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Species diversity and within-host tropism for mixed equine strongyle infections using a cytochrome c oxidase subunit I metabarcoding approach ☆

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ABSTRACT

Strongyles, especially non-migratory Strongylidae, are the most common equine gut parasites, and typically occur in mixed infections with 10 – 20 species per host. Current knowledge on strongyle species prevalence and within-host tropism is sparse. Herein species composition of mixed strongyle infections of 12 naturally infected untreated young horses, based on strongyle eggs extracted from faeces, cultured third stage larva and luminal worms collected from ventral and dorsal colon was examined. Species were identified using a cytochrome *c* oxidase I (COI) metabarcoding approach allowing differentiation of operational taxonomic units (OTU) in small strongyle species complexes. In total, 16 small strongyle and one large strongyle species were identified. Eleven small strongyles were detected in all equines, including three cryptic species: *Cylicostephanus calicatus* OTU II, *Cylicostephanus minutus* OTU II and III. Coherence of detection rate, alpha and beta diversity showed high agreeance between data obtained using DNA from faecal eggs and cultured larvae. Diversity, but not observed richness, was higher in eggs and larvae compared to luminal worms (Inverse Simpson index, Shannon index, all $P < 0.05$). Bray-Curtis and Jaccard dissimilarity showed overlapping beta diversity clusters for eggs and larva, while ventral and dorsal colon samples clustered separately and differed significantly according to PERMANOVA ($P < 0.001$). Five small strongyle species showed significantly higher occurrence in the ventral than dorsal colon, including cryptic species *Cylicostephanus calicatus* OTU II. This study provides novel prevalence data for five cryptic species, documents species tropism within intestinal compartments and demonstrates high strongyle species composition agreement between faecal eggs and cultured larvae.

1. Introduction

Today, the most prevalent gut parasites in equines are non-migratory species from the family Strongylidae, commonly referred to as small strongyles (Bellaw and Nielsen, 2020). They cause multi-species infections, typically with 10–20 species detected in a single host (Bull et al., 2025; Halvarsson et al., 2024; Lyons et al., 2011; Sallé et al., 2018; Sargison et al., 2022). While most small strongyle infections remain subclinical, they have been linked to diarrhoea, colic, weight loss and poor growth (Love et al., 1999; Mair et al., 2000). In rare cases, they can cause life-threatening disease known as larval cyathostominosis, which

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usually affects young horses (Lawson et al., 2023; Love et al., 1999; Mair et al., 2000; Peregrine et al., 2006). In addition to small strongyles, three migratory Strongylidae species (large strongyles), *Strongylus (Str.) vulgaris, Str. edentatus* and *Str. equinus* remain relevant to equine management (Nielsen et al., 2022b; Sallé et al., 2020), of which only *Str. vulgaris* is considered highly pathogenic (Pihl et al., 2018). Because both small and large strongyles share egg morphology, individual species cannot be detected using faecal egg tests. Broad-spectrum diagnostic tools are therefore required for species-level monitoring.

Based on morphological identification, 47 small strongyle species (morphospecies) are recognized and known to infect equines (*Equus ferus caballus*) (Lichtenfels et al., 2008). Of those, 35 species have been identified globally in domestic equines since 1975, with some regional differences in species composition (Bellaw and Nielsen, 2020). For North America, *Cyathostomum* (*Cya.*) *catinatum, Cylicocyclus* (*Cyc.*) *nassatus, Cylicostephanus* (*Cys.*) *longibursatus, Coronocyclus* (*Cor.*) *coronatus* and *Cylicostephanus* (*Cys.*) *goldi,* followed by *Cylicostephanus* (*Cys.*) *calicatus, Cylicostephanus* (*Cys.*) *minutus, Cylicocyclus* (*Cyc.*) *leptostomus, Cylicocyclus* (*Cyc.*) *insigne* and *Cyathostomum* (*Cya.*) *pateratum* have been reported as 'core' small strongyle species, accounting for up to 98 % of all identified specimens (Bellaw and Nielsen, 2020; Reinemeyer et al., 1984). In addition to morphospecies, phylogenetic studies have identified three lineages each within *Cys. minutus* (Bredtmann et al., 2019b; Gao et al., 2020; Hung et al., 1999) and *Cys. calicatus* (Bredtmann et al., 2019a; Louro et al., 2021), shown to represent six separate cryptic species named *Cys. minutus* OTU I – III and *Cys. calicatus* OTU I – III (Bredtmann et al., 2019b; Diekmann et al., 2025; Louro et al., 2021). Distinguishing these cryptic species is important, as collapsing them under two single morphospecies obscures true species composition and could confound ecological and epidemiological analyses.

Species-specific investigations of equine small strongyles have been hampered by the non-availability of feasible cost-effective sensitive and specific methods in the past, which has resulted in limited availability of species-specific prevalence reports (Bredtmann et al., 2017; Gasser et al., 2004). Prior to the rise of high-throughput sequencing (HTS) technologies, small strongyle species were assessed through laborious and highly specialised morphological identification of adult worms, invasively collected during necropsy or expelled after anthelmintic treatment (Bellaw and Nielsen, 2020; Kuzmina et al., 2005). Currently, HTS metabarcoding approaches present the most feasible solution for species differentiation and for investigating species-specific characteristics (Abbas et al., 2023; Bull et al., 2025; Byrne et al., 2024; Courtot et al., 2023; Diekmann et al., 2025; Ghafar et al., 2023; Hedberg Alm et al., 2023; Mitchell et al., 2019; Nielsen et al., 2022a; Poissant et al., 2021; Rinaldi et al., 2022; Sargison et al., 2022). Moreover, these approaches continue to refine our taxonomic understanding of this diverse nematode subfamily (Bredtmann et al., 2019a; Diekmann et al., 2025; Louro et al., 2021). Metabarcoding targets for mixed strongyle infections currently include the internal transcribed spacer 2 (ITS-2) sequence (Gasser et al., 2004), a short 450 bp cytochrome *c* oxidase I (COI) sequence (Courtot et al., 2023) and a long 650 bp COI sequence (long COI) (Diekmann et al., 2025). The ITS-2 and long COI methods have been used to identify large strongyles as well (Diekmann et al., 2025; Gasser et al., 2004). The ITS-2 approach, while widely applied (Bull et al., 2025; Byrne et al., 2024; Courtot et al., 2023; Ghafar et al., 2023; Halvarsson et al., 2024; Poissant et al., 2021), cannot discriminate between all small strongyle species. Most notably, two 'core species' *Cor. coronatus* and *Cys. calicatus* cannot be reliably differentiated using ITS-2 (Bredtmann et al., 2019a; Byrne et al., 2024; Diekmann et al., 2025; Louro et al., 2021). In contrast, the 650 bp COI method is able to discriminate these species (Diekmann et al., 2025) as well as the three cryptic species *Cys. calicatus* OTU I – III (Bredtmann et al., 2019a; Louro et al., 2021). In a recent methods comparison, the long COI approach showed higher power for nemabiome analysis compared to ITS-2 (Diekmann et al., 2025) and demonstrated its suitability for differentiating recognized morphospecies as well as cryptic species.

A further consideration in equine nemabiome studies is the choice of biological sample type. Metabarcoding approaches enable species differentiation regardless of developmental stage (Avramenko et al., 2015; Courtot et al., 2023; Gasser et al., 2004; Miller et al., 2024), which offers more flexibility in study design and reduces the need for invasive approaches used to collect luminal worms (Bucknell et al., 1995; Collobert-Laugier et al., 2002). Faecal eggs are the most accessible sample type and can be directly isolated from faeces, but they contain the smallest amount of DNA per specimen. Depending on egg-shedding levels and retention during egg isolation, samples may fall short of necessary thresholds for HTS library preparation. Moreover, because egg recovery depends on gravid females, it is possible that some species may go undetected due to differences in infection timing, prepatent period, and egg production numbers (Kuzmina et al., 2012). Larval culture, during which faecal eggs are developed to the L3 stage, may offer a suitable alternative with increased DNA content per specimen. However, development rates may differ between equine strongyle species. In ruminants, for instance, substantial efforts were made to account for uneven development of mixed gastrointestinal nematodes (Avramenko et al., 2015; Borkowski et al., 2020; Redman et al., 2019; Rinaldi et al., 2022). While the suitability of larval culture and faecal eggs has been investigated in many host species (Pafčo et al., 2018), only one other metabarcoding study compared larval cultures to other biological sample types for equines strongyles (Courtot et al., 2023). That study, however, did not include cryptic species and data for many species are still missing. Further studies are needed to validate this approach, particularly with respect to cryptic species.

All small strongyle species are considered to share similar characteristics, including a direct non-migratory life cycle, during which third-stage larvae mature to adults within the host's hindgut. However, several morphological studies have described differences in species abundance and tropism between different sections of the hindgut (Bellaw et al., 2018; Gasser et al., 2004; Nielsen et al., 2022a; Ogbourne, 1976). Necropsy studies report three main parasitism sites of small strongyles within the host: caecum, ventral ascending colon (ventral colon) and dorsal ascending colon (dorsal colon) (Love and Duncan, 1992; Ogbourne, 1978). Moreover, while large strongyle larval stages undertake a migration through several organs within the host, they are found as preadult and adult stages in the caecum, ventral and dorsal colon, making these three sections optimal sampling targets for luminal worm stages of mixed strongyle infections. Worm burdens are lowest in the caecum, estimated at only 10 % of the total worm burden, while ventral and dorsal colon harbour similar amounts (Collobert-Laugier et al., 2002; Ogbourne, 1976). To date, it remains unclear why certain small strongyle species prefer one location over another and implications for the host are unknown. Species' tropism within the host could affect their pathogenicity, which may have implications for the clinical relevance of strongyle infections, if characterized to species-level. For the ventral colon, *Cys. minutus, Cya. catinatum and Cyc. nassatus* have been described as most abundant (Collobert-Laugier et al., 2002; Love and Duncan, 1992; Mfitilodze and Hutchinson, 1985; Ogbourne, 1976), while two publications also reported *Cyc. labiatus* (Collobert-Laugier et al., 2002; Mfitilodze and Hutchinson, 1985). In the dorsal colon, *Cys. longibursatus* has been unanimously described as most abundant (Collobert-Laugier et al., 2002; Love and Duncan, 1992; Mfitilodze and Hutchinson, 1985; Ogbourne, 1976). Furthermore,

multiple studies reported high abundances of *Cys. goldi* and *Cyc. insigne* in the dorsal colon as well (Collobert-Laugier et al., 2002; Mfitilodze and Hutchinson, 1985; Ogbourne, 1976). Studies based on genetic species differentiation have also reported differences in anthelmintic response (Bull et al., 2025; Hedberg Alm et al., 2023), including shortened egg-reappearance periods following macrocyclic lactone treatment (Bellaw et al., 2018; Kooyman et al., 2016; Nielsen et al., 2022a; Van Doorn et al., 2014) and evidence of pyrantel- and macrocyclic lactone-resistant populations (Bull et al., 2025; Hedberg Alm et al., 2023). Resistance to benzimidazoles is considered widespread in small strongyles (Bellaw et al., 2018; Lester et al., 2013; Lyons, 2003). However, detailed data for many species are lacking and our understanding of their biology is still incomplete. Furthermore, there is a lack of metabarcoding data for species composition in different hindgut sections.

Considering that geographic differences, treatment history and husbandry conditions shape strongyle species composition (Bellaw and Nielsen, 2020), the present study population deserves special consideration, as several aspects are unique. 'Population S' is a long-term research herd at the Gluck Equine Research Center of the University of Kentucky and was established in 1974 (Lyons, 2003). It consists of a herd of Shetland-type ponies and has remained closed, except for the occasional replacement stallion. The history in terms of gastrointestinal parasite infections, anthelmintic treatments, treatment efficacy and husbandry data since its establishment is well documented, as the population has been investigated in several studies over the years (Drudge et al., 1985, 1983, 1974, 1963; Lyons, 2003; Lyons et al., 2001, 1996, 1994; Scare et al., 2020, 2018). Over the course of various anthelmintic treatment regimens, the herd has become subject to small strongyles that are now resistant to both the benzimidazole and tetrahydropyrimidine drug classes, demonstrated in consecutive critical tests (Lyons et al., 2001, 1996; Scare et al., 2018). While the only remaining available drug class, macrocyclic lactones, has been widely used for decades in horses worldwide and increasing reports of resistance in small strongyles have emerged (Nielsen, 2022), it was first used on this herd in 2018 (Scare et al., 2020). This offers a unique opportunity for strongyle community investigations.

Strongyle communities in this herd have been well characterized in morphological studies, which found 14 to 28 small strongyle species (Lyons, 2003; Lyons et al., 2001, 1996). Four species dominated between 1977 and 1999: *Cya. catinatum, Cyc. nassatus, Cys. goldi and Cys. longibursatus*. More recently, *Cor. coronatus* and *Cys. calicatus* have also shown consistently high prevalence and abundance (Lyons, 2003). In addition, *Cys. minutus* was among the proven benzimidazole and tetrahydropyrimidine double-resistant species and showed increased occurrence since the introduction of tetrahydropyrimidine drug classes (Lyons et al., 2001). In contrast to the consensus 'core species' described for North America, prevalence for *Cys. leptostomus* and *Cyc. insigne* were reported low in this population (Lyons et al., 2001). Both *Str. vulgaris* and *Str. edentatus* were evident in earlier studies (Drudge et al., 1983) but were subsequently not identified for several decades (Lyons, 2003; Lyons et al., 1996). A recent study, however, detected *Str. edentatus* through morphological identification of cultured larvae (Scare et al., 2018). The species composition of the strongyle community infecting 'Population S' has not been investigated since 2003 (Lyons, 2003) and no metabarcoding approach has been used on this population to date.

The objectives of this study were to differentiate mixed strongyle infections in equines using a novel COI metabarcoding approach, to (1) obtain prevalence data for individual strongyle species, (2) characterize species composition in eggs, cultured larvae and luminal worms, (3) compare detection rates between different sample types, and (4) investigate species within-host tropism across caecum, ventral colon, and dorsal colon.

2. Materials and methods

This study was approved by the University of Kentucky's Institutional Animal Care and Use Committee (IACUC), USA, under protocol number 2012–1046 and conducted between February 26th and March 17th, 2020.

2.1. Study design, sample collection and storage

This study included 12 domestic Shetland-type ponies (*Equus ferus caballus)* from 'Population S'. As elaborated in the introduction, the herd is known to be infected with small strongyles resistant to the benzimidazole and tetrahydropyrimidine drug classes (Lyons, 2003). It was last treated in January 2018 with moxidectin and oxibendazole (Scare et al., 2020). The subset of equines included in this study, however, had never received treatment, as they were all born in 2019. The group consisted of two females and ten intact males, which were clinically healthy at the time of sample collection and humanely euthanized for reasons unrelated to this study. Samples were collected in February and March 2020 during necropsy. Faecal samples were collected from the rectum for faecal egg counts, extraction of strongyle-type eggs and larval culture. Furthermore, at necropsy, luminal nematode specimens were collected in duplicates from 5 % aliquots of the ingesta of each of three hindgut sections: caecum, ventral colon and dorsal colon, as described by Nielsen et al. (2022a, b). Faecal egg counts (FEC) were assessed using Mini-FLOTAC (Cringoli et al., 2017). Strongyle-type eggs were extracted from faecal samples by mixing faeces with tap water and filtering through gaze, centrifuging the filtrate, resuspending the pellet with saturated sodium chloride solution and washing the top portion through a series of 200 μm, 85 μm and 30 μm sieves. Finally, contents left on the last sieve were collected by resuspending them with tap water and suctioning them off using a pipette (Demeler et al., 2013; Steuer et al., 2022). Strongyle eggs in this extract were enumerated and frozen in duplicates at −20 °C. To obtain larval samples, faeces was loosely placed in culture glasses at ambient temperature (approximately 22 °C) for 10 days (Jürgenschellert et al., 2022). Then, culture glasses were filled with tap water, inverted onto a petri dish and tap water was added to the petri dish. This setup was kept overnight, to allow L3 larvae to migrate into the petri dish. Larvae were then collected, counted and frozen in duplicates at −20 °C. Luminal nematode specimens were collected from each hindgut section, counted and stored in ethanol (70 %) at −20 °C. For strongyle eggs, DNA was extracted using the NucleoSpin Soil Kit (Macherey-Nagel, Germany, #740780), as this is very effective for use on nematode eggs (Demeler et al., 2013). For larvae and luminal worms, the NucleoSpin Tissue Kit (Macherey-Nagel, Germany, #740952) was used. A 28S PCR was run on all samples as qualitative verification of DNA extraction and visualized using a gelectrophoresis (1.5 % agarose) prior to library preparation (Demeler et al., 2013). The library preparation was split into two PCR reactions. First, a target PCR (PCR1) was run to amplify the target region and attach an Illumina adaptor overhang (Forward Primer: GAAAGTTCTAATCATAARGATATTGG; Reverse Primer: ACCTCAGGATGACCAAAAAAYCAA) (Diekmann et al., 2025; Duscher et al., 2015). The PCR reaction contained 2 μl of template DNA, 0.75 μl each of forward and reverse primer (4 nmole Ultramer® DNA Oligo, TruGrade®, IDT Technologies, Skokie, Illinois, USA), 12.5 μl of 2 × Kapa HiFi HotStart ReadyMix (Roche Molecular Systems, Peasanton, CA, USA) and 9 μl of double-distilled water. The cycling conditions consisted of 3 min at 95 °C, followed by 34 cycles of 20 s

at 98 °C, 15 s at 60 °C and 40 s at 72 °C, and finally, 2 min at 72 °C. PCR products were purified through magnet-bead cleaning (CleanNGS, GC biotech, Netherlands), following the manufacturer's instructions, using a 10:8 sample to bead ratio. This was followed by the indexing PCR (PCR2), to attach unique combinations of 8 bp indexes at p7 and p5 and to complete the Illumina adaptor. Index sequences were based on NuGen DNA library preparation for Illumina, but obtained from biomers.net (Ulm, Germany). For PCR2, the reactions contained 10 µl of PCR1 product, mixed with 1.25 µl each of p7 and p5 indexes (10 µM) and 12.5 µl of 2 × Kapa HiFi HotStart ReadyMix (Roche Molecular Systems, Peasanton, CA, USA). Cycling conditions for PCR2 consisted of 98 °C for 45 s, followed by 7 cycles of 98 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s, and finally, 72 °C for 1 min. PCR2 was run in duplicates, which were combined to a single sample during the following magnet bead clean-up, which was conducted twice to ensure complete removal of primer dimers and following the same procedure as previously described. Following purification, the amplicon content was measured using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Darmstadt, Germany) on a Qubit 4 Fluorometer (Thermo Fisher Scientific, Darmstadt, Germany). An additional magnet bead cleaning was performed prior to sequencing. Amplicon sequencing was performed on an Illumina MiSeq using a V3 600 cycle kit (Illumina, 2 × 300 cycles). An initial spike-in (~1 mio reads) was run and the data was used to create new sample pools according to read numbers. Samples were then sequenced again until at least 10,000 reads per sample were obtained.

2.2. Species assignment via DADA2 pipeline

Preprocessing of sequencing results was conducted as described by (Krücken et al., 2025). Key programming scripts are available in Supplementary data. Briefly, raw sequences from multiple runs of N = 48 samples were merged. Primers and indices were removed with cutadapt version 4.4 (Martin, 2011), followed by quality control measures and filtering based on the DADA2 pipeline version 1.26.0 (Callahan et al., 2016). Based on read quality plots, reads were trimmed to 200 bp and 180 bp for forward and reverse reads, respectively. Since the amplicon length considerably exceeds the sum of the paired-end reads, the "justConcatenate" mode of DADA2's mergePairs function was used, which does not calculate overlaps for the denoised forward and reverse reads but concatenates them separated by 10 "N". BLASTn (Altschul et al., 1990) was used to filter out ASVs that did not match at least ≥80 % matching identity and ≥90 % query coverage inside the Strongylidae family, based on the COI-database (Diekmann et al., 2025). After filtering, all 48 samples remained for further analysis. The COI-database (Diekmann et al., 2025) was also used to assign species. Where appropriate, multiple ASVs were assigned to a single species. Species assignment was conducted with two different methods: DADA2's implementation of *rdp* (minBoot = 0.8) and *vsearch sintax* version 2.27 (sintax_cutoff = 0.8). Matches were only included, if they were located ≤30 basepairs from the 5' / 3' end of the target sequence. The taxonomic assignments from both tools were used to calculate the consensus, with conflicting assignments between the two tools considered as 'unclassified'. Finally, all ASVs with less than 0.1 % relative read abundance across all samples and samples with fewer than 500 reads were removed to obtain the final dataset for further analyses. All descriptive statistics were calculated and visualised using R (Version 3.1.4 or 4.4.1). Colour selection for visualization was based on (Crameri et al., 2020).

2.3. Species prevalence

Prevalence of species was calculated at the individual equine level, as presence or absence of detection in any sample obtained at necropsy (eggs, luminal worms: dorsal colon, ventral colon) and subsequent larval cultures. Unclassified ASVs were excluded for prevalence analysis. All ASVs assigned to a species were aggregated to a binary outcome for each sample. Furthermore, all samples (N = 48) were aggregated to a binary outcome, to assess prevalence. Species prevalence was categorized as high (>75 %), moderate (25–75 %) or low (<25 %). Binomial Wilson score confidence intervals (95 %) were calculated (R package: epitools, version 0.5–10.1). In addition, species-specific differences in proportional occurrence between ventral and dorsal colon samples were assessed at the individual horse level. Presence/absence data were aggregated to binary outcomes per sample type and species. Species with low prevalence, defined as detection in less than three horses, were excluded from analysis. Pairwise comparisons between ventral and dorsal colon were performed using Fisher's exact test (two-sided), and odds ratios with 95 % confidence intervals were reported (R package: stats, version 4.4.1). Resulting *P*-values were adjusted for multiple testing using the 'false discovery rate'.

2.4. Detection rate for species across biological sample types

For each strongyle species and individual equine, presence or absence was recorded for four sample types: faecal eggs, cultured larvae, ventral colon luminal worms and dorsal colon luminal worms. For this analysis, both luminal worm sample types were combined as 'luminal worms'. Composite presence at the individual host level was defined as positive if the species was detected in any of the samples from that equine. Species were included if detected in at least three sample types and at least three individuals. For each included strongyle species, the detection rate for each sample type was calculated as the proportion of composite positive horses in which that sample type was also positive. Binomial Wilson score confidence intervals (95 %) were calculated (R package: epitools, version 0.5–10.1). Sample types were compared using a binomial generalised linear mixed model with detection as a binary outcome, sample type as a fixed effect, and random intercepts for equine identifier (horse_id) and species, followed by pairwise contrasts with multivariate t adjustment (R packages: lme4, version 1.1.35–1; emmeans, version 1.11.2). Coherence between sample types was assessed using pairwise agreement for all horses and species using Cohen's kappa and McNemar's test on discordant pairs with exact binomial p values (R packages: DescTools, version 0.99.60; stats, version 4.4.1).

2.5. Species richness, alpha and beta diversity

Alpha diversity was measured using three indices: observed richness, Inverse Simpson index and Shannon index. For each index, a linear mixed-effects model was fitted (R package: lme4, version 1.1.35–1). The respective alpha diversity index was set as dependant variable (Observed, Inverse Simpson, or Shannon), while sample type (levels: eggs, larva, ventral colon, dorsal colon) was the independent variable. Equine identifier (horse_id) was included as a random effect to account for repeated measures within individuals. For beta diversity, PERMANOVA was used to calculate species composition variance between sample types, based on Bray-Curtis dissimilarity (based on relative abundance) and Jaccard distance (based on presence / absence) of species and ASVs (R packages: vegan, version 2.6–8). In addition, non-metric multidimensional scaling (NMDS) was used to produce ordination plots using both Bray-Curtis and Jaccard measures (R packages: phyloseq version 1.44.0, vegan version 2.6–8) (McMurdie and Holmes, 2013).

2.6. Data accessibility

All sequences generated in this study are available in GeneBank under accession numbers specified in Supplements Table S2. All programming scripts used to analyse data and produce figures are publicly available under https://doi.org/10.6084/m9.figshare.29085455 and https://doi.org/10.6084/m9.figshare.29099111.

3. Results

3.1. Enumeration of faecal eggs, cultured larvae and luminal worm specimens

All equines included in this study were shedding strongyle-type eggs (N = 12). Faecal egg counts (FEC) ranged from 605 to 3775 eggs per gram (mean (x) = 1456; median (M) = 1380). Eggs of *Parascaris* spp. were identified in two equines. Due to a human error, the strongyle FECs were only recorded for 11 of 12 horses. Larval culture yielded 1500 – 15,950 larvae (x = 6851; M = 6175). Luminal nematode specimens were recovered from three hindgut sections, the caecum (range = 1 – 14; x = 5; M = 5), ventral colon (range = 142 – 598; x = 327; M = 295) and dorsal colon (Range = 33 – 332; x = 176; M = 128). An overview of FECs and specimens per sample type for each equine are presented in Supplementary Table 1. Based on the very low number of luminal worms recovered from caecum samples, all caecum samples were excluded from further analysis in this study.

3.2. Eleven strongyle species identified in all equines, including three cryptic species

Considering all included samples (N = 48), 1486 unique amplicon sequence variants (ASVs) were produced, of which 1125 ASVs (75.6 %) remained after filtering. Of those, 1098 ASVs were classified to species level and matched to 16 small strongyle species and one migratory large strongyle, *Str. edentatus* (Figs. 1 and 2). This included five species cryptic to morphologically recognized species *Cys. minutus* (Bredtmann et al., 2019b; Hung et al., 1999) and *Cys. calicatus* (Bredtmann et al., 2019a; Louro et al., 2021). Specifically, *Cys. minutus* OTU I, II and III as well as *Cys. calicatus* OTU I and II were identified in this study and considered as individual cryptic species, as suggested by Gao et al. (2020) and Louro et al. (2021). One ASV was manually assigned to *Cor. labratus*, after matching with a *Cor. sagittatus* (SAGI_F01, rdp: 100 %, vsearch: 100 %) as well as two *Cor. labratus* reference sequences (LABR_F12, rdp: 99.0 %, vsearch: 98.9 %; LABR_F13, rdp: 98.5 %, vsearch: 98.9 %). Including all sample types, 12 to 17 strongyle species were found per equine, with a x = 14.6 and M = 14.5 species per individual host. Eleven small strongyle species were detected in all equines: *Cor. coronatus, Cor. labratus, Cya. catinatum, Cyc. insigne, Cyc. nassatus, Cys. calicatus* OTU II, *Cys. goldi, Cys. longibursatus, Cys. minutus* OTU II, *Cys. minutus* OTU III, and *Pot. imparidentatum*. Furthermore, *Cyc. leptostomum* (91.6 %). and *Cor. labiatus* (83.3 %) had high prevalence (Fig. 1). *Cylicostephanus longibursatus* had the highest number of ASVs (n = 396), while only two ASVs were identified for *Str. edentatus* (Table 1).

3.3. Five small strongyles showed significant differences in occurrence between ventral and dorsal colon

Across 16 species tested, Fisher's exact test indicated that proportional occurrence differed significantly between ventral and dorsal colon for five species. In all cases, these findings reflected complete or near-complete separation, resulting in infinite odds ratios (Supplementary Table 3). Two species, *Cyc. leptostomus* and *Cyc. ashworthi*, showed identical results. Both were not detected in any dorsal colon samples (OR = Infinite, $P = 0.019$). Furthermore, *Cya. catinatum* (OR = Infinite, $P = 0.047$), *Cyc. nassatus* (OR = Infinite, $P = 0.019$) and *Cys. calicatus* OTU II (OR = Infinite, $P = 0.019$) showed significant differences, with higher proportional occurrence in the ventral colon compared to the dorsal colon. One species, *Str. edentatus* was excluded from this analysis as it was only detected in two horses. (Figs. 1 and 2, Supplementary Table 3).

3.4. Species detection rate similar between eggs, cultured larvae and luminal worms

The binomial generalized linear mixed model showed no significant differences in detection rates between sample types for any included individual species (n = 15) or when comparing the sample types (eggs-larvae, eggs-luminal worms, larvae-luminal worms; all $P > 0.05$). Two species, *Str. edentatus* and *Cyc. ashworthi*, were excluded from this analysis as they were detected in fewer than three horses or sample types. Pairwise agreement analyses supported these findings. Agreement between eggs and larvae was 81.4 % ($\kappa = 0.578$), with McNemar's exact test showing no significant discordance ($P = 0.256$). Agreement between eggs and luminal worms was 70.6 % ($\kappa = 0.330$; McNemar's exact $P = 0.245$), and between larvae and luminal worms was 74.5 % ($\kappa = 0.398$; McNemar's exact $P = 0.890$) (Supplements Fig. 1).

3.5. Alpha diversity, but not richness, significantly different between luminal worms and other sample types

Luminal worm samples showed several significant differences to eggs and larva in diversity, but not richness. Larva and ventral colon worms (Inverse Simpson index, $P = 0.001$; Shannon, $P < 0.001$) as well as larva and dorsal colon worm samples (Inverse Simpson, $P = 0.051$; Shannon, $P = 0.034$) differed significantly in diversity, but not richness (Observed, larva-ventral colon worms $P = 0.086$, larva-dorsal colon worms $P = 0.086$). Similarly, eggs and ventral colon worm samples differed significantly in both alpha diversity measures (Inverse Simpson, $P = 0.002$; Shannon, $P = 0.002$), but not in richness (Observed richness, $P = 0.086$). Dorsal colon worm and egg samples did not show statistically significant differences in richness or diversity (Observed richness, $P = 0.086$; Inverse Simpson, $P = 0.146$; Shannon, $P = 0.106$). Eggs and larva did not differ significantly in richness (Observed richness, $P = 0.939$) or diversity (Inverse Simpson index, $P = 0.458$; Shannon index, $P = 0.450$). Moreover, ventral and dorsal colon worm samples did not differ in richness (Observed richness, $P = 0.939$) or diversity (Inverse Simpson index, $P = 0.06$; Shannon index, $P = 0.06$)(Fig. 3 A-C).

3.6. Beta diversity significantly different between hindgut locations and some sample types

A PERMANOVA showed that for luminal worms within-host location (sample type) was a significant factor of species composition. According to Jaccard dissimilarity scores (JD), 16–22 % of variance could be explained by sample type, while Bray-Curtis dissimilarity scores (BCD) saw 44–73 % explained by sample type. The opposite observation was made for egg and larval samples. Sample type was not a significant factor between these groups. In addition, NMDS plots were used to visualise and analyse beta diversity for both dissimilarity measures. Ordination plots revealed clustering of species compositions by sample type and location within the host among luminal worm samples, with Bray-Curtis showing tighter clusters than Jaccard (Fig. 4). At ASV level, both dissimilarity measures showed pronounced clusters, especially

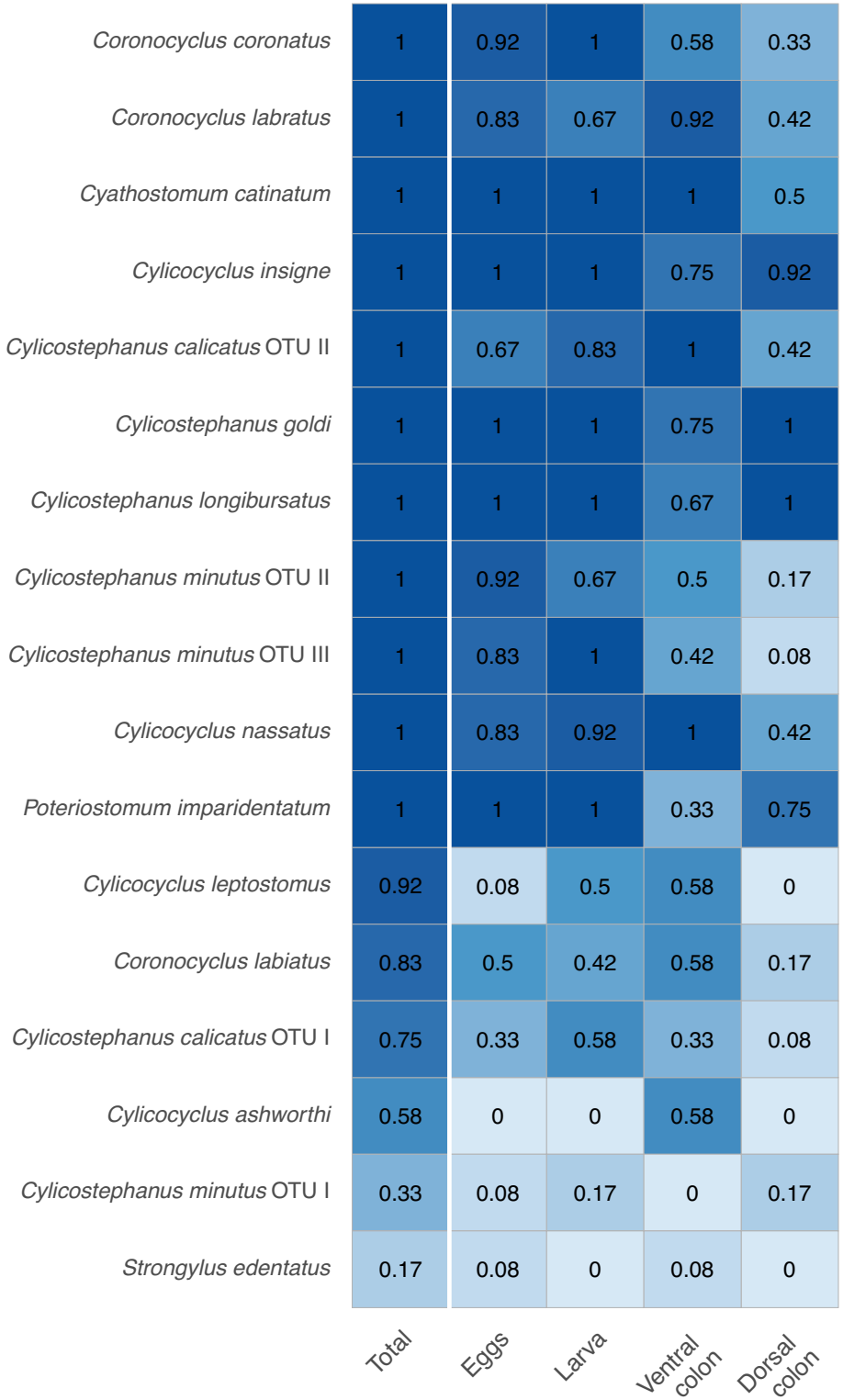

Fig. 1. Strongyle species prevalence at host level (N = 12) and species occurrence in different sample types. Species prevalence was calculated as proportional presence / absence of species at individual host level, including all sample types. Species occurrence per sample type was determined as proportional presence / absence of a species in the given sample type. For this purpose, all amplicon sequence variants (ASVs) matched to species level were aggregated to a binary result. Unassigned ASVs were excluded. Colours indicate the proportional prevalence/presence (0 to 1), depicted on a grey scale from white (0 = absent) to medium grey (0.5) to dark grey (1). Prevalence, species prevalence at individual host level including all sample types; Eggs, faecal eggs; Larva, cultured larvae; Ventral colon, luminal worms collected from the ventral colon; Dorsal colon, luminal worms collected from the dorsal colon; OTU, operational taxonomic unit.

Fig. 2. Species detection (presence/absence) per sample type shown for each individual equine (N = 12). Filled fields indicate presence of a species, whereas white fields show absence. Eggs, faecal egg samples; Larva, cultured larvae, Ventral colon, luminal worms collected from the ventral colon; Dorsal colon, luminal worms collected from the dorsal colon; H1-H12, individual equine identifier.

Table 1
Strongyle species prevalence at individual host level (N = 12).

Species	ASVs	Prevalence	95 % CI	
Coronocyclus coronatus	22	100.00	75.75	100.00
Coronocyclus labratus	18	100.00	75.75	100.00
Cyathostomum catinatum	120	100.00	75.75	100.00
Cylicocyclus insigne	59	100.00	75.75	100.00
Cylicocyclus nassatus	152	100.00	75.75	100.00
Cylicostephanus calicatus OTU II	51	100.00	75.75	100.00
Cylicostephanus goldi	152	100.00	75.75	100.00
Cylicostephanus longibursatus	396	100.00	75.75	100.00
Cylicostephanus minutus OTU II	39	100.00	75.75	100.00
Cylicostephanus minutus OTU III	18	100.00	75.75	100.00
Poteriostomum imparidentatum	13	100.00	75.75	100.00
Cylicocyclus leptostomus	21	91.67	64.61	98.51
Coronocyclus labiatus	12	83.33	55.20	95.30
Cylicostephanus calicatus OTU I	7	75.00	46.77	91.11
Cylicocyclus ashworthi	13	58.33	31.95	80.67
Cylicostephanus minutus OTU I	3	33.33	13.81	60.94
Strongylus edentatus	2	16.67	4.70	44.80

ASV, amplicon sequencing variant; CI, binomial confidence interval.

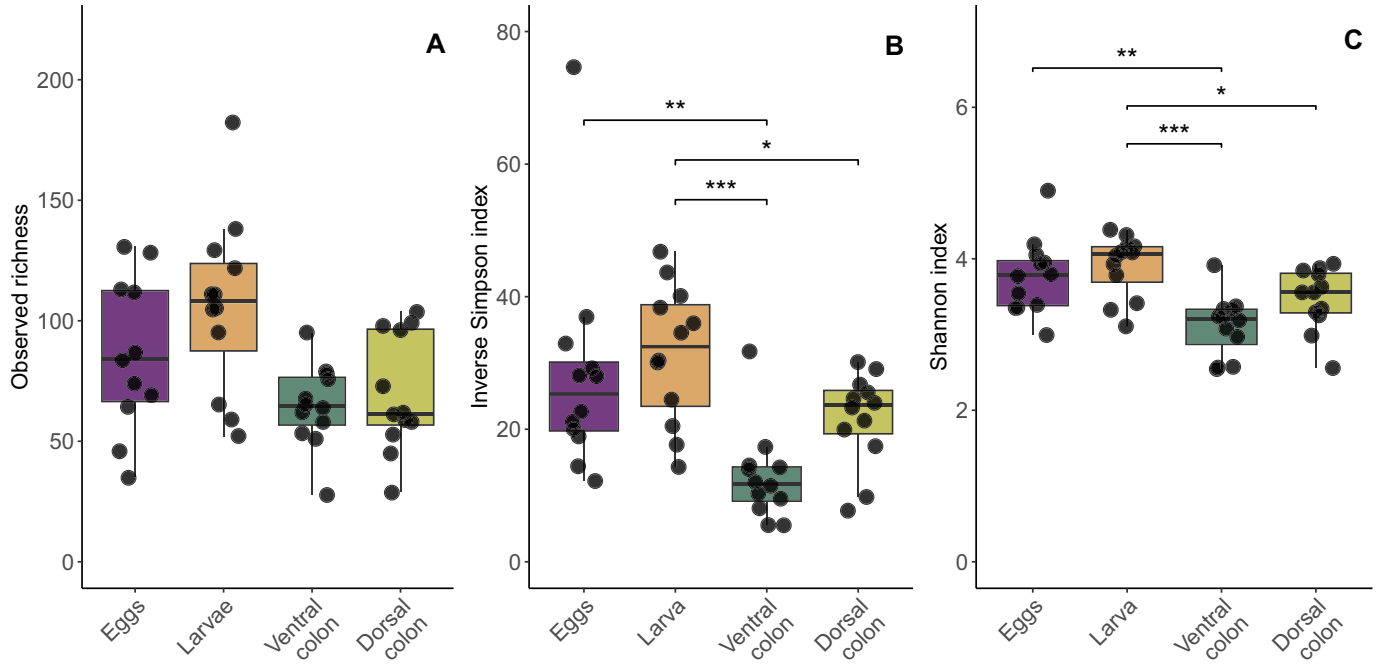

Fig. 3. Alpha diversity comparison between faecal egg samples (Eggs, n = 12), cultured larvae (Larva, n = 12), luminal worms collected from the ventral colon (Ventral colon, n = 12) and the dorsal colon (Dorsal colon, n = 12), based on amplicon sequence variants (ASVs). Unassigned ASVs were excluded from this analysis. Observed Richness (A) compares number of ASVs per individual. Inverse Simpson Index (B) and Shannon Index (C) measure diversity by a combination of richness and evenness of ASVs distribution between samples. A linear mixed model using the equine identifier as random effect variable found significant differences for several combinations, indicated as *, $P < 0.05$; **, $P < 0.01$ or ***, $P < 0.001$.

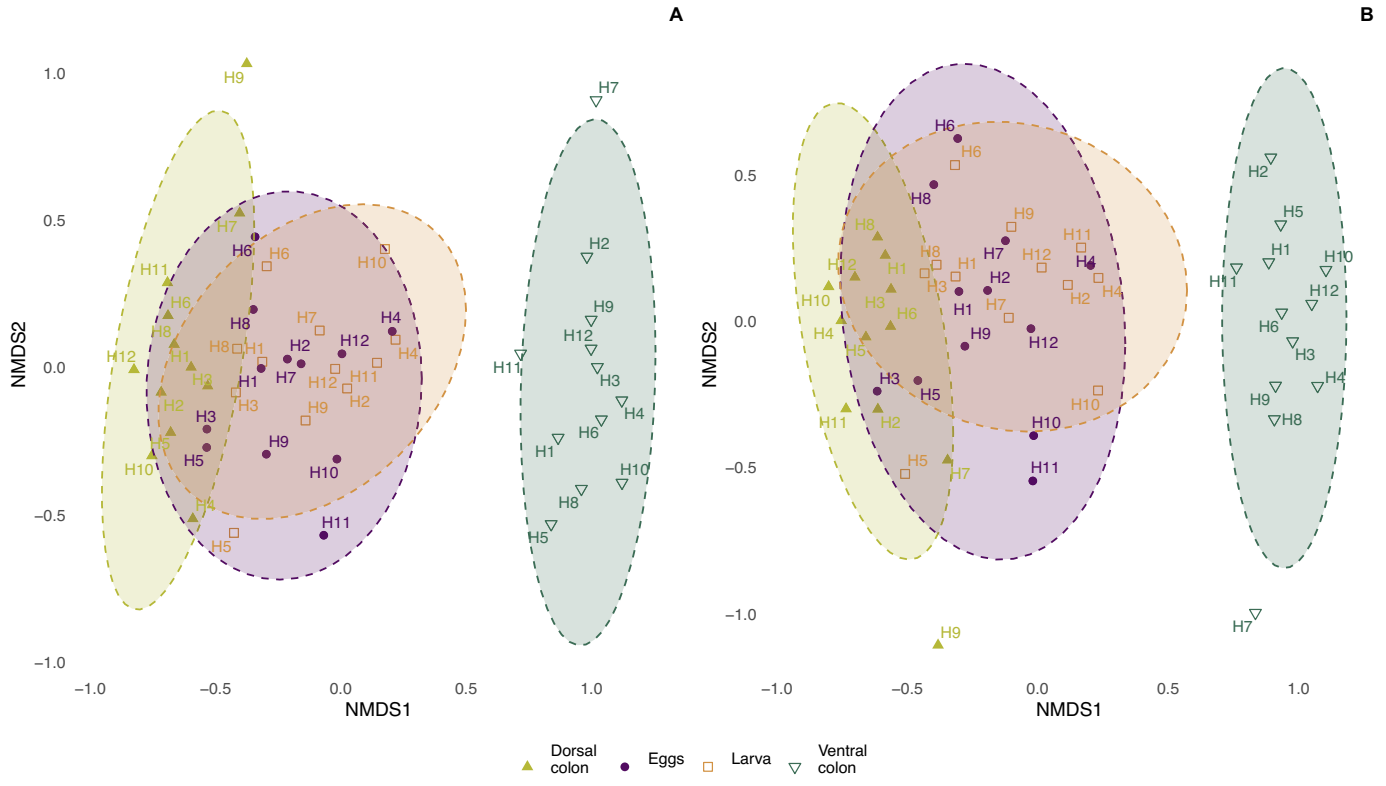

Fig. 4. Beta diversity comparison between sample types, based on species assignment. Non-metric multidimensional scaling (NMDS) ordination plots show Bray-Curtis dissimilarity (A) and Jaccard distance (B) of t-distribution-normalised species assignments for different sample types. Symbols represent individual samples. For each sample type the 95% data ellipse (stat_ellipse) is based on a multivariate t-distribution. Stress values were 0.13 for Bray-Curtis and 0.14 for Jaccard dissimilarity suggesting a fair representation of the dissimilarity patterns in the two-dimensional plots. Eggs, faecal egg samples; Larva, cultured larvae; Ventral colon, luminal worms from the ventral colon; Dorsal colon, luminal worms from the dorsal colon. OTU, operational taxonomic unit.

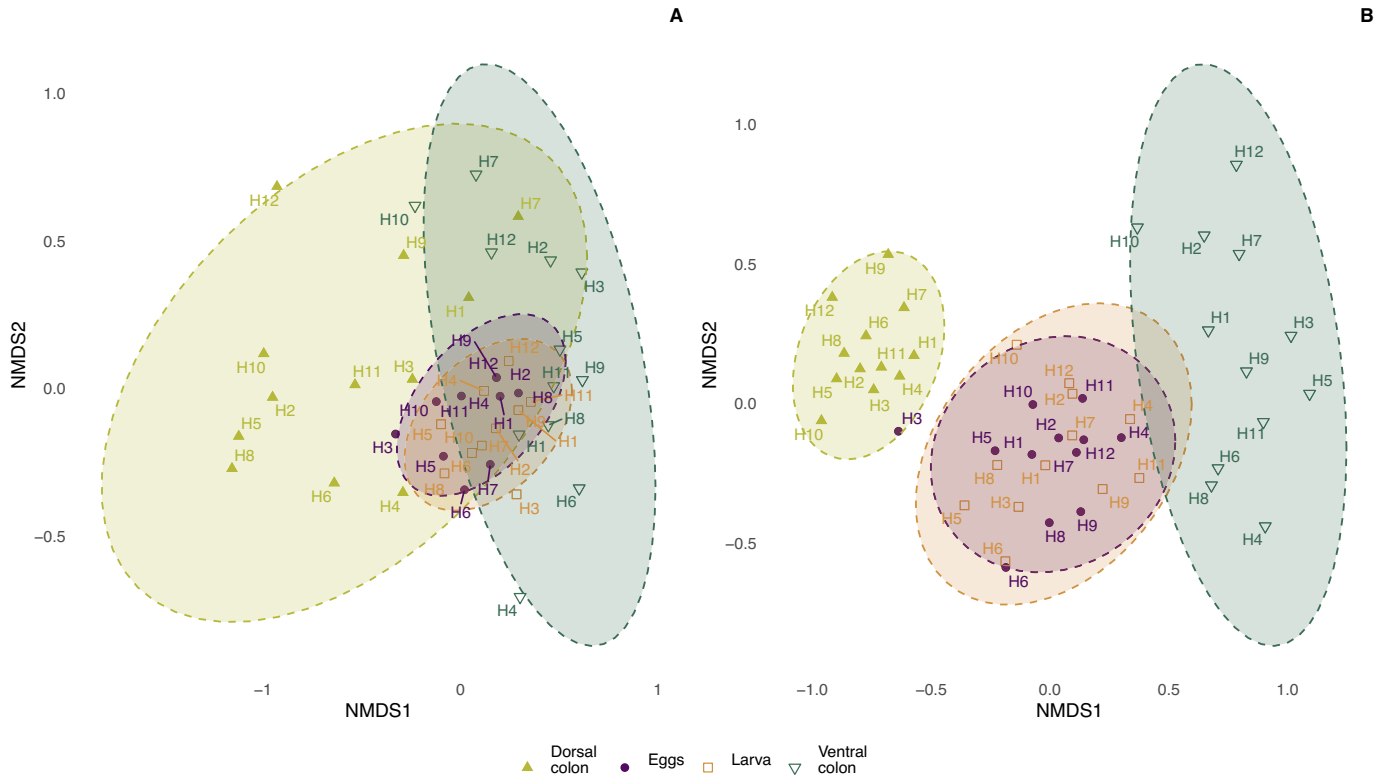

Fig. 5. Beta diversity comparison between sample types, based on amplicon sequence variants (ASVs). Non-metric multidimensional scaling (NMDS) ordination plots show Bray-Curtis dissimilarity (A) and Jaccard distance (B) of t-distribution-normalised ASVs for different sample types. Symbols represent individual samples. For each sample type the 95% data ellipse (stat_ellipse) is based on a multivariate t-distribution. Stress values were 0.18 for Bray-Curtis and 0.18 for Jaccard dissimilarity suggesting a fair representation of the dissimilarity patterns in the two-dimensional plots. Eggs, faecal egg samples; Larva, larval culture from faeces; Ventral colon, luminal worms from the ventral colon; Dorsal colon, luminal worms from the dorsal colon. OTU, operational taxonomic unit.

for ventral colon, which clustered separately from other samples (Fig. 5). Eggs and larvae showed a strong overlap in all NMDS plots.

4. Discussion

The species composition analysis in this study offers new information on mixed strongyle infections in naturally infected equines, based on COI metabarcoding data. Prevalence data for several species, including the second report for five cryptic species is shown. Moreover, detection rates and species tropism across different biological sample types are presented for several small strongyle species. Finally, faecal eggs and cultured larvae were examined as sample types for strongyle community characterization.

Species prevalence data was clearly dominated by 11 small strongyle species that occurred in all equines, including three cryptic species. Aside from the novel identification of cryptic species, the dominant species largely match previous reports (Bellaw and Nielsen, 2020; Lyons, 2003). Among the cryptic species, *Cys. calicatus* OTU II, *Cys. minutus* OTU II and *Cys. minutus* OTU III were most prevalent at host level, followed by *Cys. calicatus* OTU I and *Cyc. minutus* OTU I, which showed high and moderate prevalence, respectively. While the study population was of modest size, this data constitutes one of the first prevalence reports for cryptic species prevalence in equines. To date, only one epidemiological study has differentiated the cryptic species of *Cys. calicatus* (OTU I – III) and *Cys. minutus* (OTU I – III) (Krücken et al., 2025), although both morphospecies have been named as core species (Bellaw and Nielsen, 2020; Lyons, 2003). This discrepancy points to a limitation of morphological studies, as prevalence estimates for *Cys. calicatus* and *Cys. minutus* are likely inflated when cryptic species are collapsed under single morphospecies. The broader implications of not differentiating cryptic species remain unclear. They may extend beyond prevalence accuracy to aspects of species biology and anthelmintic response, although this remains speculative. Differences in prepatent periods, host age preferences, climatic adaptation, pathogenic potential, and drug response have been described among small strongyles (Halvarsson et al., 2024; Lyons, 2003; Lyons et al., 1996; Scare et al., 2020, 2018), underscoring the value of species-level resolution in future studies.

Four recognized species were more prevalent than expected in this study. *Cylicocyclus insigne* was among the dominant species, although earlier studies of 'Population S' described rare to moderate prevalence, with fluctuations linked to the introduction of new drug classes (Lyons, 2003; Lyons et al., 2001, 1996). This is the first report after macrocyclic lactones were introduced to 'Population S' in 2018. In the same region, *Cyc. insigne* was rare in an untreated control group (Bellaw et al., 2018) but showed moderate prevalence in a later study (Nielsen et al., 2022a), alongside shortened egg-reappearance periods following macrocyclic lactone treatment. Comparable findings were reported in resistant UK cyathostomin populations (Bull et al., 2025) and in regularly treated horses across several countries (Krücken et al., 2025). The core species *Cyc. leptostomus* had high prevalence in this study, in contrast to its low prevalence in previous 'Population S' investigations (Lyons, 2003). Anthelmintic resistance to both benzimidazoles and macrocyclic lactones has been suggested for this species in North America (Bellaw et al., 2018).

Unexpectedly high prevalences were also observed for *Cor. labratus* and *Pot. imparidentatum*, both generally classified as 'low' or 'very low' worldwide (Bellaw and Nielsen, 2020) and specifically in 'Population S' (Lyons, 2003). Earlier studies of long-term untreated populations reported moderate to high preva-

lence (Chapman et al., 2003; Lyons et al., 1997), while more recent data showed high *Cor. labratus* prevalence in untreated horses and significantly higher *Pot. imparidentatum* prevalence in regularly treated horses (Krücken et al., 2025). The manual assignment of a single ASV to *Cor. labratus*, after a stalemate match between two *Cor. labratus* sequences and one *Cor. sagittatus* sequence, was a judgement call based on findings of a recent study from our group (Diekmann et al., 2025) and warrants further investigation into these two rare species. Since only a single specimen of *Cor. sagittatus* was available for reference sequencing, the reliability of species matching is limited. Future studies should try to identify and sequence additional specimens to help clarify this close genetic relationship.

Interestingly, *Str. vulgaris* was not detected in the present study, while *Str. edentatus* was found in two equines. It should be noted that read numbers were low compared to other species. Early surveys of 'Population S' reported both species (Drudge et al., 1983). Between 1983 and 2003, the herd received regular anthelmintic treatments with benzimidazoles and tetrahydropyrimidines, and consecutive investigations during this period did not detect any large strongyles (Lyons, 2003; Lyons et al., 2001, 1996, 1994). In contrast, *Str. edentatus* reappeared in 2017 as the only large strongyle in untreated controls (Scare et al., 2018) and the same was seen in post-mortem examinations conducted in February 2025 (unpublished data, personal communication by MK Nielsen). While no recent studies have specifically tested anthelmintic efficacy in large strongyles, incomplete efficacy of several drug classes has been known for decades (Drudge et al., 1984, 1974, 1963). The reappearance of *Str. edentatus* in this long-term treated, closed herd could suggest reduced anthelmintic susceptibility and warrants further investigations.

Taken together, these observations indicate possible shifts in species composition in this population, including the reappearance of *Str. edentatus*. These changes could potentially be linked to changes in anthelmintic susceptibility, although this goes beyond the scope of this investigation. The interpretation of prevalence data remains limited by the small sample size and the restriction to a single time point.

Data for five species suggested differences in tropism between the two colon sections, with higher proportional occurrence in the ventral colon. For *Cya. catinatum, Cyc. nassatus, Cor. leptostomus*, this corroborates findings of morphological studies (Lyons et al., 2011; Ogbourne, 1978, 1976). For *Cys. ashworthi* and *Cys. calicatus* OTU II this has not been previously described. However, the morphospecies *Cys. calicatus* has been associated with the ventral colon (Ogbourne, 1978, 1976). In contrast, the dorsal colon has been described as the most species diverse section, dominated by *Cys. longibursatus*, *Cyc. goldi* and *Cyc. insigne* (Ogbourne, 1976), although these differences were not significant in the present study. While constrained by sample size, these observations extend current knowledge by providing the first description of within-host tropism for five cryptic species, with possible implications towards their proposed status as individual species.

At the individual host level, faecal eggs and cultured larvae showed good coherence in detection rates as well as alpha and beta diversity. Small strongyles were consistently detected across both sample types. *Strongylus edentatus* was detected in two egg samples but not in the corresponding larval samples, likely reflecting low larval development for this species in mixed strongyle cultures (Bellaw and Nielsen, 2015; Cao et al., 2013; Scare Kenealy and Steuer, 2021). None of the alpha or beta diversity measures showed significant differences between eggs and larva, further suggesting high agreeance between the two sample types. At the time of preparing this manuscript, this is only the second study to compare diversity of equine strongyle egg and cultured larva samples based on nemabiome data and corroborates findings of Courtot et al. (2023), who concluded developmental stage did not confound species diversity in ASV-based species identification. This finding is somewhat unexpected, considering that differences in development rates are a known source of bias in ruminant gastrointestinal nematodes (Rinaldi et al., 2022). While this has not been specifically investigated, findings from the present and previous studies (Bull et al., 2025; Courtot et al., 2023) suggest that this does not appear to be a relevant factor for small strongyles. These observations are of practical relevance as they allow for some flexibility in study design concerning sample type selection, depending on the studies' specific interests or requirements.

From a practical perspective, both faecal egg extracts and cultured larvae have advantages and limitations. While larval cultures require ten days of incubation, they are technically simple, inexpensive and samples do not need to be cooled after collection (Jürgenschellert et al., 2022). However, culture conditions may bias nemabiome investigations by disadvantaging some species. In contrast, faecal egg extraction requires less time but more resources, including a centrifuge and several DNA-free sieves of different sizes, depending on the protocol (Demeler et al., 2013; Steuer et al., 2022). The repeated wash steps may bias recovery, as little is known about interspecific differences in egg size. Only one study has compared egg sizes and egg numbers between morphologically identified species (Kuzmina et al., 2012). In the present study, some eggs may have been retained on the medium sieves (85 μm). Additionally, since only gravid females produce eggs, both faecal egg samples and larval cultures may underestimate nemabiome composition at the host level. Both faecal eggs and larval cultures appear to be representative sample types, each with practical advantages and limitations that should be considered in study design.

Beta diversity analysis indicated that ventral and dorsal colon had a characteristic species composition. PERMANOVA and both Bray-Curtis and Jaccard measures showed hindgut sections clustering separately from one another as well as from eggs and larvae (Figs. 4 and 5). The sample type clustering was more distinct for ASV-based than species-based analyses, which was consistent in both dissimilarity measures. Species-based NMDS plots (Fig. 5A-B) showed some overlap between sample types, particularly regarding Jaccard dissimilarity (Fig. 5B). Overall, these findings point to both inter- and intra-species differences in 'ASV-diversity' between hindgut sections. Potential drivers such as anatomical and physiological differences between hindgut sections may play a role, but this remains speculative and requires targeted investigations.

When interpreting these results, it must be considered that, except for Jaccard dissimilarity, all diversity calculations were based on read numbers, which do not directly reflect relative abundance or luminal worm counts. PCR-based amplicon sequencing biases, such as primer mismatches, GC content and copy number variations can influence read numbers (Krehenwinkel et al., 2017; Stadhouders et al., 2010). Moreover, the COI copy number remains unknown for small strongyles and could vary according to developmental stage and species. Species differences in size (Lichtenfels et al., 2008), abundance (Collobert-Laugier et al., 2002; Nielsen et al., 2018; Ogbourne, 1976) and eggs produced (Kuzmina et al., 2012) could affect total DNA content. By extension, this is expected to have an impact on absolute sequence frequency and all subsequent analyses.

This study provides prevalence and within-host tropism data for five cryptic species, three of which were among the dominant taxa, and reports the reappearance of *Str. edentatus* in a long-term treated herd. It also applies a 650 bp COI metabarcoding marker across different biological sample types, with comparable results for faecal eggs and cultured larvae. The modest sample size, single time point, and technical limitations of metabarcoding must

be acknowledged, but the findings contribute to a more detailed picture of strongyle community composition in equines.

CRediT authorship contribution statement

Luise Grace Klass: Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Irina Diekmann:** Writing – review & editing, Investigation, Conceptualization. **Sandro Andreotti:** Writing – review & editing, Visualization, Methodology, Formal analysis, Conceptualization. **Susan Mbedi:** Writing – review & editing, Resources, Methodology, Investigation. **Sarah Sparmann:** Writing – review & editing, Resources, Methodology, Investigation. **Thore Schenk:** Writing – review & editing, Investigation. **Haley P. Anderson:** Writing – review & editing, Resources. **Jennifer Bellaw:** Writing – review & editing, Resources, Investigation. **Martin K. Nielsen:** Writing – review & editing, Writing – original draft, Supervision, Resources, Data curation. **Jürgen Krücken:** Writing – review & editing, Writing – original draft, Supervision, Formal analysis, Conceptualization. **Georg von Samson-Himmelstjerna:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2025.09.010.

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