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Nemabiome metabarcoding reveals differences between gastrointestinal nematode species infecting co-grazed sheep and goats

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Abstract

Our current understanding of differences in the epidemiology of gastrointestinal nematode (GIN) species in co-grazed sheep and goats is inadequate with reference to the development of sustainable control strategies. The next-generation metabarcoded sequencing method referred to as the ‘nemabiome’ allows some of these differences to be explored to describe the intensity of co-infecting GIN species. We applied this platform to study sheep and goats that were co-grazed on Guinea grass pasture in northeastern Brazil. Co-grazed goats and sheep were treated with a monepantel anthelmintic, then exposed to the same gastrointestinal nematode species. Overall, there were differences in the prevalence of GIN species identified in the sheep and goats; *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* predominated in goat kids, while *Haemonchus contortus* predominated in adult does, ewes and lambs once

burdens became re-established after anthelmintic treatment. Description of the pattern of re-infection following anthelmintic treatment was prevented by the unpredicted poor efficacy of 2.5 mg/kg and 5 mg/kg, respectively, of monepantel against *O. columbianum* and *T. circumcincta* in lambs, and *T. circumcincta* adult does. Differences in drug efficacy between host age and species groups may be important when considering sustainable GIN control strategies for co-grazed animals. The aggregated FECs of the adult does and goat kids representing re-established GIN burdens, were higher than those of the co-grazed adult ewes and lambs. This implies that there are inherent differences in GIN species adaptation to the two naïve small ruminant host species, and shows the need for better understanding of the factors giving rise to this situation associated with exposure to infective larvae and host responses. At the start of the study, the adult does were co-infected with several GIN species, with the highest intensity of *T. circumcincta*, contrasting with the situation in the adult ewes, in which *H. contortus* predominated. However, once burdens became re-established after treatment, *H. contortus* predominated in both adult does and ewes. This demonstrates the potential for host burdens of *H. contortus* to establish and predominate after anthelmintic treatment when burdens of co-infecting GIN species are low.

1. Introduction

Sheep and goats are the mainstay of both smallholder and larger-scale livestock agriculture throughout Asia, the middle-east, Africa and South America, where the two species are commonly co-managed on the same pastures. Despite their physical similarities, domestic sheep (*Ovis aries*) and goats (*Capra hircus*) have genetically different wild progenitors; based on mitochondrial DNA sequence analysis, sheep are more closely related to cattle, buffalo and pigs than they are to goats (MacHugh and Bradley, 2001). The physiology of the two species is underpinned by different metabolic pathways, hence their nutritional requirements and responses to infectious disease challenge vary. However, understanding of the consequences of these differences on gastrointestinal nematode (GIN) infections in co-managed groups of sheep and goats is rudimentary.

In natural environments, goats feed mainly by browsing on shrubs and trees, while sheep and other closely related domesticated ruminants feed by grazing on short herbage, such as grasses. This may result in different levels of exposure to nematode larvae, and have given rise to evolutionary differences in host responses to GIN parasites (Hoste et

al., 2016). From an evolutionary perspective, it is thought that this difference in feeding behaviour may partly explain the poorer ability of goats to develop effective protective immune responses against GINs, when compared to other domesticated ruminants (Hennessy, 1997; Gonzalez-Canga et al., 2009; Hoste et al., 2011). Because of these immunological, physiological and behavioural differences, advice on sustainable GIN control based on studies with sheep cannot be freely extrapolated to goats, and *vice versa* (Torres-Acosta et al., 2004; Lamy et al., 2008; Hoste et al., 2010), or to co-grazed animals.

GINs constitute a major constraint to the production of sheep and goats around the world, in particular in tropical and subtropical environments that are conducive to the development and survival of infectious larvae (L₃). In general, sheep and goats can be infected by the same GIN species, mainly belonging to the Trichostrongylidae family (Vieira, 2005). However, the effects of immunological, physiological and behavioural differences in co-managed sheep and goats on the abundance of co-infecting GIN species are poorly understood. The rates of emergence of anthelmintic resistance mutations differ between GIN species infecting different hosts; hence accurate characterisation of the diversity and seasonal abundance GIN species infecting co-managed sheep and goats is relevant to the development of sustainable control strategies.

The value of conventional gross parasitological and molecular methods in the study of co-infecting GIN species is limited by their poor reproducibility, accuracy and scalability. These limitations can now be overcome by combining a post-genomic deep amplicon sequencing method with faecal egg count (FEC) data to reliably quantify parasite community species diversity, referred to as the ‘nemabiome’ (Avramenko et al., 2015; 2017). The present study aimed to use this method to describe the intensity of different GIN species infecting co-managed adult and growing sheep and goats in a tropical environment in northeastern Brazil.

2. Materials and methods

All procedures were approved through the Ethics Committee for Animal Experimentation of the Federal University of Maranhão, Brazil, under number 23115.005441/2017-62.

2.1. Experimental management

Thirteen lambs <1 year-old (10 male and 3 female; body weight (BW) 23.8 ± 4.8 kg), eleven adult ewes between 3.5 and 5 years-old (BW 55.6 ± 7.7 kg), ten goats kids < 1 year-old (2 male and 8 female; BW 16.7 ± 6.7 kg) and eight adult does between 3.5 and 5 years-old (BW 47.3 ± 7.7 kg) were managed under a semi-intensive breeding system and kept on an irrigated pasture comprising of Guinea grass (*Panicum maximum* cv. Massai) in northeastern Brazil. The sheep were crossbreed Santa Inês and the goats were cross-bred Anglo-Nubian. The experimental animals were individually identified and maintained at a stocking rate of 5.2 animals per ha, along with equivalent proportions of other sheep and goats to ensure efficient grazing management and maintain GIN contamination. All animals grazed between 8 am to 6 pm, when they were housed in pens according to their experimental group and were provided with *ad libitum* access to fresh water. The diet was supplemented daily with different formulations of concentrates fed to goat kids and lambs (68% corn, 26% soybean meal, 2% wheat bran, 2% limestone and 2% others minerals) and to adult does and ewes (85% corn, 3% soybean meal, 9% wheat bran, 1% limestone and 2% others minerals). The crude protein components of the concentrates fed to the young and adult animals at a rate of 2% of body weight were estimated to be 13.2% and 8.2%, respectively. This ration was fed from three months before and throughout the experimental period

All of the animals had been grazing on the study pasture for at least three months before the start of the study period (23rd October; Day 0); when the experimental animals were weighed and orally dosed with monepantel (Zolvix; Elanco), which has no reported persistent activity against GINs. The sheep were treated at the manufacturer's recommended rate of 2.5 mg/kg, and in the absence of a data sheet recommendation, the goats were treated at the generally accepted higher rate of 5.0 mg/kg. Faecal samples were collected from each study animal before treatment on Day 0, and on Days 15, 18, 21, 24, 27, 30, 34, 37, 40, 43, 46, and 48 post-treatment. FECs were performed on each sample using a modified McMaster method (Gordon and Whitlock, 1939) with a limit of detection of 50 eggs per gram (epg). Approximately equal amounts of faeces from days 0, 15, 18, 46 and 48 were pooled into four groups (ewes, lambs, does and kids) for L₃ culture and recovery using the method described by Ueno and Gonçalves (1998). The L₃ collected were fixed in 70% ethanol and stored at 4°C until required for genomic DNA extraction.

2.2. Genomic DNA lysate preparation

Pools of approximately 2,000 L₃ from Days 0, 15, 18, 46 and 48 were centrifuged for two minutes at 7,200 x g and the supernatant was discarded. The pellet was re-suspended in 25 µL of lysis solution prepared with 22.72 µL of Direct PCR lysis reagent (Viagen Biotech, California, USA), 1.14 µL of proteinase K solution at 10 mg/mL (New England BioLabs, Massachusetts, USA) and 1.14 µL of DTT at 1M (Invitrogen, Massachusetts, USA). The samples were incubated at 60°C for two hours to lyse the larvae, followed by 85°C for 15 minutes to inactivate the proteinase K. This lysate was used as a template for subsequent PCRs.

2.3. Meta-barcoded PCR amplification and Illumina Mi-Seq run

Deep amplicon sequencing was performed to show the frequency of each nematode species in each host group. A 321bp fragment of the rDNA ITS-2 region was amplified using a universal forward primer complementary to 5.8S rDNA coding sequence and a universal reverse primer complementary to the 28S rDNA coding sequence. A second round PCR was performed by using adapter primers, where each sample was amplified with a unique nucleic acid combination barcode. Adapter/barcoded PCR amplification and magnetic bead purification were performed as previously described by Avramenko et al. (2015). 10 µl aliquots of each barcoded PCR product were combined to make a pooled library.

The purified products were pooled and visualised in a 1.4% agarose gel. Bands were excised and purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. The eluted DNA was purified using AMPure XP Magnetic Beads (1X) (Beckman Coulter, Inc.) to produce a single 20 µL pooled purified DNA library. The library was checked with the KAPA qPCR library quantification kit (KAPA Biosystems, USA), and then run on an Illumina MiSeq Sequencer using a 500-cycle pair-end reagent kit (MiSeq Reagent Kits v2, MS-103-2003) at a concentration of 15 nM with the addition of 15% Phix Control v3 (Illumina, FC-11-2003).

2.4. Illumina MiSeq data handling

The MiSeq separated all sequences by metabarcoded sample identity during post-run processing by recognised indices to generate two FASTQ files for each sample.

Analysis of the FASTQ files was performed with a bespoke pipeline using Mothur v.1.39.5 software (Schloss et al., 2009) and the Illumina Miseq standard operating procedure (Kozich et al., 2013), with modifications in the Command Prompt pipeline described by Avramenko et al. (2015). Briefly, the raw paired read-ends were run in the ‘make.contigs’ command to combine the two sets of reads from each sample. This command extracts the sequences and quality score data from the FASTQ files, creating the complement of the forward and reverse reads, which it then combines into contigs. Suspicious reads that did not match the expected length of the target region (being <200 bp, or >450 bp), were excluded from the dataset using the ‘screen.seqs’ command. The ‘summary.seqs’ command was then used to identify fragments encompassing parts of the ITS-2 rDNA. The data were aligned with the ITS-2 rDNA reference database using the ‘align.seqs’ command previously described by Avramenko et al. (2015). Sequence data that did not align with the rDNA ITS-2 consensus sequence taxonomy library were discarded as being non-specific amplification. The FASTQ file analysis was completed by classifying the sequences into GINs species by using the ‘classify.seqs’ command and creating a taxonomy file by using the ‘summary.tax’ command. Samples with <2,000 reads were discarded as being unreliable, because they could relate to errors in sample preparation. Any GIN species represented by <0.05% of the total reads for each sample was removed, because this cut-off might represent amplification from less than one worm, hence probably represented contamination or misidentification of reads.

Data were processed and presented using R Studio (R Development Core Team, 2015). Species-specific sequencing biases were corrected by multiplying the ITS-2 read proportions by correction factors that were previously validated by Avramenko et al. (2015) and Redman et al. (2019). The total number of reads for the sample and percentages for the other species were then re-calculated. The relative percentage of species composition of each sample was calculated by dividing the total number of corrected reads assigned to each species by the total number of reads per sample to obtain the relative percentage of each species (Avramenko et al., 2015).

Correction factors were applied to reduce potentially species-specific sequencing bias, by multiplying the number of raw reads per species by published factors: *Ostertagia ostertagi* and *Haemonchus placei* (Avramenko et al. 2015); other species, except *Oesophagostomum columbianum* (Redman et al., 2019). The *Oesophagostomum venulosum* correction factor (Redman et al., 2019) was applied to *O. columbianum*; and no correction factor was applied to unclassified *Trichostrongylus* spp.

2.5. Data analysis

Beta diversity estimations were calculated using ‘amova’ and ‘metastats’ commands in Mothur (Schloss et al., 2009), based on a random subsample of the sequences equal in size to the smallest sample. Bonferroni correction was used for each of these analyses, dividing the intended alpha of 0.05 by the number of pairwise comparisons in each analysis. Dendograms of diversity and frequencies of GIN species were calculated using dendroUPGMA software available at <http://genomes.urv.es/UPGMA/>. The Pearson correlation coefficient (r) with transformation $[(1 - r) * 100]$ was used to obtain similarity and clustering indices using the unweighted pair group method with arithmetic mean (UPGMA).

The efficacy of monepantel treatment in each experimental animal group was determined using the formula: $[(\text{mean of FEC Day 0} - \text{mean of FEC Day 15}) / \text{mean of FEC Day 0}] * 100$. The FECs were aggregated by group, transformed to $\text{Log}(X + 1)$ and the D’Agostino and Pearson normality test was used. Kruskal-Wallis with Dunn’s multiple comparisons tests were used to analyse the FEC differences among the groups. 95% confidence intervals (CI) were calculated using the formula in the RESO FECRT, version 2.0 (CSIRO, Australia) analytical software (Wursthorn and Martin, 1990). The level of significance used was 5% and the statistical analyses were carried out using GraphPad Prism 7.0 software (GraphPad Inc., CA, USA).

3. Results

3.1. Faecal eggs counts and treatment efficacy

The FECs of each animal on each sampling day are shown in Figure 1. For the subsequent analyses, FECs were aggregated into three different time periods: pretreatment (Day 0); six sampling dates between Day 15 and Day 30; and six sampling dates between Day 34 and Day 48 (Figure 2). These periods were chosen to represent the nematode contamination that would have been on the pasture at the start of the study; host burdens resulting from initial post-treatment re-infection from pasture L_3 ; and re-established burdens. The mean ($\pm$ SEM) FECs of the goat kids, adult does, lambs, and adult ewes on Day 0 (representing established GIN burdens arising from pasture L_3 contamination at the start of the experimental period) were $1,645 \pm 2,682$, 928 ± 994 , $900 \pm 1,464$, and $1,014 \pm 2,005$ epg, respectively. There were no significant differences

between any of the groups ($P > 0.05$). The aggregated FECs for each of the sampling dates between Day 15 and Day 30 after treatment with monepantel (representing GIN burdens arising from initial re-infection post-treatment, but confounded in the adult does and lambs by survivors of anthelmintic treatment) were 26 ± 57 , 104 ± 173 , 176 ± 633 , and 13 ± 41 epg, respectively. During this period, there were significant differences between comparable aged host species groups (goat kids vs. lambs; $P = 0.02$: adult does vs. ewes; $P < 0.001$); and between age groups of the same host species (goat kids vs. adult does; $P = 0.01$: lambs vs. adult ewes; $P < 0.001$) (Figure 2A). The aggregated FECs for each of the sampling dates between Day 34 and Day 48 after treatment with monepantel (predominantly representing re-established GIN burdens arising from pasture L_3 contamination) were $607 \pm 1,062$, 344 ± 419 , 350 ± 687 , and 135 ± 565 epg, respectively. During this period, the aggregated FECs were significantly higher in the goat kids ($P < 0.001$), adult does ($P < 0.001$) and lambs ($P = 0.01$) when compared to the adult ewes (Figure 2B), but no significant differences were found among the other groups ($P > 0.05$).

Monepantel was chosen as the anthelmintic drug in this study with an expectation of high efficacy against most GIN species (Hosking et al., 2009a) and no prior evidence of resistance in the study region. The reduction in FECs between Day 0 and aggregated Day 15 and Day 18 allowed the efficacy of 2.5 mg/kg and 5 mg/kg monepantel to be calculated in the sheep and goats, respectively. The FEC reductions were: 99% (95% CI: 96% and 100%) in the goat kids; 89% (95% CI: 59% and 97%) in the adults does; 68% (95% CI: 0 and 95%) in the lambs; and 100.0% in adult ewes. The mean pre and post treatment FECs are shown in Figure 3, and the results show drug inefficacy in the adult does and lambs.

3.2. Analysis of metabarcoded ITS-2 rDNA

The Illumina MiSeq was used to sequence metabarcoded fragments of ITS-2 rDNA from pools of about 2,000 GIN L_3 , recovered from the coprocultures from co-grazed adult ewes, lambs, adult does and goat kids before (Day 0) and on Days 15, 18, 46 and 48 after treatment with monepantel. The most abundant species identified in the sheep and goats were *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus colubriformis* and *O. columbianum*. When the GIN species were shown as proportions of the total FEC, the highest relative abundances of *T. colubriformis* were apparent in the goat kids, *T. circumcincta* in the adult does, and *H. contortus* in the sheep

(Figure 3). However, despite the differences in the proportions of GIN species between different host and age groups shown in supplementary figure S1, the diversity index in all aggregated analyses was only significantly different for *H. contortus* between goat kids and adult ewes (Table 2). On Days 15 and 18 after monepantel treatment, *T. circumcincta* predominated at low intensity (nemabiome proportion x FEC) in the goat kids, adult does and adult ewes, while *O. columbianum* predominated in the lambs (Figure 3), representing survivors of drug treatment (Table 1). On Days 46 and 48 after monepantel treatment, representing the period of reinfection from L₃ on the pasture, the proportions of *T. colubriformis* and *H. contortus* increased in the adult ewes ($P < 0.05$), while the proportions of *O. columbianum* and *T. circumcincta* decreased in the lambs and adult ewes ($P < 0.05$) (Supplementary Table S1).

The frequency and diversity of nematode species on Day 0, aggregated Days 15 and 18, and aggregated Days 46 and 48 were described using dendograms to provide a visual representation of similarities and differences between host species and ages (Supplementary Figure S1).

4. Discussion

Sheep and goats are generally co-infected by several species of Trichostrongylid nematode, each with direct life cycles alternating between free-living eggs and developing larvae and parasitic larval and adult stages. This study aimed to describe patterns of GIN species infection intensity in sheep and goats, co-grazed on a Guinea grass monoculture. This represented the commonplace scenario around the world, whereby keeping the two small ruminant species on the same pastures enables transmission of GIN infection from one host to the other (Hussain et al. 2014; Cerutti et al., 2010; Dey et al., 2019). There were three phases of the study. During the first phase (three months prior to Day 0) the pasture was grazed by sheep and goats, including the study animals, to allow for natural L₃ contamination with a representative GIN species composition; FECs and the nemabiome on Day 0 would reflect this challenge. The sheep and goats were treated with an anthelmintic drug on Day 0, so that the second phase (Day 15 to Day 30) would show the progression of intensity of re-infection with different GIN species (Gaudin et al., 2019). However, this description was not possible due to the poor efficacy of monepantel in some groups, hence FECs between Day 15 and Day 30, and the nemabiomes on Days 15 and 18 were used to describe responses to drug treatment. The third phase (Day 34 to Day 48) was to show the re-establishment and stabilisation of

naturally acquired GIN burdens. The aggregated FECs of the adult does and goat kids between Day 34 and Day 48 after treatment, representing re-established GIN burdens, were higher than those of the co-grazed adult ewes and lambs, respectively. Similar findings are commonly reported in pasture co-grazing systems (Le Jambre et al., 1976; Pomroy et al., 1986; Jallow et al., 1994; Huntley et al., 1995; Hoste et al., 2010).

ITS2 amplicons were generated from pools of L₃ harvested from faecal samples of different ages of goats and sheep and then sequenced at depth using the Illumina MiSeq platform. This approach generated a huge amount of sequence data that were used to identify and quantify the GIN species in each group. Nevertheless, misrepresentation of the proportions of different GIN species could potentially have arisen due to specific differences in: the rate and efficiency of egg hatching and larval development to the fixed L₃ stage and larval survival in the coprocultures; the efficiency of DNA extraction and amplification; copy numbers of the target genomic loci; or the generation of sequence reads within the deep amplicon sequencing platform. The molecular biology causes of species misrepresentation were addressed by the application of correction factors (Avramenko et al., 2017). There are no published correction factors for *O. columbianum*, hence the published factor for the closely related *O. venulosum* (Redman et al., 2019) was used. This was considered appropriate because the correction factors for closely related *T. colubriformis* (0.7), *Trichostrongylus vitrinus* (0.81), *Trichostrongylus axei* (1.28, and for *O. ostertagi* (1.29) and *T. circumcincta* (0.84) are within similar ranges, albeit not identical. The published correction factors were used in this study show an approximately four-fold difference in the efficiency of sequence read generation between *H. contortus* (0.63), *T. circumcincta* (0.84) and *T. colubriformis* (0.7) and *O. venulosum* (3.85) (Redman et al., 2019). This implies technical challenges in the generation of *Oesophagostomum* spp. sequence reads, hence it is possible that *O. columbianum* may have been missed when only present at low proportions of the total nemabiome. The low sensitivity of the McMaster egg counting method that was used with a detection threshold of 50 epg, could compound inaccuracies in describing the intensity of infection with different GIN species (Levecke et al., 2012).

Analysis of metabarcoded ITS-2 rDNA allowed for description of proportions of the same GIN species in different age and host species groups at different times. However, the method did not allow species compositions to be quantified, because the concentration of nematode eggs in the faeces may not have a consistent, linear relationship to the number of adult female nematodes in the gastrointestinal tract. Furthermore, differences

in intensity and distribution of GIN species infection among co-grazed animals are common due to variation in individual species biology, density-dependence and host immunity (Seyoum et al., 2018) that could not all be accounted for in the study design.

Our experimental design involved pooling equal amounts of faeces from the four sample groups (ewes, lambs, does and kids) for L₃ culture and recovery, and subsequent DNA extraction. Because of this design it is possible that the proportions of each GIN species were skewed by one or two individual samples with much higher larval recoveries than the others in each pool. Overdispersed population distributions make this scenario common, even with pools of ten or more samples (Cornell et al., 2004). If this has been the case, then our observed differences in relative species abundances between the four host species and age groups could be stochastic. One way around this problem could have been to recover larvae from individual samples, and then pool an equal number of larvae from each in the group. However, while this approach may be helpful in cattle studies, the amounts of faeces that can be recovered from small ruminants, lambs and kids in particular, is small. The coprocultures would, therefore, be susceptible to differential levels of dessication, resulting in further inaccuracies in describing the true GIN species composition.

The predominant GIN species in the goat kids at the start of the study and once burdens were re-established and stabilised after treatment was *T. colubriformis*, with similar infection intensities during both periods. The goat kids were also infected with *T. circumcincta* once burdens were re-established and stabilised. This contrasted with the situation in the lambs, where the predominant GIN species at the start of the study and once burdens were re-established and stabilised was *H. contortus*, also with similar infection intensities during both periods. The results might imply inherent differences in GIN species adaptation to different host species (Silvestre et al., 2009; Brasil et al., 2012). The variability in the intensity of host infection with different GIN species might also have arisen due to environmental (climate and management), or host factors. Sheep naturally feed by grazing, whereby they are exposed to GIN L₃ that have migrated onto the lower sward, while goats naturally feed by browsing, thereby largely avoiding L₃ ingestion. The differences in GIN species infection intensities between the goat kids and lambs grazing on Guinea grass pasture could, therefore, have arisen due to differences in host challenge associated with the height of migration of L₃ (Wang et al., 2018) of different species. Sheep have evolved strong protective immune responses against GINs (Schallig, 2000) that are slow in onset, hence dependent on age and exposure; while goats

of all ages generally have poorly developed protective immune response mechanisms against GINs (Huntley et al., 1995; Macaldowie et al., 2003; Hoste et al., 2001, 2008, 2010). GIN species-specific immune responses might also account for differences in the dynamics of infection intensities, albeit for some species both the goat kids and the lambs would have been immunologically naïve relative to the adult does and ewes. The situation could have been different for *H. contortus*, in which infections in adults are generally attributed to more short-lived host immunity.

In general host resistance and resilience are strongly influenced by the age and nutrition of animals. While nutrition does not appear to influence the initial establishment of GINs in young, non-immune animals, it can affect the expression of immunity at the later stages of growth (Kambara et al., 1993; Van-Houtert and Sykes, 1996; Hoste et al., 2016). These aspects were considered in the present study, where the young and adult animals all received supplementary concentrate fed, commensurate with the higher nutritional requirements of ruminants in a tropical climatic area (Salah et al., 2014) to ensure that maintenance nutritional requirements were met. To overcome any constraints of concentrate feeding on GIN larval intake, high levels of pasture contamination were encouraged by having more than 50 experimental animals co-grazed with others non-treated animals (Cornell et al., 2004).

At the start of the study, the adult does were co-infected with several GIN species, with the highest intensity of *T. circumcincta*. This contrasted with the situation in the adult ewes, which had similar FECs, but the predominant GIN species at the start of the study was *H. contortus*. Similar findings were reported in the Ivory Coast, where *H. contortus* was present in co-infections with other GIN species in goats, and in near-monospecific infections in sheep (Achi et al., 2003). However, *H. contortus* predominated in both adult does and ewes once burdens were re-established after treatment. These results provide further support for differences in GIN species adaptation to different hosts, grazing behaviour and immune responses; but also imply that in the absence of co-infections with other GIN species, parasitic stages of *H. contortus* can establish and predominate, regardless of host-adaptation, host species, host age and immunity. *H. contortus* populations show marked local genetic differentiation, suggesting that there are severe genetic bottlenecks, arising due to their reduced abundance during unfavourable periods and their high reproductive rate, which allows the species to exploit favourable opportunities, even after severe population reduction (Silvestre et al., 2009).

High levels of genetic differentiation have been shown between *T. circumcincta* infecting sheep and goats, suggesting the presence of sympatric parasite populations, one of which could infect both sheep and goats and the other could only infect goats (Grillo et al., 2007), and providing a possible explanation for the host species predisposition of *T. circumcincta* in the present study. Similar genetic differentiation between *H. contortus* populations infecting sheep and goats has not been demonstrated (Gharamah et al. 2012), consistent with the predominance of *H. contortus* in both adult ewes and does once burdens were re-established after anthelmintic treatment. The near-absence of *H. contortus* in the goat kids is intriguing.

H. contortus is the most widespread Trichostrongylid GIN species in the tropics (Costa et al., 2011), whereas *T. circumcincta* is present in all sheep and goat farms in temperate areas (O'Connor et al., 2006; Silvestre et al., 2009). The identification of *T. circumcincta* infecting small ruminants in a tropical setting with an all year round mean daily temperature 27°C was unexpected. *T. circumcincta* egg hatching and larval development has been reported at temperature up to 30°C (Salih and Grainger, 1982), but following rapid development L₃ survival is short. The identification of *T. circumcincta* demonstrates the power of the nemabiome sequencing method in discriminating between abomasal GIN species such as *T. circumcincta* and *T. axei*, with similar L₃ morphology (Van Wyk and Mayhew, 2013). It might also suggest parasite adaptation to tropical conditions.

Monepantel treatments were given to enable the analysis of patterns of GIN species re-infection from pasture L₃, with the *a priori* assumption that they would be effective. The aim of the study was not to evaluate monepantel efficacy; but the FEC reductions of 89% in the adult does 68% in the lambs on days 15 and 18 post-treatment presented an opportunity to describe the efficacy of this anthelmintic drug class. The treatment efficacies differed between host age and species groups: *T. circumcincta* survived treatment in the adult does, and *O. columbianum* and *T. circumcincta* survived treatment in the lambs. The reduced efficacy of several anthelmintics in goats compared with sheep is often attributed to lower absorption and rapid hepatic metabolism and clearance due to higher activities of biotransformation enzymes in goats (Sangster et al., 1991; Hennessy et al., 1997; Szotáková et al., 2004). In the present study, the dose of monepantel used in goats was twice the manufacturer's recommended sheep dose (Scott et al., 2013; Costa-Junior et al., 2015), hence the treatment survival of *T. circumcincta* could also imply monepantel resistance in this species. Similarly, very low infection

intensity of *T. circumcincta* in the lambs 15 and 18 days after monepantel treatment might imply anthelmintic resistance, albeit confirmatory statistics could not be calculated because the species was not identified in the pre-treatment samples. Monepantel resistance to *T. circumcincta* has been reported in sheep (Hamer et al., 2018) and goats (Scott et al., 2013), having arisen after few rounds of drug treatment. Monepantel had been used therapeutically on a few occasions on the study farm. High efficacy of monepantel against *O. venulosum* fourth stage larvae and adults has been demonstrated (Hosking et al., 2009a; 2009b), but there are no specific data for *O. columbianum*. Poor activity has been reported for monepantel against *Oesophagostomum* spp. in Brazil (Bustamante et al., 2009; Brito et al., 2018). The pre-patent period for *O. columbianum* is 36 to 46 days (Dash, 1973); hence the high infection intensity of *O. columbianum* in the lambs 15 and 18 days after monepantel treatment is highly suggestive of anthelmintic resistance. Failure to identify *O. columbianum* in the pre-treatment coprocultured L₃ pools might reflect aforementioned technical challenges in the generation of sequence reads when the species is only present at low proportions of the total nemabiome.

This study describes the dynamics of GIN species co-infections in goats and sheep co-grazed on a Guinea grass pasture in a tropical region of northern Brazil. The infection intensities of different GIN species suggest that goats are naturally infected with predominantly *T. colubriformis* and *T. circumcincta*, while sheep are naturally infected with *H. contortus*; and that in the absence of co-infecting GIN species, *H. contortus* can rapidly establish in both host species. Further studies are now needed to explore the adaptation of different GIN species and strains to different hosts, and the impact of host immunity, anthelmintic drug treatments and grazing behaviour on the intensity of co-infecting GIN species. Improved understanding of the population genetics of GIN species co-infections in co-grazed sheep and goats kept in different environments is needed to inform sustainable control strategies.

Conflict of interest statement

The authors have no conflicts of interest to disclose.

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Figures

Figure 1. Log FECs (FEC + 1) of groups of co-grazed goat kids (A), adult does (B), lambs (C) and adult ewes (D). The sheep were orally dosed with 2.5 mg/BW of monepantel and goats were treated with 5.0 mg/kg monepantel on Day 0.

Figure 2. Aggregate Log (FECs + 1) of groups of co-grazed adult ewes, lambs, adult does and goat kids for each of six sampling dates between Day 15 and Day 30 (A) and for each of six sampling dates between Day 34 and Day 48 (B). The sheep were orally dosed with 2.5 mg/kg monepantel and goats were treated with 5.0 mg/kg monepantel on Day 0.

Figure 3. Aggregate FECs by GIN species in groups of co-grazed adult ewes, lambs, adult does and goat kids, identified by analysis of metabarcoded ITS-2 rDNA for sampling dates from pretreatment (D0), from Day 15 and Day 18 (D15) and from Day 46 and Day 48 (D46).

The ‘others’ nematode species identified at low frequencies were: *Oesophagostomum venulosum*, *Cooperia curticei*, *Ostertagia ostertagi*, *Haemonchus placei*, *Trichostrongylus axei*, *Trichostrongylus vitrinus* and *Trichostrongylus* sp.

Supplementary Figure S1. Dendrogram based on UPGMA analysis of composition and frequencies of GIN species identified by Illumina MiSeq in co-grazed goat kids (GK), adult does (AD), lambs (La) and adult ewes (AE) on Day 0 (A), Day 15 and Day 18 aggregated (B) and Day 46 and Day 48 aggregated (C) after monepantel treatment. The dendrograms show that on Day 0, GIN infections were similar within and different between host species, independent of age. During the pre-patent period after monepantel treatment, representing low intensity of GIN species surviving drug treatment, goat kids and lambs had similar frequencies and diversity of GIN species. Thereafter, during the period representing established reinfection from L_3 on the pasture, GIN infections were again similar within and different between host species, independent of age.

Table 1. Monepantel efficacy (lower and upper 95% CI) based on pre-treatment (Day 0) and aggregated Day 15 and Day 18 post-treatment FECs.

Nematode species	Goat kids	Adult does	Lambs	Adult ewes[#]
<i>Haemonchus contortus</i>	77% (0 - 96)	69% (0 - 92)	100%	100%
<i>Teladorsagia circumcincta</i>	93% (60 - 99)	89% (59 - 97)	0*	100%
<i>Trichostrongylus colubriformis</i>	100%	n.a.	100%	100%
<i>Oesophagostomum columbianum</i>	n.a*	0 (0 – 31)	n.a*	100%
Unspeciated FEC reduction	99% (96 - 100)	89% (59 - 97)	68% (0 - 95)	100%

n.a. – Not applicable because this species was not found in pre-treatment or post-treatment samples

* - Species not found in the pre-treatment samples, but identified post treatment

- Although the FEC (using a McMaster method with a detection threshold of 50 epg) from adult ewes post treatment were zero, L₃ were cultured and identified as *H. contortus* (1.6%), *T. circumcincta* (51.8%), *O. columbianum* (21.0%), *O. venulosum* (10.2%), *Cooperia curticei* (9.3%), and others species (15.4%).

Table 2. Beta-diversity (% mean $\pm$ standard error) significance for individual GIN species from co-grazed goat kids, adult does, lambs and adult ewes on aggregated analysis from samples collected five time points (Days 0, 15, 18, 45 and 48).

Nematode species	Goat kid	Adult does	Lambs	Adult ewes
<i>Haemonchus contortus</i>	13.6 $\pm$ 8.0 ^b	31.6 $\pm$ 11.7 ^{a,b}	29.4 $\pm$ 11.6 ^{a,b}	43.8 $\pm$ 11.8 ^a
<i>Teladorsagia circumcincta</i>	36.3 $\pm$ 10.0	31.0 $\pm$ 9.7	23.9 $\pm$ 9.2	22.3 $\pm$ 8.0
<i>Trichostrongylus colubriformis</i>	24.3 $\pm$ 11.7	11.1 $\pm$ 7.5	8.8 $\pm$ 7.5	7.6 $\pm$ 4.7
<i>Oesophagostomum columbianum</i>	6.5 $\pm$ 5.2	3.6 $\pm$ 2.4	14.4 $\pm$ 8.5	8.6 $\pm$ 5.9
Other GIN species	19.3	22.7	23.5	17.7

The other gastrointestinal nematode species identified at low frequencies were: *Oesophagostomum venulosum*, *Cooperia curticei*, *Ostertagia ostertagi*, *Haemonchus placei*, *Trichostrongylus axei*, *Trichostrongylus vitrinus* and *Trichostrongylus* sp.

Different superscript letters show statistically significant differences ($p < 0.05$) among goat kids, adult does, lambs and adult ewes.

Supplementary Table S1. Beta-diversity (% mean $\pm$ standard error) significance for individual GIN species from co-grazed goat kids, adult does, lambs and adult ewes on aggregated Days 15 and 18 and aggregated Days 46 and 48 post monepantel treatment.

Nematodes Species	Days 15 - 18				
	Goat kid	Adult does	Lambs	Adult ewes	Goat kid
<i>Haemonchus contortus</i>	18.2 $\pm$ 15.6	17.4 $\pm$ 16.1	17.0 $\pm$ 16.3	7.7 $\pm$ 6.0 ^B	12.2 $\pm$ 10.7 ^b
<i>Teladorsagia circumcincta</i>	40.6 $\pm$ 13.9	42.3 $\pm$ 13.6	25.9 $\pm$ 12.5	43.2 $\pm$ 12.2 ^A	38.0 $\pm$ 16.7 ^a
<i>Trichostrongylus colubriformis</i>	1.7 $\pm$ 1.2	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	34.7 $\pm$ 20.2
<i>Oesophagostomum columbianum</i>	14.0 $\pm$ 10.9	7.6 $\pm$ 5.0	30.3 $\pm$ 16.8 ^A	18.6 $\pm$ 12.0	0.0 $\pm$ 0.0

Different superscript capital letters show statistically significant differences ($p < 0.05$) between the two time periods. Different superscript lower case letters show statistically significant differences ($p < 0.05$) among goat kids, adult does, lambs and adult ewes.