2022

The microbiome of the equine roundworm, *Parascaris* spp.

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Digital Object Identifier: https://doi.org/10.13023/etd.2022.254

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The microbiome of the equine roundworm, *Parascaris* spp.

DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Agriculture, Food and Environment
at the University of Kentucky

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ABSTRACT OF DISSERTATION

The microbiome of the equine roundworm, *Parascaris* spp.

Parasitic nematodes, including the large roundworms colloquially known as ascarids, affect the health and well-being of livestock animals worldwide. The equine ascarid, *Parascaris* spp., was the first ascarid parasite to develop wide-spread anthelmintic drug resistance, with other species slowly following suit. There are no new classes of anthelmintics currently in development, and a solution to the ever-increasing prevalence of resistance is desperately needed. The microbiome has been shown to be an important factor in the fitness and health of many organisms and changes to microbiome composition have been associated with a plethora of diseases. The microbiome is also important to the health of parasitic nematodes, and the endosymbiotic bacterium *Wolbachia*, whose presence is essential for the viability of filarial nematodes, has been exploited for treatment of filariasis in humans by using both broad-range and, more recently, specific anti-*Wolbachial* antimicrobial treatments. Despite this success, parasite microbiomes are understudied. The overarching goal of this dissertation was to characterize the microbiome of *Parascaris* spp. by identifying a common core microbiota, by comparing microbiota diversity metrics for the whole worm at different life stages and in individual organs in male and female parasites, and by assessing the female gonad microbiota in greater detail.

Worms, along with jejunal content samples, were collected from foals at necropsy and used for both the whole worm study, which utilized a total of 27 parasites (9 male, 9 female, 9 immature), and in the organ study, which utilized a total of 46 adult parasites (24 male, 22 female). DNA extracted from these samples was used to produce a library using a 16S rRNA metagenomic sequencing protocol, and this library was sequenced using the Illumina MiSeq platform. A bioinformatics pipeline was developed to identify taxa and their relative abundance in the samples, and subsequent data analysis was carried out using R packages including Vegan, DESeq2, corncob, metagenomeSeq, and ANCOM.BC. The 22 female gonad samples were further analyzed using next generation metagenomic sequencing following the same protocol as the other two studies, and then using a kit that targeted to multiple regions and that allowing consensus sequences to be assembled. Additionally, another female worm was also collected, immediately fixed, dissected, and submitted for sectioning and examination by transmission electron microscopy.

A common core microbiota consisting of eleven genera was established for *Parascaris* spp. and consisted of: *Acinetobacter*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (ANPR), *Clostridium senso stricto 1*, *Gemella*, *Janthinobacterium*, *Lactobacillus*, *Reyranella*, *Sarcina*, *Sphingomonas*, *Streptococcus*, and *Veillonella*. When comparing organs, *Veillonella* was differentially abundant when using DESeq2 and ANCOM-BC ($p < 0.0001$), corncob ($p = 0.0008$), and metagenomeSeq ($p = 0.0118$) and *Sarcina* was differentially abundant across all four analytical methods ($p < 0.0001$).

Alpha and beta diversity for the whole worm microbiota was similar across groups for all three taxonomic levels. Alpha diversity for the organ microbiota was significantly different based upon both sex and location at all three taxonomic levels. Simpson alpha diversity was significantly different between the female intestine (FI) and male gonad (MG) at the phylum ($p < 0.0001$), family ($p = 0.0058$) and genus ($p = 0.0018$) levels, and between both the female gonad (FG) and FI ($p < 0.0001$) and the FI and male intestine (MI; $p = 0.0072$) at the phylum level. Shannon alpha diversity was significantly different

between the FI and the FG ($p < 0.0001$), the horse jejunum (HJ; $p = 0.0483$), the MG ($p < 0.0001$) and the MI ($p = 0.0007$) at the phylum level, between the FI and MG ($p = 0.0003$) at the family level and between the FG and MG ($p = 0.0130$), the FI and HJ ($p = 0.0383$) and the FI and MG ($p = 0.0001$) at the genus level. Beta diversity was significantly different between FI and FG ($p = 0.0377$) at the phylum level, MG and FG ($p = 0.0010$), FI ($p = 0.0174$), and HJ ($p = 0430$), and FG and MI ($p = 0.0061$) at the family level, and MG and FG ($p = 0.0006$), MI and FG ($p = 0.0093$), and MG and FI ($p = 0.0041$) at the genus level.

Twelve species were identified in the female gonad, and phylogenetic trees were created for the genera *Aminobacter*, *Reyranella*, *Limosilactobacillus* and *Ligilactobacillus*. Cladograms indicated that consensus sequences from members of these genera were related to species found in soil and water, and to those that had previously been found in horses, and thus the presence of related bacteria in parasites makes biological sense. Finally, morphological structures identified as candidate bacteria were found in the cells of *Parascaris* spp. female gonad sections, indicating that there are also possibly endosymbionts associated with these parasites.

In summary, the overarching goal of this research was met. A common core microbiota was established for *Parascaris* spp., diversity metrics were compared for different life stages and organs, and the female gonad was explored in more detail. This research lays the groundwork for future studies involving the *Parascaris* spp. microbiome and provides more data to the effort to understand parasite microbiomes.

KEYWORDS: Microbiome, *Parascaris*, equine parasite control, transmission electron microscopy, nematode

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DEDICATION

To my husband, Chris, for his unending support, brainstorming sessions, and always knowing how to make me laugh.

ACKNOWLEDGMENTS

Science is a collaborative endeavor, and without a supportive, intelligent, and overwhelmingly helpful group of people, the following work would not have been possible. I am grateful to my advisor and dissertation chair, Dr. Martin K. Nielsen, for his unwavering support and allowing me to explore new areas of research in our field, continually propose ideas for research projects, write grant proposals, and present at conferences. The Nielsen Laboratory is an incredible group of people, and I am grateful to them – particularly Nichol Ripley, Constance Finnerty, Jamie Norris, and Holli Gravatte – for all of their help, support, lunch dates, and words of encouragement.

I also want to thank the groups that have supported this project both technically and financially. First, the University of Kentucky Core facilities for all of their technical support and guidance throughout this project, Dr. John Shields at the University of Georgia for his expertise, and the faculty and staff at the University of Kentucky Veterinary Diagnostic Laboratory; this thesis would not have been possible without them. Generous financial support from the National Center for Veterinary Parasitology and Zoetis, LLC made all of this work possible. Finally, I want to thank the horses and the University of Kentucky farm crew. None of this work would have happened without the sacrifice of many foals to collect samples, and the farm crew who tirelessly cares for these horses, and who helped me when I really needed it.

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CHAPTER 1. INTRODUCTION

Equids have been an important partner to humans for over 32,000 years (Valladas et al., 2001) as food sources, a means of transportation, agricultural tools, weapons of warfare and, more recently, as sporting partners and companions. There is an estimated horse population of 58 million globally (Gilbert et al., 2018), with over 7.2 million of those residing in the United States, where they have an economic impact of $122 billion (American Horse Council Foundation, 2018). The health and welfare of such impactful animals is paramount and involves many facets, one of which is parasite control. There are four major groups of parasitic nematodes affecting horses globally: cyathostomins, large strongyles, ascarids, and anoplocephalids. These parasites can cause a range of clinical symptoms including diarrhea, weight loss, various types of colic, and in rare cases, death (Love, 1992; Cribb et al., 2006; Getachew et al., 2008; Trotz-Williams et al., 2008; Getachew et al., 2010; Ghelen et al., 2020).

Anthelmintic treatments were first developed at the turn of the 20th century to treat human hookworms and involved a variety of options ranging from Epsom salts to dyes and other synthetic compounds, many of which caused devastating side effects such as blindness, diarrhea, organ damage, and even death (Faust, 1937; Horton, 2003). The major nematocidal anthelmintic classes currently used in horses are benzimidazoles, introduced in 1961 (Brown et al., 1961), tetrahydropyrimidines, introduced in 1966 (Austin et al., 1966), and macrocyclic lactones, introduced in 1980 (Campbell et al., 1983).

Anthelmintic resistance quickly developed in parasites affecting agricultural animals; in some cases, within only three years of a drug first entering the market (Drudge et al., 1964; Kotze & Prichard, 2016). Equine cyathostomins and ascarids both exhibit widespread resistance to at least one available drug class, and resistance is emerging to all three. Cyathostomin resistance to phenothiazine was first reported in 1960 (Gibson, 1960), benzimidazoles in 1965 (Drudge & Lyons, 1965), tetrahydropyrimidines in 1996 (Chapman et al., 1996), and macrocyclic lactones in 2008 (Molento et al., 2008). Equine ascarid resistance to macrocyclic lactones was first reported in 2002 (Boersema et al., 2002), tetrahydropyrimidines in 2007 (Craig et al., 2007), and benzimidazoles in 2014 (Armstrong et al., 2014). Clearly, anthelmintic resistance in equine parasites is a serious issue, but despite its ever-increasing prevalence, no new anthelmintic drugs have been introduced since 1992 and no new drug classes since 1981. There are no drugs currently being developed for commercial use.

Drug development can take decades, and it is therefore imperative to search for new treatment options now, when at least some of the currently available options still have some efficacy. The microbiome has become a focus within human medicine over the past decade and has now been characterized for various body sites within humans, as well as in some animals, plants, and the environment. The microbiome is associated with various health outcomes in humans (Zheng et al., 2016; Ni et al., 2017; Helmink et al., 2019) and animals (Kennedy et al., 2016; Lima et al., 2016). As a result, there has been a push towards investigating microbiome manipulation for environmental, health, and agricultural purposes (Correa-García et al., 2018; Clemmons et al., 2019; Rosado et al., 2019; Song et al., 2019; Deng et al., 2021; Peixoto et al., 2021; Santoro et al., 2021). The endosymbiont *Wolbachia,* found in filarial nematodes, is perhaps the most relevant with respect to the potential of the microbiome as an anthelmintic drug target in parasites. These bacteria play an important role in parasite survival and reproduction (Hoerauf et al., 2000; Casiraghi et al., 2002; Hoerauf et al., 2003; Arumugam et al., 2008; Mand et al., 2009; Landmann et al., 2011 Foray et al., 2018), an observation that led to the development of anti-*Wolbachia* drugs that target the bacteria and thereby kill their parasitic host. This important case highlights the potential importance of the parasite microbiome and the role that it might play for the development of future anthelmintic treatments.

This dissertation focuses on the equine ascarid, *Parascaris* spp., and on characterizing its microbiome. This type of foundational research could, by seeking to understand the basic biology of the organism, provide the necessary background information for the development of parasite control options in the future. First, background literature on *Parascaris* spp. will be reviewed in order to highlight the lack of understanding, not only of basic biology of this parasite, but also its anthelmintic resistance. Next, helminth microbiomes and the generation and analysis of microbiome data will be discussed in order to set the scene for the research projects presented herein. Finally, research projects characterizing the *Parascaris* spp. microbiome, and the implications of their results, will be described.

1.1 Aims and Hypotheses

The objective of this research was to characterize the microbiome of *Parascaris* spp. in order to increase the knowledge base of parasite microbiomes. Such knowledge could provide a first step towards understanding the microbial community that may play an important role in parasite health and so could be a potential target for the development

of novel anthelmintic treatments. In order to achieve this objective, the following specific aims (SA) and hypotheses (H) were addressed:

SA 1: Determine the common core microbiome associated with *Parascaris* spp. at different life stages and between sexes.

H 1.1: There are bacteria taxa within the *Parascaris* spp. microbiome that are present in all groups within the study population.

SA 2: Compare microbiome diversity metrics for the whole worm at different life stages and between adult individual organs and sexes.

H 2.2: Alpha diversity is higher in the equine jejunum contents than within the parasites.

H 2.2: Beta diversity dissimilarity is high between *Parascaris* spp. life stages.

H 2.3: Alpha diversity is higher in the intestine than the gonad in both male and female parasites.

H 2.4: Beta diversity dissimilarity is high between *Parascaris* spp. gonads and intestines.

SA 3: Determine differentially abundant bacterial genera between groups for the whole worm at different life stages and between individual organs in members of both sexes.

H 3.1: There are bacterial genera that are differentially abundant in the whole worm microbiomes between groups.

H 3.2: There are bacterial genera that are differentially abundant in the organ microbiomes between groups.

SA 4: Assess the female *Parascaris* spp. gonad microbiome with higher resolution by identifying bacterial species present with this organ and determining whether their presence makes biological sense.

H 4.1: Bacterial species found in the *Parascaris* spp. female gonad will make biological sense based upon parasite and host life cycles and feeding habits and upon previous microbiome studies.

SA 5: Visualize bacteria within cells of the *Parascaris* spp. female gonad using transmission electron microscopy (TEM).

H 5.1: Endosymbiotic bacteria are located within the cells of the *Parascaris* spp. female gonad.

CHAPTER 2. THE EQUINE ASCARID: *PARASCARIS* SPP.

2.1 Ascarid Parasites

2.1.1 A brief history

Helminth parasites have been known to humans for millennia. The Egyptian Ebers Papyrus, circa 1550 BCE, refers to intestinal worms; the Greeks wrote about helminths infecting other species; and the Romans clearly described *Ascaris* parasites, including symptoms of clinical disease (Cox, 2002). Carl Linnaeus described and named six helminths in 1758, including *A. lumbricoides*, which ultimately led to an increasing number of helminths being described and formally named (Cox, 2002).

Equine ascarids in particular have an important place in the history of scientific discovery. German zoologist Johann August Ephraim Goeze was the first to name *Ascaris equorum* in 1782. Several decades later in 1824, Hippolyte Cloquet coined the name *Ascaris megalocephala*, which was commonly used for the equine ascarid throughout the late 1800s and early 1900s. The genus name *Parascaris* was introduced in 1926, leading to the modern name *Parascaris equorum* (Yorke & Maplestone, 1926). Belgian embryologist Édouard van Beneden used *Ascaris megalocephala* as a model species and showed that fertilization consisted of the union of haploid gametes to form a diploid zygote with a full set of chromosomes, and that chromosome number is consistent for every cell within a species (van Beneden, 1883; Hamoir, 1992). Over the course of the following five years, at least twenty-seven papers – four by van Beneden – were published featuring the use of *A. megalocephala* for studying phenomena such as cell division, chromosome organization, and chromatin diminution (Boveri, 1887; Boveri, 1888).

2.1.2 Notable species

Ascarid parasites belong to the order Ascaridida and infect a wide range of hosts, causing clinical disease. Many species are also zoonotic, infection with which has serious consequences to human health. Important members of this order include: ascarids of poultry, *Ascaridia galli* and *Heterakis gallinarum*; fish, *Anisakis* sp.; canids, *Toxocara canis*; felids, *Toxocara cati*; felids and canids, *Toxascaris leonina*; cattle, *Toxocara vitulorum*; mustelids and bears, *Baylisascaris* spp.; pigs, *Ascaris suum*; and humans, *Ascaris lumbricoides*. Many of these parasites can cause severe clinical disease including high mortality in cattle (Borgsteede et al., 1992; Gundran & More, 1999; Chelladurai et

al., 2015), loss of appetite and weight, anorexia, depression, and increased mortality in chickens (Kaufmann et al., 2011; Thapa et al., 2015; Sharma et al., 2019), and pneumonitis, dyspnea, and coughing in pigs (Yoshihara et al., 1983; Curtis et al., 1987; Stewart & Hale, 1988; Holland, 2013; Mateus et al., 2015). All of these pathologies can lead to millions of dollars in agricultural economic losses. An estimated 807 million to 1.2 billion humans globally are infected with *A. lumbricoides*, many of them children in impoverished countries, causing retardation of physical and mental growth, pneumonia, asthma, abdominal distension, intestinal obstruction, pancreatitis, and death (Bethony et al., 2006). Zoonotic infections can also lead to a condition known as visceral larva migrans, where the parasite larvae migrate through the intestinal wall but are unable to complete their life cycle. Cases have been reported in *Toxocara cati* (Eberhard & Alfano, 1998; Zibaei et al., 2014), *Toxocara canis* (Hill et al., 1985; Xinou et al., 2003; Gakosso et al., 2020), *Ascaris suum* (Volk & Tormey, 2017; Avery et al., 2018), *Baylisascaris* (Saffra et al., 2010; Kelly et al., 2012), and *Anisakis* (Kojima et al., 2013; Sohn et al., 2015), resulting in various clinical manifestations including coughing, rash, myalgia, liver lesions, myocarditis, visual impairment, neurological symptoms, and, in rare cases, death.

2.1.3 Introduction to *Parascaris*

The equine ascarids, *Parascaris* spp., are considered the most pathogenic parasites infecting juvenile horses globally and can cause coughing, nasal discharge, lethargy, poor appetite, diarrhea and colic (Reinemeyer, 2009; Nielsen, 2016). Fibrotic liver lesions (Brown & Clayton, 1979), lung lesions, hyperpnea, bronchiolitis, and lobular pneumonia (Clayton & Duncan, 1978; Nicholls et al., 1978) have been reported in experimentally infected foals. Poor body condition has been associated with *Parascaris* spp. infection in working equids (Getachew et al., 2008; Getachew et al., 2010), however infected foals raised under parasite management programs have not exhibited these signs in recent studies (Bellaw et al., 2016; Nielsen et al., 2021). Small intestine impaction is one of the largest concerns with this parasite, which often requires hospitalization and surgery, and can ultimately lead to death (Southwood et al., 1996; Cribb et al., 2006; Tatz et al., 2012; Nielsen, 2016). In 37 published cases where surgical intervention was necessary for impaction colic due to *Parascaris* spp., 31 horses survived until discharge, but only 11 survived more than one year (Nielsen, 2016). While the cause of death was not confirmed in these cases, long-term complications resulting from surgery may have contributed to mortality (Santschi et al., 2000; van Loon et al., 2020). Losing young horses

results in a direct financial loss from veterinary care and breeding fees, future losses in sales prices, which can be tens of thousands to millions of dollars, competitive winnings, and stud fees, as well as an emotional loss for the owners and caretakers associated with that horse.

2.2 Basic Biology

Two variants of *Parascaris* were described in the late 1800s and distinguished from one another by counting the number of metaphase chromosomes present in eggs prior to the first cellular division, a process known as karyotyping (van Beneden, 1883; Carnoy, 1886; Boveri, 1887; Carnoy, 1887). The karyotype of *Ascaris megalocephala univalens* was initially described in cytogenetic studies by van Beneden (van Beneden, 1883), and of *A. meg. bivalens* by Jean Baptiste Carnoy (Carnoy, 1886; Carnoy, 1887), but it was not until a few years later that Oskar Hertwig recognized them as different species (Hertwig, 1890). These species received their current names in 1978: *Parascaris univalens*, which has one chromosome pair, and *P. equorum*, which has two pairs of chromosomes (Bullini et al., 1978; Goday & Pimpinelli, 1986). Hybrids between these two species have been described (Bullini et al., 1978; Goday & Pimpinelli, 1986), although they are sterile (Goday & Pimpinelli, 1986). Another species with three pairs of chromosomes, *P. trivalens*, was described in the 1930s (Li, 1937; Tchou, 1937), but has not been described in the literature since.

The *Parascaris* species can be distinguished morphologically only by a slight difference in their spiculae, with *P. univalens* having a distally truncated spicula and *P. equorum* having a distally rounded spicula (Biocca et al., 1978). There are two additional methods that have been used in the past to distinguish the two species of *Parascaris* from one another. The first is via karyotyping primordial germ cells prior to the first cell division, which is an arduous process that requires collecting either parasites with germ cells in the proper stage (Goday & Pimpinelli, 1986) or eggs from feces at the first mitotic division (Nielsen et al., 2014; Martin et al., 2018), and the second utilizes electrophoresis of twenty-seven enzyme loci, although this method has only been employed for a single study (Bullini et al., 1978). Karyotyping is a challenging process because it requires either live parasites or viable eggs that have yet to start developing, and due to this it is rarely performed for parasitological studies utilizing equine ascarids.

In the nearly 100 years between Hertwig first naming the two species and their modern name assignment in 1978, *P. univalens* and *P. equorum* were not recognized as

separate species and were instead regarded as different variants of *P. equorum* (Lin, 1954), which may explain why *P. equorum* was thought to be the only *Parascaris* species. This ultimately led to *P. equorum* being the only species mentioned in veterinary textbooks and research for decades, with the only recognition of the existence of two species occurring in texts on cell biology and cytogenetics, highlighting a lack of communication between disciplines. The last positive identification of *P*. *equorum* via karyotyping was in 1986 (Pimpinelli & Goday, 1986), despite contemporary recognition of two species and an increased effort to karyotype specimens.

2.2.1 Phylogenetics

The phylum Nematoda consists of over 22,000 named species separated into five distinct clades (Blaxter et al., 1998; Blaxter & Koutsovoulos, 2015). The ascarid parasites fall under Clade III, which also includes pinworms, filarial nematodes, and parasites of millipedes (Blaxter et al., 1998). Within the Ascaridoidea superfamily, *Parascaris* spp. belongs to the monophyletic clade of Ascarididae along with *Baylisascaris* spp., *Toxoascaris leonina*, and *Ascaris* spp. (Nadler, 1987; Nadler & Hudspeth, 2000; Liu et al., 2016; Li et al., 2018). Parasitism in the Ascaridoidea include prehistoric host-type switches correlated with global changes in sea level (Li et al., 2018), and tissue parasitism within Clade III evolved separately at least three different times (Nadler et al., 2007). Understanding these evolutionary relationships within Clade III and the Ascarididae superfamily provides important context when comparing these parasites to other groups within the Nematoda.

Some other well-known and heavily studied species such as the model organism *Caenorhabditis elegans*, a free-living nematode, and *Haemonchus contortus*, the most pathogenic and economically significant nematode parasite of small ruminants and model organism for parasitic nematodes, fall under Clade V (Blaxter et al., 1998). Anthelmintic resistance is rampant in *H. contortus* and it has therefore been heavily studied (Kotze et al., 2014; Kotze & Prichard, 2016), along with substantial research on the same topic in *C. elegans* (Geary & Thompson, 2001; Kotze et al., 2014). This means that much of the research conducted regarding anthelmintic resistance that will be described in subsequent sections has been broadly applied to ascarids despite the work having been conducted in organisms belonging to a completely different clade. The evolutionary distance between *Parascaris* spp., *C. elegans*, and *H. contortus,* combined with distinct

differences in life cycle and parasitism, suggest that making direct comparisons and broadly applying information from one clade to another should be done with caution.

2.2.2 Life cycle

Parascaris spp. are robust, cream-colored nematode parasites with a direct life cycle whose adult stages occur primarily within the small intestine of equids. Females are typically 10 – 20 cm in length with a diameter of 5 mm, whereas their smaller male counterparts are 10 – 15 cm in length with a 3 mm diameter (Wells, 1924; Clayton & Duncan, 1979a). Males can be distinguished from females of a similar size by a curl at the posterior end and a lack of visible ovaries through the cuticle (Wells, 1924).

Adult *Parascaris* spp. reproduce sexually in the small intestine via genital pores, and females lay their 90 – 100 µm eggs in small intestinal content, from whence they are excreted into the environment (Wells, 1924). On pasture, the fertilized parasite eggs embryonate and larvae develop within the egg; it is this egg containing a second/third stage larva that is infective (Wells, 1924; Clayton & Duncan, 1979a). Once ingested by a foal, the eggs hatch in the small intestine and the larvae penetrate the intestinal wall, where they subsequently migrate to the liver within a week of initial infection (Clayton & Duncan, 1979a). Within two weeks after initial infection, the larvae enter the lungs via the pulmonary circulation, where they emerge from arterioles and capillaries. The larvae are coughed up and then swallowed by the foal, making their way back to the small intestine two to three weeks after initial infection (Clayton & Duncan, 1979a). At this stage, *Parascaris* spp. larvae are approximately 2 – 4 mm in length and over the next 4.5 months will grow 70 – 80x in size as they feed on intestinal content and mature to adults (Clayton & Duncan, 1979a). While the general life cycle of *Parascaris* spp. has been described, the biological reason for larval migration is poorly understood. It has been suggested that tissue migration may be linked to increased body size and faster growth (Read & Skorping, 1995) and may also play a role in immune system evasion (Mulcahy et al., 2005; Deslyper et al., 2016; Deslyper et al., 2019). This, however, has not been directly studied in *Parascaris* spp., and no biological signals that may be required for parasite maturation have been identified. Identifying such signals would provide valuable insight into parasite biology, possible control mechanisms, and conditions necessary to enable *in vitro* culture from egg to adult.

2.2.3 *In vitro* culturing

Helminth parasites can be difficult to maintain and grow *in vitro* because of their sometimes-complicated life cycle that is reliant on the correct host. This makes research problematic, particularly in the case of species whose hosts are either considered unethical to use as research subjects, such as humans, or too difficult to maintain as a research population, whether because of size, expense, or husbandry requirements. In the case of *Parascaris* spp., equine research herds are expensive to maintain and require a large amount of land. Furthermore, obtaining adult parasites necessitates euthanasia of healthy foals that require eleven months gestation and another approximate five months before adult parasites can be harvested. There is only one research herd known globally that is regularly used for this purpose (Lyons et al., 1990), and so many specimens are obtained elsewhere from sources such as abattoirs (Janssen et al., 2013; Martin et al., 2020b; Martin et al., 2021a; Trailovic et al., 2021) or collected opportunistically at diagnostic necropsies (Burk et al., 2014; Burk et al., 2016; Rakhshandehroo et al., 2016; Malekpour et al., 2019). Adult *Parascaris* spp. can be maintained *in vitro* for up to a week (Janssen et al., 2013; Scare et al., 2019; Martin et al., 2021a) but exhibit transcriptional stress responses to culture conditions within the first 24 hours compared to non-cultured worms, which has been demonstrated by alterations in gene expression patterns (Martin et al., 2020b). Adult *Parascaris* in general do not maintain their fitness well in culture, as evidenced by their short survival time and stress responses, compared to their *Ascaris suum* counterparts, which can be kept alive *in vitro* for at least two weeks (Islam et al., 2004). Despite these challenges, a *Parascaris* spp. fitness scoring system has been developed (Scare et al., 2019), and meaningful gene expression data have been obtained from current *in vitro* systems (Janssen et al., 2013; Scare et al., 2020; Martin et al., 2021a).

L2/L3 larvae can be hatched from eggs (Burk et al., 2014; Rakhshandehroo et al., 2017; Martin et al., 2021a), which does not require sacrificing a horse, although they cannot be grown into L4 and L5 larvae or adults. The longevity of these larvae in culture has not been reported, but they have been kept alive for at least 48 hours (Martin et al., 2021a) and have been used in drug exposure (Rakhshandehroo et al., 2017; Martin et al., 2021a) and immunology (Burk et al., 2014; Burk et al., 2016) studies. Differences in gene expression of transport proteins between adults and larvae hatched from eggs *in vitro* (Martin et al., 2021a) must be considered when interpreting data and comparing results between life stages, but the larval culturing system is a promising path forward in

Parascaris spp. research because it allows research to take place without sacrificing young horses.

2.2.4 Genetics

Genetic studies opened the door to the contemporary recognition of two distinct *Parascaris* species, although their earlier classification as variants named *Parascaris equorum* and *Parascaris equorum univalens* led to the majority of specimens in veterinary parasitology research being referred to as *Parascaris equorum*. In fact, one study utilizing electrophoresis of enzyme loci demonstrated that the opposite may be true, with 93.5% of 2238 specimens collected in an abattoir being identified as *P. univalens* (Bullini et al., 1978). This study, however, was published in Italian, and appears to have gone unnoticed for several decades. A more recent population genetics study compared equine ascarid specimens from Sweden, Norway, Germany, Iceland, Brazil, and the United States and found that all of the parasites were genetically homogenous (Tydén et al., 2013b). One of the study populations was later karyotyped and found to consist only of *P. univalens* (Nielsen et al., 2014). Additional populations in the United States (Nielsen et al., 2014), Sweden (Martin et al., 2018), Iceland (Martin et al., 2021c), and China (Han et al., 2022) were karyotyped and identified as *P. univalens*. Taken together, these studies suggest that the main species present in domestic horses globally is *P. univalens* and not *P. equorum*.

A phylogenetic analysis of *Parascaris* spp. parasites from the mountain zebra (*Equus zebra*), domestic horse, and wild ass (*Equus asinus*) using the mitochondrial genes *cox1* and *nadh1*, demonstrated that the worms from *E. asinus* formed a distinct clade compared to specimens collected from the other two *Equus* species (Peng et al., 2019). A recent whole-genome study of specimens from horses, donkeys (*Equus africanus asinus*), and zebras also indicated distinct clades for *P. univalens* specimens found within horses and those found in zebras and donkeys (Han et al. 2022). Another recent study completed a phylogenetic analysis for a select group of nuclear and mitochondrial genes across almost all *Parascaris* spp. deoxyribonucleic acid (DNA) sequences from GenBank along with data from karyotyped specimens confirmed to be *P. univalens*, and found a small group of sequences, all from parasites collected from donkeys on a single farm in China, that formed a cluster (von Samson-Himmelstjerna et al., 2021a). Due to the genetic distance between *P. univalens* specimens from North America and Europe, the specimens in this cluster may represent another genotype or

species of *Parascaris* (von Samson-Himmelstjerna et al., 2021a). This cluster could represent *P. equorum*, or even another species such as *P. trivalens*, which has only been described in a couple of studies of parasites from Chinese horses (Li, 1937; Tchou, 1937).

The first *Parascaris* spp. draft genome was published in 2017 for *P. univalens* followed by a draft genome for *P. equorum* in 2019, and both indicated the presence of over 14,000 coding genes in the parasites (Wang et al. 2017; International Helminth Genomes Consortium, 2019). When considering these genomes, however, the *Parascaris* species conundrum must be taken into consideration. The specimen reported to be *P. equorum* and included in the 50 Helminth Genomes Project was collected at necropsy from an abattoir and there is no indication that karyotyping was performed to positively identify the species (International Helminth Genomes Consortium, 2019). The previously described phylogenetic study using *Parascaris* sequences from GenBank along with karyotyped specimens indicated that nearly every sequence for the internal transcribed spacer (ITS) -1 and -2 and for cytochrome oxidase I labeled as *P. equorum* clustered with confirmed *P. univalens* specimens, indicating that they are likely all from *P. univalens* (von Samson-Himmelstjerna et al., 2021a). This information combined with the lack of karyotyping, previous research suggesting that *P. univalens* is the predominant species in domestic horses, and the fact that *P. equorum* has not been identified via karyotyping since 1986 (Pimpinelli & Goday, 1986), suggest that the specimen in WormBase ParaSite, along with many other data deposits in GenBank labelled as *P. equorum,* may well be *P. univalens* (Nielsen et al., 2014; International Helminth Genomes Consortium, 2019; von Samson-Himmelstjerna et al., 2021a). Currently, there are no GenBank deposits verified as *P. equorum* via karyotyping.

Incorrectly identified information in public repositories is detrimental to the field and can lead to misinterpretation of results. For example, one study comparing *Parascaris* mitochondrial genomes utilized fresh specimens that were not karyotyped but assumed to be *P*. *equorum* (Gao et al., 2019). These specimens were then compared to GenBank deposits of mitochondrial genomes from two karyotyped *P. univalens* isolates, and one non-karyotyped isolate assumed to be *P. equorum*. The subsequent phylogenetic analysis clustered these four specimens into a single clade, and the authors concluded that *P. equorum* and *P. univalens* may represent the same species (Gao et al. 2019). This, however, is inaccurate given that no attempt was made to identify the collected specimen to species. Instead, the clustering with the two identified specimens strongly suggests that the collected specimen was *P*. *univalens*. Correctly identifying species is

important not only for ensuring accurate results when performing future genome-wide research studies in a variety of disciplines, including the study of anthelmintic resistance, but also when developing molecular techniques, such as PCR, to identify *Parascaris* specimens to species (Doyle & Cotton, 2019; von Samson-Himmelstjerna et al., 2021a).

2.2.5 Chromatin diminution

After the first cell division, *Parascaris* spp. presomatic cells go through a process called chromatin diminution where chromosomes are fragmented and approximately 85% of the germline genome is eliminated, resulting in the creation of about 35 smaller chromosomes (Boveri, 1887; Goday & Pimpinelli, 1986; Muller & Tobler 2000; Niedermaier & Moritz 2000). The initial discovery of chromatin diminution was made with *Parascaris* spp. in 1887 and was later found to occur in other nematodes including *Ascaris suum*, *A. lumbricoides*, and *Toxocara* spp., as well as in copepods, ciliates, hagfish, lamprey, and rat fish (Boveri, 1887; Wang & Davis, 2014). The *P. univalens* germline genome has an estimated 2500 megabases (Mb), whereas the somatic genome has an estimated 250 Mb, indicating a large loss of genetic information in an organism with only a single chromosome (Wang et al., 2017). Comparisons between *Parascaris* spp. and *Ascaris* spp. indicate that the mechanism for chromatin diminution is evolutionarily conserved between the two species and so was likely present in a common ancestor (Bachmann-Waldmann et al., 2004). Comparative analysis of *Parascaris*, *Ascaris*, and *Toxocara* genomes has shown that somewhere between 1000 and 2000 genes are eliminated in chromatin diminution, with 35% of those being expressed during spermatogenesis; it has therefore been hypothesized that diminution facilitates rapid adaptation and evolutionary change in the testes without causing deleterious effects in adult worms because those genes are silenced and eliminated (Bachmann-Waldmann et al., 2004; Wang et al., 2017; Wang, 2021).

Ultimately, the process of chromatic diminution, despite its resultant large loss of genetic information, must be evolutionarily advantageous for *Parascaris* spp. It is possible that chromatin diminution helps prevent events such as population bottlenecking due to the ability of the parasites to undergo rapid evolutionary changes in the germ line and may even have played a role in the evolution of parasitism (Bachmann-Waldmann et al., 2004). Evidence from cytogenetic studies suggests that there are differences in chromosome and heterochromatin organization between the two *Parascaris* species (Goday & Pimpinelli, 1984; Goday et al., 1985; Goday & Pimpinelli, 1986). It remains to

be seen how this process differs molecularly and on a whole-genome level between the two species, particularly since the last karyotyped *P. equorum* specimen was identified in 1986, two years before the method for PCR identification was first published and six years before the first ever whole genome sequence was completed (Pimpinelli & Goday, 1986; Mullis & Faloona, 1987; Fleischmann et al., 1995). If *P. equorum* was out-competed by *P. univalens* due to a fitness disadvantage as anthelmintic use became more prevalent, understanding chromatin diminution and comparing the two species in this respect could be an important key to understanding anthelmintic resistance development in ascarid parasites (von Samson-Himmelstjerna et al., 2021a).

2.2.6 Immunology

With a few exceptions, such as adult horses in tropical regions and donkeys (Vercruysse et al., 1986; Getachew et al. 2008; Getachew et al. 2010; Lem et al. 2012), *Parascaris* spp. are generally found in juvenile horses up until the age of six to eight months when an age-dependent immunity develops (Clayton & Duncan, 1979b; Fabiani et al., 2016). Fecal egg shedding and worm counts in juvenile horses occur in an age-dependent manner, and in older foals, fewer larvae reach the small intestine, and so patent infections are less likely to develop, and fecal egg counts are lower (Clayton & Duncan, 1979b; Donoghue et al., 2015; Fabiani et al., 2016). In a study where eight worm-free foals and two yearlings were experimentally infected with *Parascaris* spp., yearlings had a more severe respiratory response but maintained their body condition, whereas foals had a mild respiratory response and lost body condition, suggesting an age-dependent immune response despite the small sample size (Clayton & Duncan, 1978). Increased titers of antibodies to whole-worm antigens have been shown to correlate with foal age and subsequent reduction in parasite prevalence (Bello, 1985), and immune responses to migrating larvae in the lungs (Nicholls et al., 1978) and liver (Brown & Clayton, 1979) have also been described. There are no studies showing direct parasite death or fitness loss as a result of equine immune responses, and molecular evidence of an immune response has yet to be demonstrated in horses despite evidence of an age-dependent response. Understanding the equine immune response to *Parascaris* spp. at an in-depth molecular level would provide invaluable information regarding host-parasite dynamics and open the door for possible vaccine development.

Helminth excretory-secretory products, including microRNAs (Sotillo et al., 2020) and extracellular vesicles (Zakeri et al., 2021) are thought to play a role in immune evasion

by eliciting host immune responses, and consequently may allow for development of vaccines and/or diagnostic tests (Lightowlers & Rickard, 1988). In *Ascaris suum*, extracellular vesicles contain immunomodulatory proteins (Hansen et al., 2019) and microRNAs may be important for parasite development (Xu et al., 2013). A recent *in vitro* analysis of larval *Parascaris* spp. excretory-secretory products identified 19 kDa, 22 kDa, 26 kDa, and 34 kDa products that were recognized by antibodies from sera of previously infected foals (Burk et al., 2014). Mares were also shown to have antibodies against these products and passed them to foals via colostrum during the first suckling (Burk et al., 2016). These antibodies are not useful for diagnosis because the foals acquire them shortly after birth, and their targets are likely are not useful for vaccination because, despite their presence, foals still become infected with *Parascaris* spp. There have been no studies to date examining *Parascaris* spp. extracellular vesicles or microRNAs, and ultimately, more research is necessary to determine the nature of equine immunity against *Parascaris* spp.

2.3 Anthelmintic Resistance

In the early 20th Century, John D. Rockefeller committed over US $1 million to hookworm control and research, and Epsom salts, thymol, carbon tetrachloride, oil of chenopodium, tetrachlorethylene, and hexylresorcinol were all either used or investigated for use as anthelmintics (Horton, 2003). Continuing into the 20th Century, various dyes and synthetic compounds were used to treat helminth infections, but many were ineffective, difficult to use, and/or toxic, causing a plethora of issues such as deafness, blindness, skin irritation, diarrhea, vomiting, organ damage, and death (Faust, 1937; Horton, 2003).

Presently, anthelmintic resistance is rampant in veterinary parasitology (Rose et al., 2005; Fleming et al., 2006; Sutherland & Leathwick, 2011; Kaplan & Vidyashankar, 2012), and understanding how it developed is important in order to slow down its progression and preserve the efficacy of current, and any future, anthelmintics for as long as possible.

2.3.1 Anthelmintic treatments

There are three available anthelmintic drug classes for the treatment of *Parascaris* spp. infection in horses: macrocyclic lactones, benzimidazoles, and tetrahydropyrimidines. Traditionally, foals were treated within their first thirty days of life, and then at either

monthly or bimonthly intervals until their first birthday (Drudge & Lyons, 1966; Ellingson & Coates-Markle, 1996; Robert et al., 2015; Nielsen et al., 2018). Early reports questioning the efficacy of ivermectin against *Parascaris* spp. (Anderson, 1984; Jones, 1985) emerged in the mid-1980s shortly after its introduction to the market, leading to a defense of the drug with claims that the parasite life cycle was misunderstood, and that errors were made during diagnostic fecal egg counts (Boraski, 1987). Formal reports of ivermectin resistance started in the Netherlands in 2002, quickly followed by Canada in 2003 (Boersema et al., 2002; Hearn & Peregrine, 2003). This was followed by reports of macrocyclic lactone resistance encompassing the global equine population and, more recently, reports of tetrahydropyrimidine and benzimidazole resistance (Table 2.1). Current recommendations reduce the overall number of anthelmintic treatments for *Parascaris* spp. in foals in an attempt to slow down the development of resistance (ESCCAP 2019; Nielsen et al., 2019; Rendle et al., 2019).

Table 2.1: Publications reporting anthelmintic resistant populations of Parascaris spp., the anthelmintic class investigated, and location by continent and country.

			Anthelmintic Class		
Continent	**Country**	**Publication**	**Macrocyclic Lactones**	**Tetrahydropyrimidines**	**Benzimidazoles**
Asia	Saudi Arabia	Alanzi et al. (2017)	X	X	X
	Turkey	Cirak et al. (2010)	X		
Europe	Denmark	Schougaard & Nielsen (2007)	X		
	Estonia	Lassen & Peltola (2014)	X		
	Finland	Näreaho et al. (2011)	X		
		Hautala et al. (2019)		X	
	France	Laugier et al. (2012)	X		
		Geurden et al. (2013)	X		
	Germany	von Samson-Himmelstjerna et al. (2007)	X		
	Iceland	Martin et al. (2021b)	X		
	Italy	Veronesi et al. (2009)	X		
		Veronesi et al. (2010)	X		
	Poland	Studzińska et al. (2020)	X		
	Sweden	Lindgren et al. (2008)	X		
		Lind & Christensson (2009)	X		
		Martin et al. (2018)		X	
		Martin et al. (2021a)			X
	The Netherlands	Boersema et al. (2002)	X		
	United Kingdom	Stoneham & Coles (2006)	X		
		Relf et al. (2014)	X		
North America	Canada	Hearn & Peregrine (2003)	X		
		Slocombe et al. (2007)	X		
	United States	Craig et al. (2007)	X	X	
		Lyons et al. (2008)	X	X	
		Lyons et al. (2011)		X	
Oceania	Australia	Armstrong et al. (2014)	X	X	X
		Beasley et al. (2015)	X		
		Wilkes et al. (2017)	X		
	New Zealand	Bishop et al. (2014)	X		
South America	Argentina	Cooper et al. (2020)	X		
	Brazil	Molento et al. (2008)	X		

Reports of anthelmintic resistance in other ascarid species of veterinary and medical importance are few and far between. Only case reports of resistance are available for a few species, including *Ascaris lumbricoides* (Krücken et al., 2017), *Ascaridia dissimilis* (Yazwinski et al., 2013; Collins et al., 2019), and *Heterakis gallinarum* (Yazwinski et al., 2013; Collins et al., 2021). Yet it is clear from widespread anthelmintic resistance of many important parasitic nematodes infecting livestock (Rose et al., 2005; Fleming et al., 2006; Sutherland & Leathwick, 2011; Kaplan & Vidyashankar, 2012; von Samson-Himmelstjerna, 2012; von Samson-Himmelstjerna et al., 2021b), including *Parascaris* spp., that evolution of resistance is a concern. Anthelmintic resistance is also an emerging concern in parasites of companion animals (Jiminez Castro et al., 2019; Jiminez Castro et al., 2021), and while there have been no reports of resistance in any companion animal ascarid species, frequent monthly treatment intervals necessitate robust anthelmintic resistance monitoring programs (von Samson-Himmelstjerna et al., 2021b). Previous reviews have discussed the need for medical parasitology to learn from veterinary parasitology and identify causes of anthelmintic resistance, modify anthelmintic treatment regimens, and monitor for resistance, in order to at minimum slow down the development of resistance (Beech et al., 2010; Vercruysse et al., 2011; Tinkler, 2020; von Samson-Himmelstjerna et al., 2021b). This One Health approach and warning to reduce treatment frequency has been mentioned for nearly two decades (Geerts et al., 1997; Geerts & Gryseels, 2000; Thompson & Roberts, 2001; Geerts & Gryseels, 2002), yet little has changed, particularly in human public health (Tinkler, 2020).

Husbandry practices and anthelmintic treatment strategies as methods to slow down the development of resistance have been discussed in detail elsewhere (Reinemeyer, 2009; von Samson-Himmelstjerna, 2012; Matthews, 2014; Nielsen, 2016; Reinemeyer & Nielsen, 2017; von Samson-Himmelstjerna et al., 2021b). Anthelmintic mechanisms of action as well as mechanisms of resistance in parasitic nematodes have also been thoroughly reviewed in the past (Prichard, 1994; Kotze et al., 2014; Whittaker et al., 2017; Kaplan, 2020). This section will briefly describe drug mechanisms of action and resistance in general, with a focus on relevant research conducted using *Parascaris* spp.

2.3.2 Benzimidazoles

Benzimidazoles are heterocyclic aromatic organic compounds, and various modifications to this structure resulted in the development of anthelmintic drugs (Townsend & Wise, 1990). The first was introduced in 1961, follow by numerous formulations in the 1960s and 1970s (Brown et al., 1961; Harder, 2002). Benzimidazoles interact with the colchicine-binding domain of β-tubulin and inhibit microtubule polymerization, disrupting vital cellular processes and causing parasite death (Friedman & Platzer, 1978; Lacey, 1988; Lacey, 1990). Microtubules are polymers made of tubulin dimers consisting of α- and β-tubulin and are essential for cellular structure and processes such as intracellular transport and cell division (Lacey, 1988; Lacey, 1990). Benzimidazoles developed as anthelmintics have a higher binding affinity for nematode β-tubulin than mammalian β-tubulin, making them safe for use in horses and other mammalian species (Lacey, 1988).

Benzimidazole resistance mechanisms are the most well-studied among the anthelmintic classes because of its rapid development in Clade V nematodes, in particular *H. contortus*, just three years after its introduction to the market (Drudge et al., 1964; Kotze & Prichard, 2016). Benzimidazole resistance is associated with mutations in isotype-1 and -2 β-tubulin genes that decrease binding affinity of the drug for its target (Lubega & Prichard, 1990; Lacey & Gill, 1994; von Samson-Himmelstjerna et al., 2007a). There are a few single nucleotide polymorphisms (SNPs) that are associated with benzimidazole resistance in *H. contortus*, with a phenylalanine-to-tyrosine substitution at codon 200 (F200Y) in the isotype-1 β-tubulin gene being the most common in wild type parasite populations (Kotze & Prichard, 2016). Other mutations in isotype-1 β-tubulin linked to benzimidazole resistance include F167Y and E198A, with the former being quite rare and the latter conferring the highest level of drug resistance of the three (Ghisi et al., 2007; Kotze et al., 2012). Limited research regarding isotype-2 β-tubulin genes has been performed, but some resistant populations of *H. contortus* show loss or decreased levels of the gene (Beech et al., 1994; Lubega et al., 1994).

Benzimidazoles are still effective anthelmintics for the treatment of *Parascaris* spp. infections, and resistance has only been reported in three studies beginning in 2014. Due to this limited emerging anthelmintic resistance, only a few studies have examined the resistance-related SNPs or transcriptional responses to benzimidazoles in *Parascaris* spp. Five studies have sequenced *Parascaris* spp. β-tubulin genes, including one using a known benzimidazole-resistant isolate, and none found any known resistance-related

SNPs, suggesting a potentially different mechanism of resistance in ascarid parasites to this anthelmintic class (Tydén et al., 2013a; Tydén et al., 2014; Malekpour et al., 2019; Martin et al., 2021b; Özben et al. 2022). Interestingly, it has been shown that isotype-1 and -2 β-tubulin genes are expressed at higher levels in *Parascaris* spp. eggs, and that, while isotype-1 remains at similar levels of expression in larvae and adults, isotype-2 gene expression is very low in adults, suggesting differing functions throughout the life cycle (Tydén et al., 2016). Additionally, *in vitro* exposure to benzimidazoles significantly increased gene expression of isotype-1 β-tubulin genes in one study using eggs (Tydén et al., 2016), whereas *in vitro* studies using adult parasites showed either down regulation of isotype-2 β-tubulin (Martin et al., 2020b) or no differential expression of β-tubulin genes (Scare et al., 2020). Previously discussed enzymes that aid in removal of xenobiotic compounds, as well as genes related to detoxification, microtubule polymerization, regulation of membrane potential, and muscle contraction were also differentially expressed following *in vitro* exposure to benzimidazoles (Martin et al., 2020b; Scare et al., 2020).

The β-tubulin genes targeted by benzimidazoles are different even within Clade V nematodes (Saunders et al., 2013), and resistance-related β-tubulin SNPs have a low frequency in benzimidazole-resistant equine cyathostomins, another Clade V parasite group, suggesting that they may not fully explain benzimidazole resistance even within the clade (Pape et al., 2003; von Samson-Himmelstjerna et al., 2003; von Samson-Himmelstjerna et al., 2007a; James et al., 2009). Considering these dissimilarities within Clade V and lack of identification of known resistance-related SNPs in benzimidazole-resistant *Parascaris* spp., it is possible that the mechanism of resistance in Clade III ascarid-type nematodes is different, and thus using these SNPs for anthelmintic resistance surveillance is inadvisable (Diawara et al., 2009; Diawara et al., 2013; Rashwan et al., 2017; Zuccherato et al., 2018; Palma et al., 2020).

2.3.3 Tetrahydropyrimidines

The tetrahydropyrimidines include two formulations of pyrantel using different salts: pyrantel pamoate and pyrantel tartrate. Drugs in this class act as agonists of acetylcholine receptors (AChRs) and cause them to stay open, leading to prolonged muscle contraction and paralysis in the parasites (Harrow & Gration, 1985; Robertson et al., 1994). Nicotinic AChRs are ligand-gated ion channels activated by the neurotransmitter acetylcholine made up of five subunits surrounding a central pore

(Beech & Neveu, 2015). The AChR repertoire of parasitic nematodes is not widely studied, with only a few subtypes having been described in nematodes in general, and even fewer when the scope is narrowed to *Parascaris*. Recently, a *Parascaris* ACR-16 receptor subunit was described, and it was found that parasitic nematodes have two AChR subunits, ACR-26 and ACR-27, that are not found in free-living nematodes (Courtot et al., 2015; Charvet et al., 2018). In *Parascaris* spp., these two subunits have a higher affinity for pyrantel than acetylcholine (Courtot et al., 2015).

Parascaris spp. resistance to pyrantel has only been reported in seven studies globally starting in 2007, and thus its mechanism has been the subject of limited research due to the lack of resistant parasite populations. There has, however, been one *in vitro* study investigating transcriptional responses in *Parascaris* spp. when exposed to pyrantel, ivermectin, and thiabendazole (Martin et al., 2020b). Eight transcript orthologs of AChR were differentially expressed, but with no clear pattern between drug classes (Martin et al., 2020b). Differential expression was also found in genes coding for enzymes that aid in the removal of xenobiotic compounds including short-chain dehydrogenases/reductases and flavin-containing monooxygenases, but these enzymes have not been characterized in parasitic nematodes and thus more research must be completed to understand their possible involvement in anthelmintic resistance (Martin et al., 2020b).

2.3.4 Macrocyclic lactones

Macrocyclic lactones are a group of drugs derived from avermectin, which are produced by *Streptomyces avermitilis* (Campbell et al., 1983; Kim & Goodfellow, 2002) or milbemycins produced by *S. hygroscopicus* (Takiguchi et al., 1980) or *S. cyaneogriseus* (Carter et al., 1988) and consist of some of the most well-known and widely used anthelmintics in the world. The avermectin derivatives – particularly ivermectin – have had a large impact in both veterinary and human medicine and the 2015 Nobel Prize in Physiology or Medicine was awarded to William C. Campbell and Satoshi Ōmura for its discovery (Nobel Prize, 2015). Ivermectin was first introduced in 1981, and by the end of the decade it was the best-selling animal health product in the world (Laing et al., 2017). Moxidectin, a milbemycin derivative, was introduced in the mid-1990s and has a longer half-life and higher potency than ivermectin (Lyons et al., 1992; Afzal et al., 1997). Macrocyclic lactones irreversibly activate glutamate-gated chloride channels (GluCls) that are present in nematode neuron and muscle cells, inhibiting neuronal and muscle activity

and ultimately causing paralysis and death (Wolstenholme, 2012; Laing et al., 2017). Within nematodes, even those that are within the same clade such as *Caenorhabditis elegans* and *Haemonchus contortus*, GluCls are highly divergent, making comparisons between species, let alone clades, difficult when studying both the mechanism of action and development of resistance (Laing et al., 2017).

Macrocyclic lactone resistance was first reported in *H. contortus* in 1987 – just six years after ivermectin hit the market – and continued to spread globally (Carmichael et al., 1987; Van Wyk et al., 1987; Prichard, 1994). Despite widespread anthelmintic resistance to ivermectin in some species of nematode parasites, the mechanism for resistance remains poorly understood. Similar to benzimidazole resistance, ivermectin resistance has been studied in *H*. *contortus*, as well as in *C. elegans* (Lespine et al., 2011; Doyle & Cotton, 2019), but little research has been conducted in ascarid parasites. P-glycoproteins (Pgp) are cell membrane efflux proteins that pump foreign substances out of cells and were first associated with ivermectin resistance in parasitic nematodes in the late 1990s (Xu et al., 1998). Subsequently, they are one of the most widely studied putative mechanisms for macrocyclic lactone resistance and the only one that has been studied in *Parascaris* spp. Similar to benzimidazole resistance, the bigger picture is complex. Macrocyclic lactone resistance is likely multigenic (Choi et al., 2017; Khan et al., 2020) and the molecular mechanism is not fully understood (Laing et al., 2016.; Rezansoff et al., 2016).

Ten *Parascaris* spp. Pgps have been identified to date, along with their tissue-specific expression levels and some evidence for interaction with ivermectin: *Pun*-Pgp-2, -3, -9, -10, -11.1, -11.2, -12, -16.1, -16.2, and -18 (Janssen et al., 2013; Chelladurai & Brewer, 2019; Gerhard et al., 2020; Martin et al., 2021a). Their role in anthelmintic resistance, however, is unclear. Transgenic expression of *P. univalens Pun*-Pgp-9 and -11 in *C. elegans* decrease susceptibility to ivermectin (Janssen et al., 2015), and *Pun*-Pgp-9 does so in a tissue-specific manner, with intestinal expression conferring a protective effect, and depends on active ingestion via pharyngeal pumping (Gerhard et al., 2021). Comparisons between ivermectin resistant and susceptible *Parascaris* spp. populations revealed overexpression of SNPs in *Pun*-Pgp-11 correlating to decreased macrocyclic lactone susceptibility (Janssen et al., 2013), but drug exposure assays showed no change in Pgp expression in response to ivermectin exposure (Gerhard et al., 2020; Scare et al., 2020; Martin et al., 2021a). Differentially expressed genes for enzymes aiding in removal of xenobiotic compounds and other cellular processes were similar to

those previously described for other drugs after ivermectin exposure, with the only exception being the upregulation of a gamma-aminobutyric acid receptor subunit (Martin et al., 2020b; Scare et al., 2020).

2.3.5 Novel Anthelmintics

Several novel anthelmintic candidates have been tested against *Parascaris* spp., many of them involving plant extracts. Wild tarragon (*Artemisia dracunculus*), pennyrile (*Mentha pulegium*), *Zataria multiflora*, cinnamon (*Cinnamomum zeylanicum*), pomegranate flower (*Punica granatum*), and pepper (*Capsicum annuum*) extracts were all lethal to L2/L3 larval *Parascaris* spp. *in vitro* (Rakhshandehroo et al., 2016; Rakhshandehroo et al., 2017). Zinc oxide nanoparticles showed *in vitro* anthelmintic efficacy against *Parascaris* spp., including changes to morphological appearance (Morsy et al., 2019). The monoterpenic phenol isomer carvacrol, isolated from herbs, also showed *in vitro* anthelmintic activity against *Parascaris* spp. by inhibiting acetylcholine-induced currents and stopping muscle contractions, suggesting that it is an antagonist of AChRs similar to pyrantel (Trailovic et al., 2021). The *Bacillus thuringiensis* crystal protein Cry5B has shown efficacy against *Parascaris* spp. when administered to foals via nasogastric tube, dropping fecal egg counts to zero, and is the only published *in vivo* experimental efficacy study of a novel drug that has been completed recently (Urban et al., 2021). While these treatments have shown some efficacy, there is little information regarding the mechanisms of action, which will be an essential piece of information if they make it to the commercial market in order to help prevent the development of resistance.

CHAPTER 3. THE MICROBIOME OF HELMINTH PARASITES

3.1 Microbiome

3.1.1 What is the microbiome?

The microbiome is the entire biome of microscopic organisms and their "theater of activity," including genetic information, environmental conditions, metabolic activity, and ecological functions (Whipps et al., 1988). The microbiota, or microbial community, is the community of microorganisms living in a specific, well-defined habitat (Whipps et al., 1988). These widely accepted definitions of "microbiome" and "microbiota" were first coined in 1988 and were recently amended slightly to include mention of the dynamic nature of the microbiome (Berg et al., 2020). Bacteria, fungi, archaea, algae, and small protists are widely accepted as members of the microbiome; however, inclusion of phages, viruses, plasmids, and mobile genetic elements is more contentious with no clear consensus of inclusion in the microbiome research community (Berg et al. 2020).

3.1.2 History of microbiome research

The history of microbiome research is also a history of scientific innovation over the past 350 years, with methodological inventions leading to a better understanding of the microbiome. Microorganisms were first discovered in the 1670s by Dutch businessman and scientist Antonie van Leewenhoeck, using a high magnification microscope that he created himself (Leewenhoeck, 1677; Lane, 2015). It was not until the mid-1800s, however, that German biologist Ferdinand Cohn laid the foundation for modern bacterial taxonomy after the advent of culture-based techniques (Drews, 2000). He also studied bacterial physiology and was an advocate for applied microbiology (Drews, 2000). Russian microbiologist Sergei Winogradsky pioneered the field of microbial ecology at the end of the 19th century, particularly the nitrogen cycle and chemosynthesis (Winogradsky, 1890; Dworkin & Gutnick, 2012).

After the central dogma of molecular biology was published in 1970 by Francis Crick, stating that sequential information is transferred residue-by-residue and cannot be transferred back from protein to either protein or nucleic acid (Crick, 1970), molecular techniques quickly gained traction from Sanger sequencing in 1977 (Sanger et al., 1977) to polymerase chain reaction (PCR) in 1986 (Mullins et al., 1986) and quantitative real-time PCR (qPCR) in 1993 (Higuchi et al., 1993), culminating with next generation

sequencing (NGS) in 2002 (Reinartz et al., 2002). The Human Microbiome Project began in 2008 and over the course of five years has characterized the microbial communities of 242 healthy individuals across multiple body sites (The Human Microbiome Project Consortium, 2012a). Currently, two more large-scale microbiome projects are in progress: The Earth Microbiome Project (Thompson et al., 2017) and the TerraGenome Project (Vogel et al., 2009).

3.1.3 The microbiome and health

Large-scale microbiome projects aimed at characterizing healthy individuals provide databases that can, and have been, used for numerous different research applications, particularly those investigating how the microbiome affects health. Microbiome research has exploded over the past few decades as its important role in health has become more and more apparent. In humans, other animals, and plants, changes in the microbiome are associated with a plethora of health outcomes including, but not limited to, Crohn's disease (Ni et al., 2017), cancer (Helmink et al., 2019), depression (Zheng et al., 2016), equine oral health (Kennedy et al., 2016), bovine respiratory disease (Lima et al., 2016), and plant growth (Bloemberg & Lugtenberg 2001). The ultimate goal of understanding host-microbiome interactions, particularly in disease, is to manipulate the microbiome in order to achieve better health outcomes and create personalized approaches for individualized medicine. This concept is currently being explored in a variety of organisms and applications, including veterinary science (Clemmons et al., 2019; Song et al., 2019; Peixoto et al., 2021), coral reefs (Rosado et al., 2019; Santoro et al., 2021), bioremediation (Correa-García et al., 2018), and agriculture (Deng et al., 2021).

3.2 Host–Microbiome Interactions

3.2.1 A brief overview

The host-microbiome relationship is bi-directional, with the microbiome playing an important role in maintaining homeostasis within the host and factors such as host environment, antibiotic use, genetics, and diet affecting the microbiome. The gut microbiome metabolizes nutrients (Russell et al., 2013), synthesizes vitamins (LeBlanc et al., 2013), and likely plays a role in mucosal permeability (Moreira et al., 2012). Host diet, including protein source, fiber, dietary fat, and carbohydrate consumption, has been

shown to affect microbiome composition (David et al., 2014), and therefore metabolite production, which has been linked to disease (Wang et al., 2011; Tang et al., 2013). Metabolites produced by microbiota also influence the immune system (Donohoe et al., 2011; Hashimoto et al., 2012; Park et al., 2015; Blacher et al., 2017) and there is also evidence that the microbiome plays a role in neurotransmitter modulation (Fung et al., 2019; Strandwitz et al., 2019; Jameson et al., 2020).

Host-microbiome interactions have also been studied in systems other than humans, albeit much less to date; a few examples of research that has been conducted follow. Microbes provide nutrients to plants both under stressful and normal growth conditions (Carbonnel & Gutjahr, 2014; Nishida & Suzaki, 2018; Rodriguez et al., 2019) and can provide protection against pathogens (Berendsen et al., 2018), while plants provide microbes with lipids and fixed carbon (Keymer & Gutjahr, 2018). The gutless oligochaete marine worms in the genus *Olavius* have an endosymbiotic relationship with sulfate-reducing and sulfite-oxidizing bacteria that provide their host with nutrients and may increase protein yields, while the worms provide the bacteria with an energy source (Dubilier et al., 2001; Blazejak et al., 2005). In cattle and other ruminants, the microbiome facilitates the digestion of plant fibers, provides volatile fatty acids, and metabolizes nitrogen to produce proteins essential for milk and muscle synthesis (Bergman, 1990; Bach et al., 2005; Malmuthuge & Guan, 2017; O'Hara et al., 2020). The mechanisms of these microbiome functions in cattle are less studied, however after a near-total ruminal content exchange, cattle rumen microbiomes revert back to their original composition rather than maintain that of the donor, suggesting host specificity (Weimer et al., 2010; Malmuthuge & Guan, 2017).

3.2.2 Genetics and the microbiome

Host genetics play an important role in microbiome composition, accounting for approximately 10% of microbiome variation in one study (Wang et al., 2016), and influence the abundance of various taxa that are essential for maintaining health (Khachatryan et al., 2008). The influence of genetics and microbiome heritability is further illustrated by twin studies showing that monozygotic twins have more similar gut (Goodrich et al., 2014b; Goodrich et al., 2016; Xie et al., 2016) and urinary (Adebayo et al., 2020) microbiomes than dizygotic twins. The host gene *Vdr*, which encodes a Vitamin D receptor (VDR), is a locus that accounted for a significant amount of microbiome variation in a cohort of over 1800 people (Wang et al., 2016). The VDR forms a

heterodimer with retinoid X receptor (RXR), and the ligands of this receptor include microbial metabolites such as secondary bile acids (Haussler et al., 2008). In a mouse model, *Vdr* knockouts had significantly different microbiome beta diversities than wild type mice (Jin et al., 2015). Genome-wide association studies have also shown associations between the gut microbiome and loci involved in conditions such as inflammatory bowel disease (Cohen et al., 2019), allergies (Bonder et al., 2016), autoinflammatory disorders (Khachatryan et al., 2008), autoimmune disorders (Montgomery et al., 2020), and atrial fibrillation (Xu et al., 2020). Using a mouse model, it has recently been shown that host genetics and the microbiome can regulate behaviors. Knockout *Cntnap2* mice exhibit social deficits and hyperactivity, but therapy with *Lactobacillus reuteri* rescues social deficits via upregulation of metabolites in the tetrahydrobiopterin pathway that improve synaptic transmission, suggesting a role for the microbiome in neurological disorders (Buffington et al., 2021).

Similar to many other aspects of microbiome research, the influence of host genetics on the microbiome have been studied more in humans and mouse models than other hosts. The microbiome of many types of plants has been shown to be heritable and affected by host genetics, including maize (Peiffer et al., 2013; Wallace et al., 2018), the plant model organism *Arabidopsis thaliana* (Horton et al., 2014), wild mustard (Wagner et al., 2016), and trees from both temperate and tropical climates (Redford et al., 2010; Kembel et al., 2014; Laforest-Lapointe et al., 2016). Pig genomes affect gut microbiome composition (Bergamaschi et al., 2020), which can in turn affect fat deposition (Tiezzi et al., 2021). The bovine rumen microbiome is heritable and associated with host genetics as well, although mechanisms remain to be studied (Sasson et al., 2017). This is generally true for microbiome research outside of humans – the mechanisms and extent of the influence of host genetics on microbiome composition have yet to be elucidated.

3.2.3 Parasites and the host microbiome

Parasites can affect the microbiome of their host, and the host microbiome can, in turn, affect the ability of a parasite to successfully infect the host (Zaiss & Harris, 2016; Fredensborg et al., 2020). In humans, infection with soil transmitted helminths (hookworms, roundworms, and whipworms) increases microbial diversity (Lee et al., 2014), and individuals able to self-clear infections had more similar microbiomes to each other than those unable to self-clear (Rosa et al., 2018). Chagas disease affects millions of humans globally and is caused by the protozoan parasite *Trypanosoma cruzi*. Infection

of mice and humans with *T. cruzi* is associated with changes in microbiome beta diversity and in relative abundance of bacteria in the family Lachnospiraceae (Robello et al., 2019; Hossain et al., 2020), and infection of its insect vector *Triatoma dimidiata* is associated with higher species richness (Orantes et al., 2018). Honeybee microbiome composition affects susceptibility to the protozoan parasite *Lotmaria passim* (Scwarz et al., 2016) and high gut microbiome diversity in bumble bees is associated with increased susceptibility to the trypanosome parasite *Crithidia bombi* (Mockler et al., 2018; Näpflin & Schmid-Hempel, 2018). Salmon infected with the myxozoan parasite *Tetracapsuloides bryosalmonae* (Vasemägi et al., 2017) exhibit a positive relationship between gut microbiome species richness and parasite burden and zebrafish infected with the nematode *Pseudocapillaria tomentosa* (Gaulke et al., 2019) exhibit a positive relationship between gut microbiome diversity and parasite burden.

Parasites can also cause various clinical signs in other large mammalian hosts and, in turn, affect their microbiomes. Infection with the amoebic parasite *Entamoeba*, some species of which cause dysentery, is associated with significant differences in the relative abundance of bacterial taxa in lowland gorillas (Vlčková et al., 2018). The ascarid parasite *A. suum* has been shown to reduce microbial diversity 54-days post infection in one study (Wang et al., 2019), whereas microbial diversity increased 14-days post infection in another study (Williams et al., 2017), differences which could be explained due to the differing infection period. *A. suum* infection also correlated to an increase in short chain fatty acids produced by bacteria, a phenomenon replicated in both mice and humans infected with intestinal helminths and resulted in attenuation of airway inflammation responses (Zaiss et al., 2015). Anthelmintic treatment against cyathostomins in domestic horses (Walshe et al., 2019) and mixed equine parasite infections in Przewalski's horses (Hu et al., 2021) resulted in a decrease in bacterial diversity, and upon natural reinfection after treatment, an increase in species richness has been observed (Clark et al., 2018), and it has also been shown that anthelmintic treatment in parasite-free animals has minimal effect on the microbiome (Kunz et al., 2019), thus the observed differences in diversity and richness are likely due to parasite infection.

3.3 The Microbiome of *Caenorhabditis elegans*

The small, free-living nematode *C. elegans* is an important model organism not only for nematode research, but also for biological research as a whole, and was the first multicellular organism to have its whole genome sequenced in 1998 (The *C. elegans*

Sequencing Consortium, 1998). This nematode has been widely used for various research applications because it is an inexpensive model, can be frozen for long-term storage, and is relatively easy to maintain *in vitro*. Due to this, the microbiome of *C. elegans* has been studied extensively, and is by far the most well-studied nematode microbiome, with the first studies being published in 2016 (Zhang et al., 2017).

3.3.1 Composition of the *C. elegans* microbiome

While microbiome composition is highly variable between individuals within a species, members of a species tend to have groups of microbes that are similar across the population, known as the core microbiome (Risely, 2020). In multiple *C. elegans* microbiome studies using worms grown in varying soil types, the gut microbiome was distinct from the soil microbiome and less diverse. Prominent microbiome members included the families Enterobacteriaceae, Pseudomonadaceae, Xanthomonadaceae, and Sphingobacteriaceae, suggesting that these are part of the *C. elegans* core microbiome across strains and environments (Berg et al., 2016a; Berg et al., 2016b; Dirksen et al., 2016). The host genotype explained 12.7% of microbiome variation in one of these initial *C. elegans* microbiome studies, and *Enterobacter cloacae* and *Pseudomonas mendocina* isolated from *C*. *elegans* conferred host-specific protection against pathogenic *Enterococcus faecalis* and *P. aeruginosa* infection, respectively (Berg et al., 2016b).

Many microbiome studies, particularly those characterizing the microbiome of a given organism for the first time, give only a snapshot of a dynamic relationship. Another recent study characterized the microbiome of *C. elegans* and their substrates for two consecutive years and found that while microbiome composition was conserved at the genus level over time, it was not conserved for amplicon sequencing variants (Johnke et al., 2020). Only three strains were present consistently over the study period (*Comamonas* ASV10859, *Pseudomonas* ASV7162 and *Cellvibrio* ASV9073), and diversity was influenced by substrate (Johnke et al., 2020). Overall, characterizing a core microbiome can be an arduous task and requires multiple studies using multiple populations to determine similarities across an entire species, even those maintained within a laboratory setting.

3.3.2 Host-microbiome interactions

As a model organism, numerous host-microbiome interactions have been described in *C. elegans* that are associated only with its standard food source, *Escherichia coli* OP50 (Stiernagle, 2006; Gerbaba et al., 2017) and bacterial signals influence various biological processes in their host. One study showed that *C. elegans* fed with a soil microbe community had higher reproduction rates, lived longer, were more tolerant to cadmium exposure, had more robust stress and immune responses, and their microbiomes were less affected by cadmium exposure than those fed only *E. coli* OP50 (Lee et al., 2020). Genetic mutants in the *C. elegans* DAF-2/insulin growth factor pathway, which is involved in innate immunity and stress responses, had a distinct microbiome from wild type specimens (Taylor & Vega, 2021). Lifespan and stress response is influenced via *E. coli* folate synthesis (Virk et al., 2016), the rapamycin complex 2 (mTORC2) and the serum- and glucocorticoid-regulated kinase 1 pathways (Mizunuma et al., 2014), and the production of nitric oxide by *Bacillus subtilis* (Donato et al., 2017). Bacterial folate synthesis also plays an important role in reproduction because folate receptor-1 is required for germ cell proliferation (Chaudhari et al., 2016). A more recent study showed that variations in signaling pathways due to natural genetic variation affects microbiome composition (Zhang et al., 2021a). Genotypes with upregulated insulin signaling activate an immune response that results in microbiomes enriched for *Ochrobactrum*, which is correlated with faster growth rates and larger adult body size (Zhang et al., 2021a).

In natural habitats, the majority of bacteria fed on by *C*. *elegans* promoted growth and, in general, microbiomes enriched with Alphaproteobacteria increased population proliferation, whereas those enriched with *Bacteroidetes* correlated with *C. elegans* that did not proliferate (Samuel et al., 2016). Recent studies have shown that the native microbiome is important for overall worm health and fitness, and that the *C. elegans* bacterial community is capable of synthesizing all essential nutrients for the worms (Zimmermann et al., 2020). Natural strains of microbiome members such as *Chryseobacterium* and *Comamonas* increase resistance to oxidative stress, increase lifespan, and upregulate cellular detoxification and signaling pathways (Haçariz et al., 2021). *Pseudomonas* isolates provide protection from pathogens by inhibiting pathogen growth, either directly via the lipopeptide massetolide E, or possibly via indirect mechanisms that have yet to be elucidated (Kissoyan et al., 2019).

3.3.3 Bacteria-bacteria interactions

Not only can the host affect its microbiome and vice versa, but the bacteria within the microbiome can also affect one another. Thus far, this effect has mainly been examined in regard to community composition. Variation in community composition may be influenced by randomness and has also been predicted by models to be influenced by early successful colonizers, whose exponential growth prevents others from forming large populations (Vega & Gore, 2017). High abundance of an *Erwinia* strain has been correlated with low alpha diversity and bacteria such as *Bdellovibrio* have been correlated with high alpha diversity (Johnke et al., 2020). Bacteria that colonize *C. elegans* well in monoculture do not all do the same in co-cultures, possibly due to bacteria-bacteria interactions (Ortiz et al., 2021).

3.4 The Notable Case of *Wolbachia*

Perhaps the most well-known example of how impactful helminth microbiomes can be is a genus of endosymbiotic bacteria called *Wolbachia* and their filarial nematode hosts. This endosymbiotic relationship and the subsequent development of anti-*Wolbachia* drugs as a treatment option for infection highlight how important characterizing and understanding helminth microbiomes is, not only for general biological knowledge, but also for identification of potential new drug targets.

3.4.1 Filarial nematodes

Filarial nematodes are a group of parasites with an intermediate arthropod host and a vertebrate definitive host that cause various important neglected tropical diseases and companion animal infections. Lymphatic filariasis, also known as elephantiasis, is caused by *Wuchereria bancrofti*, *Brugia malayi*, and, to a lesser extent *B. timori,* and is spread to humans via *Aedes*, *Anopheles*, *Culex*, or *Mansonia* mosquitoes (Taylor et al., 2010; Chandy et al., 2011). The worms accumulate in lymphatic vessels and can cause tissue swelling or thickening of the skin and tissues. Globally, over 800 million people live in areas requiring preventive chemotherapy, and an estimated 36 million people have chronic disease (World Health Organization, 2021). Onchocerciasis, also known as river blindness, affects over 20 million people globally and is caused by *Onchocerca volvulus* transmitted via black flies, resulting in severe itching, eye lesions, and sometimes blindness (Taylor et al., 2010; World Health Organization, 2019). *Dirofilaria immitis*, also

known as canine heartworm, is prevalent in the Americas and can also infect humans, albeit rarely causing symptoms (Dantas-Torres & Otranto, 2020).

3.4.2 Initial discovery and research

In the 1970s, transmission electron microscope (TEM) ultrastructure studies of *B. malayi*, *B. pahangi*, *O. volvulus*, and *D. immitis* revealed the presence of intracellular bacteria within the oocytes (Harada et al., 1970; Lee, 1975; McLaren et al., 1975; Kozek, 1977; Kozek & Marroquin, 1977); microfilariae (McLaren et al., 1975; Kozek, 1977); and lateral chords (Vincent et al., 1975; Kozek, 1977; Kozek & Marroquin, 1977). Phylogenetic studies later indicated that these bacteria were closely related to *Wolbachia pipientis*, an endosymbiotic rickettsia of arthropods (Sironi et al., 1995), and that arthropod and filarial *Wolbachia* formed distinct supergroups from one another, suggesting that they are not passed from vector to parasite (Bandi et al., 1998; Lo et al., 2007).

3.4.3 *Wolbachia* as a symbiont

In filarial nematodes, *Wolbachia* are vertically transmitted from female to offspring via egg cytoplasm (Kozek, 1977; Fenn & Blaxter, 2004) and are an important symbiont for the parasites in which they are found. They have been shown to cause inflammatory responses in the vertebrate hosts via activation of neutrophils (Brattig et al., 2001; Nfon et al., 2006; Gillette-Ferguson et al., 2007) by surface proteins (Bazzocchi et al., 2003; Brattig et al., 2004), and desensitize macrophages via toll-like receptors and adapter proteins such as MyD88 (Gillette-Ferguson et al., 2006; Turner et al., 2006). *Wolbachia* are also important for female worm development (Hoerauf et al., 2000), larval and microfilarial development, molting, survival (Casiraghi et al., 2002; Arumugam et al., 2008; Mand et al., 2009), and embryogenesis (Hoerauf et al., 2003; Foray et al., 2018). When abundance of *Wolbachia* decreases, extensive apoptosis occurs in the adult germline, embryo somatic cells, microfilariae, and larvae, highlighting their importance for parasite reproduction and survival (Landmann et al., 2011).

3.4.4 Ongoing development of anti-*Wolbachia* drugs

Due to the importance of *Wolbachia* to the survival and reproduction of filarial nematodes, antibiotics in combination with anthelmintics (Bazzocchi et al. 2008; Luck et al. 2014) have been important components of chemoprophylaxis and treatment programs

in areas affected by filarial diseases. Doxycycline induces a slow kill of adult parasites due to its indirect mechanism, which means that treatment takes multiple weeks (Hoerauf, 2008). Prophylaxis programs also tend to mass-administer drugs, thus both exposing parasites and bacteria to anthelmintics and antibiotics that they are known to develop resistance to, potentially exacerbating this issue (Taylor et al., 2014). In order to discover and develop drugs that could specifically target *Wolbachia*, are safe for children and pregnant women, and reduce treatment time, the Bill and Melinda Gates Foundation funded the formation of the Anti-*Wolbachia* Consortium in 2007 (Taylor et al., 2014).

A 10,000-compound library was subsequently screened against *Wolbachia*, with a hit rate of 0.5%, to aid in the identification of potential drug candidates (Johnston et al., 2017). As a result of this work, there are presently two candidate drugs going through clinical trials for the treatment and prevention of filariasis. The first is ABBV-4083, an antibiotic that has a spectrum of activity similar to tylosin A, but with increased potency that appears to be specific to *Wolbachia* (Taylor et al., 2019). This drug was shown to reduce *Wolbachia* load within 1 to 2 weeks in mouse and gerbil models (Taylor et al., 2019). Currently, it is undergoing Phase II clinical trials under the name TylAMac (ClinicalTrials.gov, 2021). The second drug, AWZ1066S was shown to be highly specific to *Wolbachia* and reduced parasite load within seven days using a twice per day treatment in gerbils (Hong et al., 2019). These drugs are promising, and it is important to note that it has taken nearly 50 years from the discovery of bacterial symbionts in filarial nematodes to the development of drugs specifically targeting those bacteria, which highlights the importance of starting microbiome studies in other parasitic nematodes now, while current drugs are still affective.

3.5 The *Haemonchus contortus* Microbiome

3.5.1 The barber pole worm

The trichostrongylid *H. contortus*, also known as the barber pole worm, is the most pathogenic nematode parasite affecting ruminants globally, and is responsible for millions of dollars in economic losses (Qamar et al., 2011; Lane et al., 2015). The parasite causes anemia due to blood feeding, lethargy, poor fiber quality and nutrient uptake, ill thrift, and death (Emery et al., 2016). Most importantly, *H. contortus* exhibits anthelmintic resistance to all major anthelmintic classes, having on many occasions developed widespread resistance within 10 years of a new drug being released (Kotze & Prichard, 2016).

3.5.2 Microbiome research

Four *H. contortus* microbiome studies have been completed to date. The first study, completed in 2017, investigated the microbiome of three different life stages of *H. contortus*: adult male and females, L3 larvae, and eggs using both V3-V4 and V5-V7 primer sets (El-Ashram & Suo, 2017). This study indicated that while *H. contortus* has a distinct microbiome at each of three life stages, all are dominated by *Pseudomonas*, *Ochrobactrum*, and *Eschericia-Shigella* (El-Ashram & Suo, 2017). In a subsequent study using the same three life stages, denaturing gradient gel electrophoresis, and clone libraries found that Proteobacteria, Firmicutes, and Bacteroidetes were associated with all three stages, suggesting that vertical transmission occurs (Sinnathamby et al., 2018). Additionally, *Weissella* spp. were found in all three life stages, and fluorescent *in situ* hybridization (FISH) using a probe for *Weissella* spp. indicated their presence in eggs, leading to the conclusion that they may be vertically transmitted endosymbionts (Sinnathamby et al., 2018). Another more recent study using adult *H. contortus* specimens and the V3-V4 primer set found that although male and female microbiomes were both dominated by *Escherichia*, *Shigella*, *Vibrio*, and *Halomonas*, differences in alpha and beta diversity as well as unique genera were observed between the two sexes (Mafuna et al., 2021). Finally, another study examined only female *H. contortus* specimens and found that most sequences were also found in the rumen, and an additional *in vitro* antibiotic exposure study indicated that antibiotics could kill the parasites (Bouchet et al., 2022). While there are some distinct differences in results between all three studies that may result from PCR primers, DNA extraction methods, or host geography, it is clear that there are distinct microbiomes between adult male and females of these parasitic nematodes, as well as throughout their life cycles that could possibly suggest some sort of role for the microbiome in development.

3.6 Other Parasitic Nematode Microbiomes

3.6.1 Plant parasitic nematodes

Parasitic nematodes of plants are important agricultural parasites that can cause a variety of plant diseases and result in heavy economic losses (Mota & Vieira, 2008; EFSA Panel on Plant Health et al., 2018). Due to this, their microbiomes have been studied more than many other parasitic nematodes. The pine wood nematode,

Bursaphelenchus sp., causes pine wilt disease and its microbiome has been associated with virulence (Xiang et al., 2015), life stages (Wu et al., 2016), and development (Tian et al., 2015). Its microbiome also plays an important role in α-pinene degradation where the bacteria use the chemical as a carbon source, protecting their host from the anthelmintic effects of α-pinene (Cheng et al., 2013; Wang et al., 2019). Endosymbiotic bacteria have also been identified in *Xiphinema americanum*-group species (Vandekerckhove et al., 2000), *Radopholus similis* (Haegeman et al., 2009), and *Heterodera glycines* (Noel & Atibalentja, 2006). The root knot nematodes, *Meloidogyne* spp., is thought to have obtained some of its parasitism-related genes from rhizobacteria via horizontal gene transfer (Bird et al., 2003; Scholl et al., 2003), again highlighting the importance and potential role for the microbiome in parasitic nematodes.

3.6.2 Vertebrate parasitic nematodes

There are very few studies relating to the microbiome of parasitic nematodes infecting vertebrates, although efforts in this area have increased over the past few years (Dheilly et al., 2017; Dheilly et al., 2019). In the whipworm *Trichuris muris,* which infects mice, egg hatching has been shown to be induced *in vitro* by *Escherichia coli*, *Staphylococcus aureus, Salmonella typhimurium*, and *P. aeruginosa* (Hayes et al., 2010; Wimmersberger et al., 2013), although this was not replicated in the closely related whipworm of pigs, *T. suis* (Vejzagić et al., 2015). It is, however, unclear whether this interaction is necessary *in vivo* from the only study reported (Koyama, 2013). Two more studies have been completed using *Trichuris* spp., the first of which showed that parasite infection was dependent upon host microbiota and that the parasites acquired a distinct microbiota from their host (White et al., 2018). The second more recent studied only used seven specimens and suggested that parasites could be a source of pathogenic bacteria (García-Sánchez et al., 2022).

Four microbiome studies using parasitic trematodes of fish and birds have been completed over the past four years. *Schistocephalus solidus* was shown to harbor six novel ribonucleic acid (RNA) viruses, one of which was excreted and passed to the host (Hahn et al., 2020). Additionally, it was shown that *S. solidus* had a distinct microbiome from its host, that parasite phenotype affected the host microbiome, and that the prevalence of *Chloroflexi* in the parasite microbiome correlated to the expression of host immune genes (Hahn et al., 2021). The microbiome of *Coitocaecum parvum* was analyzed throughout its life cycle and it was shown that not only did the parasite have a

microbiome distinct from that of its host and environment at every life stage, but also that a core microbiome was maintained throughout the life cycle (Jorge et al., 2020). Finally, *Philophthalmus attenuatus* was also shown to have a distinct microbiome at different life stages, but that each life stage had very few shared taxa, suggesting a high turnover in composition (Jorge et al., 2022b). Microbiome composition across geographic space was more similar than between life cycle stages, indicating that the high turnover may be due to nonrandom, stage-specific acquisition of bacteria (Jorge et al., 2022b). An additional study with *P. attenuates* demonstrated that the parasite microbiome could be altered after exposure to antibiotics while within its snail host, and that the new bacterial community post-exposure was different than that pre-exposure (Jorge et al., 2022a). Taken together, these studies suggest that parasites have varying microbiome compositions dependent upon life stage and that some of the bacteria within the microbiome may be important for development or parasite health due to similarities across geographic space, although a significant amount of research is needed in this area of study.

3.6.3 Insect parasitic nematodes

Heterorhabditis and *Steinernema* are two genera of parasitic nematodes affecting insects and are used as biological insecticides (Lacey & Georgis, 2012); they have a mutualistic relationship with *Photorhabdus* (Boemare et al., 1993) and *Xenorhabdus* (Thomas & Poinar, 1979) bacteria, respectively. Upon invasion of the insect host, the parasites release their bacteria symbionts inside the insect via regurgitation (Ciche & Ensign, 2003) or via defecation (Sicard et al., 2004). The bacteria then release natural products that allow for the parasite to infect the insect, preserve the insect cadaver, and inhibit competing bacteria, thus ensuring completion of the parasite life cycle (Shi & Bode, 2018; Heryanto & Eleftherianos, 2020). While this relationship between *Heterorhabditis* and *Steinernema* and their endosymbionts has been widely studied, the general microbiome composition of these parasites has yet to be studied in detail.

CHAPTER 4. METHODS FOR OBTAINING AND ANALYZING MICROBIOME DATA

The large number of microbiome studies being completed over the past decade, increasing from 1,822 PubMed search results in 2011 to 22,127 in 2021, means that there are also a large number of different methods used for collection, preparation, and analysis of microbiome data. There are many variations within microbiome data collection and analysis that can have downstream effects, such as differences in relative abundances and even taxa present. Ultimately, this can affect the ability to directly compare results between studies and researchers have suggested a need for standardization within the field (Sinha et al., 2015; Vandeputte et al., 2017; Dheilly et al., 2019). The following chapter outlines these various methods for collecting, preparing, and analyzing microbiome data.

4.1 Collection and Storage Methods

4.1.1 Fecal samples

Many microbiome studies focus on the gut microbiome and as such, fecal samples are the main source of data. It is generally accepted that immediate fresh collection is essential to maintain the microbiome community of the host without the introduction of contamination (Cardona et al., 2012; Vandeputte et al., 2017), but in many cases samples must be stable in field conditions and so preservation methods have been studied. Ideally, samples should be stored at room temperature and analyzed within 24 hours or immediately frozen at a minimum of -20˚C and not allowed to defrost until DNA extraction is performed (Cardona et al., 2012; Tedjoe et al., 2015). Since this is not always possible, preservation buffers have been assessed relative to freezing and significant differences in microbiome composition were observed between buffer preserved samples and frozen samples (Choo et al., 2015). Storage solutions such as RNA*later*™ (Invitrogen, Waltham, Massachusetts) and 95% ethanol are viable options, although both have advantages and disadvantages such as no need for equipment, lower DNA yield, cost, and limited storage time (Vandeputte et al., 2017). Ultimately, it is important to consider the type of downstream analysis that is necessary, as not all collection methods are suitable for metabolomics, metatranscriptomics, metagenomics, culturing, etc. (Reck et al., 2015; Vandeputte et al., 2017), and to keep in mind that the largest source of variation in fecal microbiome samples is interindividual (Wu et al., 2010; Debelius et al., 2016).

4.1.2 Tissue samples

Tissue samples are also used for microbiome analysis, including but not limited to lung (Sze et al., 2012), vagina (Bai et al., 2012), colon (Gao et al., 2015), skin (Bjerre et al., 2019), breast (Hieken et al., 2016), sinuses (Bassiouni et al., 2015), and ocular surface (Ozkan et al., 2018). These types of samples are generally acquired via surgery, biopsy, or swabs during medical procedures, from healthy volunteers, or hospital collections. Similar to fecal samples, best practice for tissue samples is considered either immediate analysis or frozen storage and the majority of variation is interindividual (Bai et al., 2012; Kim et al., 2017). Collection methods have also been analyzed for tissue samples, such as scaler versus CytoSoft™ (Medical Packaging Inc., LLC, Flemington, New Jersey) brush for dental plaque collection (Luo et al., 2016), eSwabs™ (COPAN Diagnostics, Murrieta, California) versus scrapes for skin sample collection (Bjerre et al., 2019), and mucosal biopsy versus swab for sinonasal mucosa samples (Bassiouni et al., 2015).

4.1.3 Surface Sterilization

When collecting microbiome samples, it is important to minimize contamination, and thus surface sterilization methods are used to remove potential environmental and surface contaminants, allowing for analysis of internal microbiota within the target organism. Methods vary between studies but generally involve washes with sodium hypochlorite and/or ethanol solutions (Binetruy et al., 2019; Hoffmann et al., 2020). Variations include washing with 70% ethanol followed by sterile water (Abraham et al., 2017); 10% sodium hypochlorite, 70% ethanol, and then DNA-free phosphate buffered saline (PBS) (Gofton et al., 2015); soapy water and then 70% ethanol (Lalzar et al., 2012); betadine and 80% ethanol (Hahn et al., 2021); serial washes in 70% and 99% ethanol followed by PBS (Jorge et al., 2020); and 70% ethanol followed by PBS (Jorge et al., 2021). Some studies comparing sodium hypochlorite and ethanol for surface sterilization have been conducted. It was found that when 70% ethanol was used, bacterial diversity was higher (Binetruy et al., 2019), and that washing with sodium hypochlorite may be a better method for removing surface contamination when compared to 70% ethanol (Binetruy et al., 2019; Hoffmann et al., 2020).

4.1.4 DNA extraction

DNA extraction method can affect microbiome data in both amount of DNA successfully extracted from a sample and microbial diversity found in a given sample. While variation due to DNA extraction method is generally lower than interindividual variation within a given sample (Mackenzie et al., 2015; Lim et al., 2017), one major factor in improving DNA recovery and microbial diversity is a bead beating step that effectively lyses the thick cell walls of Gram-positive bacteria (de Boer et al., 2010; Salonen et al., 2010; Yuan et al., 2012; Bag et al., 2016). A variety of commercial kits are available specifically for DNA extraction of microbiome samples, and many have been analyzed and compared in different studies (Lim et al., 2018). Ultimately, it is important to use consistent methods within a study and use caution when comparing results between studies using different extraction methods because it can affect microbial diversity findings (Kennedy et al., 2014; Lim et al., 2018; Bjerre et al., 2019; Fiedorová et al., 2019; Greathouse et al., 2019; Douglas et al., 2020; Tourlousse et al., 2021).

4.2 Microbiome Analysis

The most commonly used method for analyzing microbiome data is amplicon sequencing, typically targeting the 16S rRNA gene for prokaryotes and either the 18S rRNA or ITS genes for eukaryotes (Liu et al., 2021). PCR using primers targeting one of these genes is used to amplify the fragment of interest, and then a second PCR step is used to add index barcodes and adapters for NGS (Liu et al., 2021). There are, however, a few other methods for analyzing the microbiome that will be described in the following sections, and later sections address PCR primer choice and bioinformatic analysis for amplicon sequencing because it is so commonly used, and was the method utilized for this dissertation.

4.2.1 Microscopy

For over a century, microscopy has been used to describe bacteria living within organisms. Light microscopy in the 1920s using Giemsa staining was used to describe intracellular rickettsia-like bacteria that would later be named *Wolbachia* in insects (Hertig & Wolbach, 1924). Contemporary studies use methods such as TEM and FISH to identify bacteria and where they localize within an organism. TEM uses a high voltage electron beam within a vacuum system to produce images up to 1,000,000x. Ultrastructure studies

using TEM have shown the presence of bacteria within various organisms including insects (Kellen et al., 1981; Binnington & Hoffmann, 1989), blood (Panaiotov et al., 2018), feces (Yimagou et al., 2020), arachnids (Burgdorfer et al., 1973), and nematodes (Sinnathamby et al., 2018). While TEM can be useful for visualizing some bacteria within an organism, some disadvantages include cost of the microscope itself and time on the microscope, proper fixation of samples to obtain high enough quality for TEM and obtaining sections with bacteria positioned properly for identification. FISH utilizes a fluorescently labeled DNA probe that hybridizes to a target sequence, allowing for visualization of specific DNA or RNA sequences via fluorescent microscopy (O'Connor, 2008). In microbiome research, probes often target 16S rRNA and can either be broad spectrum at group levels such as domain, phylum, class, or order, or more specific at genus and species level (Amann & Fuchs, 2008). FISH has been used to visualize *Wolbachia* in organisms such as filarial nematodes (Bakowski et al., 2019) and *Drosphila melanogaster* (Simhadri et al., 2017), as well as various other bacterial groups in *D. melanogaster* (Akhtar et al., 2021), vaginal biofilms (Hardy et al., 2015), gastrointestinal tract (Hasegawa et al., 2017), and nematodes (Sinnathamby et al., 2018). In some cases, however, FISH has been unsuccessful when used in microbiome studies due to cross-reactivity (Huys et al., 2008; Swidsinski et al., 2017) and requires confirmation of probe specificity prior to use but can be useful for visualizing localization of bacteria within an organism when successfully applied.

4.2.2 Culturomics

Bacterial culture methods were first developed in the late 19th Century with the advent of agar plates (Koch, 1881), but largely fell to the wayside for microbiome research once modern molecular techniques entered the scene (Greub et al., 2012; Bonnet et al., 2020). Over the past decade, culturomics methods have made a resurgence as a complimentary method to metagenomic sequencing of microbiome components (Bilen, 2020) and have resulted in the discovery of taxa never previously described in human gut microbiome studies (Lagier et al., 2012; Dubourg et al., 2014; Gouba et al., 2014). High-throughput culturing techniques require a large number of cultures maintained for at least a month in many cases (Lagier et al., 2012), and also require the use of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry or a DNA sequencing method to specifically identify species present (Lagier et al., 2012; Diakite et al., 2020). Studies have used up to 212 different culture conditions in order to complete

culturomics studies (Lagier et al., 2012), although a more recent study has examined optimization strategies and suggests 25 culture conditions that can successfully isolate the majority of bacteria found in previous work (Diakite et al., 2020). Culturomics studies of the human microbiome have led to the discovery of over 100 new bacterial species and added an additional 500 species known to be associated with humans (Lagier et al., 2018). Outside of human research culturomics lags behind as a research method, although it has been used occasionally to study the microbiome of other organisms and effort is being made to develop the method for other systems and increase the number of species in international collections (Armanhi et al., 2018; Zehavi et al., 2018; Sarhan et al., 2019; Fenske et al., 2020; Pereira et al., 2020).

4.2.3 Microarray assays

Although they are not commonly used for microbiome applications, microarray assays have been developed for use in high resolution microbiome surveys. The Human Oral Microbe Identification Microarray is capable of detecting microbiome profiles at the phylum level as well as common genera found in human oral wash samples (Ahn et al., 2011). Various microarrays have been developed for the human gut microbiome, including an array targeting 1,629 bacterial and archaeal species (Palmer et al., 2007), another targeting 775 bacterial species (Paliy et al., 2009), the Human Intestinal Tract Chip targeting 1,140 bacterial species (Rajilić-Stojanović et al., 2009), and the Human Gut Chip targeting 66 bacterial families (Tottey et al., 2013). Another microarray targeting bacterial genes that encode glycoside hydrolases and lyases was able to detect low-abundance human gut bacteria that can be missed in metagenomic studies (El Kaoutari et al., 2013). Most recently, the Axiom Microbiome Array was developed to detect over 12,000 species of viruses, bacteria, fungi, protozoa, and archaea (Thissen et al., 2019). These types of microarrays are rapid, cost-effective ways to analyze large numbers of microbiome samples and may become more commonplace in microbiome research as commercial options become available.

4.2.4 Shotgun metagenomics

Shotgun metagenomic sequencing shears all DNA in a sample into small fragments and sequences all of them, providing functional gene profiles as well as a high resolution of taxonomy for organisms present in the microbiome (Sharpton, 2014; Liu et

al., 2021). This method resolves some of the drawbacks of amplicon sequencing, such as PCR biases and variance in diversity due to primer choice, but is more expensive, has a large and complex dataset, and it can be difficult to determine which genome a given read came from (Sharpton, 2014; Ranjan et al., 2016; Laudadio et al., 2018). Nevertheless, shotgun metagenomics studies allow for the identification of archaea, viruses, virophages, and eukaryotes within the same sample and provides a greater chance of identifying bacteria to the strain level (Norman et al., 2014; Jovel et al., 2016). Studies using shotgun metagenomics have shown associations between air pollution and microbial diversity (Fouladi et al., 2020), subclinical mastitis in cattle and differentially abundant bacteria (Bhatt et al., 2012), gastric adenocarcinoma, reduced species richness, and enrichment of pathways associated with biosynthesis of lipopolysaccharides and L-arginine (Hu et al., 2018), to name a few. Additionally, shotgun metagenomics can be used for more specific purposes, such as foodborne pathogen (Yang et al., 2016) and human papillomavirus (Ma et al., 2014) surveillance.

4.2.5 Metatranscriptomics

In metatranscriptomics, total RNA is extracted from an area of interest and then a cDNA library is formed with ligated adapters to form a final library that can then be amplified, sequenced, and mapped to reference genomes to determine gene expression levels (Bashiardes et al., 2016). As a relatively new field in microbiome research, the number of studies using metatranscriptomics has increased, however there is a lack of reference genomes that can make these studies challenging, and data in public repositories sometimes lack metadata that limits the conclusions that can be drawn (Shakya et al., 2019). Nevertheless, metatranscriptomics studies have provided insight into the microbiome of many different systems. In humans, disease-associated oral microbiomes have been shown to have distinct, conserved differences in metabolism compared to healthy oral microbiomes (Jorth et al., 2014), inflammatory bowel disease-associated microbiomes had differentially expressed genes that may influence clinical symptoms such as inflammation (Schirmer et al., 2018), among other findings (Bikel et al., 2015; Zhang et al., 2021b). Studies have also been completed in other systems such as the rumen microbiome (Jiang et al., 2016; Söllinger et al., 2018; He et al., 2019), foods (de Filippis et al., 2016; Jiang et al., 2016), and plants (Turner et al., 2013; Gonzalez et al., 2018).

4.2.6 Metabolomics

Metabolomics is the comprehensive analysis of metabolites within a given biological specimen (Clish, 2015) and is another relatively new method used in microbiome research to provide functional data of microbe activity (Peisl et al., 2018; Zierer et al., 2018; Lee-Sarwar et al., 2020). This approach allows for a greater understanding of host-parasite interactions and dynamics such as transformation of nutrients and pollutants and can also provide biomarkers that predict or help diagnose disease and environmental stress (Aguiar-Pulido et al., 2016). Variances in differential abundances within the metabolome have been shown to correlate with visceral fat mass in humans (Zierer et al., 2018), inflammatory bowel disease status (Franzosa et al., 2019), and arsenic exposure (Lu et al., 2014). Changes in metabolite levels can also increase host susceptibility to pathogens (Theriot et al., 2014), and recently it was shown that the microbiome has the ability to affect the chemistry of all organs in mice (Quinn et al., 2020). Combining metabolomics analysis with the other described methods for assessing the microbiome will be an important path forward in order to gain an understanding of both microbiome form and function within the host organism.

4.2.7 Metaproteomics

Another method for studying the functional aspects of the microbiome is metaproteomics, which investigates the entire protein repertoire of a microbial community (Kolmeder & de Vos, 2014). After protein extraction and fractionation, mass spectrometry is used to analyze the masses of peptides and their fragments (Kolmeder & de Vos, 2014; Salvato et al., 2021). Protein identification can be challenging, however, due to incomplete databases for the millions of proteins that are identified in metaproteomic studies (Qin et al., 2010; Kolmeder & de Vos, 2014; Salvato et al., 2021). Nevertheless, as an emerging field in microbiome research, databases are only growing larger and the number of studies is steadily increasing (Maron et al., 2007; Salvato et al., 2021). Upregulation of microbial proteins related to oxidative antimicrobial activities has been shown to correlate with changes to microbial functions that are associated with inflammatory bowel disease (Zhang et al., 2008). In colorectal cancer patients, altered abundance of microbial proteins related to oxidative stress, iron intake and transport, and DNA replication, repair, and recombination have been shown to have differential abundances compared to healthy patients (Long et al., 2020). Mycotoxin ingestion in piglets has also been shown to alter

the metaproteome, altering the abundance of different proteins such as those related to oxidative stress and metabolism (Saenz et al., 2021). Along with the other methods, combining metaproteomics with metagenomics and other microbiome research methods can provide a deeper insight into the composition and function of the microbiome.

4.3 PCR Primers

There is currently an array of different PCR primers available for microbiome analysis, particularly for the 16S rRNA gene in bacteria (Liu et al., 2021), and there is not a consensus on which primers are the best to use. Generally, referring to studies that one would like to compare results to and using the same PCR primers is a good method in order to avoid differences in results that can arise from using different primer sets (Wasimuddin et al., 2020; Liu et al., 2021). The following section discusses the various primer sets that are available and used for microbiome studies.

4.3.1 Bacteria

The bacterial 16S rRNA gene has nine hypervariable regions, V1-V9, that alternate with highly conserved regions, thus allowing for the development of universal PCR primers for these regions (Gray et al., 1984; Soergel et al., 2012). Nearly fifty different primers have been used for bacterial 16S rRNA gene studies (Soergel et al., 2012), making comparisons across studies difficult because different primers have different biases. For example, the 27F/338R primer set covers the V1-V2 region and is biased against bifidobacterial genes, whereas the 515F/806R primer set targets the V4 region and amplifies sequences for both bacteria and archaea (Goodrich et al., 2014a). The 341F/785R primer set, targeting the V3-V4 region, is one of the most commonly used primer sets for investigating microbial diversity due to its reproducible results and no obvious bias towards a specific species (Klindworth et al., 2013; Thijs et al., 2017; Fadeev et al., 2021). Studies have compared many of the primer sets and found that V4-V5 primer sets have more comparable results across sequencing platforms versus V1-V2 primers (Fouhy et al., 2016), and similar results compared to V3-V4 primers (Fadeev et al., 2021). Primer sets may also be combined for a single sample and aligned to make consensus sequenced contigs, allowing for higher resolution when analyzing microbiome data.

4.3.2 Archaea

The domain Archaea consists of 2,392 genomes (Rinke et al., 2021) and is unique in that as a domain, its taxonomy is based primarily on small subunit rRNA genes. Like bacteria, the 16S rRNA gene is used for archaeal identification in microbiome studies (Bahram et al., 2019). Universal primers that were supposed to be Archaea-specific were shown to have mismatches as well as bias towards taxonomic groups within the domain (Baker et al., 2003; Bahram et al., 2019). The primer pair 340F/1000R was shown to have higher specificity and coverage for Archaea than other commonly used primer sets (Gantner et al., 2011). More recently for short amplicon sequencing, the primer pairs SSU1ArF/SSU520R and 340F/806rB, covering the V1-V2 and V4-V5 regions, have been recommended for good Archaeal coverage in amplicon sequencing metagenomic studies (Bahram et al., 2019). Like bacteria, multiple regions may be sequenced from a single sample and then aligned to form consensus sequences, allowing for more in-depth analysis of microbiome data.

4.3.3 Fungi

Fungal ITS genes are used for amplicon analysis because they are able to amplify a large range of targets within the diverse kingdom Eumycota (Martin & Rygiewicz, 2005). Specifically, the highly variable ITS1 and ITS2 sequences found between the small and large subunit coding sequences of the ribosomal operon are used for identification of fungi within microbiome samples (Martin & Rygiewicz, 2005; Liu et al., 2021). Ideally, the entire ITS would be sequenced in order to provide higher resolution date, but generally only the variable regions are analyzed. It has been found that using ITS1 alone may overestimate diversity and richness and when compared, ITS1 and ITS2 showed differences in taxa identified (Yang et al., 2018). Novel primers to increase taxonomic coverage for ITS1 have been developed (Usyk et al., 2017), but the ITS2 sequence may be a more suitable marker for richness and taxonomic identification (Yang et al., 2018). Overall, primer selection for identifying fungi in microbiome samples determines overall composition and diversity, which makes primer choice – particularly when comparing to other studies or combining data sets – essential (Li et al., 2020b).

4.4 Sequencing and Bioinformatics

4.4.1 Sequencing Platforms

The next steps in amplicon metataxonomic analysis are sequencing and bioinformatic analysis, both of which have various available platforms and databases. Sequencing platforms include the Roche 454 Genome Sequencer FLX+, Illumina MiSeq™, Illumina HiSeq, PacBio® RS, and Life Technologies Ion Torrent PGM™. Sequencing platforms have intrinsic biases, such as AT-rich sequence bias (Quail et al., 2012), errors such as poly-base and substitution (Shendure & Ji, 2008; Liu et al., 2012; Ferrarini et al., 2013), quality scores, and read lengths (Allali et al., 2017). The most commonly used platforms for short read amplicon metataxonomic analysis in microbiome studies include Illumina MiSeq™, Roche 454 Genome Sequencer FLX+, and Life Technologies Ion Torrent PGM™ (Lear et al., 2018). When compared, microbiome composition was consistent between all three platforms, although relative abundances varied (Allali et al., 2017). Again, as is a common theme with microbiome research, it is important to use consistent methods for combined datasets and take methods into consideration when comparing results between studies because many factors, including sequencing platform, can have an effect on the results.

4.4.2 Sequencing Depth

It is also important when choosing a sequencing method to ensure that enough sequencing depth will be achieved. Both the Illumina MiSeq™ and Life Technologies Ion Torrent PGM™ are capable of producing over 5 million reads in a single sequencing run (Di Bella et al., 2013). More reads are generally better, and thus studies should be set up to achieve the maximum number of reads that fit within a budget. Read number ranging from 25,000 to 75,000 reads per sample has been shown to effect composition and predictions of factors affecting the microbiome (Ramakodi et al., 2021) and it has also been shown that as read number increases, so does species richness (Dickie, 2010; Gihring et al., 2012). The Earth Microbiome Project only used samples with at least 50,000 reads (Thompson et al., 2017) and the initial Human Microbiome Project had a mean of 6,212 sequences per sample (The Human Microbiome Project Consortium, 2012b). On the Illumina MiSeq™ platform, a sequencing depth of 50,000 to 100,000 reads per sample is generally obtained, which is considered acceptable for most microbiome studies (Liu et al., 2021).

4.4.3 Bioinformatics

A bioinformatics analysis pipeline combines numerous different software programs to complete analyses of complex datasets (Liu et al., 2021). In microbiome research this includes demultiplexing, quality control, clustering to operational taxonomic units (OTUs) or denoising to amplicon sequence variants (ASVs), and quantification (Liu et al., 2021). Several studies have compared the various software programs available for microbiome analysis. QIIME (Caporoso et al., 2010), UPARSE (Edgar, 2013), and DADA2 (Callahan et al., 2016) were compared for their ability to complete taxonomy assignments on the same dataset and it was found that UPARSE and DADA2 both yielded lower phylogenetic diversity than QIIME (Allali et al., 2017). QIIME and mothur (Schloss et al., 2009), both of which are all-in-one programs that trim, screen, align, and assign sequences to OTUs, have also been compared and it was found that both perform similarly for the most abundant taxa, but mothur produced higher richness for taxa with < 10% relative abundance (López-Garciá et al., 2018).

The database that is used for taxonomic classification can also affect clustering and diversity analysis results. There are five taxonomies available for clustering: Greengenes (McDonald et al., 2012), SILVA (Yilmaz et al., 2014), Ribosomal Database Project (RDP) (Wang et al., 2007), National Center for Biotechnology Information (NCBI) (Federhen, 2011), and Open Tree of Life Taxonomy (OTT) (Hinchcliff et al., 2015). Each of the NCBI, SILVA, RDP, and Greengenes taxonomies have many taxa that are not shared with the others – at least 63% for all classification levels from phylum to genus (Balvočiūtė & Huson, 2017). NCBI shares more taxa with SILVA than the others, and also contains most of the SILVA, RDP, and Greengenes taxonomies (Balvočiūtė & Huson, 2017). Another study using human oral microbiome data found that Greengenes, NCBI, RDP, and SILVA produced different taxa as high in ranking as phylum (Sierra et al., 2020). Greengenes retrieved the fewest unclassified OTUs and also identified uncommon genera, although misannotations can be an issue for this taxonomy as well as the others due to unverified specimen annotations (Sierra et al., 2020).

4.5 Diversity Analysis

Diversity analyses are used to compare microbiomes in order to determine differences in composition. The most commonly used are alpha and beta diversity,

although gamma and zeta diversity can also be used, albeit infrequently. There are numerous methods for determining these diversity measures, and the following section will define the diversity measures and methods of calculation.

4.5.1 Alpha

Alpha diversity, also known as within-sample diversity, is a measure of diversity within individual samples (Knight et al., 2018) and mean species diversity can be compared between groups to determine differences. There are many ways to calculate alpha diversity. The most basic alpha diversity measure is species richness, which is a strict count of the number of taxa within a given sample and does not take any other factors into consideration (Thukral, 2017). Richness indexes accounts for other factors, such as number of individuals in the sample and species abundance, and the most commonly used are Shannon's and Simpson's indexes (Thukral, 2017; Knight et al., 2018; Galloway-Peña & Hanson, 2020). Shannon's index measures evenness of abundance and a higher value equates to higher diversity (Shannon, 1948). Simpson's index measures the probability that two randomly selected individuals belong to the same group and a higher value indicates lower diversity (Simpson, 1949). Some other methods include the Chao1 index, which measures richness accounting for the number of species and the number of individuals represented by each species (Chao, 1984; Chao et al, 1993), and Faith's phylogenetic diversity, which measures phylogenetic richness (Faith, 1992).

4.5.2 Beta

Beta diversity, also known as between-sample diversity, is a measure of diversity between individuals and is used to measure differences in microbiome composition when comparing groups. Beta diversity is calculated by comparing feature dissimilarity and generating a distance matrix between all pairs of samples (Knight et al., 2018; Galloway-Peña & Hanson, 2020). The Bray-Curtis dissimilarity is a quantitative measure that takes into account taxa abundance (Bray & Curtis, 1957), and unweighted Unifrac is a qualitative measure that only accounts for presence and absence of taxa (Lozupone & Knight, 2005; Lozupone et al., 2007). Weighted unifrac takes into account phylogenetic relatedness (Lozupone & Knight, 2005) and provide biological patterns, but cannot be compared to data that does not use a phylogenetic tree (Knight et al., 2018). Beta diversity

data is visualized using principal coordinate or component analysis that reduces distance matrix to two- or three-dimensional plots (Knight et al., 2018).

4.5.3 Gamma

Gamma diversity measures species diversity on a geographic scale and measures overall diversity for different ecosystems within a region, or in the case of the microbiome, measures the diversity for large-scale samples such as ecosystems and community treatment measures (Jr. Hunter, 2002). This measure has been used for different studies such as determining diversity between habitats for samples collected via the Earth Microbiome Project (Walters & Martiny, 2020), comparison of pooled and individual samples (Ray et al., 2019), and comparison between animal populations (Couch et al., 2021).

4.5.4 Zeta

Zeta diversity is a relatively new concept and quantifies variation in taxa composition over space or time in order to show patterns in biodiversity, what causes them, and how they respond to environmental change (Hui & McGeoch, 2014; McGeoch et al., 2019). In a study of soil bacterial communities, zeta diversity was shown to decline between sample plots, indicating a low number of taxa shared between them and a high rate of turnover (Bay et al., 2020). This measure will be important for future studies regarding changes in microbiome communities over time and incorporating rare, intermediate, and common taxa into the model (Bay et al., 2020).

4.6 Differential Abundance Analysis

Differential abundance analysis aims to identify microbial taxa that explain differences between communities (Knight et al., 2018). Differences in library size after the sequencing process can cause a few problems in microbiome data analysis, particularly differential abundance analysis. More bacterial species tend to be observed with higher sequencing depth, and thus samples with smaller libraries will have inflated beta diversity measures (Weiss et al., 2017). Rarefaction is a process that is supposed to help deal with these differences by removing all samples below a minimum library size threshold and then taking a subsample of the remaining libraries so that they are equal in size (Lin & Peddada, 2020a). This method, however, has been criticized because it removes valid

data, which can include entirely removing rare taxa from the analysis (McMurdie & Holmes, 2014; Gloor et al., 2017). Zero inflation is also an issue when analyzing microbiome data. Microbiomes are unique to the individual and only a small number of taxa are shared between all members of the group while others are rare either due to truly being absent or not observed due to sequencing bias, leading to a large number of zero counts within the dataset (Xu et al., 2015). The following section describes some of the more common methods for dealing with these inherent issues in microbiome datasets.

4.6.1 Distribution based tests

A common approach for identifying differentially abundant taxa between groups is using nonparametric tests such as the Mann-Whitney *U* test or Kruskal-Wallis test, because microbiome data are not normally distributed (Weiss et al., 2017). These tests, however, are not appropriate for compositional data such as relative abundances, and so parametric models have been proposed using a generalized linear model (Paulson et al., 2013b; McMurdie & Holmes, 2014; Weiss et al., 2017). The metagenomeSeq model uses a zero-inflated Log-Gaussian mixture model to help account for zero counts in microbiome data (Paulson et al., 2013b). DESeq2 (Love et al., 2014) and edgeR (Robinson et al., 2010) are both methods that assume a negative binomial distribution, and corncob (Martin et al., 2020a) uses a beta-binomial distribution (Nearing et al., 2021).

4.6.2 Compositional data analysis

Microbiome data are inherently compositional because they are naturally described using probabilities or proportions such as relative abundance (Gloor et al., 2017). Log-ratio transformations are used to make the data symmetrical and linear, thus providing information regarding abundances of features relative to each other (Gloor et al., 2017). Isometric log ratio transformation controls for false positives by testing for changes in log ratios between abundances (Egozcue et al., 2003; Knight et al., 2018). Some other examples of methods using log-ratio transformations include ANCOM (Mandal et al., 2015), and ALDEx2 (Fernandes et al., 2014), both of which are relatively conservative at identifying significantly differential abundant taxa (Weiss et al., 2017; Nearing et al., 2021), and ANCOM-BC (Lin & Peddada, 2020b), which corrects for bias due to differences in sampling across samples.

4.6.3 Linear discriminant analysis effect size

Linear discriminant analysis effect size (LEfSe) is used to detect changes in relative abundances between two or more groups in order to discover biomarkers in metagenomic data (Segata et al., 2011). Statistically significant features are determined and then whether a feature is more abundant in all groups or just one is deciphered (Segata et al., 2011). A linear discriminant analysis is then used to estimate effect size.

4.6.4 Which differential abundance analysis is best?

With so many different methods for differential abundance analysis of microbiome data available, determining which to use can be challenging. A few papers have compared various methods using simulated and previously published data (Weiss et al., 2017; Calgaro et al., 2020; Nearing et al., 2021; Wallen, 2021), although there is no clear answer as to which is “best.” False discovery rates have been shown to be higher for edgeR, metagenomeSeq, and LEfSe and lower for corncob, ALDEx2, and ANCOM (Calgaro et al., 2020; Nearing et al., 2021). DESeq2 was shown to increase sensitivity for smaller datasets but had a high false discovery rate for larger sample sizes and uneven library size (Weiss et al., 2017). ANCOM was shown to work well for studies with more than twenty samples per group with a low false discovery rate (Weiss et al., 2017). Another study investigated agreement between methods, and it was found that ANCOM-BC and LEfSe had good agreement with other methods, whereas edgeR and metagenomeSeq did not, leading to the suggestion that using multiple methods and comparing results may be useful (Wallen, 2021).

4.7 Microbiome Genome Wide Association Studies

Genome wide association studies (GWAS) are used to compare genetic variants across genomes and determine which are associated with a specific trait or disease, such as obesity and autoimmune disease (Uffelmann et al., 2021). A microbiome GWAS (mGWAS) uses microbiome diversity metrics such as alpha and beta diversity or relative abundance as the response variable and genotype as the explanatory variable to identify genetic factors that may modulate the microbiome (Awany et al., 2019). The results of some of these types of studies were previously discussed in section 3.2.2.

Two microbiome-specific methods have been developed for completing mGWAS analysis. MicrobiomeGWAS (Hua et al., 2016) uses linear regression and beta diversity

to identify host genetic variants associated with the microbiome and environment and the microbiome-association index (Rothschild et al., 2018) quantifies the overall association of the microbiome with host phenotype. There are many challenges involved in mGWAS analysis due to the complex nature of microbiome data and the numerous factors that can affect microbiome composition. Additionally, because microbiome data consists of hundreds of bacterial taxa, a reduction in statistical power may occur due to corrections for multiple tests such as permutation tests or a Bonferroni correction (Awany et al., 2019). When conducting mGWAS studies, it may be necessary to focus on only a subset of taxa or genetic variants, and it is crucial to have sufficient sample sizes to ensure appropriate statistical power (Awany et al., 2019).

CHAPTER 5. MATERIALS AND METHODS

5.1 Parasites

All parasites for this study were collected between August 2019 and November 2021 from foals humanely euthanized as part of a regular research program under the University of Kentucky IACUC protocol 2012-1046. Intestinal content samples from the jejunum were also collected at necropsy. Parasites were placed into PBS immediately after removal from the small intestine, rinsed with water, and then placed into sterile PBS for further processing. Jejunal samples were snap frozen in liquid nitrogen and stored in an -80˚C freezer.

5.1.1 Whole worm microbiota

Three adult male, three adult female, three immature, eggs isolated from feces, and eggs isolated from female ovaries were collected from each of three foals. Parasites were serially washed in 70% ethanol and sterile water three times before being snap frozen in liquid nitrogen and stored in an -80˚C freezer until DNA extraction. Once thawed, a section from the center of adult parasites, determined by folding the worm in half and taking an approximately 2.5 cm section, was used for DNA extraction. Whole immature parasites were used for DNA extraction due to their small size.

5.1.2 Gonad and intestinal microbiota

A total of 46 adult *Parascaris* sp. (24 male and 22 female) were collected from three foals. The parasite surface was washed with 70% ethanol and then the worm was dissected using fresh, sterile scalpels. All other tools were sterilized with 70% ethanol. Gonads and intestines were dissected from each individual parasite and placed into sterile 15mL tubes, snap frozen in liquid nitrogen, and stored in an -80˚C freezer.

5.1.3 Egg isolation

Parascaris eggs were isolated from fecal samples and ovaries of freshly collected female parasites using the following protocol. In order from top to bottom, sieves with mesh sizes of 841 µm, 425 µm, 150 µm, and 75 µm were stacked, and feces placed in the 841 µm sieve. The feces were washed until the water run off ran clear, and the final content of the 75 µm sieve were collected in 50 mL falcon tubes to the 7.5 mL mark for

as many tubes as necessary to collect all content in the sieve. The tubes were subsequently filled to the 50 mL mark with sugar salt solution (specific gravity 1.25), vortexed to mix, and then centrifuged at 300 x g for ten minutes. The top 20 mL of the samples were then filtered through 200 µm and 100 µm pluriStrainer© cell strainers (pluriSelect Life Sciences, Liepzig, Germany) to collect the eggs, rinsed with distilled water, and stored in 5 mL tubes in an -20˚C freezer for further analysis.

Eggs were isolated from an additional six fecal samples using the previously described protocol, and then isolated further using the following protocol adapted from Norris et al., 2019. Samples were thawed overnight in the refrigerator at 4˚C, transferred to 15 mL conical tubes, and centrifuged at 300 x g for five minutes to form a pellet. Water was removed from the tubes down to the pellet, taking care not to disturb it. A total of 7.0 mL of sugar salt solution at specific gravity 1.12 was added to each tube, and then pipetting down the side of the tube to minimize mixing, 7.0 mL of blue-dyed sugar salt solution (specific gravity 1.07) was also added to each tube. All tubes were then centrifuged at 800 x g for twenty minutes. The top 9 mL of fluid, down to the 6 mL line, was carefully removed and discarded. From the top down to the pellet, all remaining fluid was removed using a disposable pipette and strained through a 100 µm pluriStrainer© cell strainer (pluriSelect Life Sciences, Liepzig, Germany) to remove the sugar salt solution. Eggs were then removed from the strainer and placed into a 2 mL tube with a 10% hypochlorite solution for two minutes to sterilize and decorticate the eggs. The eggs in bleach were again strained through the 100 µm cell strainer, rinsed twice with deionized water, and then placed into 2 mL tubes with deionized water. The tubes were centrifuged at 300 x g for five minutes, the top 500 µL of water removed to allow for expansion in the freezer, and then stored an -20˚C freezer for further analysis.

5.2 Next Generation Sequencing

5.2.1 DNA extraction

DNA extraction was completed using the Zymo Quick-DNA Fecal/Soil Microbe Kit (Irvine, California) with the following modifications. First, samples were placed in a 2 mL MP Biomedicals (Santa Ana, California) Lysing Matrix A tube with 750 µL of BashingBead™ Buffer and then placed in a Bead Ruptor 12 (Omni International, INC., Kennesaw, Georgia) for two 90-second rounds on high. Final elution was performed using

75 µL of 10 mM, pH 8.5, Tris-HCl Buffer (bioWORLD, Dublin, Ohio). DNA quantification was performed using the Qubit 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts).

5.2.2 Next generation sequencing library preparation and sequencing

Library preparation for gonad, intestine, and whole worm samples was completed using the Illumina 16S metagenomic sequencing protocol (Illumina, 2013). Female gonad samples were additionally prepared using the Swift Amplicon™ 16S+ ITS Panel (Integrated DNA Technologies, Coralville, Iowa) following manufacturer's instructions. Quantification was performed on an Agilent Technologies Stratagene Mx3000P (Santa Clara, California) using the Collibri™ Library Quantification Kit (Invitrogen, Waltham, Massachusetts) following manufacturer's instructions and quality analysis was performed using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California). Library pooling was completed following the respective protocols, and sequencing was performed with the Illumina MiSeq™ (San Diego, California) using the MiSeq™ reagent kit V3 2x300 (Illumina, San Diego, California) at the University of Kentucky Genomics Core Laboratory.

5.3 Transmission Electron Microscopy

5.3.1 Sample preparation

One adult female *Parascaris* spp. specimen was collected at necropsy, rinsed with PBS, and immediately placed into a solution of 3.5% glutaraldehyde and 4% paraformaldehyde in 0.1M Sorenson's buffer for 48 hours. The parasite was then dissected to remove cuticle, intestine, and gonad and these tissues were processed as follows at the University of Kentucky College of Arts and Sciences Imaging Center. First, tissues were washed in a 0.1M Sorenson's buffer and then dehydrated in graded ethanol solutions, from 50% to 100%, for ten minutes each, and finally dehydrated twice in absolute ethanol for fifteen minutes each. Samples were then placed twice in propylene oxide for fifteen minutes and infiltrated with 50% Epon-Araldite (Ted Pella Inc., Redding, California) resin and 50% propylene oxide under a 60-watt lamp overnight. The propylene oxide and resin mixture was poured off and tissues placed in fresh 100% Epon-Araldite resin in rubber molds for one hour, and finally embedded in molds for 48 hours at 60˚C.

5.3.2 Sectioning and staining

Thick sections were cut on a Leica-Reichert Ultracut E (Leica Biosystems, Wetzlar, Germany) ultramicrotome with a glass knife at approximately 1-1.5 µm thickness, floated on a drop of distilled water on a class slide, and placed on a hot plate at a medium setting until dry. Toluidine blue was added to cover the sections for 10-15 seconds and then the sections were again rinsed with distilled water and dried on the hot plate.

Thin sections were cut on the Leica-Reichert Ultracut E ultramicrotome using an ultra 45 DiATOME® diamond knife (Hatfield, Pennsylvania) at 80-90 nm thickness and placed on Ted Pella 300 mesh thin bar copper grids (Redding, California). The grids were then dried overnight in a 50˚C oven. The sample was then stained with a drop of uranyl acetate for five minutes, rinsed with distilled water, and then stained with a drop of lead citrate for two minutes followed by a final rise with distilled water. The grids were then dried and placed into a grid box for storage.

5.3.3 Imaging

All imaging took place at the University of Kentucky College of Engineering Electron Microscopy Center using the Talos™ F200X TEM (Thermo Fisher Scientific, Waltham, Massachusetts).

5.4 Bacterial Cultures

5.4.1 Sample collection

Three adult female *Parascaris* spp. were collected from each of two foals at necropsy and immediately washed in PBS and then placed into 70% ethanol. Each parasite was dissected using tools washed in 70% ethanol and a fresh sterile scalpel. The gonad was carefully removed so as not to perforate the intestine, placed into an autoclave-sterilized petri dish, rinsed with PCR-grade water, and placed into a sterile 15 mL conical tube. PCR-grade water was placed into a sterile 15 mL conical tube and all tools used for dissection were dipped into the water to act as a negative control. Samples were then submitted to the bacteriology section of the University of Kentucky Veterinary Diagnostic Laboratory for culturing.

5.4.2 Culturing

Gonads were macerated using an autoclave-sterilized mortar and pestle with 6 mL of sterile tryptic soy broth (TSB) enrichment. Approximately 10 µL of sample emulsion was inoculated onto each solid agar media plate for culture under different environmental conditions. Aerobic cultures were conducted using Remel™ blood agar, 5% SB, TSA (BAP; Thermo Fischer, Waltham, Massachusetts), Remel™ EMB agar, Levine (EMB), Eugon chocolate agar (ECA; Biomed Diagnostics, Inc., White City, Oregon) and Remel™ Columbia CNA agar (CNA) plates incubated at 28˚ +/- 2˚C; microaerophilic cultures using BAP, EMB, ECA, and CNA plates incubated at 28˚ +/- 2˚C in a humidified environment with 8% +/- 2% CO_2; anaerobic cultures using BAP, Brucella blood agar (BRU; Anaerobe Systems, Morgan Hill, California), and ECA plates in an AnaeroPouch® System anaerobic pouch jar (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan); and fungal cultures using BAP, BD BBL™ Sabouraud dextrose agar (SAB; Becton, Dickinson, and Company, Franklin Lakes, New Jersey) and Mycosel™ mycrobiotic agar (MYCO; Becton, Dickinson, and Company, Franklin Lakes, New Jersey) plates incubated at 28˚ +/- 2˚C.

Aerobic and microaerophilic plates were examined for growth daily for fourteen days followed by another examination at day 30, anaerobic were examined after five days and at day 30, and fungal cultures were examined daily for 30 days. Isolated bacteria were identified using a Bruker Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometer (Billerica, Massachusetts) and fungi were identified via direct examination using a lactophenol aniline blue wet mount.

5.5 Data Analysis

5.5.1 Sequence processing

Raw paired 16S amplicon sequence data was converted into and retrieved as fastq files from the Illumina BaseSpace (https://basespace.illumina.com) interface using an Apple Mac Pro (Cupertino, California) running macOS High Sierra 10.13.6. Unless otherwise noted, default settings were used. Fastq quality was assessed, and adapter sequences and low-quality reads were removed using dada2 (1.22.0; Callahan et al., 2016). A conservative minimum read length of 250 nucleotides was imposed for all reads. R (4.1.2; R Core Team, 2021), BiocManager (1.30.16; Morgan, 2019) and Bioconductor libraries BiocStyle (2.22.0; Oleś, 2021a), phyloseq (1.38.0; McMurdie & Holmes, 2013), DECIPHER (2.22.0; Wright, 2016), phangorn (2.8.1; Schliep et al., 2017), decontam

(1.140; Davis et al., 2018) as well as standard R libraries ggplot2 (3.3.5; Wickham, 2016), gridExtra (2.3; Baptiste, 2015), and knitr (1.37; Xie, 2020) were used in amplicon sequence analysis. The decontam package removed common contaminants in the data using control sample and DNA quantification data. The plotQualityProfile function provided by the dada2 R package was used to visualize a summary of the distribution of quality scores for a selection of forward and reverse reads and assess quality thresholds. The function filterAndTrim was used to filter paired reads. In order to reduce computation time by reducing redundant comparisons, the derepFastq function was used to dereplicate amplicon sequences contained within the filtered data, producing a series of "unique sequences" with corresponding "abundance" estimates. Error rates were estimated using the learnErrors function and plotted using the plotErrors function to assess if error rates were reasonably well-estimated. Samples were clustered and denoised using the dada function, reducing sample error, and inferring membership composition of the samples. Paired reads were merged, tabularized, and chimeric sequences were removed using the mergePairs, makeSequenceTable, and removeBimeraDenovo functions. Phyloseq and the SILVA Non-redundant rRNA Sequence Library (v132; Quast et al., 2013; Yilmaz et al., 2014) were used to analyze microbiome data and assign taxonomic rankings. All ASVs found in control samples, identified as Eukaryota, or without a named phylum were removed from downstream analysis.

5.5.2 Diversity analysis

Prior to conducing diversity analyses, all abundance counts were converted to relative abundance by aggregating ASV data to genus and then dividing genus abundance counts by total reads for a particular sample. Diversity analysis was conducted on relative abundance data using the vegan (2.5-7; Oksanen et al., 2020) package for R. Alpha diversity was calculated using both the Shannon and Simpson diversity indexes. Beta diversity was calculated using the Bray-Curtis dissimilarity and was visualized using principal coordinate analysis (PCoA). Statistical significance was tested for beta diversity using a beta-diversion calculation which was then tested for significance using an ANOVA with Tukey correction. Core microbiomes were determined using a > 0.5% relative abundance in > 20% of the samples for whole and organ microbiome samples, and a > 0.5% relative abundance in > 90% of samples for the female gonad only study, using the phyloseq, knitr, and microbiome (1.16.0; Lahti & Shetti, 2019) packages in R.

5.5.3 Statistical analysis

Alpha diversities were tested at the taxon level for normality using the Shapiro-Wilk test. All normal distributions were then tested for statistical significance using an ANOVA with Tukey adjustment, and all nonparametric distributions were tested using the Kruskal-Wallis and Dunn's tests with Bonferroni correction. Statistical analysis was completed in R with the dplyr (1.0.8; Wickham et al., 2022), lsmeans (2.30-0; Length, 2016), and FSA (Ogle et al., 2022) packages with $\alpha = 0.05$.

5.5.4 Differential abundance analysis

Differential abundance analysis was completed using four different methods that were then compared to determine commonalities in differentially abundant taxa. This analysis was completed in R using the tidyverse (1.3.1; Wickham et al., 2019), phyloseq, edgeR (3.36.0; Robinson et al., 2010), DEFormats (1.22.0; Oleś, 2021b), DESeq2 (1.34.0; Love et al., 2014), apeglm (1.16.0; Zhu et al., 2018), corncob (0.2.0; Martin et al., 2020a), ANCOMBC(1.4.0; Lin & Peddada, 2020b), eulerr (6.1.1; Micallef & Rodgers, 2014), and metagenomeSeq (1.36.0; Paulson et al., 2013a), libraries. The four differential abundance analysis methods used were ANCOM-BC, DESeq2, corncob, and metagenomeSeq, and results were compared using a Venn diagram using eulerr.

5.5.5 Consensus sequences

For the dataset produced using the Swift Panel, an in-house pipeline closely based on the Swift 16S SNAPP analysis workflow (https://github.com/swiftbiosciences/16S-SNAPP-py3) was run using the RDP Taxonomy (v18; Wang et al., 2007) database after sequence processing. The outputted consensus sequences were then searched against the curated NCBI Non-redundant 16S rRNA Database using blastn v2.9.0+ (https://blast.ncbi.nlm.nih.gov/). An in-house R script utilizing the NCBI efetch tool (16.6; Sayers, 2009) was used to convert accession numbers to genus and species classifications.

Consensus sequences were filtered to keep only those at least 500 bp in length. The BLAST E-score and percent identity were used in conjunction to determine taxonomic assignment. The E-score is the number of hits with a similar quality score that can be found by chance (Scholz, 2022), and a lower score means that there are a small number

of high-quality hits for a given sequence within the database. A genus level cut off of ≥ 95% and a conservative species-level cut off of ≥ 99% were used for percent identity (Stackebrandt & Goebel, 1994; Rossi-Tamisier et al., 2015).

5.5.6 Phylogenetics

An NCBI GenBank (https://www.ncbi.nlm.nih.gov/nuccore/) search was conducted for genera used in phylogenetic analysis using the search term "*genus name* 16S ribosomal RNA," and submissions within the 1200 – 1600 bp liner DNA range, along with an *E. coli* strain for use as an outgroup, were selected for use in phylogenetic comparisons. Sequences were aligned using Clustal Omega (McWilliam et al., 2013) and then trimmed and redundant sequences removed using Jalview (2.11.2.1; Waterhouse et al., 2009). Phylogenetic trees were created in FastME 2.0 (Lefort et al., 2015) using neighbor joining (Saitou & Nei, 1987) and Kimura two parameter model (Kimura, 1980) as previously described (Artuso et al., 2021a) with a 1,000-replicate bootstrap, and finally visualized in iTOL (6.5.2; Letunic & Bork, 2021).

5.5.7 TEM image analysis

TEM image analysis was completed in collaboration with Dr. John Shields, an expert in ultrastructure from the University of Georgia. Images from previously conducted nematode ultrastructure studies that included mitochondria were collected and can be seen in Figure 5.1 and permission for reuse information can be found in Table S8.1. These images were then used to compare to *Parascaris* spp. TEM images and to set criteria for determining whether structures were mitochondria or candidate bacteria. Criteria for identifying mitochondria included an oblong (ex. Figure 5.1K) or round (ex. Figure 5.1O) shape, and the presence of irregularly spaced tubular cristae. Candidate bacteria were objects that did not meet criteria for mitochondria and were similar in size to *Wolbachia* (~ 600 nm x 890 nm; Manoj et al., 2021) and *Weissella* (~ 710 nm x 1.4 µm; Sinnathamby et al., 2018), both of which have previously been described as intracellular symbionts of parasitic nematodes.

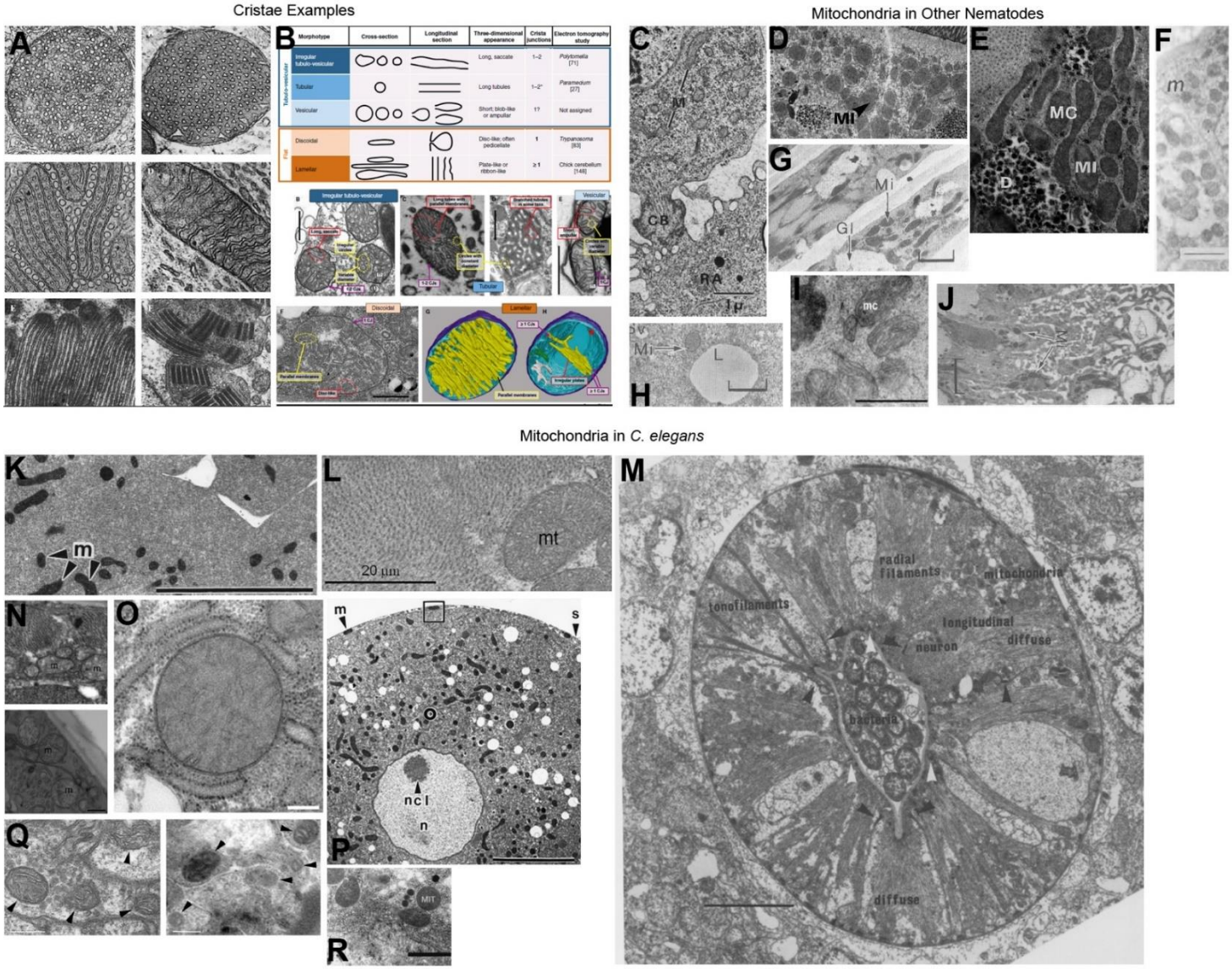
Cristae Examples
Mitochondria in Other Nematodes
Mitochondria in C. elegans
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
Morphotype
Cross-section
Longitudinal section
Three-dimensional appearance
Crista junctions
Electron tomography study
Irregular tubulo-vesicular
Tubular
Vesicular
Discoidal
Lamellar
Long, saccate
Long tubules
Short, blob-like or ampullar
Disc-like; often pedicellate
Plate-like or ribbon-like
Trypanosoma [83]
Chick cerebellum [148]
Mi
MI
MC
D
CB
RA
1μ
Gl
mc
L
m
mt
20 μm
ncl
n
MiT
radial filaments
mitochondria
tonofilaments
longitudinal
diffuse
neuron
bacteria
diffuse

Figure 5.1: Examples of mitochondria shape and cristae structure, mitochondria from *Caenorhabditis elegans,* and mitochondria found in various other nematodes. A (Zick et al., 2009) and B (Pánek et al., 2020) provide examples of different mitochondrial cristae structures in eukaryotes. C – J depict transmission electron microscopy (TEM) images of mitochondria from different species of nematode and K – S show TEM images of mitochondria in C. elegans. M also shows bacteria within C. elegans. C (Foor, 1972) scale bar = 1 µm, mitochondria = M; D (Mehlhorn & Harder, 1997) 18,000 x, MI; E (Mehlhorn & Harder, 1997) 25,000 x, MI; F (Bruňanská, 1994) 0.35 µm, m; G, H, J (Fellowes et al., 1999) 1 µm, Mi; I (Yushin & Coomans, 2005) 1 µm, mc; K, P (Hall et al., 1999) 5 µm, m; L (Sant'anna et al., 2013) 20 µm, mt; M (Hedgecock & Thomson, 1982) 2 µm; N (Tan et al., 2008) m, 500 nm; O (Byrne et al., 2019) 200 nm, m; Q (Morsci et al., 2016) 0.2 µm, arrows; R (Clayes et al., 2004) 1 µm, MIT.

CHAPTER 6. RESULTS

6.1 Sequencing Results

In total, three sequencing runs were completed on the Illumina MiSeq™. The first run included all whole worm, gonad, and intestinal specimens for a total of 129 samples and yielded 11.24 gigabases (Gb) with 87.57% passing filter. After processing through the decontam run, 7,785,001 reads remained with a mean of 60,349 reads per sample (range: 45 – 308,386).

The second run included female gonad samples run in duplicate for a total of 44 samples and yielded 12.23 Gb with 94.89% passing filter. Post-decontam, 9,060,450 reads remained with a mean of 192,776 reads per sample (range: 987 – 310,195).

The final run included the female gonad samples used with the Swift kit for a total of 26 samples and yielded 9.44 Gb with 96.33% passing filter. After running decontam, 1,548,294 reads remained with a mean of 59,550 reads per sample (range: 377 – 114,453).

6.2 Whole Worm Microbiota

After final sequencing processing, a total of 132,375 reads remained for 31 whole

Table 6.1: Most prevalent taxa for whole worm microbiota samples.

	Classification	**Count** (n = 26)	**Prevalence** (%)
Genus	*Lactobacillus*	26	100.00
	Sarcina	25	96.15
	Streptococcus	23	88.46
	Clostridium sensu stricto 1	21	80.77
	Veillonella	20	76.92
	Mycoplasma	15	57.69
	Hydrotalea	13	50.00
Family	Lactobacillaceae	26	100.00
	Clostridiaceae 1	25	96.15
	Burkholderiaceae	23	88.46
	Streptococcaceae	23	88.46
	Veillonellaceae	23	88.46
	Rhizobiaceae	20	76.92
	Chitinophagaceae	18	69.23
	Moraxellaceae	15	57.69
	Mycoplasmataceae	15	57.69
	Sphingomonadaceae	14	53.85
	Enterobacteriaceae	13	50.00
	Family XI	13	50.00
Phylum	Firmicutes	26	100.00
	Proteobacteria	26	100.00
	Bacteroidetes	22	84.62
	Actinobacteria	18	69.23
	Tenericutes	15	57.69

worm microbiota samples with a mean of 4,270 reads per sample (range: 225 – 22,940). Prior to downstream analysis, all samples with less than 1,000 reads per sample were removed, along with the single sample of isolated eggs, leaving 26 samples (3 horse, 7 male, 8 female, 8 immature) for diversity and differential abundance analysis. Overall, a total of 22 phyla, 118 families, and 232 genera were identified in the whole worm microbiota samples. The most prevalent members of each of these taxonomic levels are presented in Table 6.1.

6.2.1 Diversity analysis

Overall, there were no significant differences between life stages and the horse jejunum for either alpha or beta diversity (Figure 6.1). When comparing samples for shared genera, only 28 were common between the four groups, and each group had at least twenty unique genera (Figure 6.2). A list of taxa found only within worms or found within worms with a higher relative abundance compared to the horse are presented in Table 6.2. Only one, *Pelomonas*, had significantly different relative abundance based upon sex, with males having a significantly higher relative abundance than immatures (p = 0.0449), where the genus was not detected.

Table 6.2: Taxa found within more than one worm specimen, or within worms with a higher relative abundance in comparison to horse jejunum. RA = relative abundance, CI = confidence interval

Classification	Sex	Count	Prevalence (%)	Mean RA (%)	95% CI (%)
Bacillus	I	3	37.50	0.82	(-0.62 - 2.25)
p = 0.4351	M	2	28.57	0.17	(-0.13 - 0.48)
	F	1	12.50	0.04	(-0.05 - 0.14)
F: Mycoplasmataceae	F	4	50.00	0.09	(0.01 - 0.18)
p = 0.6185	M	2	28.57	0.21	(-0.19 - 0.61)
	I	2	25.00	0.07	(-0.03 - 0.18)
F: Veillonellaceae	F	2	25.00	0.16	(-0.13 - 0.45)
p = 0.7741	M	1	14.29	0.02	(-0.02 - 0.05)
	I	1	12.50	0.31	(-0.38 - 1.00)
Fusobacterium	F	3	37.50	0.19	(-0.06 - 0.44)
p = 0.5902	M	2	28.57	0.15	(-0.08 - 0.39)
	I	1	12.50	0.11	(-0.13 - 0.35)
Janthinobacterium	I	5	62.50	3.52	(-0.63 - 7.66)
p = 0.1552	M	3	42.86	0.55	(-0.41 - 1.50)
	F	3	37.50	0.63	(-0.13 - 1.39)
Nocardioides	I	4	50.00	0.75	(-0.07 - 1.57)
p = 0.2517	M	2	28.57	0.21	(-0.10 - 0.52)
	F	1	12.50	0.13	(-0.16 - 0.41)
Pelomonas	M	4	57.14	0.44	(0.04 - 0.84)
p = 0.0379	F	1	12.50	0.12	(-0.14 - 0.38)
Sarcina	M	7	100.00	22.13	(4.91 - 39.36)
p = 0.3540	H	3	100.00	9.29	(-3.35 - 21.94)
	F	8	100.00	27.94	(14.28 - 41.60)
	I	7	87.50	25.37	(6.60 - 44.14)

I = Immature (n = 8), M = Male (n = 7), F = Female (n = 8), H = Horse (n = 3)

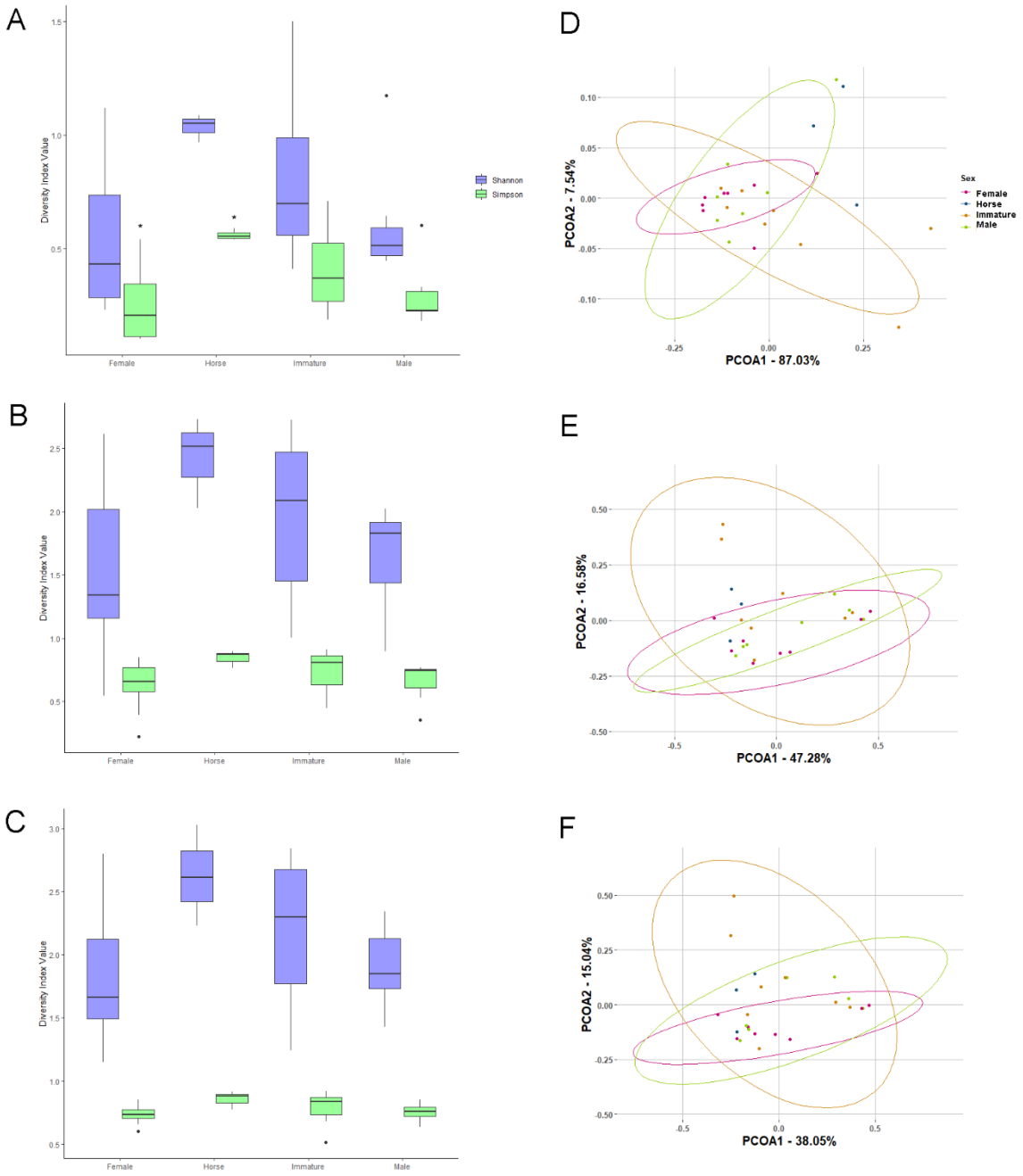

Figure 6.1: Alpha and beta diversity for whole worm microbiota. **A – C** are alpha diversity box plots showing both Shannon and Simpson alpha diversity, where • denotes outliers. **D – F** are beta diversity principal coordinate analysis plots with 95% confidence ellipses. Data is grouped by taxonomic level. **A, D** = Phylum, **B, E** = Family, and **C, F** = Genus.

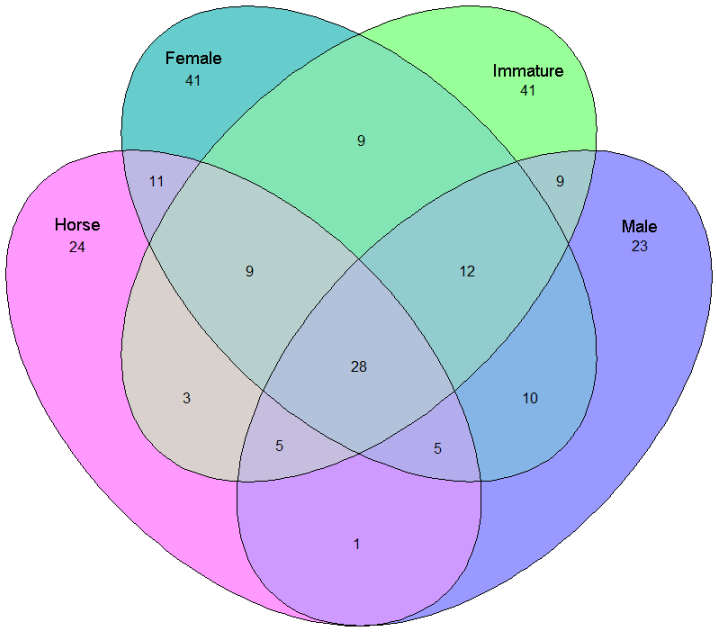

Figure 6.2: Venn diagram showing number of shared genera between all four study groups for whole worm samples.

6.2.2 Core microbiota

Heat plots generated to visualize the core microbiota for each group are shown in Figure 6.2 Comparison of these core microbiota (Figure 6.3) showed that at least two core taxa were unique to each group.

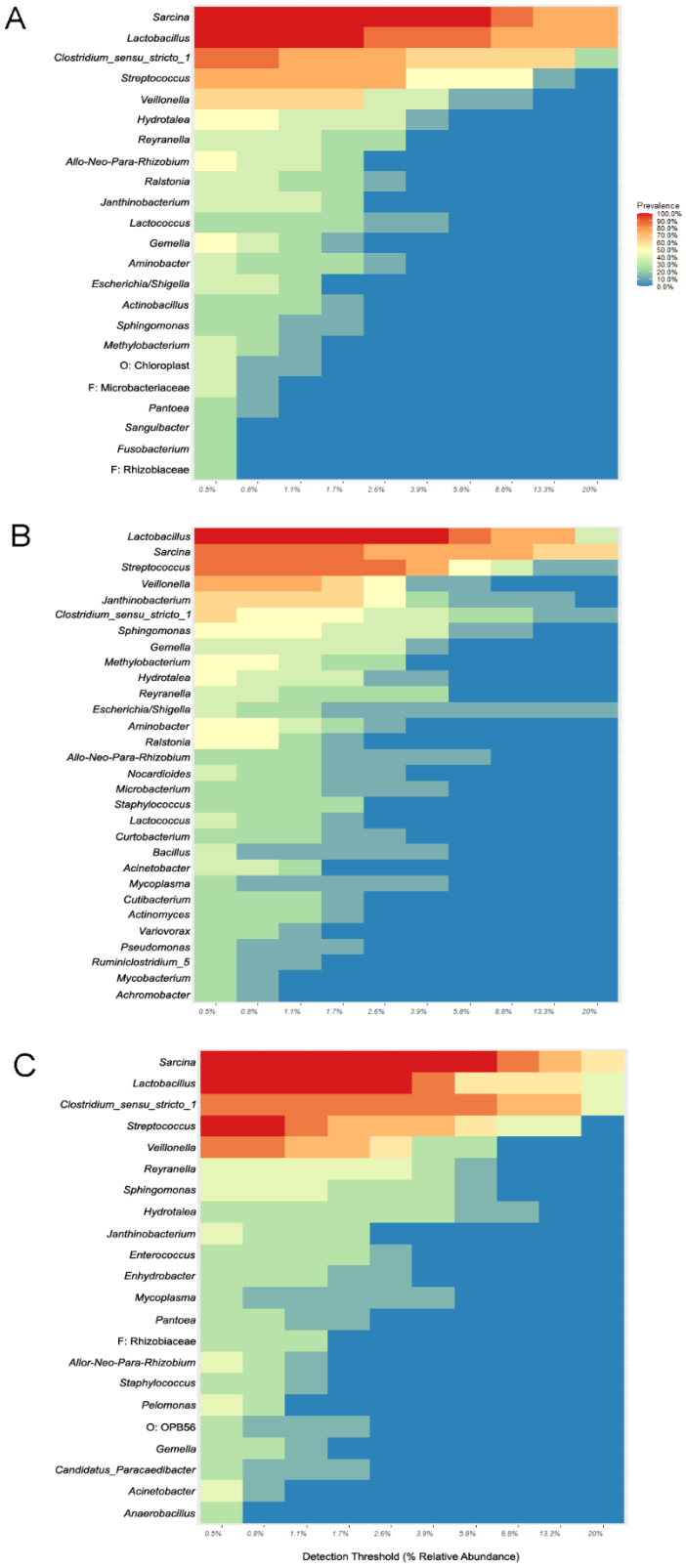

Figure 6.3: Core microbiota heat plots showing genera with a prevalence of at least 50% for **A** female parasites, **B** immature parasites, and **C** male parasites.

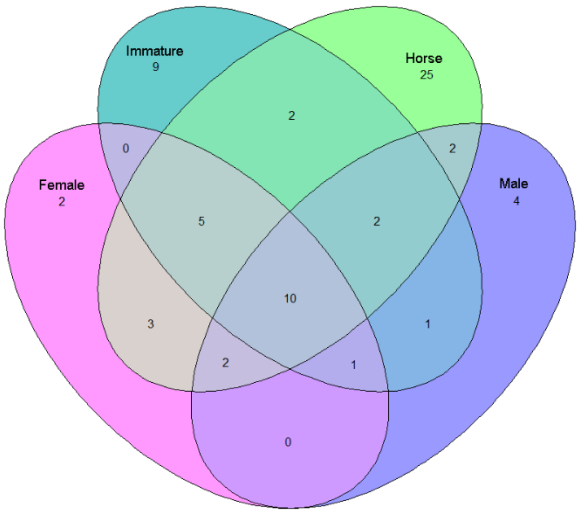

Figure 6.4: Venn diagram showing number of shared core taxa across all groups in whole worm microbiota samples.

6.2.3 Differential abundance analysis

Only ANCOM-BC and DESeq2 returned any differentially abundant taxa for the whole worm samples, and none of them were shared. The DESeq2 results indicated that *Enterococcus* was differentially abundant across samples (p = 0.0058) and ANCOMBC indicated that P: Proteobacteria (p = 0.0003) and *Sphingomonas* (p = 0.0003) were differentially abundant between female parasite and horse jejunum samples.

6.3 Gonad and Intestinal Microbiota

After final sequence processing, a total of 292,667 reads remained for 95 intestinal and gonad samples with a mean of 3,080 reads per sample (range: 0 – 11,148). Prior to downstream analysis, all samples with less than 200 reads were removed, leaving 83 samples (3 horse jejunum (HJ), 20 male gonad (MG), 23 male intestine (MI), 15 female

Table 6.3: Table of most prevalent taxa in organ microbiota study.

	Classification	Count (n = 83)	Prevalence (%)
Genus	*Lactobacillus*	77	92.77
	Mycoplasma	61	73.49
	Pseudomonas	59	71.08
	Sarcina	57	68.67
	Clostridium_sensu_stricto_1	53	63.86
	Sphingomonas	53	63.86
	Streptococcus	52	62.65
	Reyranella	51	61.45
	F: Mycoplasmataceae	50	60.24
	Veillonella	49	59.04
	Candidatus_Paracaedibacter	44	53.01
Family	Lactobacillaceae	77	92.77
	Clostridiaceae_1	66	79.52
	Burkholderiaceae	64	77.11
	Mycoplasmataceae	64	77.11
	Pseudomonadaceae	59	71.08
	Streptococcaceae	55	66.27
	Sphingomonadaceae	53	63.86
	Reyranellaceae	51	61.45
	Rhizobiaceae	51	61.45
	Veillonellaceae	51	61.45
	Enterobacteriaceae	47	56.63
	Chitinophagaceae	46	55.42
	Paracaedibacteraceae	44	53.01
Phylum	Proteobacteria	81	97.59
	Firmicutes	80	96.39
	Tenericutes	64	77.11
	Bacteroidetes	62	74.70
	Actinobacteria	45	54.22

gonad (FG), 22 female intestine (FI)) for diversity and differential abundance analysis. Overall, a total of 22 phyla, 145 families, and 294 genera were identified in the parasite gonad and intestinal microbiota samples. The most prevalent members of each of these taxonomic levels are presented in Table 6.3.

6.3.1 Diversity analysis

Alpha diversity was significantly different based upon both sex and location at all three taxonomic rankings (Figure 6.5 A-C). Simpson alpha diversity was significantly different between FI and MG at phylum ($p < 0.0001$), family ($p = 0.0058$), and genus ($p = 0.0018$) levels and FG and FI ($p < 0.0001$) and FI and MI ($p = 0.0072$) at the phylum level. Shannon alpha diversity was significantly different between FI and FG ($p < 0.0001$), HJ ($p = 0.0483$), MG ($p < 0.0001$), and MI ($p = 0.0007$) at the phylum level, FI and MG ($p = 0.0003$) at the family level, and FG and MG ($p = 0.0130$), FI and HJ ($p = 0.0383$), and FI and MG ($p = 0.0001$) at the genus level.

Beta diversity was significantly different between FI and FG ($p = 0.0377$) at the phylum level, MG and FG ($p = 0.0010$), FI ($p = 0.0174$), and HJ ($p = 0.0430$), and FG and

MI (p = 0.0061) at the family level, and MG and FG (p = 0.0006), MI and FG (p = 0.0093), and MG and FI (p = 0.0041) at the genus level. While not statistically significant based upon the alpha value set for this study, beta diversity tended to differ between MI and FI (p = 0.0560) and MG and HJ (p = 0.0578).

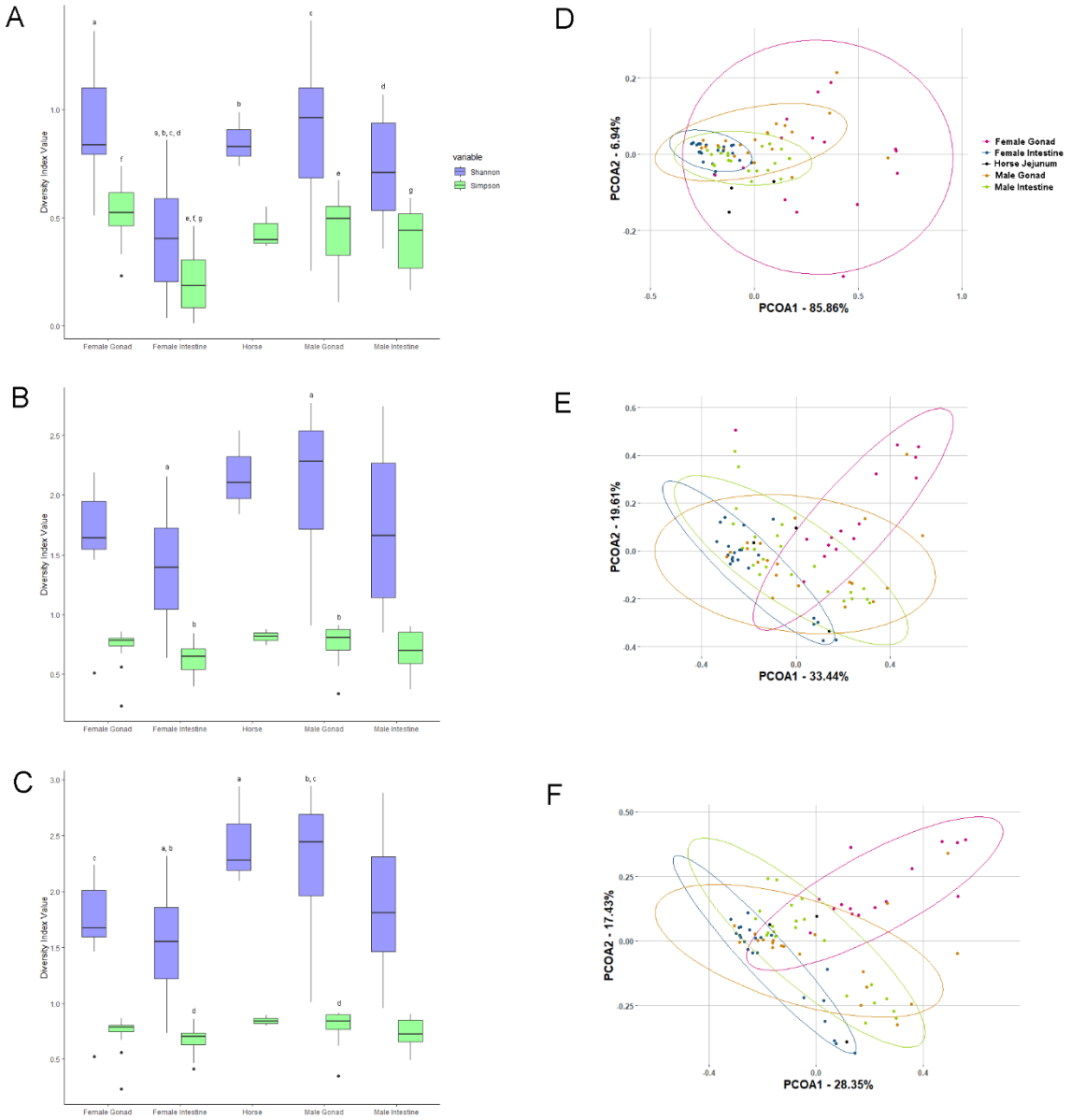

Figure 6.5: Alpha and beta diversity for worm organ microbiota. **A – C** are alpha diversity box plots showing both Shannon and Simpson alpha diversity, where • denotes outliers and same letters indicate significant differences. **D – F** are beta diversity principal coordinate analysis plots with 95% confidence ellipses. Data is grouped by taxonomic level. **A, D** = Phylum, **B, E** = Family, and **C, F** = Genus.

Comparison of all genera within groups indicated 23 shared genera across all groups and at least 8 unique genera for each group (Figure 6.6). A summary of taxa found

only within worms or found within worms with a higher relative abundance compared to the horse are presented in Table 6.4. All genera had significant differences when tested with Kruskal-Wallis. A full table of *p*-values resulting from Dunn's testing can be found in Table S8.2.

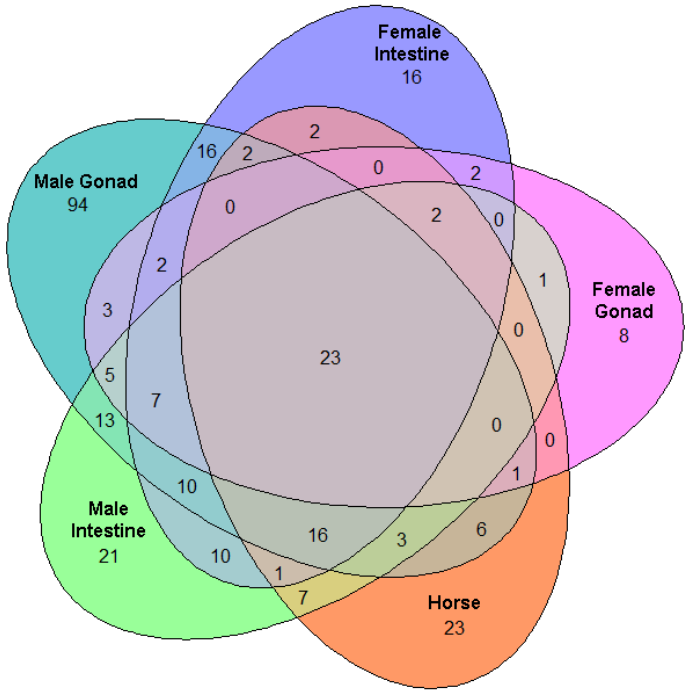

Figure 6.6: Venn diagram showing number of shared genera for each study group in the organ microbiota samples.

Table 6.4: Taxa found at a prevalence of ≥ 50% within worms only, or with a higher relative abundance compared to the horse. RA = relative abundance and CI = confidence interval.

Classification	Location	Count	Prevalence (%)	Mean RA (%)	95% CI (%)
Aminobacter	HJ	2	66.67	3.61	(3.50 - 3.71)
$p = 0.0086$	MI	13	56.52	1.47	(1.42 - 1.52)
	FG	8	53.33	5.86	(5.41 - 6.30)
	FI	9	40.91	0.40	(0.32 - 0.49)
	MG	2	10.00	1.07	(-0.34 - 2.47)
Bacillus	MG	13	65.00	1.01	(0.95 - 1.06)
$p < 0.0001$	MI	7	30.43	0.15	(0.11 - 0.18)
	FI	4	18.18	0.10	(0.03 - 0.16)
F: Mycoplasmataceae	FG	13	86.67	1.23	(0.88 - 1.59)
$p < 0.0001$	MI	18	78.26	0.67	(-0.15 - 1.49)
	FI	16	72.73	0.16	(-4.87 - 5.20)
	MG	3	15.00	0.09	(-2.07 - 2.25)
Gemella	MI	14	60.87	0.59	(0.42 - 0.75)
$p = 0.002$	FI	13	59.09	0.51	(0.37 - 0.66)
	MG	5	25.00	0.24	(0.02 - 0.47)
	FG	1	6.67	0.05	(-0.08 - 0.18)
Janthinobacterium	MG	17	85.00	5.02	(4.98 - 5.07)
$p < 0.0001$	HJ	2	66.67	0.92	(0.75 - 1.09)
	MI	9	39.13	0.72	(0.66 - 0.77)
	FG	5	33.33	4.32	(4.24 - 4.40)
	FI	4	18.18	0.24	(0.16 - 0.32)
Ralstonia	MI	16	69.57	1.06	(1.05 - 1.07)
$p = 0.0041$	FG	9	60.00	2.28	(2.27 - 2.30)
	MG	10	50.00	1.61	(1.60 - 1.63)
	HJ	1	33.33	0.11	(0.09 - 0.13)
	FI	5	22.73	0.14	(0.11 - 0.17)
Reyranella	HJ	3	100.00	3.01	(2.97 - 3.05)
$p < 0.0001$	FG	14	93.33	12.16	(12.13 - 12.19)
	MI	18	78.26	5.23	(5.20 - 5.27)
	FI	13	59.09	0.90	(0.89 - 0.90)
	MG	3	15.00	1.21	(1.21 - 1.22)
Sphingomonas	MG	17	85.00	4.91	(4.84 - 4.98)
$p < 0.0001$	MI	17	73.91	2.70	(2.65 - 2.75)
	HJ	2	66.67	1.93	(1.88 - 1.98)
	FG	9	60.00	8.67	(8.59 - 8.75)
	FI	8	36.36	0.37	(0.32 - 0.43)

FG = female gonad (n = 15), FI = female intestine (n = 22), HJ = horse jejunum (n = 3), MG = male gonad (n = 20), MI = male intestine (n = 23)

6.3.2 Core microbiota

Heat plots generated to visualize the core microbiota for each group are shown in Figure 6.7. Comparison of these core microbiota (Figure 6.8) indicate unique core taxa for MG and MI, and a total of four shared core taxa across all groups.

6.3.3 Differential abundance analysis

All four differential abundance analysis methods returned results for the organ data. ANCOM-BC and metagenomeSeq returned unique results, and there were two taxa shared across all four methods (Figure 6.9). *Sarcina* was differentially abundant across all four methods with $p < 0.0001$ and *Veillonella* was differentially abundant for DESeq2 and ANCOM-BC with $p < 0.0001$, corncob with $p = 0.0008$, and metagenomeSeq with $p = 0.0118$.

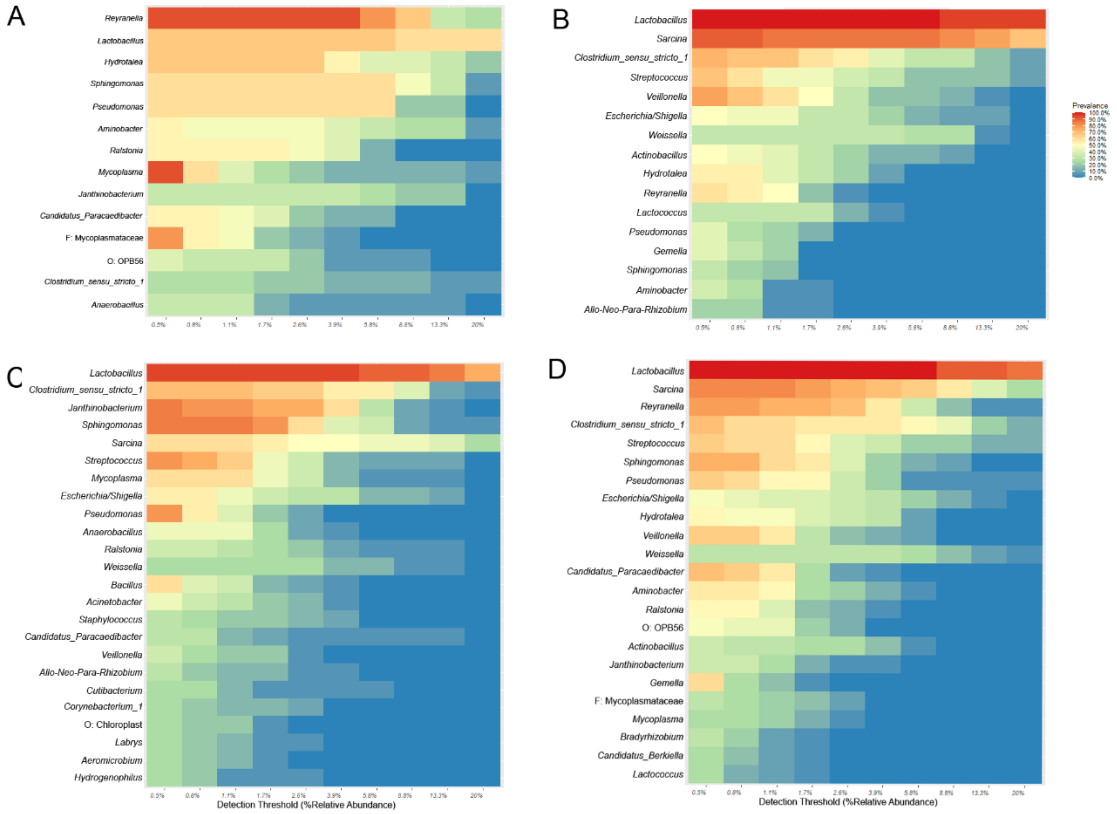

Figure 6.7: Core microbiota heat plots showing genera with at least 50% prevalence for **A** female gonad, **B** female intestine, **C** male gonad, and **D** male intestine

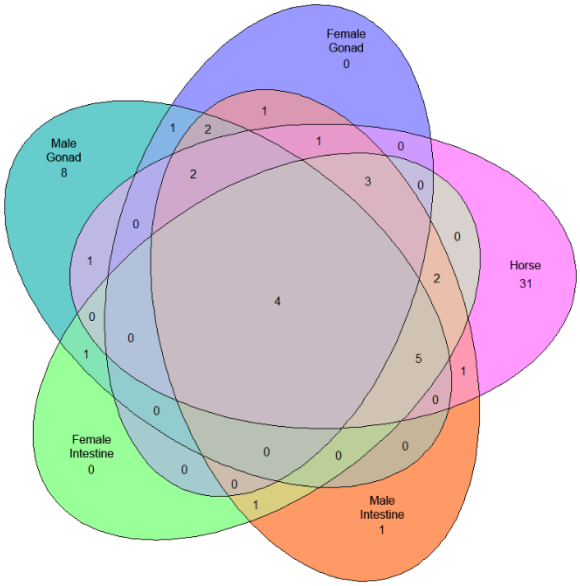

Figure 6.8: Venn diagram showing number of shared core taxa for each group in organ microbiota samples.

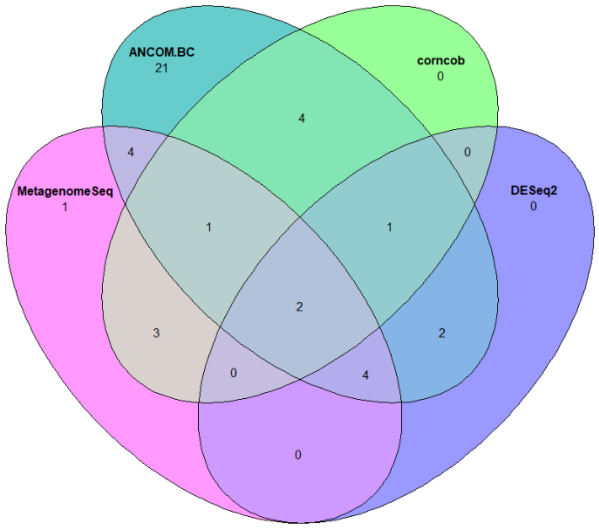

Figure 6.9: Venn diagram showing number of shared results between four different differential abundance analyses for organ microbiota samples.

6.4 Female Gonad

After final sequence processing, a total of 731,970 reads remained for 44 female gonad samples with a mean of 16,636 reads per sample (range: 0 – 26,364). Prior to downstream analysis, all samples with less than 10,000 reads per sample were removed,

leaving a total of 40 samples. Female gonad samples were run in duplicate for this particular run, and each sample was represented at least once in the sample set. Overall, a total of 15 phyla, 70 families, and 113 genera were identified in the female gonad samples.

A total of 579 consensus sequences were identified using the Swift 16S SNAPP analysis workflow, and after removing sequences shorter than 500 bp, a total of 221 (parasite = 133; horse = 88) consensus sequences remained with an average length of 609 bp (range: 522 – 944).

6.4.1 Diversity analysis and core microbiota

Alpha diversity was significantly different between samples from horse 260 and horse 66 (Figure 6.10) for both Shannon (p = 0.0073) and Simpson (p = 0.0098) diversity indices at the genus level. A heat plot was generated to visualize the core microbiota for the female gonad samples and is shown in Figure 6.11.

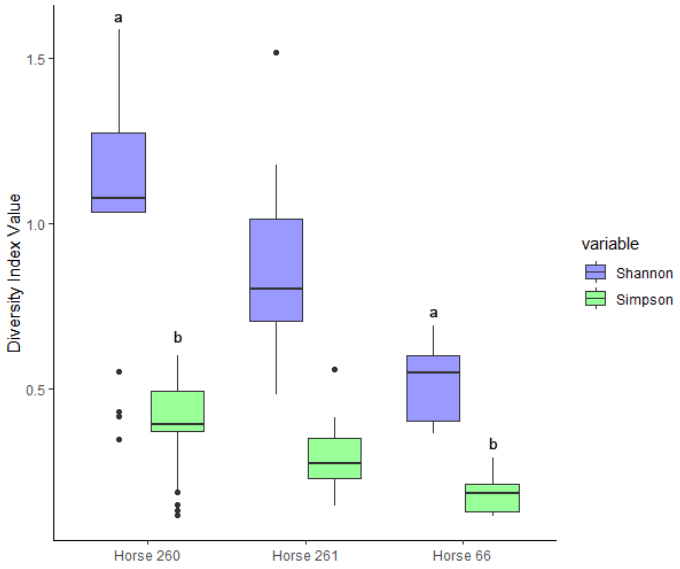

Figure 6.10: Box plot showing alpha diversity of female gonad samples grouped by horse. Same letters indicate significant differences and • indicate outliers.

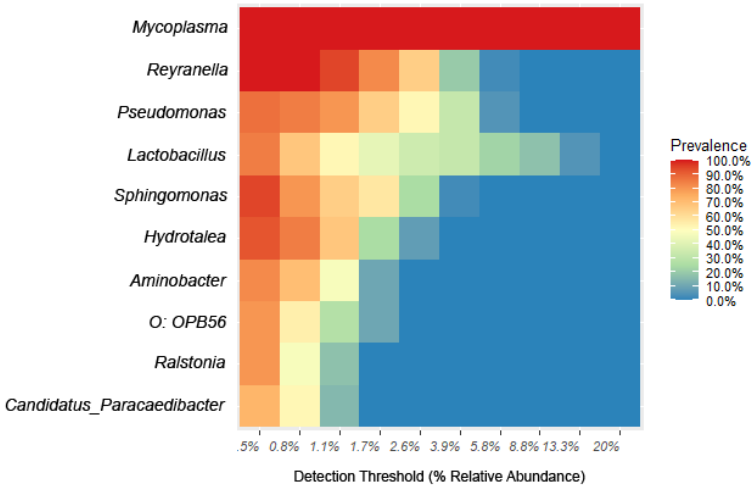

Figure 6.11: Heat map showing core genera for female gonad samples.

Table 6.5: Genus and species level identifications from female gonad consensus sequences.

Classification		Mean Length (bp)
Genus	*Aminobacter*	532
	Aquabacterium	546
	Brachybacterium	532
	Clostridium	582
	Kocuria	525
	Lactobacillus	748
	Ligilactobacillus	638
	Limosilactobacillus	604
	Mesorhizobium	576
	Mycobacterium	525
	Pantoea	685
	Pseudomonas	757
	Ralstonia	576
	Reyranella	547
	Sarcina	659
	Staphylococcus	525
	Streptococcus	524
Species	*Acinetobacter schindleri*	525
	Aquabacterium parvum	525
	Clostridium butyricum	525
	Clostridium paraputrificum	525
	Hydrotalea flava	525
	Labrys wisconsinensis	525
	Ligilactobacillus equi	525
	Ligilactobacillus hayakitensis	525
	Limosilactobacillus equigenerosi	525
	Ralstonia pickettii	532
	Reyranella aquatilis	529
	Sarcina maxima	532

6.4.2 Consensus sequences

After processing parasite consensus sequences based on E-score and percent identity, 31 sequences were identified to species level, 75 to genus level, 22 with a percent identity < 95%, and five that were unable to be identified due to an abundance of identical hits. Genus and species level identifications are summarized in Table 6.5 and full data can be found in Table S8.3.

6.4.3 Phylogenetic comparisons

Phylogenetic trees were constructed for consensus sequences identified as belonging to the genera *Limosilactobacillus*, *Ligilactobacillus*, *Aminobacter*, and *Reyranella* (Table 6.6). After trimming and removing redundant sequences, 27 specimens remained for *Limosilactobacillus*, 32 for *Ligilactobacillus*, 27 for *Aminobacter*, and 22 for *Reyranella*. The final sequences encompass the V3, V4, and V6-V9 hypervariable regions

Table 6.6: Summary of consensus sequences used for phylogenetic analysis.

ASV	ID	Length (bp)
S001567179	*Aminobacter* sp. ParFG	511
S003063129	*Reyranella* sp. ParFG	506
S000901488	*Ligilactobacillus* sp. ParFG01488	469
S001792808	*Ligilactobacillus* sp. ParFG92808.11	469
S001792808	*Ligilactobacillus* sp. ParFG92808.8	469
S003560971	*Ligilactobacillus* sp. ParFG60971.5	469
S003560971	*Ligilactobacillus* sp. ParFG60971.4	469
S003560971	*Ligilactobacillus* sp. ParFG60971.6	469
S003560971	*Ligilactobacillus* sp. ParFG60971.1	469
S003560971	*Ligilactobacillus* sp. ParFG60971.61	469
S003560971	*Ligilactobacillus* sp. ParFG60971.11	469
S003561013	*Ligilactobacillus* sp. ParFG61013	469
S000964154	*Limosilactobacillus* sp. ParFG64154	510
S001060020	*Limosilactobacillus* sp. ParFG60020	510
S003560979	*Limosilactobacillus* sp. ParFG60979	510

of the bacterial 16S rRNA gene, and a summary of sequences pulled from GenBank for the final phylogenetic analysis can be found in Table S8.4.

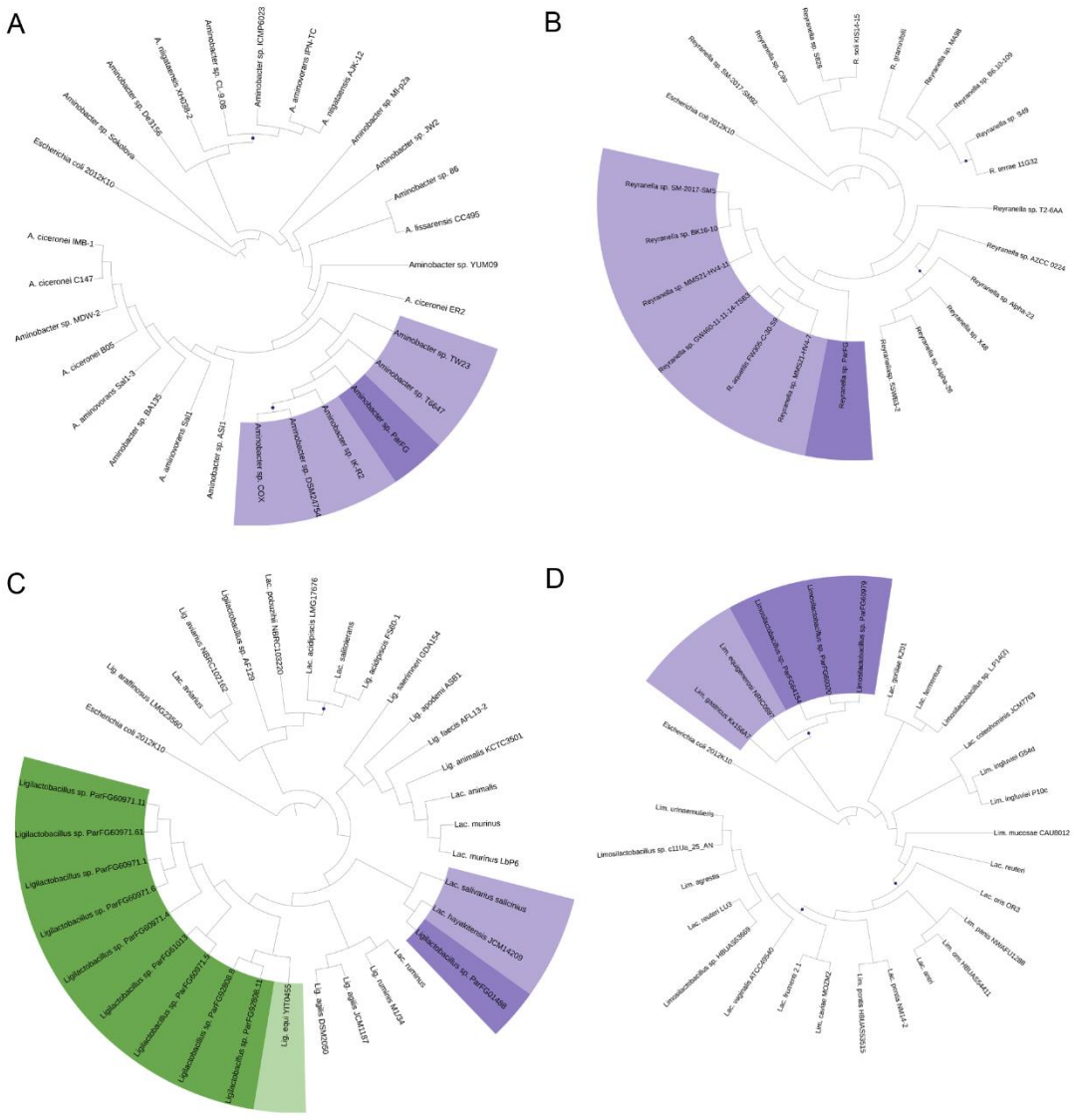

Figure 6.12: Phylogenetic trees for **A** *Aminobacter*, **B** *Reyranella*, **C** *Ligilactobacillus*, and **D** *Limosilactobacillus* specimens obtained from consensus sequences of bacteria from the *Parascaris* spp. female gonad. The • represents branches with > 70% bootstrapping values. Colored groups represent the clade formed with *Parascaris* spp. consensus sequences. The darker green or purple highlight the consensus sequences and the lighter green or purple highlight the rest of the members of the clade.

Phylogenetic trees were compared to those previously published using whole genome and 16S rRNA approaches in order to assess quality of tree construction using partial 16S rRNA sequences (Zheng et al., 2020; Artuso et al., 2021b). The *Aminobacter* tree did not closely resemble a tree recently made using whole genome sequences (Artuso et al., 2021a), although it contained more and different specimens. *Aminobacter* sp. ParFG formed a clade with five other unnamed species (Figure 6.12A) that have previously been isolated from soil and sediment. There are not any large-scale phylogenies that have been completed for *Reyranella*, however there is some agreement

in terms of clade formation for the named species (Ciu et al., 2017; Lee et al., 2017). *Reyranella* sp. ParFG formed a clade with six other *Reyranella* strains, including *R. aquatilius* FW305-C-30-S9 (Figure 6.12B).

Limosilactobacillus and *Ligilactobacillus* trees were compared to those recently published (Zheng et al., 2020) using long-read 16S rRNA sequences and both showed good agreement with the short read phylogenies. *Ligilactobacillus* sp. ParFG01488 formed a clade with *Lac. hyakitensis* JCM14209 and *Lac. salivarius salicinius* and nine additional *Ligilactobacillus* sp. ParFG specimens (Table 6.6) formed a clade with *Lig. equi* YIT0455 (Figure 6.12C). *Limosilactobacillus* sp. ParFG60979, ParFG60020, and ParFG64154 formed a clade with *Lim. equigenerosi* NRIC0697 and *Lim. gastricus* Kx156A7 (Figure 6.12D).

6.5 Transmission Electron Microscopy

A total of 270 images were taken of female *Parascaris* spp. gonad using the TEM. Representative images of toluidine blue stained thick sections and their corresponding thin sections can be seen in Figure 6.13. Using the criteria outlined in section 5.5.7., candidate bacteria were observed in two images (Figure 6.14).

6.6 Bacterial Culture

Three bacterial species were isolated from all cultures: *E. coli* and *Enterococcus faecalis* from one aerobic culture and *Fusobacterium varium* from one anaerobic culture.

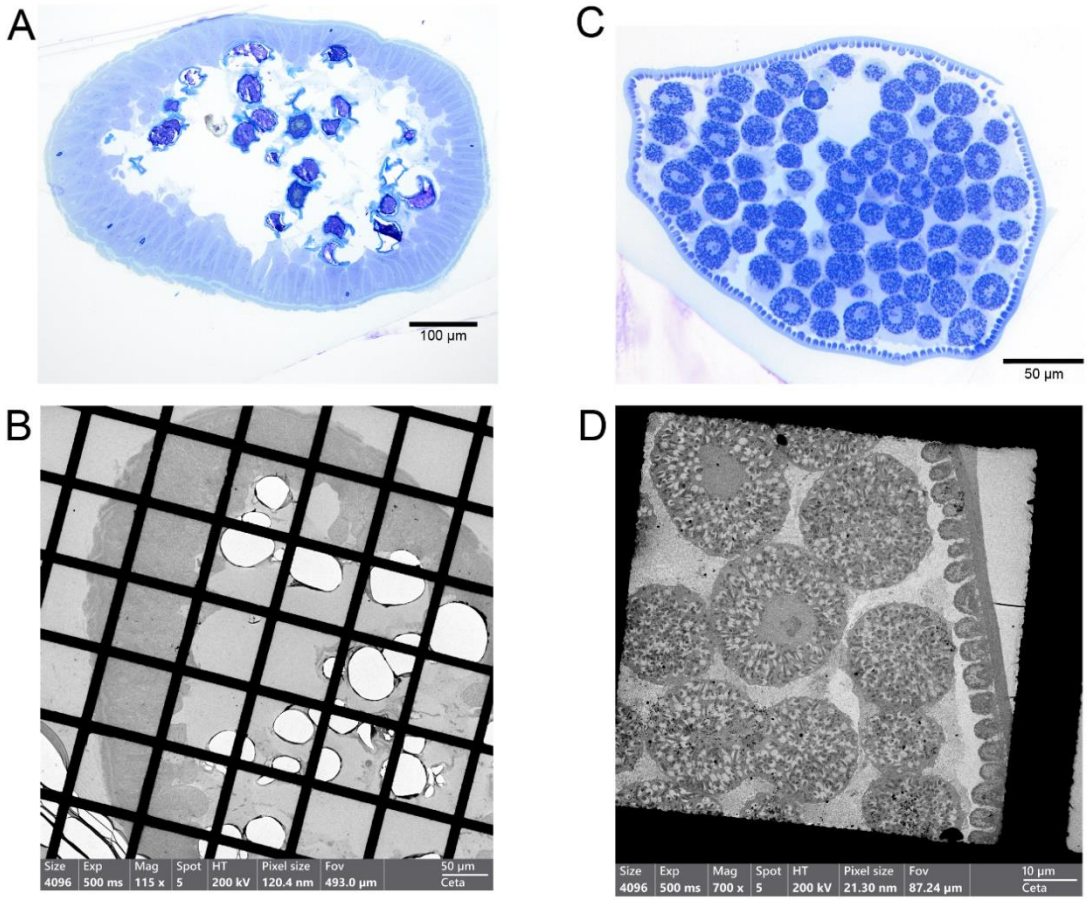

Figure 6.13: Representative transmission electron microscopy (TEM) images of *Parascaris* spp. female gonad. **A, C** are toluidine blue-stained thick sections and **B, D** are TEM images. **A, B** and **C, D** are the same specimens.

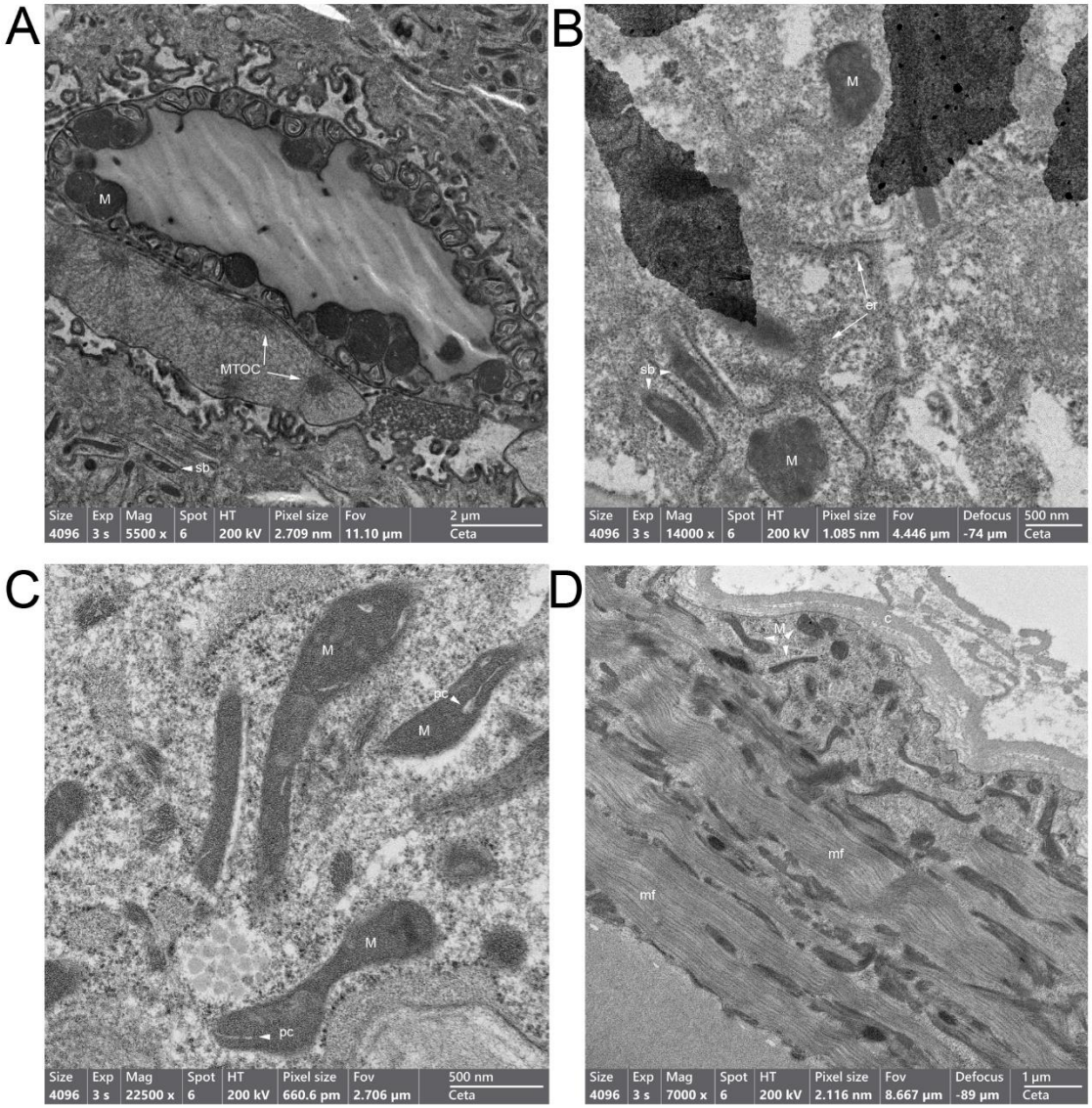

Figure 6.14: Transmission electron microscopy (TEM) images of *Parascaris* spp. female gonad. **A** and **B** present the two examples of possible bacteria, while **C** and **D** illustrate more examples of mitochondria. M = mitochondria MTOC = microtubule organizing centers sb = suspected bacteria er = endoplasmic reticulum pc = possible cristae mf = muscle fibers c = cuticle

CHAPTER 7. DISCUSSION AND FUTURE DIRECTIONS

Despite the importance of parasitic nematodes in global health – both in veterinary and human medicine – their microbiomes, and that of ascarids in particular, have been largely unstudied. The few exceptions include studies that: examined colonization of *Ascaris lumbricoides* by *Vibrio cholerae* (Nalin & McLaughlin, 1976); cultured nineteen facultative anaerobes from the intestine of *A. suum* (Hsu et al., 1986); and examined bacterial flora as a source of serotonin in *A. suum* (Shahkolahi & Donahue, 1993). Outside of these types of studies and the previously described research, however, the microbiome of parasitic nematodes remains a mystery. In 2019, a paper was published highlighting one hundred questions that need addressed in livestock helminthology research. These were chosen by vote from 385 questions submitted by veterinary parasitology researchers in an effort to help close knowledge gaps and direct research efforts in the field; the nematode microbiome was number 73 on that list (Morgan et al., 2019). Subsequently, a few papers have been published calling for more research and highlighting potential challenges, reinforcing the notion that helminth microbiome research is not only important, but possibly an up-and-coming area of study (Dheilly et al., 2017; Dheilly et al., 2019; Jenkins et al., 2019; Formenti et al., 2020).

The purpose of the work reported in this dissertation was to establish a core microbiota for *Parascaris* spp. and compare the microbiota at different life stages and between the sexes and organs of the worms. Overall, a few broad goals were accomplished with this work in addition to the original aims. First, a methodology for collection and analysis of microbiota data for parasitic helminths collected from horses was established. This sets the framework for future studies not only in horse parasites, but in parasites affecting other large animals hosts as well. Second, gaps in the knowledge gained from this study highlight the necessity for more studies using various methods to firmly establish a microbiota for these parasites. Finally, this research further highlights the necessity for microbiota research in a broader range of helminth parasites and the possibility that their microbiota could be utilized as a control mechanism in the future.

7.1 *Parascaris* spp. Common Core Microbiota

The common core microbiota consists of taxa that are shared by all, or most, members of a given group (Hamady & Knight, 2009; Shade & Handelsman, 2011).

Prevalence and abundance of a given microbiota member are not necessarily tied to function, and rare taxa can be essential for host survival (Jousset et al., 2017; Hammer et al., 2019). Identification of the common core, however, does facilitate an understanding of host-microbiome, phylogenetic, and population level microbiome compositions (Risely et al., 2020).

The combination of core microbiota results from both whole worm and organ microbiota studies utilized a total of 68 worms and indicated that there are eleven shared genera between the combined male, female, and immature whole worm core microbiomes and the combined male and female organ core microbiomes. The eleven genera are: *Acinetobacter*, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (ANPR), *Clostridium senso stricto* 1, *Gemella*, *Janthinobacterium*, *Lactobacillus*, *Reyranella*, *Sarcina*, *Sphingomonas*, *Streptococcus*, and *Veillonella*. Among these, differential abundance of *Sarcina* and *Veillonella* was observed in the organ microbiota study. All of these genera were also identified in the horse jejunal microbiome in this study, and all except *Reyranella* have previously been identified in equine gastrointestinal microbiomes (Costa et al., 2012; O'Donnell et al., 2013; Paßlack et al., 2020; Ang et al., 2022; Gilroy et al., 2022; Mach et al., 2022; Voss et al., 2022). *Reyranella*, however, has previously been identified in other microbiomes such as in human neonates and vaginas (Li et al., 2020a), fish (Zhang et al., 2016; Méndez-Pérez et al., 2020; Mondal et al., 2022), and shrimp (Zhang et al., 2016), as well as in the *Trichuris* spp. microbiome (García-Sánchez et al., 2022).

While is it not possible to discern any functional implications of this core microbiota from the present research, some of the members may be worth investigating further in future studies. *Reyranella*, for example, has been previously identified in the human vaginal microbiome and was also identified in this study at a higher prevalence and relative abundance in the female gonad compared to the other investigated sites, suggesting a possible function in the female reproductive tract. Additionally, the two differentially abundant genera, *Sarcina* and *Veillonella*, are also worth deeper investigation since they represent a differentiating factor between different organ compartments of *Parascaris* spp. Overall, this information provides a jumping-off point for future studies aimed at comparing various populations, identifying common members, and selecting microbiota members for studies seeking to identify functional aspects of the microbiome.

7.1.1 Whole worm, intestine, and gonad results

Comparisons between whole worm and organ results suggest that whole organism analysis of microbiota for helminths may mask nuances in microbiota structure within an organism. The organ study identified 27 more families and 62 more genera than were found in the whole worm study, including many that were shown to be unique to different sexes and organs (Figure 6.6). It also highlighted differences in relative abundance, such as the higher relative abundance of *Aminobacter*, F: Mycoplasmataceae, *Ralstonia*, and *Reyranella* in the female gonad and of *Sphingomonas* and *Janthinobacterium* in both male and female gonads (Table 6.4). Such nuances were not evident in the whole worm study and are significant because the microbiome can play an important role in parasite reproduction, as was discussed previously in sections 3.4.3 and 3.6.2. Future studies establishing relative abundance trends in the global *Parascaris* spp. population as well as metabolomic, metaproteomic, and *in vitro* studies will be essential for determining the roles that these genera play in the overall reproductive success of the parasite.

Additionally, these comparative results highlight both the need for larger sample sizes and for dissection as a method of studying parasite microbiomes. It is possible that more differences between groups would have been evident in the whole worm study had larger sample sizes been available. Nuances found in the organ study could also be unique to the parasites within the single horse population studied, and a larger sample size could either have uncovered different variances between groups, or that there are no discernible differences between them. Regardless, larger sample sizes, including more populations of *Parascaris* spp. from other horses, would provide a deeper insight into the parasite's microbiome. Dissection is also important to incorporate into parasite microbiome studies where possible. The microbiota varies between different organs, and thus it is important to consider the microbiome of different parasite organs when conducting a microbiome study. While the organ study herein only included the intestine and gonad, future studies could also include the cuticle and further subdivision of different parts of the male and female gonad to further characterize the parasite microbiome.

One aspect of the whole microbiota study that was unsuccessful was the analysis of more life stages than just those found in the small intestine. Immature parasites in the small intestine range in size and, in some cases, were difficult to extract DNA from. The same issue occurred with eggs from both the female gonad and extracted from feces; there was not enough DNA extracted to obtain any useable results. Future studies into finding the best ways to break up the cuticle for whole worm microbiota studies and to

break through the coat of the eggs in order to successfully extract DNA will be necessary before more life stages can be studied. Dissecting immature intestinal stages and analyzing the gonad, intestine, and cuticle separately are a good option moving forward and will provide more detailed information than simply extracting DNA from the whole worm for microbiome analysis. Another avenue is to explore the *Parascaris* spp. microbiome at different life stages by using an egg hatch protocol, as previously discussed in section 2.2.3; in this way, the microbiome of freshly hatched larvae could be assessed.

7.1.2 Comparison to previous Haemonchus contortus studies

Out of the few helminth parasite microbiome studies completed to date, *H. contortus* is the only parasite of veterinary importance to have had its microbiota analyzed in multiple studies. As discussed previously, there have been four studies completed and two of those used a 16S rRNA hypervariable region and Illumina NGS to analyze the microbiome of different life states as well as male and female *H. contortus* (El-Ashram & Suo, 2017; Mafuna et al., 2021). Comparing microbiome studies with different methods is not always ideal as many different factors can have an effect on results as previously discussed, and these two studies differed from one another in DNA extraction, library preparation, and taxnonomic database. The more recent Mafuna et al. (2021) study used a different DNA extraction method from the one described herein but used the same database for taxonomic assignment and the same library preparation protocol, and thus results can be compared with a good level of confidence.

In the first study, two different primer sets targeting the V3-V4 and V5-V7 regions were used to examine adult, L_3, and egg microbiota of *H. contortus* (El-Ashram & Suo, 2017). Alpha diversity was found to be higher for L_3, which is similar to the finding in this study that immature *Parascaris* spp. tended to have a higher alpha diversity than adult males or females. There were only three genera – *Escherichia/Shigella*, *Bacillus*, and *Pseudomonas* – that were highly prevalent in *H. contortus* and also found in individual *Parascaris* spp. core microbiota. None of these, however, were shared with the *Parascaris* spp. common core. While there are few similarities between the *H. contortus* microbiota and that of *Parascaris* spp., the methods for these two studies highly differed and thus comparisons must be interpreted with caution.

In the more recent study (Mafuna et al., 2021), male specimens had a higher alpha diversity than females, but both sexes clustered together for beta diversity. This is similar to results found in the present study, where there were no discernible clusters specifically

for male or female worms for beta diversity. Alpha diversity was similar for the whole worm study, however male *Parascaris* spp. gonad and intestine both had higher alpha diversity than female organs when parsed out. Out of the most prevalent genera in the *H. contortus* study, only *Acinetobacter* was shared with the *Parascaris* spp. common core microbiota, and out of the unique genera found in either male or female *H. contortus*, only *Corynebacterium* 1 and *Prevotella* were shared between the two parasite species. They were found in the male gonad and female and male intestine, and the male gonad, respectively, both with prevalences ≤ 25.0%.

The other two studies used either PCR denaturing gradient gel electrophoresis (Sinnathamby et al., 2018) or PCR (Bouchet et al., 2022) and clone libraries to identify members of the *H. contortus* microbiome. Neither of these methods allows for diversity or abundance analyses, and so the only way to compare results is to assess which genera may be shared between *H. contortus* and *Parascaris* spp. Between the two studies, a total of 15 genera were found in *H. contortus* that were also identified in individual *Parascaris* spp. core microbiomes: *Achromobacter*, *Acinetobacter*, ANPR, *Bradyrhizobium*, *Enterococcus*, *Escherichia/Shigella*, *Lactobacillus*, *Lactococcus*, *Methylobacterium*, *Mycoplasma*, *Pelomonas*, *Pseudomonas*, *Ralstonia*, *Streptococcus*, and *Veillonella*. Five of those were also found within the *Parascaris* spp. common core microbiome: *Streptococcus*, *Lactobacillus*, *Veillonella*, *Acinetobacter*, and ANPR.

Overall, there were some similarities between the *Parascaris* spp. and *H. contortus* microbiota despite the wide range of methods used for assessing the *H. contortus* microbiota. Future studies assessing microbiome function and localization will be necessary to determine whether some of these bacterial genera are important for parasite fitness, what role they play in parasite biology, and whether they are passed down via vertical transmission or acquired from the environment.

7.2 *Parascaris* spp. Female Gonad

Female *Parascaris* spp. gonads were chosen for more in-depth analysis for a couple of reasons. As previously discussed in section 3.4.3, the bacterial symbiont *Wolbachia* in filarial nematodes has been shown to play an important role in reproduction, including embryogenesis, indicating the important role of the microbial composition of the female parasite gonad. The organ portion of the study discussed herein identified a larger number of unique bacteria within the male gonad, however many of those were only found in a single organism. There were, however, some unique bacteria found within the female

gonad that not only had a prevalence greater than 50%, but also had significantly higher relative abundance than other organs within the worms (Table S8.2). Due to these findings, the female gonad was chosen for more in-depth analysis including TEM imaging, 16S rRNA consensus sequence construction, and phylogenetic analysis of a few bacteria genera of interest.

7.2.1 Comparison with previous studies

The female gonad specific analysis returned 15 phyla, 70 families, and 113 genera compared to 12, 42, and 53, respectively, for the female gonad samples in the organ dataset. This difference is likely due to a few different factors. First, more samples were analyzed, with only 15 individual female gonad samples in the organ dataset versus 22 samples for the female gonad only analysis. Another important consideration is the number of reads per sample. The read cutoff for female only samples was 10,000 reads (range 12,170 – 26,364) versus a cutoff in the organ dataset of 200 reads (female gonad range 237 – 2,061). A lower cutoff was used for the organ dataset in order to prevent removal of a large number of samples from individual categories. This issue could be rectified in the future by increasing overall sample size to maintain group integrity when samples must be removed, and also by decreasing the number of samples sequenced per NGS run in order to increase the number of reads obtained for each sample.

A total of ten genera were identified as belonging to the female gonad core microbiome, and all ten were also found in the organ dataset female gonad core: *Aminobacter, Candidatus Paracaedibacter*, *Hydrotalea*, *Lactobacillus*, *Mycoplasma*, O: OPB56, *Pseudomonas*, *Ralstonia*, *Reyranella*, and *Sphingomonas*. Three of these (*Lactobacillus*, *Reyranella*, *Sphingomonas*) were also part of the common core. One previous study using *H. contortus* investigated the whole worm microbiota and also identified *Ralstonia*, *Pseudomonas*, *Mycoplasma* (Bouchet et al., 2022) within female specimens. Another identified *Lactobacillus* within females, but as a unique genus, which was not the case in this study (Mafuna et al., 2021).

Alpha diversity differed significantly between horse 260 and horse 66 (Figure 6.11) for both Shannon (p = 0.0073) and Simpson (p = 0.0098), suggesting a role for both sample size and environment in affecting alpha diversity. In this case, sample size is an important consideration because there were twelve samples from horse 260 and only three from horse 66. Ideally, an equal number of parasites would be obtained from each horse, however that is not always possible due to the variability in number of adult female

parasites found within an individual horse jejunum (Fabiani et al., 2016). Limiting the number of parasites taken from each horse (e.g., three adult females from each horse) would greatly limit the number of overall worms collected because there is not always a large number of horses with an infection of adult horses at necropsy. This was the case for the samples collected for samples collected for the organ study, where only three horses had a *Parascaris* spp. infection with adult parasites of both sexes.

As discussed in section 3.2, various factors including host genetics, environment, and diet can affect microbiome composition, with variation between individuals accounting for a large amount of variation between microbiomes. Parasite microbiomes are not only affected by the microbiome composition of their equine host – a.k.a. their environment – but also may be affected by their own genetics, dietary preferences, and in the case of *Parascaris* spp., location within the small intestine. Due to all of these factors, it is unsurprising that alpha diversity would vary based upon environment. A larger *Parascaris* spp. microbiome study using multiple worms, from multiple horses in a variety of locations would allow for a more in-depth analysis of microbiome composition based upon environment for both global location and individual horse.

7.2.2 Intracellular bacteria

In the first half of the 20th Century, *Wolbachia* was first described (Hertig, 1936) and due to the advent of TEM, more species were discovered as endosymbionts of mosquitos, other insects, and filarial nematodes during the latter half of the century (Wright & Wang, 1972; Mclaren et al., 1975). TEM has also been used to show the presence of bacteria in the intestine of *C. elegans* (Hedgecock & Thomson, 1982) as well as the intracellular presence of bacteria in *H. contortus* (Sinnathamby et al., 2018). The small TEM study conducted for this study consisted of the gonad of a single female specimen and 270 images. Despite the low number of samples, candidate bacteria were found in two of the TEM images (Figure 6.14). The candidate bacteria were not shaped like the mitochondria identified in other TEM images from *Parascaris* spp. (Figure 6.14C & D), nor did they contain cristae. They were also within the size range for bacteria, where one candidate bacterium was ~ 700 nm x 180 nm Figure 6.14A) and the other two were ~ 700 nm x 250 nm (Figure 6.14B)

This TEM finding suggests that there may be endosymbiotic bacteria within *Parascaris* spp., however the objects found in TEM images are described as “candidate bacteria” because bacteria can be difficult to distinguish from mitochondria. More

research, including a larger volume of TEM images from multiple individuals, as well as confirmation using FISH staining specifically for bacteria in *Parascaris* spp. histology sections, would be necessary to add confidence to identifying candidate bacteria. Previous research in *H. contortus* used FISH to identify bacteria within the gut, eggs, and uterus of female parasites (Sinnathamby et al., 2018). Using a universal bacterial probe such as Eub338 could help determine whether there are intracellular bacteria, and then more specific probes could be used to narrow down which bacterial groups localized where within *Parascaris* spp. Additionally, including multiple specimens, including males, as well as including the intestine would broaden knowledge and allow for more stringent visualization of bacteria within the parasites.

7.3 Notable Bacterial Taxa

A few bacterial taxa were unique in *Parascaris* spp. in that they were in a higher prevalence in the female gonad compared to other compartments and/or they had not previously been found in parasites or horses. Additionally, short-read 16S rRNA metagenomic sequencing does not allow for identification to the species level, and so an additional study was conducted to create longer 16S rRNA reads in order to specifically identify bacteria within the *Parascaris* spp. female gonad. Using longer reads also allowed for phylogenetic analysis of some bacteria to determine their relationship to other identified members within the genus.

Overall, this study allowed for the identification of 17 bacterial genera and 12 bacterial species (Table 6.5). A total of 133 consensus sequences were constructed from sequences within the *Parascaris* spp. samples, and ultimately four genera, *Aminobacter*, *Reyranella*, *Limosilactobacillus*, and *Ligilactobacillus* were used for phylogenetic analysis. *Aminobacter* was chosen due to either some of the consensus sequences having low identity and thus potentially representing a new species, and *Reyranella* was chosen because there were sequences identified to both genus and species level and this particular genus has not been identified in parasites or horses previously. The latter two were chosen because they were not identified in earlier studies, likely due to the recent changes to Lactobacillaceae phylogeny (Zheng et al., 2020), and as a method to confirm that the consensus sequences were adequate for phylogenetic analysis. Neither *Reyranella* nor *Aminobacter* have as in-depth phylogenetic analyses as the Lactobacillaceae, although some of the limited analyses were also used for comparison.

The following sections describe these three groups and the findings of phylogenetic analyses.

7.3.1 Aminobacter

Aminobacter are aerobic, rod-shaped, gram-negative bacteria with rounded ends that use subpolar flagella for locomotion and produce ammonia (Urakami, 2015). The type species, originally called *Pseudomonas aminovorans*, was first described in 1926 (den Dooren de Jong) and the genus *Aminobacter* was described in 1992 (Urakami et al.). These bacteria are capable using trimethylamine (Rappert & Müller, 2005), a chemical responsible for many unpleasant industrial odors, as a carbon and energy source and they can also break down the industrial pollutant herbicide 2,6-dichlorobenzamide (T'Syen et al., 2015). Due to this, *Aminobacter* has mainly been investigated for use in bioremediation at industrial sites and in biofilters (Schultz-Jensen et al., 2014; Aguirre et al., 2018).

Although they are generally known as a soil-swelling bacteria (Table S8.4), *Aminobacter* has been identified in both plant and animal microbiomes including cattle (Scarsella et al., 2021), medicinal leeches (Grafskaia et al., 2020), zebrafish (Almeida et al., 2019), mites (Zheng et al., 2022), ants (Green & Klassen, 2021), planaria (Arnold et al., 2016), truffles (Liu et al., 2021), and the plant rhizosphere (Gnangui et al., 2021). *Aminobacter* has also been identified as a reagent contaminant (Salter et al., 2014); however, that is unlikely to be the case in this study because a negative reagent control was used, all sequences found in the control were removed from analysis, and the decontam (Davis et al., 2018) pipeline was also used which takes into account sample concentration to remove contaminant sequences.

While not identified as a member of the common core, *Aminobacter* was identified in the core microbiota of whole worm male and female samples (Figure 6.3), female gonad and intestine, and male intestine (Figure 6.7). In the organs, the female gonad had a significantly higher relative abundance of *Aminobacter* than the male gonad ($p = 0.0239$). In the female gonad specific study, *Aminobacter* was identified in 100% of samples with a mean relative abundance of 1.1%. The phylogenetic analysis including the *Aminobacter* sp. ParFG consensus sequence (Figure 6.12A) placed it in a clade with species that have previously been identified in soil and sediment samples (Table S8.4). This is unsurprising given that *Aminobacter* was also identified in jejunal samples, since horses feed on grass and frequently ingest soil when grazing, and the horses used in this study were on 24/7

turnout. Much of the *Parascaris* spp. microbiota is likely acquired from the host gut content considering that is the main food source for the parasites, which means that it makes biological sense for this bacterium to be present in both the horses and parasites. *Parascaris* spp. also spends a portion of its lifecycle, albeit still within the egg, in the environment, providing another possible route of microbiome acquisition.

Previous studies, however, have indicated that 16S rRNA is not high enough resolution to decipher phylogenetic details for *Aminobacter* outside of the genus grouping together, and few conclusions can be drawn from this tree (Artuso et al., 2021b). More research and analysis, preferably using shotgun sequencing and/or culturomic methods, is necessary to identify the *Aminobacter* species present in the *Parascaris* spp. microbiome and its relationship to other members of the genus.

7.3.2 Reyranella

Reyranella are gram-negative, non-motile, microaerophilic rod-shaped bacteria that have weak urase activity, oxidize CO to CO_2, and are generally found in water and soil samples (Table S8.4; Pagnier et al., 2011). *Reyranella* is a relatively new genus, with the type species *R. massiliensis* first being described in 2011 (Pagnier et al.), and there are presently five named species within the genus. As previously discussed, *Reyranella* has been previously described in some microbiome studies, however it has not previously been described in horses or parasites.

Reyranella was identified as a member of the common core microbiota and had an overall prevalence of 46.2% for the whole worm study, 61.5% for the organ study, and 100% for the female gonad study. While relative abundances were similar between groups in the whole worm study, the female gonad had significantly higher *Reyranella* relative abundance than the female intestine ($p < 0.0001$), male gonad ($p < 0.0001$), and the male intestine had a higher relative abundance than the male gonad ($p = 0.0011$). In the female gonad study, *Reyranella* had a mean relative abundance of 3.0%, making it one of the most prevalent genera in the female gonad. The species *R. aquatilis* was identified and the phylogenetic analysis placed the consensus sequence *Reyranella* sp. ParFG with a *R. aquatilis* strain (Figure 6.12B) along with specimens found in the soil, rhizosphere, and freshwater (Table S8.4). Similarly to *Aminobacter*, this is unsurprising and makes biological sense due to the feeding habits and life cycle of the host and parasite.

There is limited phylogenetic data for *Reyranella*, particularly any that includes unnamed species found in public repositories. When comparing phylogenetic results from this study to those using 16S rRNA sequences from named species in previous studies, there is similar clade formation (Cui et al., 2017; Lee et al., 2017). This shows that conclusions drawn from the phylogenetic tree created for *Reyranella* in this study are supported by the current best-available data.

7.3.3 Limosilactobacillus and Ligilactobacillus

Limosilactobacillus and *Ligilactobacillus* are both lactic acid bacteria that were recently split from the genus *Lactobacillus* after a whole genomic phylogenetic analysis (Zheng et al., 2020). In general, the lactobacilli are gram-positive fermenters, facultative anaerobes, acid tolerant, and either rod or sphere shaped. *Limosilactobacillus* is a genus of heterofermentative bacteria that often form biofilms in the upper respiratory tract, and *Ligilactobacillus* is a genus of homofermentative bacteria, some of which are motile, that express urease and are common in fermented foods, starter cultures, and probiotics (Zheng et al., 2020). Neither of these genera were identified in the earlier microbiome studies, likely because they have yet to be added to the SILVA database, and so the general *Lactobacillus* results will be discussed whole worm, organ, and female gonad V3-V4 16S rRNA results.

Members of the genus *Lactobacillus* are commonly found in microbiomes, and changes in relative abundance of *Lactobacillus* in the microbiome have been associated with various diseases (Heeney et al., 2018). In horses, *Lactobacillus* has been considered as a beneficial probiotic and is commonly found in equine microbiomes (O'Donnell et al., 2013; Costa et al., 2015; Kauter et al., 2019). This genus has been identified in parasite microbiomes (El-Ashram & Suo, 2016; Sinnathamby et al., 2018; Jorge et al., 2020; Hahn et al., 2021; Mafuna et al., 2021). In this study, *Lactobacillis* was one of the most prevalent (range 92.77 – 100%) and abundant (range 3.47 – 41.34%) genera, as well as a member of the *Parascaris* spp. common core. The species *Lig. equi*, *Lig. hyakitensis*, and *Lim. equigenerosi* were identified from female gonad consensus sequences. Subsequent phylogenetic analysis correlated this identification, with consensus sequences forming clades with these three species (Figure 6.12C & D). The phylogenetic tree for *Ligilactobacillus* has good agreement with the whole genome tree and the tree for *Limosilactobacillus* has some differences in clade formation from the whole genome tree (Zheng et al., 2020). All three species, some still bearing the *Lactobacillus* genus name

in GenBank, have previously been found in horses (Table S8.4) and thus it, again, makes biological sense for them to be present in the parasites.

7.4 Future Directions

Understanding parasite microbiomes and parasite-host-microbiome interactions opens up many doors for improving health outcomes for animals and development of parasite control options. Little research has been done thus far on parasite microbiomes, but there are numerous paths forward from initial characterization of the microbiome. While some future directions have been mentioned in brief already, the following section discusses future research directions for the *Parascaris* spp. microbiome in more detail.

7.4.1 Increased sample size and population inclusion

As was mentioned multiple times in previous sections, an increased population size and including a broader range of equine populations will be essential for determining a common core microbiome for all *Parascaris* spp., rather than those from just one population of horses. Ideally, this would include parasite collected from horses globally. A group of scientists from fifteen countries has previously expressed interest in submitting *Parascaris* spp. specimens for research. This included diagnostic laboratories in North America (Canada, United States of America), South America (Argentina, Brazil), Europe (Denmark, Finland, France, Germany, Iceland, Ireland, Italy, Netherlands, Sweden, Switzerland), and Australia. Collecting a minimum of ten parasites from each of those locations, as well as additional specimens from the two research herds at the University of Kentucky, would greatly increase the sample size and allow for a comparison between individual parasites, horses, and global populations. Dissecting these parasites to individually assess and compare intestine and gonad based upon sex would also provide further insight into the shared core microbiomes between sexes and organs within the parasites. Ultimately, finding commonalities between global *Parascaris* spp. microbiomes provides better insight into which bacterial taxa may be important for parasite health and may also be appropriate targets for new anthelmintics.

7.4.2 In vitro and in vivo studies

Another important factor when determining whether the *Parascaris* spp. microbiome can be exploited for anthelmintic control is determining whether current

antibiotics have any effect on parasite health. The first step of this would be an *in vitro* study where *Parascaris* spp. maintained in culture flasks were exposed to different antibiotics and changes to fitness based upon a scoring system were observed (Scare et al., 2019). The microbiome of the parasites would be analyzed at each fitness score to determine whether there was a correlation between fitness decline and a decrease in relative abundance of bacterial taxa. A similar study was conducted *in vitro* with *H. contortus* (Bouchet et al., 2022), although this study did not include any negative controls, nor was the microbiome analyzed, it was simply observed that the parasites died after exposure to antibiotics.

Affecting the microbiome *in vitro* is one thing, but affecting the microbiome *in vivo* is another. Jorge et al. (2022) examined indirect exposure of the parasite *Philophthalmus attenuates* to antibiotics by treating its snail host and found that not only were parasite bacterial communities altered, but they also stabilized with a different structure over three months post-treatment compared to the pre-treatment community structure. The same type of treatment could be conducted with *Parascaris* spp., where foals are split into three groups for necropsy at five months of age: no antibiotic exposure, antibiotic treatment when eggs first appear in feces, and antibiotic treatment one week prior to necropsy. All parasites from those foals would be collected, and the microbiome assessed via 16S rRNA to determine changes in community structure. Together, these two studies would shed light on whether broad-spectrum antibiotics have an effect on parasite fitness and whether indirect exposure via the host affects the *Parscaris* spp. microbiome, both of which are important if a novel anthelmintic treatment is to be developed.

7.4.3 Diversification of methods

Finally, another important factor for future *Parascaris* spp. microbiome research is diversification of methods. Using 16S rRNA short reads can give insight into community structure but does not indicate which species are present and whether there are novel species, gives no data on localization, and does not provide functional insight. Combining various methods such as culturomics, shotgun sequencing, and metabolomics for studying a single dataset would provide more detail and information regarding microbiome composition and function. Additional microscopy techniques, such as FISH, could also be used to determine where different bacterial taxa localize within the parasite. Using a variety of methods helps address some of the shortfalls of only a single method, such as low resolution and biases in 16S rRNA studies and provides a sturdy foundation on which

to build future studies examining the function of the microbiome and how it might be exploited for parasite treatment options.

7.5 Specific Aims and Hypotheses

All specific aims were addressed within this dissertation, and hypotheses were either rejected or accepted based upon the presented data as follows:

SA 1: Determine the common core microbiome associated with Parascaris spp. at different life stages and between sexes.

H 1.1: There are bacteria taxa within the Parascaris spp. microbiome that are present in all groups within the study population. **ACCEPTED**

SA 2: Compare diversity metrics for the whole worm at different life stages and between individual organs and adult male and female specimens.

H 2.2: Alpha diversity is significantly higher in the equine jejunum than within the parasites. **REJECTED**

H 2.2: Beta diversity dissimilarity is high between *Parascaris* spp. life stages. **REJECTED**

H 2.3: Alpha diversity is significantly higher in the intestine than gonad in both male and female parasites. **REJECTED**

H 2.4: Beta diversity dissimilarity is high between *Parascaris* spp. gonad and intestine. **ACCEPTED**

SA 3: Determine differentially abundant bacterial genera between groups for the whole worm at different life stages and between individual organs and adult male and female specimens.

H 3.1: There are bacterial genera that are differentially abundant between groups for the whole worm microbiome. **REJECTED**

H 3.2: There are bacterial genera that are differentially abundant between groups for the *Parascaris* spp. organ microbiome. **ACCEPTED**

SA 4: Assess the female *Parascaris* spp. gonad microbiome with higher resolution by determining bacterial species present with the organ and whether their presence makes biological sense.

H 4.1: Bacterial species found in the *Parascaris* spp. female gonad will make biological sense based upon parasite and host life cycles and feeding habits, and previous microbiome studies. **ACCEPTED**

SA 5: Visualize bacteria within cells of the *Parascaris* spp. female gonad using TEM.

H 5.1: Endosymbiotic bacteria are located within the cells of the *Parascaris* spp. female gonad. **ACCEPTED**

7.6 Conclusions

Overall, the research studies described herein met the goals of determining a common core microbiota for *Parascaris* spp. and exploring the community structures of the parasite microbiota between whole worm life stages, adult organs, and delving into the female gonad microbiota in some detail. The *Parascaris* spp. common microbiota core consists of eleven members, one of which, *Reyranella*, has not previously been described in the equine host microbiota, and two of which, *Sarcina* and *Veillonella*, were differentially abundant. While the different life stages did not have any significant differences in community composition at the whole worm level, adult organs showed differences in alpha diversity, a slight deviance of the female gonad microbiome in beta diversity, and significant differences in relative abundance for some bacterial genera. The female gonad study identified twelve bacteria to species and candidate bacteria in TEM images, providing foundation to further explore the importance of the microbiome in parasite gonads. Finally, this research provides a backbone for future studies, highlighting areas that need more research and also providing a methodology that can be used in future studies for exploring not only the microbiome of *Parascaris* spp., but also other parasitic nematodes.

APPENDIX 1: SUPPLEMENTAL TABLES

Table S7.1: Permissions information for images from Figure 5.1. Full citations can be found in the references section.

Year	Authors	Journal	Figures	Publisher	License ID
1994	Bruňanská, M.	Folia Parasitologica	7	Folia Parasitologica	Email
2019	Byrne, et al.	Cellular and Molecular Life Sciences	2	Birkhaeuser Verlag AG	1207457-1
2004	Claeys, et al.	Nematology	2B	Brill	1207869-1
1999	Fellowes, et al.	Journal of Comparative Neurology	2	John/Wiley & Sons, Inc.	1207457-6
1972	Foor, W. E.	The Journal of Parasitology	1	Allen Press	1212235-1
1999	Hall, et al.	Developmental Biology	2D, 9B	Academic Press	1207457-2
1982	Hedgecock & Thomson	Cell	6, 9	Cell Press	1207457-7
1997	Mehlhorn & Harder	Parasitology Research	7, 15	Springer-Verlag	1207457-10
2016	Morsci, et al.	Journal of Neuroscience	1F, 1G	Society for Neuroscience	1208282-1
2020	Pánek, et al.	Current Biology	2	Cell Press	1207457-5
2013	Sant'anna, et al.	Experimental Parasitology	6	Academic Press	1207457-8
2008	Tan, et al.	Journal of Cell Science	1E	Company of Biologists Ltd.	1207457-9
2005	Yushin & Coomans	Acta Zoologica	1C, 3A, 3C	Blackwell Publishing	1207457-4
2009	Zick, et al.	Biochimica et Biophysica Acta (BBA) - Molecular Cell Research	1	Elsevier BC	1207457-3

Table S7.2: Results of Dunn's tests with Bonferroni correction for bacterial taxa found in *Parascaris* spp. organs, presented as *p*-values.

	Aminobacter	***Bacillus***	**F: Mycoplasmataceae**	***Gemella***	***Janthinobacterium***	***Ralstonia***	***Reyranella***	***Sphingomonas***
FG - FI	0.3892	1.0000	0.0074	0.0155	0.5203	0.0084	< 0.0001	0.0017
FG - MG	0.0239	< 0.0001	< 0.0001	1.0000	0.0382	1.0000	< 0.0001	1.0000
FI - MG	1.0000	0.0010	0.2123	0.2486	< 0.0001	0.2722	1.0000	0.0002
FG - MI	1.0000	0.5265	0.3178	0.0069	1.0000	1.0000	0.1564	1.0000
FI - MI	0.6156	1.0000	0.8418	1.0000	1.0000	0.0153	0.0528	0.0251
MG - MI	0.0320	0.0163	0.0022	0.1315	0.0010	1.0000	0.0011	0.9760

FG = Female Gonad FI = Female Intestine MG = Male Gonad MI = Male Intestine

Table S7.3: Summary of GenBank hit data for consensus sequences.

ASV	Sample	Length	Reference Genome	%Identical	Overlap	Mismatch	Gaps	Start Q	End Q	Start S	End S	E Value	Bit Score	Species
S001567179	260fg11	532	NR_028876.1	100	239	0	0	1	239	465	703	1.02E-123	442	*Aminobacter aganoensis*
S001567179	260fg4	532	NR_028876.1	100	239	0	0	1	239	465	703	1.02E-123	442	*Aminobacter aganoensis*
S001567179	260fg5	532	NR_028876.1	100	239	0	0	1	239	465	703	1.02E-123	442	*Aminobacter aganoensis*
S001567179	260fg6	532	NR_028876.1	100	239	0	0	1	239	465	703	1.02E-123	442	*Aminobacter aganoensis*
S001567179	261fg6	532	NR_028876.1	100	239	0	0	1	239	465	703	1.02E-123	442	*Aminobacter aganoensis*
S001567179	261fg7	532	NR_028876.1	100	239	0	0	1	239	465	703	1.02E-123	442	*Aminobacter aganoensis*
S001567179	260fg5	532	NR_025302.1	100	239	0	0	1	239	464	702	1.02E-123	442	*Aminobacter niigataensis*
S004415243	260fg5	546	NR_024874.1	96.65	269	7	1	280	546	1079	1347	1.04E-128	459	*Aquabacterium parvum*
S004415243	260fg9	546	NR_024874.1	96.65	269	7	1	280	546	1079	1347	1.04E-128	459	*Aquabacterium parvum*
S004415243	261fg6	546	NR_024874.1	96.65	269	7	1	280	546	1079	1347	1.04E-128	459	*Aquabacterium parvum*
S001914556	261fg5	532	NR_113401.1	100	251	0	0	1	251	496	746	2.18E-130	464	*Brachybacterium paraconglomeratum*
S004117832	260fg11	532	NR_112170.1	97.29	258	0	1	1	258	493	743	2.22E-120	431	*Clostridium butyricum*
S004117832	260fg5	532	NR_112170.1	97.29	258	0	1	1	258	493	743	2.22E-120	431	*Clostridium butyricum*
S004117832	260fg8	532	NR_112170.1	97.29	258	0	1	1	258	493	743	2.22E-120	431	*Clostridium butyricum*
S004117832	66fg1	532	NR_112170.1	97.29	258	0	1	1	258	493	743	2.22E-120	431	*Clostridium butyricum*
S001070107	66fg2	784	NR_042144.1	95.8	524	8	1	1	510	338	861	0	846	*Clostridium butyricum*
S000964173	260fg5	525	NR_148610.1	100	251	0	0	1	251	471	721	2.15E-130	464	*Kocuria arsenatis*
S001684812	260fg1	576	NR_119274.1	95.6	318	7	1	266	576	1087	1404	6.46E-146	516	*Lactobacillus crispatus*
S001684812	260fg11	938	NR_119274.1	96.67	420	7	1	526	938	1087	1506	0	704	*Lactobacillus crispatus*
S001684812	260fg12	576	NR_119274.1	95.6	318	7	1	266	576	1087	1404	6.46E-146	516	*Lactobacillus crispatus*
S001684812	260fg4	699	NR_119274.1	98.16	381	0	1	1	381	549	922	0	658	*Lactobacillus crispatus*
S001400072	260fg5	836	NR_119274.1	100	374	0	0	145	518	549	922	0	691	*Lactobacillus crispatus*
S001684812	260fg6	801	NR_119274.1	96.67	420	7	1	389	801	1087	1506	0	704	*Lactobacillus crispatus*
S001684812	260fg7	576	NR_119274.1	95.6	318	7	1	266	576	1087	1404	6.46E-146	516	*Lactobacillus crispatus*
S001684812	260fg8	938	NR_119274.1	96.67	420	7	1	526	938	1087	1506	0	704	*Lactobacillus crispatus*
S001684812	260fg9	836	NR_119274.1	98.16	381	0	1	138	518	549	922	0	658	*Lactobacillus crispatus*
S001684812	66fg3	699	NR_119274.1	98.16	381	0	1	1	381	549	922	0	658	*Lactobacillus crispatus*
S001792808	260fg8	672	NR_113259.1	95.72	421	11	2	259	672	1065	1485	0	686	*Ligilactobacillus agilis*
S001792808	260fg9	672	NR_113259.1	95.72	421	11	2	259	672	1065	1485	0	686	*Ligilactobacillus agilis*
S001792808	260fg11	569	NR_028623.1	95.28	318	8	1	259	569	1009	1326	2.97E-144	510	*Ligilactobacillus equi*
S003560971	260fg11	706	NR_028623.1	95.28	318	8	1	396	706	1009	1326	3.71E-144	510	*Ligilactobacillus equi*
S001792808	260fg5	569	NR_028623.1	95.28	318	8	1	259	569	1009	1326	2.97E-144	510	*Ligilactobacillus equi*
S003560971	260fg5	569	NR_028623.1	95.28	318	8	1	259	569	1009	1326	2.97E-144	510	*Ligilactobacillus equi*
S000901488	260fg1	569	NR_041498.1	95.6	318	7	1	259	569	1064	1381	6.38E-146	516	*Ligilactobacillus hayakitensis*
S000901488	260fg10	569	NR_041498.1	95.6	318	7	1	259	569	1064	1381	6.38E-146	516	*Ligilactobacillus hayakitensis*
S000901488	260fg11	706	NR_041498.1	95.6	318	7	1	396	706	1064	1381	7.98E-146	516	*Ligilactobacillus hayakitensis*
S000901488	260fg4	569	NR_041498.1	95.6	318	7	1	259	569	1064	1381	6.38E-146	516	*Ligilactobacillus hayakitensis*
S000901488	260fg5	569	NR_041498.1	95.6	318	7	1	259	569	1064	1381	6.38E-146	516	*Ligilactobacillus hayakitensis*
S000901488	260fg6	569	NR_041498.1	95.6	318	7	1	259	569	1064	1381	6.38E-146	516	*Ligilactobacillus hayakitensis*
S000901488	260fg8	809	NR_041498.1	96.68	421	7	1	396	809	1064	1484	0	706	*Ligilactobacillus hayakitensis*
S000901488	260fg9	809	NR_041498.1	96.68	421	7	1	396	809	1064	1484	0	706	*Ligilactobacillus hayakitensis*
S000964154	260fg1	627	NR_041566.1	96.01	376	8	1	259	627	1103	1478	5.23E-177	619	*Limosilactobacillus equigenerosi*
S000964154	260fg11	671	NR_041566.1	96.43	420	8	1	259	671	1059	1478	0	701	*Limosilactobacillus equigenerosi*
S000964154	260fg8	627	NR_041566.1	96.01	376	8	1	259	627	1103	1478	5.23E-177	619	*Limosilactobacillus equigenerosi*
S001060020	260fg9	525	NR_041566.1	100	251	0	0	1	251	521	771	2.15E-130	464	*Limosilactobacillus equigenerosi*
S001059989	260fg4	569	NR_029084.1	95.6	318	7	1	259	569	1078	1395	6.38E-146	516	*Limosilactobacillus gastricus*
S001567179	260fg1	576	NR_114124.1	95.6	318	7	1	266	576	988	1305	6.46E-146	516	*Mesorhizobium plurifarium*
S001567179	261fg5	576	NR_114124.1	95.6	318	7	1	266	576	988	1305	6.46E-146	516	*Mesorhizobium plurifarium*
S001866572	260fg1	525	NR_044791.1	100	251	0	0	1	251	479	729	2.15E-130	464	*Mycobacterium triplex*
S001866572	260fg11	525	NR_044791.1	100	251	0	0	1	251	479	729	2.15E-130	464	*Mycobacterium triplex*
S004593428	261fg3	685	NR_114735.1	95.3	319	7	1	1	311	1041	1359	4.36E-145	512	*Pantoea agglomerans*
S004484017	260fg1	583	NR_044974.1	95	320	7	1	273	583	1038	1357	3.94E-143	507	*Pseudomonas chlororaphis*
S004484017	260fg10	822	NR_044974.1	96.21	422	7	1	410	822	1038	1459	0	695	*Pseudomonas chlororaphis*
S004484017	260fg12	583	NR_044974.1	95	320	7	1	273	583	1038	1357	3.94E-143	507	*Pseudomonas chlororaphis*
S004484017	260fg5	822	NR_044974.1	96.21	422	7	1	410	822	1038	1459	0	695	*Pseudomonas chlororaphis*
S004484017	260fg6	944	NR_044974.1	96.21	422	7	1	532	944	1038	1459	0	695	*Pseudomonas chlororaphis*
S004484017	260fg7	583	NR_044974.1	95	320	7	1	273	583	1038	1357	3.94E-143	507	*Pseudomonas chlororaphis*
S004484017	260fg8	807	NR_044974.1	96.21	422	7	1	395	807	1038	1459	0	695	*Pseudomonas chlororaphis*
S004484017	260fg9	944	NR_044974.1	96.21	422	7	1	532	944	1038	1459	0	695	*Pseudomonas chlororaphis*
S004484017	261fg6	685	NR_044974.1	96.21	422	7	1	273	685	1038	1459	0	695	*Pseudomonas chlororaphis*
S004484017	261fg7	685	NR_044974.1	96.21	422	7	1	273	685	1038	1459	0	695	*Pseudomonas chlororaphis*
S004484017	260fg11	705	NR_115642.1	96.38	387	0	2	1	387	492	864	1.27E-178	625	*Pseudomonas fluorescens*
S004484017	260fg3	842	NR_115642.1	96.38	387	0	2	138	524	492	864	1.52E-178	625	*Pseudomonas fluorescens*
S004484017	260fg4	842	NR_115642.1	96.38	387	0	2	138	524	492	864	1.52E-178	625	*Pseudomonas fluorescens*
S001132542	260fg5	576	NR_118984.1	95	320	7	2	259	576	1035	1347	3.89E-143	507	*Ralstonia solanacearum*
S001132542	260fg6	576	NR_118984.1	95	320	7	2	259	576	1035	1347	3.89E-143	507	*Ralstonia solanacearum*
S003992595	260fg9	525	NR_158037.1	99.6	251	1	0	1	251	423	673	1.00E-128	459	*Reyranella aquatilis*
S003063129	260fg1	569	NR_116005.1	95.3	319	7	1	259	569	1003	1321	8.25E-145	512	*Reyranella massiliensis 521*
S001070446	260fg4	659	NR_026147.1	96.36	385	0	2	1	385	481	851	1.53E-177	621	*Sarcina maxima*
S001070446	260fg5	659	NR_026147.1	96.1	385	1	2	1	385	481	851	7.12E-176	616	*Sarcina maxima*
S004172337	260fg11	525	NR_118450.1	100	251	0	0	1	251	515	765	2.15E-130	464	*Staphylococcus petrasii*
S003370226	260fg11	524	NR_152063.1	98.8	250	3	0	1	250	544	793	7.79E-125	446	*Streptococcus halotolerans*
S003560976	260fg5	524	NR_152063.1	98.8	250	3	0	1	250	544	793	7.79E-125	446	*Streptococcus halotolerans*
S003560976	260fg8	524	NR_152063.1	98.8	250	3	0	1	250	544	793	7.79E-125	446	*Streptococcus halotolerans*
S000979950	260fg9	524	NR_152063.1	98.8	250	3	0	1	250	544	793	7.79E-125	446	*Streptococcus halotolerans*
S002113818	261fg5	525	NR_025412.1	100	251	0	0	1	251	492	742	2.15E-130	464	*Acinetobacter schindleri*
S003051855	260fg5	525	NR_024874.1	100	251	0	0	1	251	497	747	2.15E-130	464	*Aquabacterium parvum*
S001070107	260fg1	525	NR_112170.1	100	251	0	0	1	251	493	743	2.15E-130	464	*Clostridium butyricum*
S001070107	260fg4	525	NR_112170.1	100	251	0	0	1	251	493	743	2.15E-130	464	*Clostridium butyricum*
S001070107	260fg6	525	NR_112170.1	100	251	0	0	1	251	493	743	2.15E-130	464	*Clostridium butyricum*
S001070107	261fg7	525	NR_112170.1	100	251	0	0	1	251	493	743	2.15E-130	464	*Clostridium butyricum*
S001060396	66fg2	525	NR_113021.1	99.2	251	2	0	1	251	499	749	4.66E-127	453	*Clostridium paraputrificum*
S002033100	260fg6	525	NR_117026.1	100	251	0	0	1	251	472	722	2.15E-130	464	*Hydrotalea flava*
S002033100	261fg5	525	NR_117026.1	100	251	0	0	1	251	472	722	2.15E-130	464	*Hydrotalea flava*
S000806423	260fg6	525	NR_116004.1	100	251	0	0	1	251	472	722	2.15E-130	464	*Labrys wisconsinensis*
S001792808	260fg1	525	NR_028623.1	100	251	0	0	1	251	471	721	2.15E-130	464	*Ligilactobacillus equi*
S003560971	260fg1	525	NR_041498.1	99.2	251	2	0	1	251	526	776	4.66E-127	453	*Ligilactobacillus hayakitensis*
S003560971	260fg10	525	NR_041498.1	99.2	251	2	0	1	251	526	776	4.66E-127	453	*Ligilactobacillus hayakitensis*
S003560971	260fg4	525	NR_041498.1	100	251	0	0	1	251	526	776	2.15E-130	464	*Ligilactobacillus hayakitensis*
S003560971	260fg6	525	NR_041498.1	99.2	251	2	0	1	251	526	776	4.66E-127	453	*Ligilactobacillus hayakitensis*
S000901488	66fg1	525	NR_041498.1	99.2	251	2	0	1	251	526	776	4.66E-127	453	*Ligilactobacillus hayakitensis*
S003560971	66fg1	525	NR_041498.1	99.2	251	2	0	1	251	526	776	4.66E-127	453	*Ligilactobacillus hayakitensis*
S000901488	66fg2	525	NR_041498.1	100	251	0	0	1	251	526	776	2.15E-130	464	*Ligilactobacillus hayakitensis*
S003561013	66fg2	525	NR_041498.1	100	251	0	0	1	251	526	776	2.15E-130	464	*Ligilactobacillus hayakitensis*
S003560979	260fg10	525	NR_041566.1	100	251	0	0	1	251	521	771	2.15E-130	464	*Limosilactobacillus equigenerosi*
S001060020	260fg5	525	NR_041566.1	100	251	0	0	1	251	521	771	2.15E-130	464	*Limosilactobacillus equigenerosi*
S001059968	260fg6	525	NR_041566.1	100	251	0	0	1	251	521	771	2.15E-130	464	*Limosilactobacillus equigenerosi*
S001060020	260fg7	525	NR_041566.1	100	251	0	0	1	251	521	771	2.15E-130	464	*Limosilactobacillus equigenerosi*
S000964154	261fg5	525	NR_041566.1	100	251	0	0	1	251	521	771	2.15E-130	464	*Limosilactobacillus equigenerosi*
S000964154	261fg6	525	NR_041566.1	100	251	0	0	1	251	521	771	2.15E-130	464	*Limosilactobacillus equigenerosi*
S003560979	66fg1	525	NR_041566.1	100	251	0	0	1	251	521	771	2.15E-130	464	*Limosilactobacillus equigenerosi*
S003560979	66fg3	525	NR_041566.1	100	251	0	0	1	251	521	771	2.15E-130	464	*Limosilactobacillus equigenerosi*
S001132542	261fg5	532	NR_114126.1	100	251	0	0	1	251	507	757	2.18E-130	464	*Ralstonia pickettii*
S003063129	260fg8	525	NR_158037.1	99.6	251	1	0	1	251	423	673	1.00E-128	459	*Reyranella aquatilis*
S002336367	261fg7	532	NR_158037.1	99.6	251	1	0	1	251	423	673	1.02E-128	459	*Reyranella aquatilis*
S003674095	260fg6	532	NR_026147.1	100	251	0	0	1	251	481	731	2.18E-130	464	*Sarcina maxima*

Table S7.4: Summary of samples from GenBank used for phylogenetic analysis.

GenBank Accession	Identification	Length (bp)	Environment	Country
KP165414.1	*Aminobacter aminovorans* IPN-TC	1402	soil/gas contaminated	Colombia
KC767641.1	*Aminobacter aminovorans* Sal1	1443	soil/agricultural	France
KC767642.1	*Aminobacter aminovorans* Sal1-3	1443	soil/agricultural	France
EU876581.1	*Aminobacter ciceronei* B05	1383	soil/oil contaminated	China
AF246220.1	*Aminobacter ciceronei* C147	1453	soil/agricultural	Canada
L20802.1	*Aminobacter ciceronei* ER2	1410	soil/agricultural	Canada
AF034798.1	*Aminobacter ciceronei* IMB-1	1407	soil/agricultural	United States
AF107722.1	*Aminobacter lissarensis* CC495	1435	soil/agricultural	United States
MN715368.1	*Aminobacter niigataensis* AJK-12	1238	-	-
KF424786.1	*Aminobacter niigataensis* XH038-2	1408	sediment/deep sea	China
EU304289.1	*Aminobacter* sp. 86	1376	mining site	Switzerland
DQ401866.1	*Aminobacter* sp. ASI1	1485	soil	Denmark
EU748914.1	*Aminobacter* sp. BA135	1433	root nodule	Argentina
HQ113207.1	*Aminobacter* sp. CL-9.08	1406	agricultural waste	-
*AY307924.1	*Aminobacter* sp. COX	1450	soil	United States
HQ183833.1	*Aminobacter* sp. De3156	1447	sediment/leachate	China
*MK511828.1	*Aminobacter* sp. DSM24754	1452	-	-
MK382451.1	*Aminobacter* sp. ICMP6023	1375	soil	New Zealand
*MH900189.1	*Aminobacter* sp. IK-R2	1433	-	Japan
MN372076.1	*Aminobacter* sp. JW2	1353	soil	China
KX387895.1	*Aminobacter* sp. MDW-2	1407	soil	China
DQ196478.1	*Aminobacter* sp. MI-p2a	1317	cave	United States
FJ907162.1	*Aminobacter* sp. Sokolova	1366	soil	Russia
*MT568546.1	*Aminobacter* sp. T6647	1386	sediment	China
*AY934494.1	*Aminobacter* sp. TW23	1367	soil	United Kingdom
MT259661.1	*Aminobacter* sp. YUM09	1330	cave	Papua New Guinea
JQ951604.1	*Escherichia coli* 2012K10	1462	human	China
AF049743.1	*Lactobacillus acidipiscis* LMG17676	1473	food/chili bo	Malaysia
M58807.1	*Lactobacillus animalis*	1507	baboon dental plaque	Unknown
AY253659.1	*Lactobacillus antri*	1520	human stomach	Sweden
AB001836.2	*Lactobacillus aviarius*	1510	-	-
AB911495.1	*Lactobacillus coleohominis* JCM7763	1517	chicken	-
U62624.1	*Lactobacillus fermentum*	1496	sediment/leachate	United States
JX272061.1	*Lactobacillus frumenti* 2.1	1370	food/sourdough	Denmark
AB904716.1	*Lactobacillus gorillae* KZ01	1525	gorilla feces	Japan
*LC480804.1	*Lactobacillus hayakitensis* JCM14209	1500	horse feces	-
M58826.1	*Lactobacillus murinus*	1514	rat intestine	-
EF445114.1	*Lactobacillus murinus* LbP6	1446	dog feces	-
KC561105.1	*Lactobacillus oris* sOR3	1528	chicken	Iran
AB326359.1	*Lactobacillus pobuzihii* NBRC103220	1489	food/pobuzihi	Taiwan
HM218071.1	*Lactobacillus pontis* NM14-2	1487	food/fermented	China
L23507.1	*Lactobacillus reuteri*	1535	human intestine	-
AY735406.1	*Lactobacillus reuteri* LU3	1531	food/fermented	South Korea
M58828.1	*Lactobacillus ruminus*	1567	bovine rumen	-

LC127508.1	*Lactobacillus salitolerans*	1488	mushroom bed	Japan
*M59054.1	*Lactobacillus salivarius salicinius*	1512	saliva	-
AF243177.1	*Lactobacillus vaginalis* ATCC49540	1541	human vagina	United States
NR_024718.1	*Ligilactobacillus acidipiscis* FS60-1	1406	food/fermented	Thailand
NR_044700.2	*Ligilactobacillus agilis* DSM20509	1511	sewage	-
NR_113259.1	*Ligilactobacillus agilis* JCM1187	1485	sewage	-
NR_041610.1	*Ligilactobacillus animalis* KCTC3501	1489	baboon dental plaque	-
NR_042367.1	*Ligilactobacillus apodemi* ASB1	1532	mouse feces	Japan
NR_104979.1	*Ligilactobacillus araffinosus* LMG23560	1515	chicken	-
AB326355.1	*Ligilactobacillus aviarius* NBRC102162	1488	-	-
*NR_028623.1	*Ligilactobacillus equi* YIT0455	1413	horse feces	Japan
NR_114391.1	*Ligilactobacillus faecis* AFL13-2	1545	jackal feces	South Africa
KX826967.1	*Ligilactobacillus ruminis* M1/34	1514	biogas reactor	Germany
NR_029085.1	*Ligilactobacillus saerimneri* GDA154	1568	pig feces	Sweden
LC655142.1	*Ligilactobacillus* sp. AF129	1486	alfalfa silage	Japan
MT823154.1	*Limosilactobacillus agrestis*	1569	vole	-
KT343143.1	*Limosilactobacillus caviae* MOZM2	1487	guinea pig mouth	Czech Republic
*NR_041566.1	*Limosilactobacillus equigenerosi* NRIC 0697	1519	horse feces	Japan
*NR_029084.1	*Limosilactobacillus gastricus* Kx156A7	1550	human stomach	Sweden
MZ889575.1	*Limosilactobacillus ingluviei* G54d	1496	goose feces	Poland
MZ889573.1	*Limosilactobacillus ingluviei* P10c	1496	pigeon feces	Poland
MF425027.1	*Limosilactobacillus mucosae* CAU8012	1419	food/pickle	China
MH819644.1	*Limosilactobacillus oris* HBUAS54411	1496	human intestine	China
MG462197.1	*Limosilactobacillus panis* NWAFU1288	1434	food/fermented	China
MZ749587.1	*Limosilactobacillus pontis* HBUAS53515	1497	acidic gruel	China
MW567700.1	*Limosilactobacillus* sp. c11Ua_25_AN	1437	human urine	Portugal
MZ787740.1	*Limosilactobacillus* sp. HBUAS53669	1498	acidic gruel	China
OM841498.1	*Limosilactobacillus* sp. L.P14(2)	1400	poultry	Iran
MW016377.1	*Limosilactobacillus urinaemulieris*	1563	human urine	Portugal
*MT538333.1	*Reyranella aquatilis* FW305-C-30-S9	1313	-	-
AB839882.1	*Reyranella graminifolii*	1453	bamboo litter	South Korea
JX260424.1	*Reyranella soli* KIS14-15	1429	soil/forest	South Korea
MG818310.1	*Reyranella* sp. 5SWB3-2	1411	freshwater	South Korea
MH686075.1	*Reyranella* sp. Alpha-23	1530	soil	South Korea
MH686078.1	*Reyranella* sp. Alpha-26	1470	soil	South Korea
MK875880.1	*Reyranella* sp. AZCC 0224	1358	soil/forest	United States
JX458408.1	*Reyranella* sp. B6.10-109	1395	bottled water	Portugal
*KY319057.1	*Reyranella* sp. BK16-10	1418	freshwater	South Korea
MT756085.1	*Reyranella* sp. C99	1439	soil	Vietnam
*OM867428.1	*Reyranella* sp. GW460-11-11-14-TSB3	1311	-	United States
KY445620.1	*Reyranella* sp. MA98	1447	soil	-
*MW197410.1	*Reyranella* sp. MMS21-HV4-11	1436	soil	-
*MW197420.1	*Reyranella* sp. MMS21-HV4-7	1425	soil	-

KY176929.1	*Reyranella* sp. S49	1453	soil	-
MH688823.1	*Reyranella* sp. S826	1424	-	South Korea
*LC218367.1	*Reyranella* sp. SM-2017 SM5	1361	rice rhizosphere	Japan
LC218395.1	*Reyranella* sp. SM-2017 SM92	1421	rice rhizosphere	Japan
KU713087.1	*Reyranella* sp. T2-6AA	1317	water treatment plant	United States
KF003176.1	*Reyranella* sp. X48	1447	grass carp gut	China
KP185143.1	*Reyranella terrae* 11G32	1420	soil/agricultural	South Korea

* denotes shared clade with *Parascaris* spp. female gonad specimens

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VITA

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Education

2017	University of Nebraska–Kearney, Kearney, Nebraska Master of Science, Biology
2013	Westminster College, Salt Lake City, Utah Bachelor of Science, Biology

Professional Experience

2022 – Present	Research Parasitologist, Parasight System, Inc.
2021 – 2022	Laboratory Technician Senior, University of Kentucky Veterinary Diagnostic Laboratory
2017 – 2021	Graduate Research Assistant, University of Kentucky Department of Veterinary Science
2016 – 2017	Forensic Biologist I, Kentucky State Police Central Forensics Laboratory
2013 – 2016	Preventive Medicine Specialist (68S), United States Army

Awards

2022	American Association of Veterinary Parasitologists Outstanding Graduate Student

Publications

*Denotes mentored student

Cain, J. L., Norris, J. K., Ripley, N. E., Suri, P., Finnerty, C. A., Gravatte, H. S., Nielsen, M. K. (2022). The microbial community associated with *Parascaris* spp. infecting juvenile horses. *Parasites & Vectors*. **Under Peer Review.**

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Cain, J. L., Slusarewicz, P., Rutledge, M. H., McVey, M. R., Wielgus, K. M., Zynda, H. M., Wehling, L. M., Scare, J. A., Steuer, A. E., Nielsen, M. K. (2020). Diagnostic performance of McMaster, Wisconsin, and automated egg counting techniques for enumeration of equine strongyle eggs in fecal samples. *Veterinary Parasitology*. **284**, 109199.

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Presentations

Cain, J. L. (2022). Presentation: American Association of Veterinary Scientists, Snowbird, UT. *From dealing with crap to playing in it: My journey to parasitology.*

Cain, J. L., Norris, J. K., Shields, J. P., Nielsen, M. K. (2022). Presentation: American Association of Veterinary Scientists, Snowbird, UT. *Girl power: The microbiome of Parascaris spp. female gonads*.

Cain, J. L., Peters, K. T., Slusarewicz, P., Suri, P., Roher, A., Rutledge, M. H., Wielgus, K. M., Zynda, H. M., McVey, M. R., Wehling, L. M., Nielsen, M. K. (2021). Presentation: 11th Equine Infectious Disease Conference, Online. *Automated parasite fecal egg counts: Limiting the room for error*.

Cain, J. L., Norris, J. K., Gravatte, H. S., Nielsen, M. K. (2021). Presentation: 11th Equine Infectious Disease Conference, Online. *Characterizing the Parascaris spp. microbiome*.

Cain, J. L., Norris, J. K., Gravatte, H. S., Nielsen, M. K. (2021). Presentation: World Association for the Advancement of Veterinary Parasitology, Online. *Profiling parasites: the Parascaris spp. microbiome*.

Cain, J. L., Peters, K. T., Suri, P., Roher, A., Rutledge, M. H., Nielsen, M. K. (2021). Presentation: American Association of Veterinary Parasitologists, Lexington, KY. *Too cool for school: How analyst training affects fecal egg count variability*.

Cain, J. L., Norris, J. K., Gravatte, H. S., Shaffer, C. L., Nielsen, M. K. (2020). Presentation: American Association of Veterinary Parasitologists, Online. *The microbiome of Parascaris spp.: A pilot study*.

Cain, J. L., Peters, K. T., Suri, P., Roher, A., Nielsen, M. K. (2020). Poster: American Association of Veterinary Parasitologists, Online. *Analyst variability at the counting step for McMaster, Wisconsin, and automated equine fecal egg count methods*.

Cain, J. L., Foulk, D., Jedrzejewski, E., Stofanak, H., Nielsen, M. K. (2019). Presentation: World Association for the Advancement of Veterinary Parasitology, Madison, WI. *The importance of anthelmintic efficacy monitoring: Results of an outreach effort.*

Cain, J. L., Slusarewicz, P., Wehling, L. M., Wielgus, K. M., Zynda, H. M., McVey, M. R., Roemmele, E., Nielsen, M. K. (2019). Presentation: World Association for the Advancement of Veterinary Parasitology, Madison, WI. *Precision, sensitivity, and specificity analysis of an automated parasite fecal egg counting system in comparison to McMaster and Wisconsin methods*.

Cain, J. L., Slusarewicz, P., Wehling, L. M., Wielgus, K. M., Zynda, H. M., McVey, M. R., Nielsen, M. K. (2018). Presentation: American Association of Veterinary Parasitologists, Denver, CO. *Precision analysis of an automated parasite fecal egg counting system in comparison to McMaster and Wisconsin methods.*

Cain, J. L., Roe, K., Macaluso, K., Luedtke, B. (2017). Presentation: American Association of Veterinary Parasitologists, Indianapolis, IN. *The effect of fecal egg count data and presence of Strongylus vulgaris on body score of feral horses on Fort Polk, Louisiana.*

Funding

June 2021 – April 2022. Zoetis. Characterizing the microbiome of *Parascaris* spp.: Life stages and localization.
Award Amount: $20,191

September 2018 – September 2020. National Center for Veterinary Parasitology. Mapping the intestinal microbiome of *Parascaris* spp.: A pilot study.
Award Amount: $11,925