Male	0.107	0.001	2.37	**0.025**					
Residual	8.758	0.763	-	-	Total	6.424	1.000	-	-
Total	11.481	1.000	-	-					

	PERMDISP: Unweighted Unifrac Ordination					
Groups	Sum sq	F	p value	Sum sq	F	p value
Infection status	0.013	2.20	0.108	0.001	0.36	0.572

Chapter 5

5. General discussion

5.1. Thesis summary

This thesis aimed to explore the interaction between the gut microbiota, nutrition and helminth infection using wood mice *(Apodemus sylvaticus*) as a model species, in both the laboratory and wild environments. Using a unique lab-to-wild model, I characterised and compared the gut microbiota of wood mice with wild and lab mouse counterparts, to gain a greater understanding of how the environment shapes the gut microbiota. Moreover, I used a helminth perturbation experiment to determine how the gut microbiota and immune response of wood mice is altered throughout the course of a natural, co-evolved, parasite infection and to establish if infection outcome is microbiota mediated. Finally, I investigated the impact of supplemented nutrition during helminth infection in a paired laboratory and wild study, with specific focus on how diet and infection impact the gut microbiota and if this follows a similar pattern across environments.

In Chapter 2, I describe the establishment and maintenance of two wood mouse (*A. sylvaticus*) colonies that are differentiated only by their gut microbiota exposure. I then go on to characterise the gut microbiota of these wood mice cohorts and compare their microbiota diversity and composition with a cohort of wild, wood mice and a conventional strain of lab mice (*Mus musculus*). Here, I find that our Wild-like:As colony, which consists of wild-derived but now laboratory reared wood mice, maintain a wild-like gut microbiota, when compared to free-living wild wood mice (Wild:As) populations sampled across Scottish woodlands. Whilst Wild:As mice had a greater within sample diversity compared to Wild-like:As mice, there was a significant overlap in the composition of the gut microbiota of mice across these cohorts, as measured through beta-diversity distance metrics and differential abundance analysis. This highlights that whilst the Wild-like:As mice may have lost some rare bacterial taxa over the duration of their time housed under laboratory conditions, overall, they have a remarkably stable gut microbiota composition that still closely resembles their wild, free-living counterparts. Conversely, our second wood mouse colony, Lab-like:As,

consist of mice taken from our Wild-like:As colony but caesarean re-derived and cross-fostered by a conventional lab mouse strain (CD1: *M. musculus*), therefore, these Lab-like:As have been reared and thus, had environmental exposure to a CD1 mice gut microbiota. Interestingly, when we compared the gut microbiota of these Lab-like:As mice to both the Wild-like:As mice and a cohort of CD1 mice (Lab:Mm), we found that the composition of their microbiota was distinct from both these other mouse cohorts. Thus, suggesting the gut microbiota composition is driven by a combination of both environmental and genetic factors among these Lab-like wood mice.

In Chapter 3, I expand upon the work in Chapter 2, by assessing how the gut microbiota of three mouse cohorts; Wild-like:As, Lab-like:As and Lab:Mm responds to experimental perturbation via infection with the gastrointestinal helminth, *Heligmosomoides polygyrus,* which is a natural parasite of wood mice within the wild. I assessed how the diversity and stability of the microbiota composition was altered over the course of infection through alpha and beta diversity metrics, plus, differential abundance analysis of faecal samples collected pre-infection, 7 days post infection (p.i), 14 days p.i and 21 days p.i. Moreover, I assessed how infection dynamics differed between the three mouse cohorts, through (i) *H.polygyrus* worm burden counts in the small intestine, (ii) concentration of total faecal IgA and (iii) four commonly used histopathology metrics, at two key experimental time points, day 14 p.i (peak infection and day 21 p.i (post-peak infection).

Here, I found that *H.polygyrus* was able to establish patent infection in both Wild-like:As and Lab-like:As mice at day 14 p.i, whilst 50% of the mice in each of these cohorts had successfully cleared their infections by day 21 p.i. However, all mice among the Lab:Mm cohort, were able to clear their *H.polygyrus* burdens by both day 14 and 21 p.i. In general, Wild-like:As mice had significantly higher levels of total faecal IgA compared to the Lab-like:As mice throughout the duration of infection, and this appeared to be *H.polygyrus* driven, whereby Wild-like:As mice with a higher total number of *H. polygyrus* had higher IgA antibody concentrations. However, levels of pathology caused by *H.polygyrus* infection was similar between both wood mouse cohorts. In regard to the gut microbiota of the three mouse cohorts, we observed that whilst the Lab:Mm mice had the highest within sample diversity overall, this was stable over the duration of *H.polygyrus* exposure, whereas in Wild-like:As mice, within

sample diversity significantly increased over the duration of infection. Further, I found that the microbiota composition of both wood mice cohorts (Wild-like:As and Lab-like:As), was similar regardless of infection, but a decrease in the diversity of the microbiota composition of Wild-like:As mice was associated with *H.polygyrus* worm burden. Finally, I investigated the differential abundance of taxa within faecal samples at day 7, 14 and 21 p.i compared to samples collected pre-infection, of all three mouse cohorts. I found that across all cohorts, there was a trend of bacterial taxa being significantly depleted at each timepoint compared to pre-infection, suggesting that exposure to *H.polygyrus* leads to a depletion in specific bacterial taxa including *Staphylococcus* and *Jeotgalicoccus.*

In Chapter 4, I used faecal samples collected during a paired study, in which both a wild and laboratory population of wood mice received experimental supplementation with an enriched diet. Here, I investigated the interaction between diet-helminths-microbiota among wood mice across these two environmental settings. More specifically, I explored how dietary supplementation impacted the diversity and composition of the gut microbiota and determined if this was influenced by infection with *H.polygyrus* and if findings were consistent between the lab and the wild wood mouse populations. I found that the environment played a key role in shaping the gut microbiota of wood mice, explaining almost one quarter of the variation within the faecal samples and that wild wood mice had significantly higher levels of 21 bacterial taxa compared to wood mice reared within the lab. Next, I found that a higher quality, supplemented diet significantly increased the within sample diversity of wood mice microbiota in both the lab and wild, compared to mice given control diets. This finding was more pronounced among wood mice reared within the lab environment, however, I also observed that there were significant differences in the abundance of the same four bacterial taxa in both the lab and wild samples, in response to diet treatment regimens.

Upon examining the impact of *H.polygyrus* infection on the microbiota diversity and composition of wood mice, we found that within the lab environment, a primary infection led to a reduction of the within sample microbiota diversity. Although, this appeared to be mediated by diet, whereby mice on supplemented diet had increased alpha diversity during secondary *H.polygyrus* infection when compared to mice on

control diets. Among wood mice within the lab environment, differential abundance analysis highlighted that the specific microbiota composition was altered during *H.polygyrus* infection and that this was impacted by the diets mice received, whereby mice on a supplemented diet had increased abundance of several *Lachnospiraceae* genera and *Helicobacter.* Overall, *H.polygyrus* infection had a more subtle impact on the gut microbiota of wood mice within the wild population. Here, I found no statistical difference of the within sample diversity between infected and uninfected mice, but, when examining the microbiota composition, mice on a supplemented diet had significantly higher between sample diversity. Moreover, the differential abundance of specific bacterial taxa differed significantly between infected and uninfected wild wood mice and some of these differences were associated with a supplemented diet.

In this final chapter, I will discuss the broader impacts of my research in contributing to our understanding of how helminth infection and dietary changes can affect the gut microbiota of wood mice, in both a laboratory and wild context. In addition, I will discuss how our establishment of a lab-to-wild model system with wood mice provides a real-world, ecologically relevant model for future research, that allows for the controlled study of immunity and disease in a more natural and contextually relevant setting. I will also discuss the key limitations of the research presented throughout this thesis and the potential for future research that will expand upon and complement my findings.

5.2. Broader impacts

5.2.1. Naturalising mouse models

For almost 100 years, research using the lab mouse (*M. musculus*) as a model organism has been fundamental to our understanding of mammalian anatomy, disease pathophysiology and immunity (Ericsson et al. 2013; Morse 2007; Perlman 2016). Such studies have provided key insights that have aided the development of disease treatments, antimicrobial agents, and effective vaccines (Gaynes 2017; King 2012; Racaniello 2006; Zaragoza et al. 2011; Zhang et al. 2011). However, in many cases treatments that appear promising in pre-clinal mouse trials do not translate successfully to human trials. There are several factors that account for the lack of

translational success, however, differences between the mouse and human immune responses to exogenous stimuli undoubtably plays a large role here (Mestas & Hughes 2004). Unlike humans and other free-living mammalian populations, the lab mouse inhabits standardised, specific-pathogen free environments with minimal dietary variation, which is necessary to limit variation within and between experiments (Home Office 2014). However, this has resulted in lab mice developing a somewhat naïve immune system, in part due to lack of environmental antigenic and microbial exposure (Beura et al. 2016; Bowerman et al. 2021; Kohl et al. 2014; Thomson et al. 2022), whereas wild populations have much greater levels of immunophenotypic diversity (Graham 2021). Consequently, there has been a gradual shift among the research community to increase the ecological, genetic, and environmental variation within lab mouse studies and thus, to create a more naturalised mouse model.

As this remains an emerging field, there is not yet a consensus about the best way to achieve this, therefore there have been several different approaches adopted. These range from relatively simple changes such as the use of outbred mouse strains (Churchill et al. 2012; Whary et al. 2015), altering housing temperature or light/dark exposure (Ganeshan & Chawla 2017; Graham 2021; Karp 2012)and even encouraging increased movement (Goh & Ladiges 2015; Graham 2021; Knudsen et al. 2020; Meijer & Robbers 2014). More comprehensive approaches include, transfer of faecal material from wild-caught mice to lab-strains (Rosshart et al. 2017), co-housing conventional lab mice with mice bought from pet shops (Beura et al. 2016; Pierson et al. 2021) and infecting lab mice with viruses and parasites prior to vaccination (Reese et al. 2016). However, to date, the two most thorough approaches of creating more natural mouse models have been either through embryo transfers of lab mice into wild mice, creating a mouse cohort referred to as “wildlings” (Rosshart et al. 2019) or the process of “rewilding” lab mice by housing in outdoor enclosures for several months (Leung et al. 2018a). Both wildlings and rewilded mice have been shown to exhibit enhanced immune system maturation with more varied phenotypes and a more diverse gut microbiota (Leung et al. 2018a; Rosshart et al. 2019).

The research I conducted for Chapter 2 and 3 and the results presented, contributes to this body of work in a complementary, yet unique manner. To the best of my knowledge, the establishment of a wild-derived, laboratory-reared wood mouse

colony, that has been caesarean re-derived to a conventional lab-mouse strain, thus possessing a lab-like gut microbiota, is a first of its kind. In addition, the maintenance of a second wood mouse colony, consisting of mice with a wild-like gut microbiota, enables direct comparisons of wild, genetically outbred mice, under controlled laboratory conditions. In contrast to field studies within wild populations, this allows for the control of various environmental and ecological variables that can influence findings and make causal inferences difficult, such as differences in exposure to helminths, reproductive status, and life history. With these unique wood mouse colonies, I was able to elucidate how unique microbial exposures both shape the gut microbiota and impact the response to infection with a co-evolved helminth parasite among these mice. As such, these two wild-derived wood mouse colonies provide not only an additional approach to the creation of a more natural, lab-to-wild mouse model, but also provide a new resource for the research community allowing for the controlled study of immunity and disease in a more natural and contextually relevant setting.

5.2.2. Diet-helminth-microbiota interactions

To date, much of the current work investigating the complex interactions between diet, helminth infections and the gut microbiota have either studied just two factors at a time and/or used experimental studies in controlled, laboratory conditions or observational studies of humans. However, as the mammalian gastrointestinal tract is the site of diet-ingested nutrient absorption, whilst also home to the gut microbiota communities and a niche for intestinal helminths, it is almost inevitable that interactions between these three components of the gut ecosystem are occurring, and such interactions may have the potential to impact host health. For instance, these interactions may be particularly important in populations where both high levels of malnutrition and endemic helminth infections coincide.

As such, to gain a greater understanding of how these three factors interact and what consequences this may have for the host, we need conduct studies using a more holistic approach where all factors are considered and/or manipulated in a more real-world, ecologically relevant system. In Chapter 4, I was able to expand upon the handful of studies currently published within the literature exploring the three-way diet-

helminth-microbiota interactions. I used samples collected during our previous work in this system, whereby we experimentally manipulated both nutrition (with a high-quality whole-diet supplementation) and infection in either wild or a controlled laboratory experiment with H.*polygyrus.* Here, we found in both lab and wild environments, that mice given access to the supplemented high-quality diet were more resistant to *H. polygyrus* infection, shed fewer eggs, had higher parasite specific and nonspecific immunological responses and had higher efficacy when given anthelmintic drug treatment (Sweeny et al. 2021). As such, my work adds another piece of the puzzle to this study, whereby I characterise how diet and infection are impacting the gut microbiota and possibly driving diet-induced helminth resistance here. There have been a few studies recently that have also explored how these three factors - helminths, diet and the gut microbiota interact and potentially impact host health (Kang et al. 2021; Liu et al. 2020; Myhill et al. 2018). However, they have all been conducted in controlled settings, without incorporating the variation we see in hosts, the gut microbiota and environment of natural systems, therefore we provide novel findings with a novel approach to the literature here.

5.3. Methodological Limitations

For each data chapter within this thesis, to determine how the gut microbiota composition differed between mouse cohorts and experimental groups, I estimated beta diversity using three widely used ordination metrics, namely Bray-Curtis dissimilarly and Weighted and Unweighted UniFrac distances. To then test for statistically robust differences between the distance matrices generated for each mouse cohort or experimental group, I used permutational multivariate analysis of variance (PERMANOVA; (Anderson 2001)) for each beta diversity metric.

Whilst PERMANOVA is a robust and flexible analyses for testing differences between groups, as with any statistical analyses it has limitations (Anderson 2001; Anderson & Walsh 2013). For instance, PERMANOVA's currently do not allow for random effects within the analysis approach. An alternative restricted PERMANOVA can be used to account for this, however, when experimental groups have unequal sample sizes this cannot be used. Due to the study design of each of the data chapters within this thesis,

sample sizes often differed slightly between mouse cohorts and experimental groups and samples were often highly dispersed and not homogenous between groups. Therefore, restricted PERMANOVA's could not be fit and it is important to note that in such instances, psuedoreplication (in the form of multiple samples collected from the same individual mouse) could not be accounted for during these analyses. This has the potential to lead to an incorrect rejection of the null hypothesis, thus, Type 1 errors. For future analyses and to reach more robust conclusions regarding differences between experimental groups, more flexible models, such as Multiple Membership Multilevel Models, including Bayesian Regression Models (Bürkner 2018; Shafiei et al. 2015) or Multi-membership Linear Mixed Effects Models (Browne et al. 2001; van Paridon J 2023), should be used alongside or to replace PERMANOVAS. These are able to account for structural differences and repeated measured within the data and avoid Type 1 errors.

5.4. Concluding Remarks

Overall, this thesis both develops an exciting new lab-to-wild mouse model that will enable both mechanistic studies in the lab, and fitness-relevant experiments in the field to better understand the complex interactions between nutrition, infection, the gut microbiota, and health. Importantly, my results show that the gut microbiota is an important player in the gut ecosystem, and my results provide a greater understanding of how the interplay between nutrition, immunity and helminthiasis can impact host health and infection dynamics.

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