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Beyond parasitism: Exploring the microbial profile of *Haemonchus contortus* and its predilection site (abomasum) in Kashmir Merino sheep

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Abstract

Gastrointestinal helminth infection, particularly by *Haemonchus contortus*, poses significant challenges to sheep farming worldwide. While anthelmintic drugs have been traditional control measures, the emergence of resistance calls for alternative strategies. Understanding the interaction between parasites, host, and their microbiome is crucial. This study intricately explored the interactions between microbial communities in Kashmir Merino sheep infected with *H. contortus*, to understand the complex interplay between host, parasite, and their microbiome. Metagenomic sequencing unveils distinct microbial patterns, where Proteobacteria were dominant in *H. contortus*, while Bacteroidota and Firmicutes prevailed in the sheep abomasum. The revelation of unique genera and shifts in diversity indices underscored helminth-induced disruptions in the host. Beta diversity analysis further showed significant variations in bacterial profiles, providing insights into the intricate host, parasite, and microbiome dynamics. Additionally, this study elucidated the presence of pathogenic bacteria within *H. contortus*, accentuating their potential role in exacerbating sheep health issues. This finding underscored the complexity of the host-parasite-microbiome interaction showing helminth-induced microbiome alterations of the host, and points towards potential alternative strategies for effective control of helminths while focusing on the microbiome particularly.

Keywords: Kashmir Merino sheep, Metagenomics, *Haemonchus contortus*, Host-Parasite relationship, Microbiome, Bacteria

Introduction

Gastrointestinal helminth infestation of sheep is a pervasive and economically significant health concern in the global livestock industry¹. These helminths include nematodes, cestodes, and trematodes, leading to a wide array of health problems that can substantially impact the well-being of the animals and the profitability of sheep farming operations². Among helminths, nematodes cause considerable damage to sheep health and productivity. *Haemonchus contortus*, *Ostertagia ostertagi*, *Cooperia oncophora*, *Teladorsagia circumcincta* and *Trichostrongylus* spp. are highly pathogenic nematodes of sheep³. The damage caused to the gastrointestinal tract by the feeding and migration of these worms can result in chronic gastro-intestinal inflammation and digestive disturbances, which can cause reduced growth rates, decreased milk production and even mortality. The effective sustainable management and control of gastrointestinal helminth infestations in sheep have become a critical aspect of modern animal husbandry practices⁴⁻⁷. Anthelmintic drugs have been a cornerstone of control strategies for decades. However, the persistent and widespread use of these drugs has given rise to a pressing concern: the emergence of anthelmintic resistance among gastrointestinal helminths⁸. Thus, alternative approaches from the use of botanicals to nanoparticles are being used to target helminth infections among livestock⁹.

To better characterize the pathophysiology of these infections and the effects of the control strategies used, recent attention has been focused on the impact of these parasites on the microbiome of their ecological niche¹⁰. Comparable to other metazoans, the intestinal ecosystem of sheep gut encompasses a rich diversity of microorganisms that play crucial physiological functions, including nutrient digestion and absorption the development and the modulation of the immune system associated to the intestinal mucosa in mammals¹¹. Various factors are known to alter the gut microbiome of the host including diet, habitat, age, sex, health status, genetic makeup of host¹²⁻¹⁴. Infection with helminth parasites is also known to alter host gastrointestinal microbiome composition, favouring an increase in parasite-associated bacteria while decreasing those beneficial to the host^{6,15-17}. Helminths also have an associated microbiome which helps in different physiological functions including acclimatation and survival in the host gut, egg production, and hatching¹⁸⁻²⁰. The nematode microbiome is being studied to delve deeper into the tripartite relationship of host, parasite, and microbiome. Numerous studies have investigated the microbiome of *H. contortus* and other nematode parasites^{19,21-23}. In this study, we investigate the impact of *H. contortus* infection on the

microbiome of Kashmir Merino sheep in relation to the microbiome associated with *H. contortus*.

Results

Identification of worms

The collected parasitic worms were identified as *H. contortus* through a combination of morphometric measurements. On average, males were 12mm (SEM = 0.23) in length with a spicular length of 0.39mm (SEM = 0.01) whereas females were 21mm (SEM = 0.56) in length with the distance between the vulva and posterior end equal to 3.3mm (SEM = 0.08). Morphological identification was validated with nBlast results (Table 1).

Microbiome associated with *H. contortus*, infected and non-infected abomasum at phylum level

A total of 20 different phyla, dominated by Proteobacteria (57%), Firmicutes (25%), Bacteroidota (15%), Actinobacteriota (3%) were associated with *H. contortus*. The abomasal microbiota from infected sheep showed 17 different phyla including Bacteroidota (38%) followed by Firmicutes (30%), Proteobacteria (12%), Fibrobacterota (8%), Actinobacteriota (5%), Verrucomicrobiota (2%), and other phyla. In non-infected sheep, the abomasal microbiota showed 22 phyla including Bacteroidota (52%), Firmicutes (26%), Verrucomicrobiota (12%), Proteobacteria (2%), Planctomycetota (2%), Spirochaetota (1%) and other phyla (Fig. 1).

Microbiome Composition of worms at Genus level

The total number of OTUs found in all samples was 757 (Table S2), with 211 OTUs unique to *H. contortus* microbiota, 37 OTUs unique to the abomasal microbiota from infected sheep, and 104 OTUs unique to the non-infected abomasum. With 121 OTUs present in all, 23 OTUs were shared between *H. contortus* and infected abomasum, 79 OTUs were shared between *H. contortus* and non-infected abomasum, and 182 were shared between Infected and non-infected abomasum (Fig.2).

Predominant genera in *H. contortus* included *Acinetobacter* (25%), *Massilia* (10%), *Prevotella* (9%), *Faecalibacterium* (4%), *Staphylococcus* (4%), *Streptococcus* (3%), *Stenotrophomonas* (3%), *Variovorax* (3%), *Phyllobacterium* (3%), and other genera. Infected abomasum showed genus *Prevotella* (18%) followed by *Fibrobacter* (7%), *Acinetobacter* (3%), *Prevotellaceae-*

unclassified (4%), *Ruminobacter* (4%), *Succinivibrio* (3%), *Lachnospiraceae_unclassified* (2%), *Erysipelatoclostridiaceae-UCG-004* (2.6%), *Ruminococcus* (2.3 %) and other genera. Non-infected abomasum was predominated by *Rikenellaceae_RC9_gut_group* (12 %), *Prevotella* (11%), *WCHB1-41_ge* (10%), *F082_ge* (5%), *Prevotellaceae_unclassified* (5%) and other genera (Fig. 3).

Major unique bacterial genera

H. contortus showed 211 unique genera of which predominant ones include *Massilia*, *Faecalibacterium*, *Staphylococcus*, *Variovax*, *Spiroplasma*, *Achromobacter*, *Sphingobacterium*, and *Collinsella* among others. Among 37 OTUs unique to the infected abomasum, major ones were *Asteroleplasma*, *Anaerosporobacter*, *Erysipelotrichaceae_UCG-007*, and *Victivallis*. Non-infected abomasum showed 104 unique OTUs including *Suttonella*, *Pelospora*, and *Isosphaeraceae_UCG* among others.

Bacterial diversity statistics

Shannon diversity index and evenness indices were higher in infected abomasum followed by non-infected abomasum and *H. contortus*. However, Ace, Chao, and Simpson indices show higher bacterial diversity in non-infected abomasum followed by infected abomasum and *H. contortus*. These indices depict greater richness and higher dominance of certain bacteria in non-infected abomasum than in infected abomasum and *H. contortus*. In the Kruskal-Wallis test, while comparing groups, a significant difference in microbiome diversity was found in all indices between *H. contortus* and infected abomasum. However, diversity indices did not differ significantly between infected abomasum and non-infected abomasum except Observed values, Chao and Shannon evenness indices (Table 2, Fig. 4).

Beta Diversity

The application of Non-Metric Multidimensional Scaling (NMDS) utilizing the Bray-Curtis dissimilarity measure elucidated distinctive clustering patterns among the bacterial profiles associated with *H. contortus*, infected abomasum, and non-infected abomasum (Fig. 5). These findings suggest distinct differences in microbial communities among the specified groups. Furthermore, the heatmap representations (Fig. 6) accentuate the observed dissimilarities in bacterial profiles between infected and non-infected abomasum. Notably, the infected

abomasum showed a slightly darker blue coloration, suggesting increased within-group variation within its bacterial community compared to the non-infected abomasum.

To validate these disparities, a PERMANOVA (Permutational Multivariate Analysis of Variance) analysis was conducted on Bray-Curtis and Jaccard dissimilarity distances, employing 1000 permutations. The results of the PERMANOVA underscored statistically significant differences in bacterial profiles between infected and non-infected abomasum, as well as between *H. contortus* and infected abomasum, yielding a p-value of 0.009. This robust statistical examination provides compelling evidence that the observed variations in bacterial composition among these groups are not attributable to random chance but are statistically significant.

Discussion

In this study, we investigated both, the microbiome of *H. contortus* and the one of its ecological niche, the sheep abomasum, in the Kashmir Merino breed. We showed distinct patterns in the microbial communities of *H. contortus*, the infected and the non-infected abomasum of Kashmir Merino sheep. The Non-Metric Multidimensional Scaling (NMDS) analysis based on Bray-Curtis and Jaccard dissimilarity measures indicated significant clustering, highlighting dissimilarities in bacterial profiles across these groups. Mafuna et al. ²³ using Principal coordinate analysis (PCoA) based on unweighted UniFrac distances also showed distinct clustering of bacterial profiles of *H. contortus* and sheep abomasal microbiome. Altogether, this result suggests that, even in close interaction, microbial communities exhibit underlying specificity stemming from specific functional determinants.

The microbiome composition at the phylum level showed variations in the abundance of bacterial taxa. In keeping with previous studies, in *H. contortus*, Proteobacteria dominated, followed by Firmicutes and Bacteroidota ^{19,22-24}. The abomasal microbiome were characterized by a predominance of Bacteroidota, followed by Firmicutes and Proteobacteria, in both infected and non-infected sheep. Interestingly, a higher abundance of Firmicutes, Proteobacteria, Actinobacteriota, and Fibrobacterota and a lower abundance of Verrucomicrobiota was observed in infected abomasum compared to the non-infected abomasum. These findings suggested nuanced microbial dynamics associated with parasitic infection. Our results are in agreement with other studies which also showed an increase in Firmicutes and Proteobacteria proportion with *H. contortus* infection ^{17,25}. Bacteroidota and Firmicutes are considered to be important bacterial phyla for mammals including small

ruminants. The disturbance in their relative proportion would contribute to the indirect deterioration of abomasal homeostasis and the detrimental effect on the physiology of the host²⁶. At the genus level, *H. contortus* is marked by the prevalence of *Acinetobacter*, *Massilia*, and *Prevotella*, among others. In our previous study, we compared the microbiome of *H. contortus* with *Trichuris ovis* and found significant differences in bacterial communities across the two nematode species²¹. Infected abomasum showed a dominance of *Prevotella*, *Fibrobacter*, and *Ruminobacter* while non-infected abomasum is characterized by Rikenellaceae_RC9_gut_group, *Prevotella*, and WCHB1-41_ge. The unique bacterial genera identified in each group further underscore the distinct microbial profiles. Other studies also reported *Prevotella* as the predominant genera in sheep abomasum with an increase in its abundance in infected samples^{25,27}. In addition to *Prevotella*, we reported an increase in the abundance of *Fibrobacter*, *Ruminobacter*, *Succinivibrio*, and *Clostridia* like genera in infected abomasum.

We tried to find any consistency between the microbiome of *H. contortus* and that of its ecological niche to investigate the interaction between the worm microbiome and the abomasal one. Our results suggested the transmission of bacteria like *Prevotella*, *Ruminococcus*, *Escherichia/Shigella*, and *Lachnospiraceae_unclassified*, common to *H. contortus* and the abomasum. However, bacteria like *Acinetobacter*, *Massilia*, *Phyllobacterium*, *Variovax*, *Sphingomonas*, and *Spiroplasma* were unique to *H. contortus*, suggesting a level of specificity of the microbiome of *H. contortus* compared with the microbiome of abomasum. Notably, some pathogenic bacteria like *Pseudomonas*, *Streptococcus*, and *Staphylococcus* were also associated with worms depicting their role as carriers of secondary infections to the host and potentially antimicrobial resistant strains *Fibrobacter*, *Succinivibrio*, *Aeriscardovia*, and *Pseudocardovia* were found only in the abomasum. These findings underscore a complex tripartite relationship among the host, parasite, and their microbiomes, characterized by selective transmission of bacteria between the host and parasite, each maintaining its distinct core microbiome.

Our study reveals significant alpha diversity differences among *H. contortus*, infected and non-infected abomasum. Shannon diversity index and evenness indices were higher in infected abomasum, followed by non-infected abomasum and *H. contortus*. However, Ace, Chao, and Simpson indices show higher bacterial diversity in the non-infected abomasum, emphasizing an impact of *H. contortus* infection on the richness and dominance of specific phyla. Other

studies also showed similar results depicting that *H. contortus* infection tends to increase the bacterial diversity of sheep abomasum^{17,25}. Statistical analysis showed a significant difference in microbial profile of infected and non-infected abomasum depicting that *H. contortus* infection tended to alter the sheep abomasal microbiome. It has also been showed that other helminths like *Teladorsagia circumcincta*, *Trichostrongylus colubriformis*, *Ostertagia ostertagi*, *Trichuris suis*, *Opisthorchis viverrine*, altered the host microbiome^{6,28-31}. Beta diversity analysis through NMDS highlighted unique clustering patterns, emphasizing dissimilarities in bacterial profiles. An increased within-group variation was observed, reinforcing the hypothesis of a disturbed microbial community induced by *H. contortus* infection, in accordance with previous relevant investigations^{16,25,31,32}.

In conclusion, this study provided a comprehensive examination of the microbiome associated with *H. contortus* and its impact on the abomasum in Kashmir Merino sheep. The investigation revealed specific microbial patterns in infected and non-infected abomasum, emphasizing the dynamics of parasitic infection. The dominance of Proteobacteria, Firmicutes, and Bacteroidota in *H. contortus* aligns with prior research, showcasing the consistency of these findings across different studies. At the genus level, the prevalence of *Acinetobacter*, *Massilia*, and *Prevotella* in *H. contortus*, along with the unique bacterial genera identified in infected and non-infected abomasum, underscores the complex tripartite relationship among the host, parasite, and their respective microbiomes. Here, we showed that pathogenic bacteria could be carried by *H. contortus*, and suggested a bacterial flux between *H. contortus* and the abomasum. Additionally, we found that *H. contortus* and the abomasum have distinct core microbiomes. The changes we observed in diversity indices and the differences in diversity patterns highlighted the significant impact of *H. contortus* infection on the abomasal microbiome of Kashmir Merino sheep. These findings provided valuable insights on the disruption of the normal microbial balance by of gastrointestinal parasite infection, suggesting new ways to manage sheep health, especially in the face of anthelmintic resistance.

Materials and Methods

Collection of abomasal contents and identification of parasites

Multiple Sheep gastrointestinal tracts were collected from a local slaughterhouse and brought to Parasitology lab, Department of Zoology, University of Kashmir. In the lab, guts were dissected open and screened thoroughly for helminth infection. Only those guts that were either infected with *Haemonchus contortus* or uninfected were selected. Abomasal contents were

collected and squeezed through a four-layered cheesecloth to remove large particulate matter. The filtrate was put in 90% alcohol for further processing. In addition, live worms were collected from the abomasum and washed with PBS (pH 7.4) to remove residual gut contents from the parasites. To prevent postmortem alterations in the microbiome communities, sheep intestines were processed immediately after sheep death. *H. contortus* was identified morphologically through vulvar and spicular characteristics^{33,34} and further validated with molecular characterization. A total of 10 guts were selected, 5 were infected with *Haemonchus contortus* only, and 5 were devoid of any helminth infection. In total, 15 samples were prepared 5 each of *H. contortus* worms, infected abomasum, and non-infected abomasum. A single worm sample contained 10 to 16 adult worms with an equal male to female ratio.

DNA extraction

For worm samples, adult worms were rinsed with PBS, then with antibiotic solution (Streptomycin/Erythromycin 20mg/ml) for 2-3hrs and then washed with 4% sodium hypochlorite 5 times, and finally again with PBS to remove surface contamination^{23,24}. The sterilized worms were then homogenized and put in 2ml Eppendorf tubes and centrifuged at 8000rpm for 5 minutes. Discarding the supernatant, 400µl of lysis buffer (20mM Tris HCL pH 8, 100mM disodium EDTA) and 200µl of 10% SDS were added to the Eppendorf tubes. The mixture was vortexed and then 15 µl Proteinase K solution (20mg/ml) was added to the tube. After 5 minutes of vortexing again, samples were incubated at 65°C for 2hrs and then lysozyme (20mg/ml) was added to the tube and kept for incubation at room temperature overnight. Lysis buffer, SDS, Proteinase K, and lysozyme were also added to distilled water for negative control. Parasite and bacterial DNA from these samples was isolated using PureLink Microbiome DNA Purification Kit Catalog number: A29790 as per the manufacturer's protocol. The DNA eluted was stored at -20 °C until further processing. For abomasal contents, the filtrate was first centrifuged, and the supernatant containing ethanol was discarded. The pellet was subjected to 2-3 washings and centrifuge cycles to remove any traces of ethanol from the samples followed by the addition of lysis buffer, SDS, Proteinase K, and lysozyme as in worm samples. Similar steps were followed to extract DNA from abomasal contents and eluted DNA was stored at -20 °C until further processing.

DNA amplification, library preparation and sequencing

For the molecular validation of *H. contortus*, the ITS-2 region was amplified using 25 ng of worm DNA³⁵. The reaction included Promega Master Mix catalog number: M7122 and 1µl

(1 μ M concentration) of each NC1 and NC2 primers (NC1: ACGTCTGGTTCAGGGTTGTT, NC2: TTAGTTTCTTTTCCTCCGCT). The PCR setup involved a denaturation step (95°C for 5 min) followed by 35 cycles of elongation step (95°C for 30s, annealing at 55°C for 30s and 72°C for 1 min) and a final extension step (72°C for 5 min). The PCR products (350 bp) were screened by gel electrophoresis in 1.5 % agarose gel run at 100 V for 30 min, and the product size was confirmed with a 100bp Promega DNA marker. The PCR products were purified using AMPure beads (1.6X) and sent for sequencing.

For the microbiome, 16S rRNA (V3-V4 hypervariable region) was amplified from DNA samples. The reaction included Promega Master Mix catalog number: M7122 and 1 μ M final concentration of forward and reverse primers (V3V4F: CCTACGGGNGGCWGCAG, V3V4R: GACTACHVGGGTATCTAATCC)^{36,37}. PCR setup included a denaturation step (95°C for 5 min) followed by 35 cycles of elongation step (95°C for 30s, 55°C for 45s, and 72°C for 30s) and a final extension step (72°C for 5 min). The PCR products were screened by gel electrophoresis in 1.5 % agarose gel run at 100 V for 30 min, and the product size was validated with a 100bp Promega DNA marker. AMPure beads (1.6X) were used to purify PCR products. To prepare the sequencing libraries, 8 cycles of PCR were performed using Illumina barcoded adapters as per Illumina 16s Metagenomic Sequencing Library Preparation guide. The sequencing was then carried out in the Illumina MiSeq 2x300bp PE chemistry.

Data processing and analysis

The data was put through quality control checks, including assessments of base quality, the percentage of bases with quality above a Q25 threshold, %GC content, and the presence of sequencing adapter contamination. Low-quality bases and adapter/primer sequences were removed using FastQC v.0.12.0 and Trimgalore v.0.6.7^{38,39}. Subsequently, the high-quality forward and reverse reads were merged into contigs using Mothur v.1.48.0⁴⁰. These contigs were scrutinized for ambiguous bases, and duplicate sequences, and were processed to eliminate gaps and overhangs. Any chimeric sequences were also removed. Taxonomic classification of the filtered contigs was performed using the SILVA_v138.1 database, and they were grouped into Operational Taxonomic Units (OTUs)⁴¹.

To assess the diversity of the data, both alpha and beta diversity measures were calculated in mothur. Alpha diversity included indices like Shannon, Simpson, Ace, Chao, Simpson evenness, and Shannon evenness. Beta diversity was evaluated using Bray-Curtis and Jaccard distances. The relative abundance of OTUs was computed based on the taxonomy information and OTU count table generated by Mothur. The normality of the data was checked using the

Shapiro-Wilcoxon test in R studio 2023.03.0 Build 386, and as it was found to be non-normal, the Kruskal-Wallis test was employed to determine statistical significance for alpha diversity. Beta diversity results were analyzed for statistical significance using PERMANOVA (Permutational Multivariate Analysis of Variance) ⁴².

For data visualization and plotting, R studio 2023.03.0 Build 386 was used with packages such as phyloseq, vegan, tidyverse, dplyr, phangorn, and ggplot2 packages ⁴³⁻⁴⁹.

Ethics declarations

The study was approved by Institutional Ethical Committee, Department of Pharmaceutical Sciences, University of Kashmir under approval number KU/2022/23.

Data availability

The datasets presented in this study can be found on NCBI SRA repository under BioProject ID: PRJNA1014978.

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Author Contributions

Bhat A. H, Tak H, and Ganai B. A conceptualized the study design and methodology. Bhat A. H, and Malik I. M conducted the practical aspects of the research. Bhat A. H carried out the data analysis. Bhat A. H, Tak H, and Bambou J. C, contributed to the writing and reviewing of the manuscript. All authors reviewed and provided approval for the final version of the manuscript.

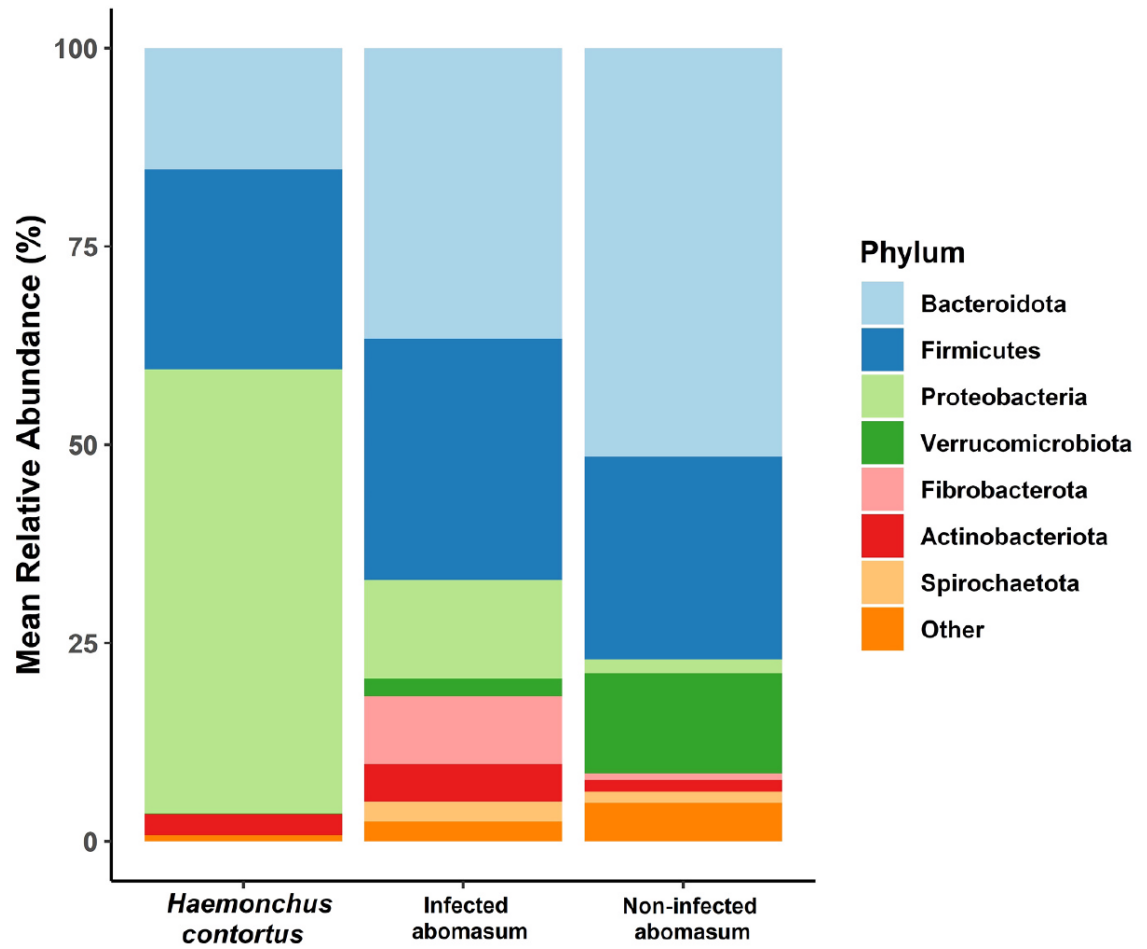
Competing interests

The authors have disclosed that there are no potential conflicts of interest related to the research, authorship, and/or publication of this article.

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Fig. 1. Stacked Bar Chart showing mean relative abundance of microbiome diversity of *Haemonchus contortus*, infected and non-infected abomasum at phylum level



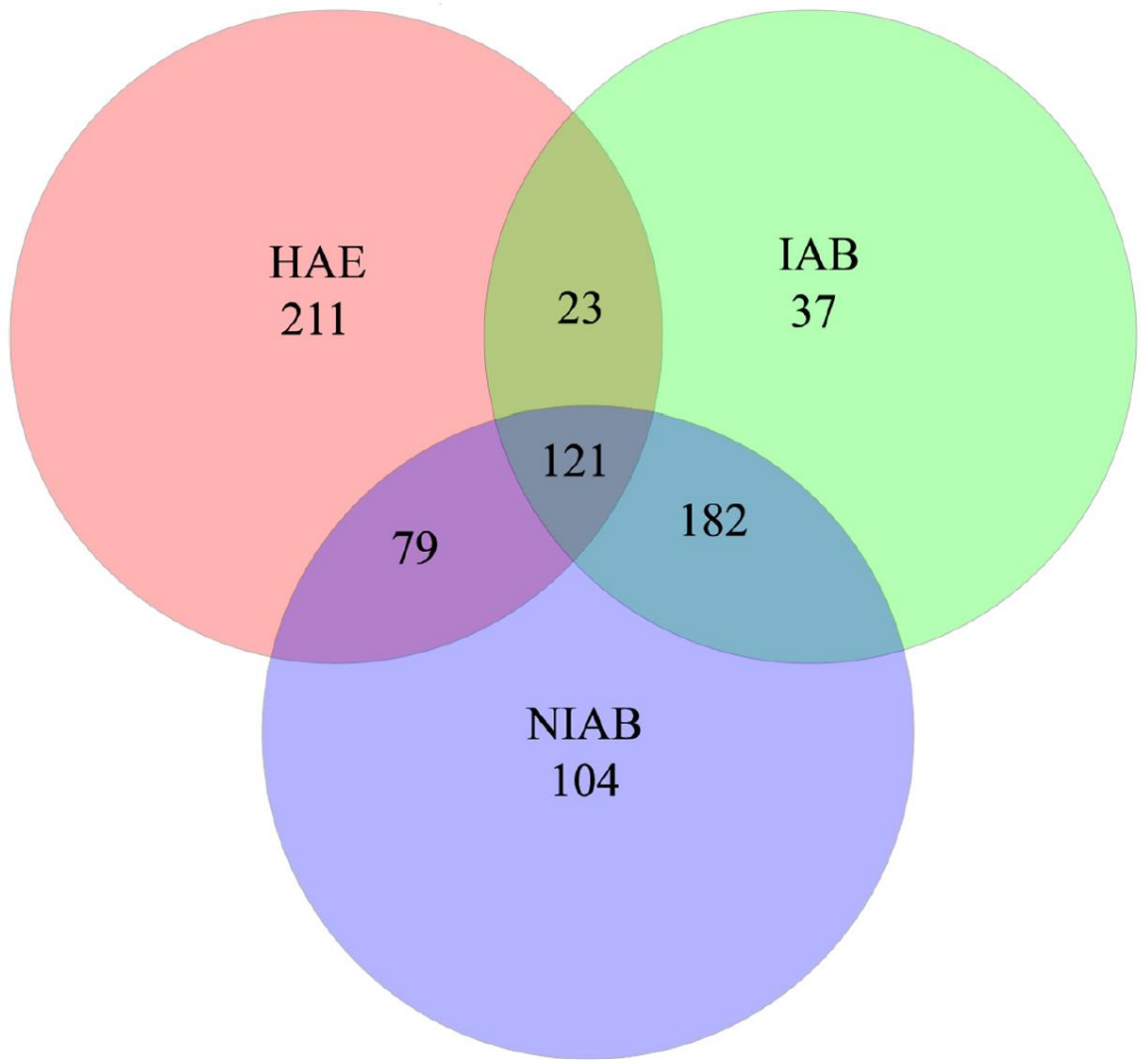


Fig. 2. Venn diagram showing the shared and unique number of OTUs in HAE: *Haemonchus contortus*, IAB: infected abomasum, and NIAB: non-infected abomasum

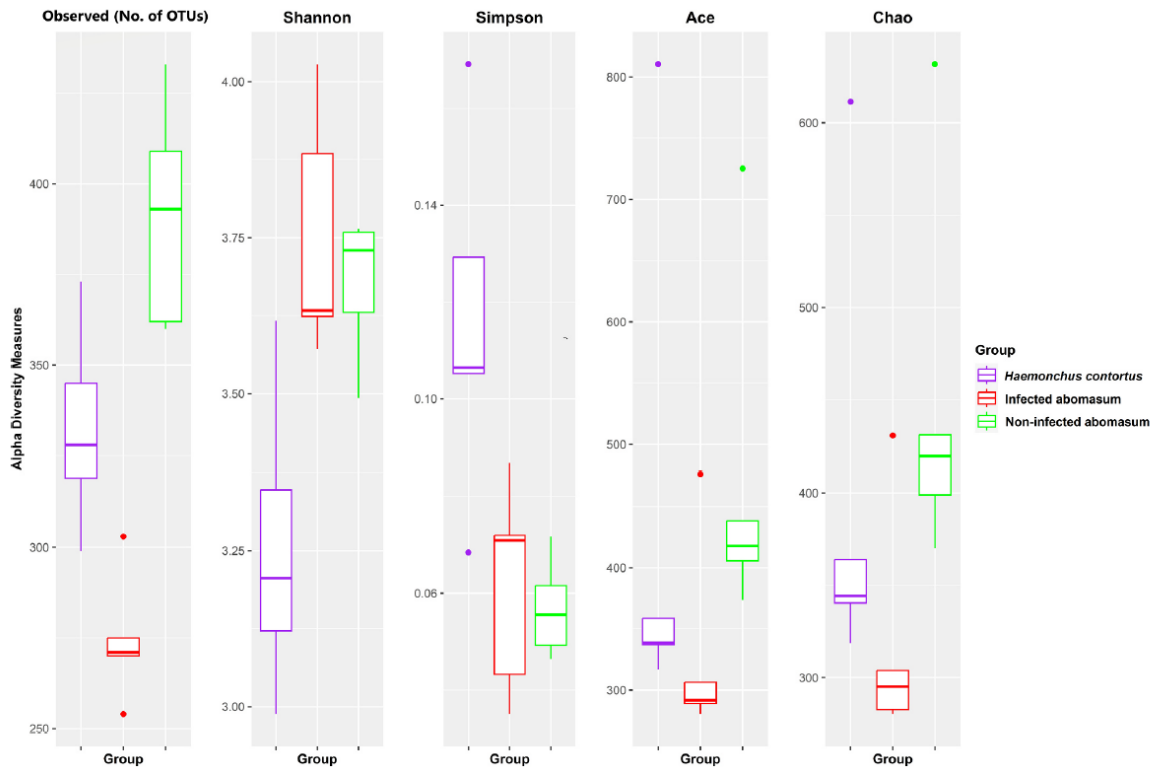


Fig. 4. Alpha diversity measures: Boxplot of alpha diversity indices of *Haemonchus contortus*, infected abomasum, and non-infected abomasum

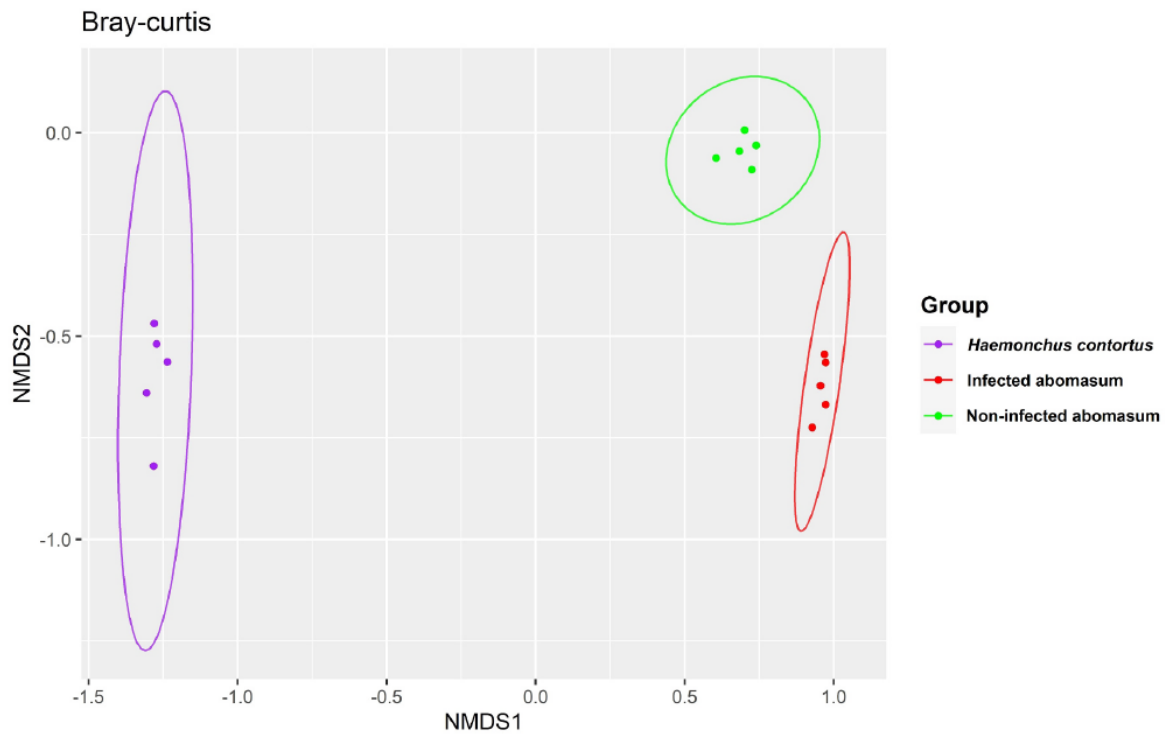


Fig. 5. Nonmetric multidimensional scaling (NMDS) ordination of microbiota in *Haemonchus contortus*, infected and non-infected abomasum based on Bray-Curtis distances; Each dot in the visualization represents the microbiome profile of an individual sample, while the ellipse illustrates the extent of the microbiome community spread within a specific group.

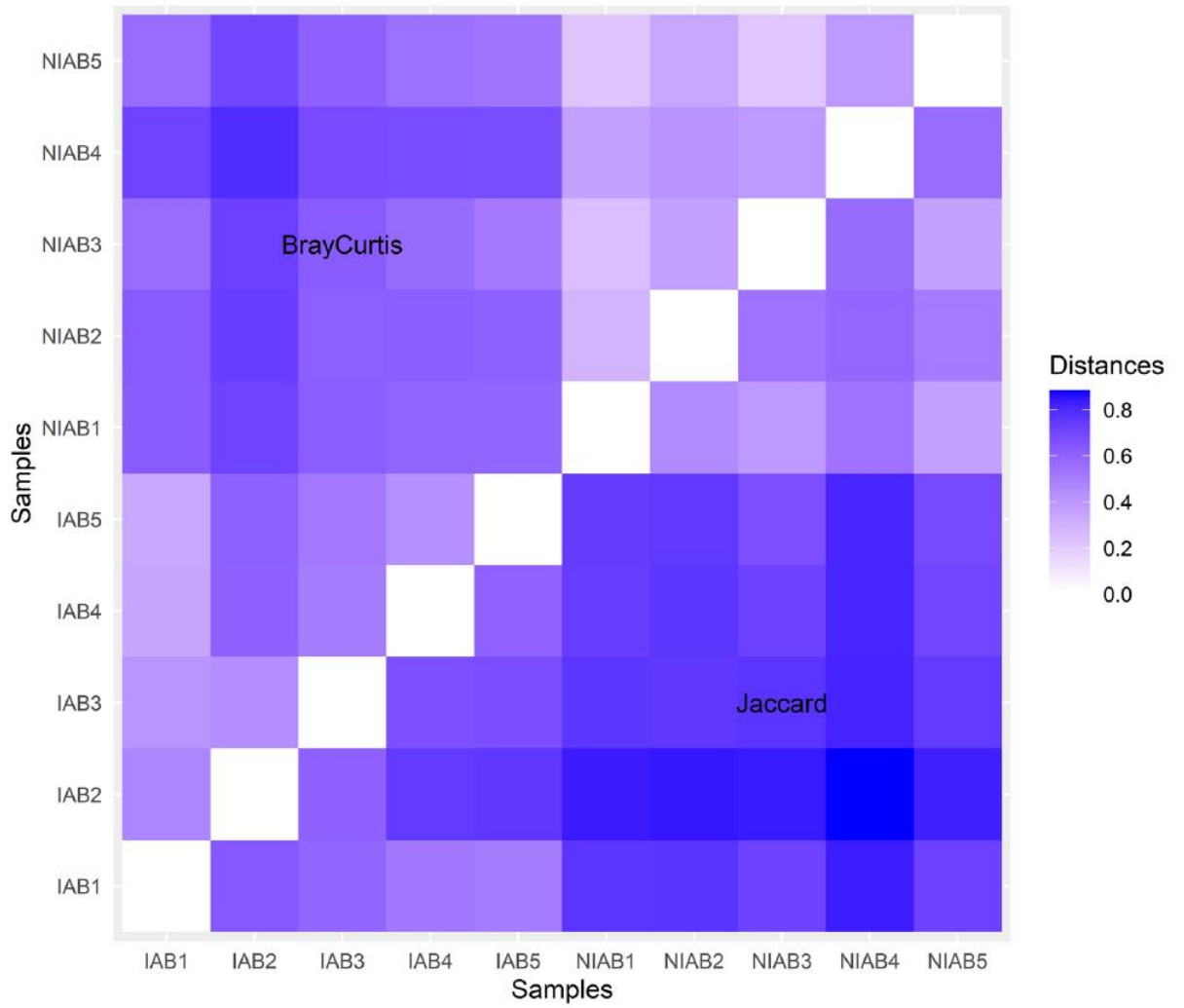


Fig. 6. Heatmap of Microbiome profile of *Haemonchus contortus*, infected and non-infected abomasum based on Bray-Curtis and Jaccard distances; Each small box represents the distance between two samples.

Tables

Table 1. GenBank accession number of *H. contortus* samples; Known sequences with >95% match and overlap were used to identify worm species in nBlast search.

Species	Gene amplified	GenBank Accession number
<i>Haemonchus contortus</i>	ITS2	OQ788264; OQ788265; OQ788266; OQ788267; OQ788268

Table 2. Alpha diversity measures for *H. contortus*, infected abomasum and non-infected abomasum; Statistical significance was calculated with the Kruskal-Wallis test at $P < 0.05$ (* represents significant difference).

Diversity Indices	<i>H. contortus</i> Mean±SD	Infected Abomasum Mean±SD	Non-Infected Abomasum Mean±SD	Infected Abomasum to Non-Infected Abomasum		<i>H. contortus</i> to Infected Abomasum	
				H-value	p-value	H-value	p-value
Observed	332.8±27.93	274.6±17.78	391.4±31.19	6.81	0.009*	5.77	0.01*
Shannon	3.25±0.24	3.74±0.19	3.67±0.11	0.09	0.75	5.77	0.01*
Simpson	0.11±0.04	0.06±0.02	0.05±0.008	0.09	0.75	3.93	0.04*
Ace	432.3±211.9	328.7±82.97	472.1±143.4	3.15	0.07	3.15	0.07
Chao	395.7±121.6	318.5±63.3	450.2±104	3.93	0.04*	3.15	0.07
Shannon Evenness	0.56±0.041	0.66±0.036	0.61±0.016	5.77	0.01*	6.81	0.009*