International Journal for Parasitology 47 (2017) 893–902

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

The use of nemabiome metabarcoding to explore gastro-intestinal nematode species diversity and anthelmintic treatment effectiveness in beef calves

Russell W. Avramenko [a], Elizabeth M. Redman [a], Roy Lewis [b], Murilo A. Bichuette [c], Bruna M. Palmeira [a], Thomas A. Yazwinski [d], John S. Gilleard [a,*]

[a] Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada
[b] Merck Animal Health, Calgary, Alberta, Canada
[c] São Paulo State University, Jaboticabal, São Paulo, Brazil
[d] Department of Animal Science, University of Arkansas, Fayetteville, AR, United States

ARTICLE INFO

Article history:
Received 28 March 2017
Received in revised form 31 May 2017
Accepted 1 June 2017
Available online 7 August 2017

Keywords:
Nemabiome
Metabarcoding
Ivermectin
Ostertagia
Cooperia
Anthelmintic resistance
Beef cattle

ABSTRACT

Next-generation deep amplicon sequencing, or metabarcoding, has revolutionized the study of microbial communities in humans, animals and the environment. However, such approaches have yet to be applied to parasitic helminth communities. We recently described the first example of such a method – nemabiome sequencing – based on deep-amplicon sequencing of internal transcribed spacer 2 (ITS-2) rDNA, and validated its ability to quantitatively assess the species composition of cattle gastro-intestinal nematode (GIN) communities. Here, we present the first application of this approach to explore GIN species diversity and the impact of anthelmintic drug treatments. First, we investigated GIN species diversity in cow-calf beef cattle herds in several different regions, using coproculture derived L3s. A screen of 50 Canadian beef herds revealed parasite species diversity to be low overall. The majority of parasite communities were comprised of just two species; *Ostertagia ostertagi* and *Cooperia oncophora*. *Cooperia punctata* was present at much lower levels overall, but nevertheless comprised a substantive part of the parasite community of several herds in eastern Canada. In contrast, nemabiome sequencing revealed higher GIN species diversity in beef calves sampled from central/south-eastern USA and Sao Paulo State, Brazil. In these regions *C. punctata* predominated in most herds with *Haemonchus placei* predominating in a few cases. *Ostertagia ostertagi* and *C. oncophora* were relatively minor species in these regions in contrast to the Canadian herds. We also examined the impact of routine macrocyclic lactone pour-on treatments on GIN communities in the Canadian beef herds. Low treatment effectiveness was observed in many cases, and nemabiome sequencing revealed an overall increase in the proportion of *Cooperia* spp. relative to *O. ostertagi* post-treatment. This work demonstrates the power of nemabiome metabarcoding to provide a detailed picture of GIN parasite community structure in large sample sets and illustrates its potential use in research, diagnostics and surveillance.
© 2017 The Authors. Published by Elsevier Ltd on behalf of Australian Society for Parasitology. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

There is a need to develop better tools with which to qualitatively and quantitatively assess the species composition of gastro-intestinal nematode (GIN) communities in both humans and animals. Approaches utilizing next-generation sequencing technologies, similar to those used to study the microbiome, hold massive potential in this regard but have yet to be seriously explored for helminths (Avramenko et al., 2015). GIN infections of livestock are an excellent system in which to explore such approaches because complex co-infections are common and cause both clinical disease and sub-clinical production loss worldwide (Stromberg and Gasbarre, 2006; Grisi et al., 2014). Control is largely reliant on the routine application of anthelmintic drugs, the high effectiveness of which has allowed farmers to maximize stocking rates and increase production returns for many years. However, these parasites are still often poorly controlled (Gasbarre et al., 2015). Results of a recent survey of the National Animal Health Monitoring System (NAHMS) in the United States (US), suggest that GINs are as preva-

* Corresponding author at: HSC 2531, 3330 Hospital Drive NW, Calgary, AB T2N 4N1, Canada. Fax: +1 403 210 6693.
E-mail addresses: rwavrame@ucalgary.ca (R.W. Avramenko), jsgillea@ucalgary.ca (J.S. Gilleard).

http://dx.doi.org/10.1016/j.ijpara.2017.06.006
0020-7519/© 2017 The Authors. Published by Elsevier Ltd on behalf of Australian Society for Parasitology.
This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

lent in cattle today as they were before the wide-scale introduction of broad-spectrum anthelmintic use over 30 years ago (Stromberg et al., 2015). Furthermore, cattle parasites resistant to the macrocyclic lactone drugs are now emerging, making the reliance on chemical control increasingly unsustainable (Gasbarre et al., 2009; Kaplan and Vidyashankar, 2012).

There are many different GIN species that infect cattle, and many of these differ in their pathogenicity, production impact, epidemiology and drug sensitivity (Taylor et al., 2015). However, there is remarkably little contemporary data published on the prevalence and infection intensities of different parasite species in different geographical regions and production systems. Most information for North America is based on a small number of regional studies, many of which are over 40 years old, or on anecdotal information from veterinarians, parasitologists and diagnostic laboratories (Hitchcock, 1956; Zimmerman and Hubbard, 1961; Ciordia, 1975; Stromberg et al., 2015). Additionally, there is a need for more information regarding which parasite species are developing resistance to each drug class and how drug treatments are impacting parasite species diversity and community composition.

One of the main barriers to obtaining large-scale quantitative data on parasite species prevalence, infection intensities and the impact of anthelmintic use is the lack of appropriate tools. Although conventional PCR assays are increasingly used to detect cattle parasites, these are not quantitative and only indicate the presence or absence of different parasite species in a sample (Zarlenga et al., 2001). The more commonly available PCR assays also only allow discrimination to the genus level (Stromberg et al., 2015). A real time PCR assay has been developed for *Ostertagia ostertagi* and *Cooperia oncophora* but these tests require significant optimization between laboratories and only detect two of the many species commonly present in cattle parasite communities in different regions (Roeber et al., 2012; Höglund et al., 2013).

We have recently developed and validated a new approach, nemabiome sequencing or metabarcoding, which accurately quantifies the relative proportions of parasitic nematode larvae isolated from cattle feces (Avramenko et al., 2015). It is based on deep amplicon next generation sequencing of the internal transcribed spacer 2 (ITS-2) rDNA locus and is equivalent to 16S rDNA sequencing of bacterial communities used in microbiome studies (Avramenko et al., 2015). In this paper, we apply nemabiome metabarcoding to investigate the species composition of parasite communities, and how those are impacted by macrocyclic lactone treatments, in calves from 50 Canadian cow-calf beef herds. We also compare the species composition of parasitic nematode communities in Canadian beef cattle with those from the mid/southern US, as well as Sao Paulo State, Brazil. This work illustrates how nemabiome metabarcoding provides a powerful new approach to obtain an unprecedented amount of quantitative information on parasite community composition with many applications, not only in cattle, but in a variety of different host-parasite systems.

2. Materials and methods

2.1. Collection of fecal samples

2.1.1. Canada

Samples were collected from 50 beef herds from across Canada from June through December of 2012; British Columbia (BC) (1), Alberta (23), Saskatchewan (7), Manitoba (2), Ontario (16) and Quebec (1). 20 individual animal fecal samples were collected, either per rectum or as freshly voided on pasture, from groups of calves from 50 different herds either at pasture or at feedlot entry. An additional 20 fecal samples were collected from the same group (but not necessarily from the same animals) 14 days after anthelmintic treatments were applied by the producer using their own protocols. Fecal samples were collected under an approved Animal Use Protocol (Animal Care Committee, Study #AC13-0157, University of Calgary, Canada), which is in accordance with the principles outlined in the Guidelines of the Canadian Council on Animal Care. Fecal samples were placed in plastic bags, which following exclusion of air were sealed and shipped with cool packs. Fecal egg counts (FECs) were performed using the Modified Wisconsin Technique (Ito, 1980). Three grams of feces were used for each FEC with all eggs being counted providing a detection threshold of 0.33 eggs per gram of feces (epg). Coprocultures were set up as described in Roberts and O'Sullivan (1950), however vermiculite was used in place of sawdust, and incubated at room temperature (~20 °C) for 21 days. Larvae harvested from each individual fecal sample were pooled by herd (keeping pre-treatment and post-treatment samples separate). Larvae were fixed in 70% ethanol and stored at −80 °C in aliquots of ~1000–2000 larvae where possible. In the case of post-treatment fecal samples larvae were obtained from only 42 out of 50 herds assessed and, in some cases, fewer than 1000 larvae were obtained (range 100–1000).

2.1.2. US

Fecal samples from the US were collected from 38 individual stocker cattle entering feedlot operations throughout 2014 in Oklahoma (22), Arkansas (3) and Nebraska (13). Each calf was from a different stocker cattle delivery from farms across the mid/southern US, and thus likely to be derived from different farms. Fecal samples were collected per rectum, stored and subsequently cultured for 14 days using the same protocol as for the Canadian fecal samples. Larvae were fixed in 70% ethanol until needed.

2.1.3. Brazil

Fecal samples were collected from 26 farms, from animals 6–9 months of age, across Sao Paulo State in Brazil during August and September, 2015. Three fecal samples from each farm were collected, set up individually for coproculture and the resulting L3s pooled together. Coprocultures were set up as previously described at room temperature (~25 °C). Larvae were collected and fixed in 70% ethanol until needed.

2.2. Calculation of drug treatment effectiveness

For the Canadian samples, mean egg counts of each group were compared pre- and post-anthelmintic treatment. Drug treatment effectiveness was calculated using the eggCounts R Statistical package, using the web interface with default parameters (Torgerson et al., 2014). The percentage reduction in egg counts was calculated separately for both strongyle and *Nematodirus* egg counts. This produced a FEC reduction for each farm with 95% confidence intervals (CIs). In order to assess the effectiveness of the anthelmintic treatments against each nematode species, we partitioned the strongyle FECs into separate species by multiplying the total egg count by the fraction of each species in the sample as determined by the nemabiome sequencing data from the L3 cultures. A similar approach has been used by others using species proportions determined by visual morphological analysis of L3s from fecal cultures (Waghorn et al., 2006; McMahon et al., 2013). Drug treatment effectiveness was not assessed for species that had fewer than 1 epg pre-treatment. Percent effectiveness tests for each species were calculated by the following formula: (EggsPre − EggsPost)/(EggsPre) * 100. As the species analysis was completed on pooled data, calculation of S.D. and/or CIs was not possible on a per farm basis. Statistical differences between effectiveness for each species, across all farms, were determined with a repeated-measures ANOVA, with a Sidak confidence interval adjustment.

2.3. Deep amplicon sequencing of the ITS-2 rDNA region

Bulk DNA lysates were obtained from larval populations, and the ITS-2 rDNA fragment was PCR amplified and sequenced by deep amplicon sequencing as previously described and validated for the nemabiome assay (Avramenko et al., 2015). Sequencing was completed on the Illumina MiSeq platform with the 2x250 v2 Reagent Kit (Illumina Inc., San Diego, CA, USA). An average read depth of ~45,000 was obtained for each sample. Samples were removed if they did not have at least 2000 reads, as this is indicative of poor amplification, which can diminish the accuracy of the assessment of species proportions (Avramenko et al., 2015). All samples were prepared and sequenced in duplicate from a single DNA lysate preparation.

2.4. Bioinformatic analysis

Samples were analyzed using the Mothur bioinformatic tool version 1.36.1 (Schloss et al., 2009). Reads from each sample duplicate were merged to create a single file for analysis. Raw paired-end reads were assembled to create single contigs. Reads were filtered and removed if they were <200 bp or >450 bp or if there were ambiguities between the overlapping paired-end reads. A database containing available ITS-2 sequences from all relevant nematodes was created from the public databases and our own reference samples, and can be found in Supplementary Data S1. Detailed information regarding analysis protocols can be viewed at www.nemabiome.ca. Reads were aligned to this ITS-2 database and discarded if they did not align to at least 10% of any ITS-2 amplicon with at least 90% sequence similarity. The remaining sequences were classified as corresponding to reference sequences in the database, using the k-nearest-neighbor method with k = 3. Sequences in which the three nearest matches did not map to a single species were then assessed at a higher taxonomic level, until an agreeable classification could be made. Read counts were adjusted to account for biases that might be introduced by species-specific differences in DNA content, extraction efficiency, ITS-2 amplification and/or copy number as previously described in Avramenko et al. (2015). Observed read counts were multiplied using each of the previously validated correction factors: *Nematodirus helvetianus* = 0.68871028, *C. oncophora* = 1.183576925, *Trichostrongylus axei* = 1.296071418, *Trichostrongylus colubriformis* = 0.584257283, *Cooperia punctata* = 2.779407405, *O. ostertagi* = 1.294841767 and *Haemonchus placei* = 0.919208106 (Avramenko et al., 2015). The percentage species composition of samples was calculated by dividing the total reads assigned to each species by the total number of reads per sample to obtain the relative percentage of each species. All species with prevalence below 0.05% were removed, as this relates to fewer than one worm per sample, thus counts this low are likely to be due to contamination or misidentification of reads as previously described (Avramenko et al., 2015).

2.5. Statistical analysis

The alpha diversity of each assessed sample was calculated through the inverse Simpson index (Simpson, 1949). The calculations were performed in Mothur v.1.36.1 using the built-in inverse Simpson calculation. All samples were subsampled to 2000 reads to ensure an equal comparison during diversity estimations. Samples that did not meet this threshold were removed from analysis. Each sample was individually assessed, taking into account the evenness and richness of the species distribution. To assess whether the inverse Simpson index differed significantly between populations, a one-way ANOVA, assuming non-equal variances and using a Games-Howell post-hoc test, was performed using SPSS Statistics (IBM Corp. Released 2012. IBM SPSS Statistics for Macintosh, Version 21.0. Armonk, NY: IBM Corp).

Beta diversity calculations between different groups and populations were calculated using the weighted Bray-Curtis dissimilarity index (Bray and Curtis, 1957). Calculations were performed in Mothur v.1.36.1, using the weighted-UniFrac function (Lozupone and Knight, 2005). This calculation is used to assess the level of dissimilarity between the compositions of two groups, and is used to answer the question whether two communities differ in their composition. A score of 0 means they are identical, while a score of 1 indicates they are completely different (i.e. no species are shared).

Beta diversity estimations between populations on a species and genus basis were accomplished with the MetaStats plugin in Mothur v. 1.36.1, using 1000 permutations and default parameters (White et al., 2009). This method highlights which features (i.e. species) differ in abundance between two different populations or groups. This method assesses how two communities differ in their species composition. The underlying assumption made by MetaStats is that the data is not normally distributed, and thus uses a modified non-parametric *t*-test to assign a *P*-value of significance (White et al., 2009). As this can overestimate the significance of lowly abundant features, we refrain from claiming significance if the species are present at less than 2% on average in one of the comparable groups. $P < 0.05$ was considered significant for all statistical tests implemented.

The bar charts, dot plots and boxplots displayed in figures were created in R Studio version 0.99.489.

3. Results

3.1. Species composition of GIN communities in Canadian beef cattle

We performed FECs and nemabiome sequencing of L3s cultured from feces to assess the species composition of GIN communities in 50 beef herds across Canada: British Columbia (1), Alberta (23), Saskatchewan (7), Manitoba (2), Ontario (16) and Quebec (1) (Fig. 1A). The mean strongyle FECs ranged from 0.5 to 54 epg with an overall mean of 12.7 epg. *Nematodirus* eggs were detected in 33/50 herds ranging from 0.02 to 26.8 epg with an overall mean of 2.8 epg in positive herds. *Trichuris* eggs were detected in 10/50 herds, ranging from 0.02 to 0.12 epg, with an overall mean of 0.045 epg in positive herds.

The two most prevalent species of the parasite community overall were *O. ostertagi* and *C. oncophora* with these two species making up the majority of parasite communities in all herds (59.1% and 37.6%, respectively) (Fig. 1A). The next most prevalent species was *C. punctata*, which was detected in some, but not all, herds and showed a marked regional distribution, only being present at high levels in some Ontario herds. *Haemonchus* spp., *Oesophagostomum* spp. and *Trichostrongylus* spp. were also present on several farms but comprised a low percentage of the community in all cases and thus did not contribute significantly to the observed species diversity (Fig. 1A). The samples were grouped into prairie provinces (BC, Alberta, Saskatchewan and Manitoba) and eastern provinces (Ontario and Quebec) to assess geographical differences in parasite community composition. The average inverse Simpson diversity index for the prairie provinces was 1.74, while the average diversity for the eastern provinces was 2.00, which was significantly different ($P = 0.004$, one-way ANOVA). *Cooperia* spp. were significantly differently represented in the eastern provinces compared with the prairie provinces ($P < 0.001$) when assessed with MetaStats (White et al., 2009).

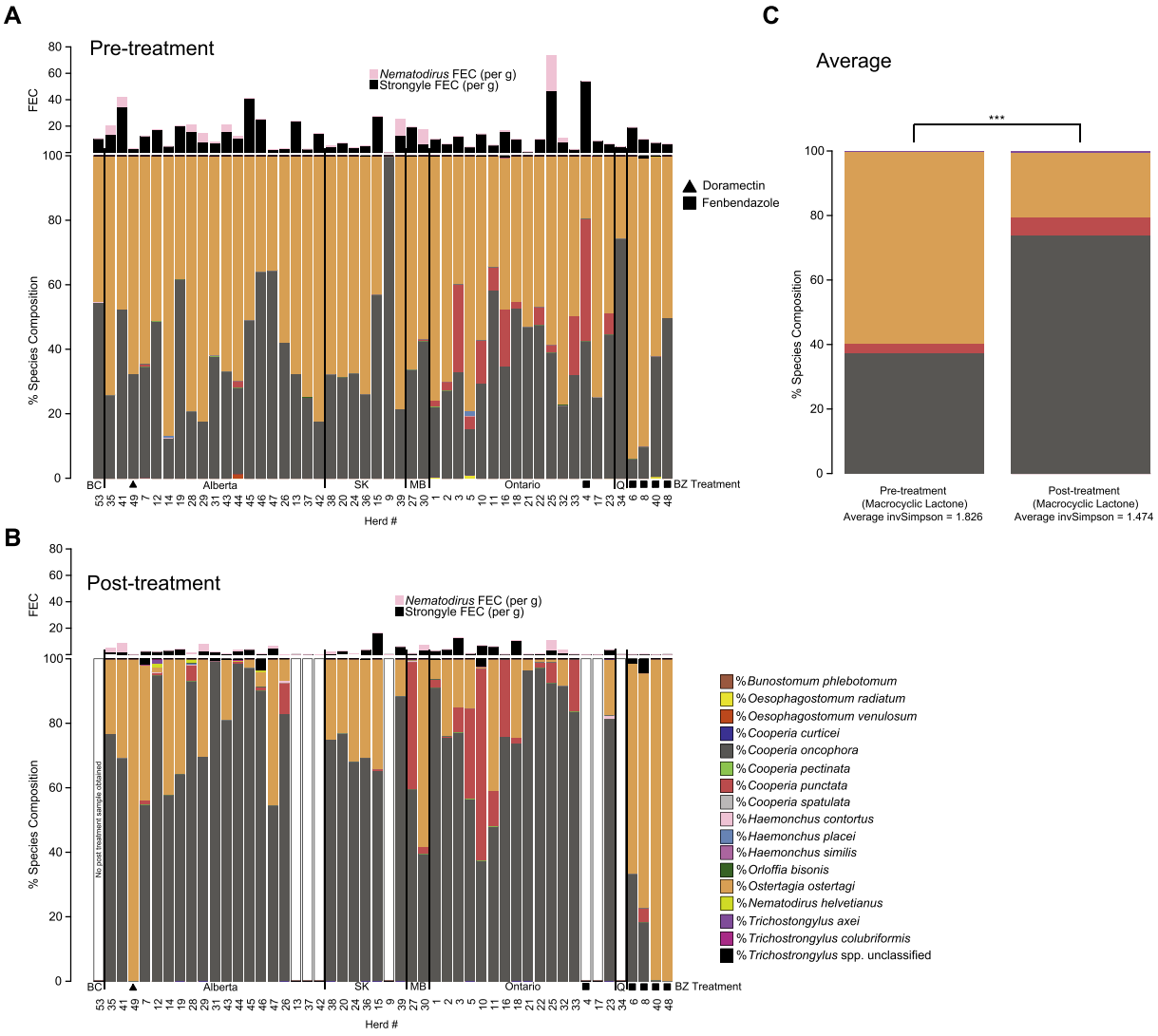

Fig. 1. Infection intensities and species composition of gastro-intestinal parasitic nematode communities in calves from Canadian beef cattle herds before and after anthelmintic treatment. (A) The upper portion of the chart shows the mean fecal egg counts (FEC) of 20 calves (for both strongyle and *Nematodirus* eggs) from each of 50 beef cattle herds across Canada (y-axis units are eggs per gram of feces). The lower portion of the chart shows the relative proportion of each parasite species present in each herd as determined by nemabiome sequencing (represented by the colour indicated in the inset legend). Sequencing was performed on ~1000 L3s extracted from cultures of feces pooled from the 20 sampled animals from each herd. The province of origin (British Columbia (BC), Saskatchewan (SK), Manitoba (MB), Quebec (Q)) and the respective herd number is shown on the x-axis. (B) The upper portion of the chart shows the mean FEC from 20 calves 14 days after anthelmintic treatment from 50 the herds shown in A. The lower portion of the chart shows the proportions of the different parasite species present in each herd as determined by nemabiome sequencing of ~100–1000 L3s extracted from cultures of feces pooled from the 20 sampled animals from each herd (species represented by the colour indicated in the inset legend). Blank bars indicate the seven herds from which larvae were not obtained from post-treatment samples, due to high treatment effectiveness. The province of origin and herd number is shown on the x-axis. The square symbols indicate the four farms that used fenbendazole whereas all other farms used a pour-on macrocyclic lactone drug (further details in Supplementary Table S1). (C) Proportion of different parasite species present overall pre- and post- macrocyclic lactone treatment when based on the mean of data from A and B, respectively. The diversity as measured by the inverse Simpson index decreased significantly after treatment (from 1.826 to 1.472: $P < 0.001$; one-way ANOVA). MetaStats was used to calculate significant differences in abundance. *Cooperia oncophora* and *Ostertagia ostertagi* were significantly differently abundant after treatment ($P < 0.001$). *Cooperia punctata* was not found to be significantly differently abundant after treatment. Significance values were not calculated for fenbendazole treatment samples, as there were too few observations to draw meaningful conclusions. BZ, fenbendazole.

3.2. Effectiveness of anthelmintic treatments and their impact on GIN communities

FECs, per gram of feces, were obtained for 20 animals per herd before and after anthelmintic treatment and the mean percentage reduction in egg count was determined (results summarized in Figs. 1 and 2). Drug treatment history by farm can be viewed in Supplementary Table S1. Strongyle eggs were still detected in the feces 14 days post-treatment in 45 out 49 herds. However, L3s were only obtained from 42 out of 49 herds. The mean percentage reductions in the strongyle egg counts were 69.3% (median 85.1%, range −100 to +100%) and 98.6% (median 99.4%, range 96.0–99.7%) for macrocyclic lactone and fenbendazole treatment, respectively (Figs. 1A, 2). *Nematodirus* eggs were still detected in feces 14 days post-treatment in 23 out 49 herds. The mean percentage reduction in the *Nematodirus* egg count was 64.8% (median 84.3%, range −100 to +100%) for macrocyclic lactone treatments (Figs. 1A, 2). Only one sample had *Nematodirus* eggs before treatment with fenbendazole and this was 100% effective. There were no significant regional differences between the provinces in the percentage reduction of either strongyle or *Nematodirus* egg counts following anthelmintic treatment (data not shown).

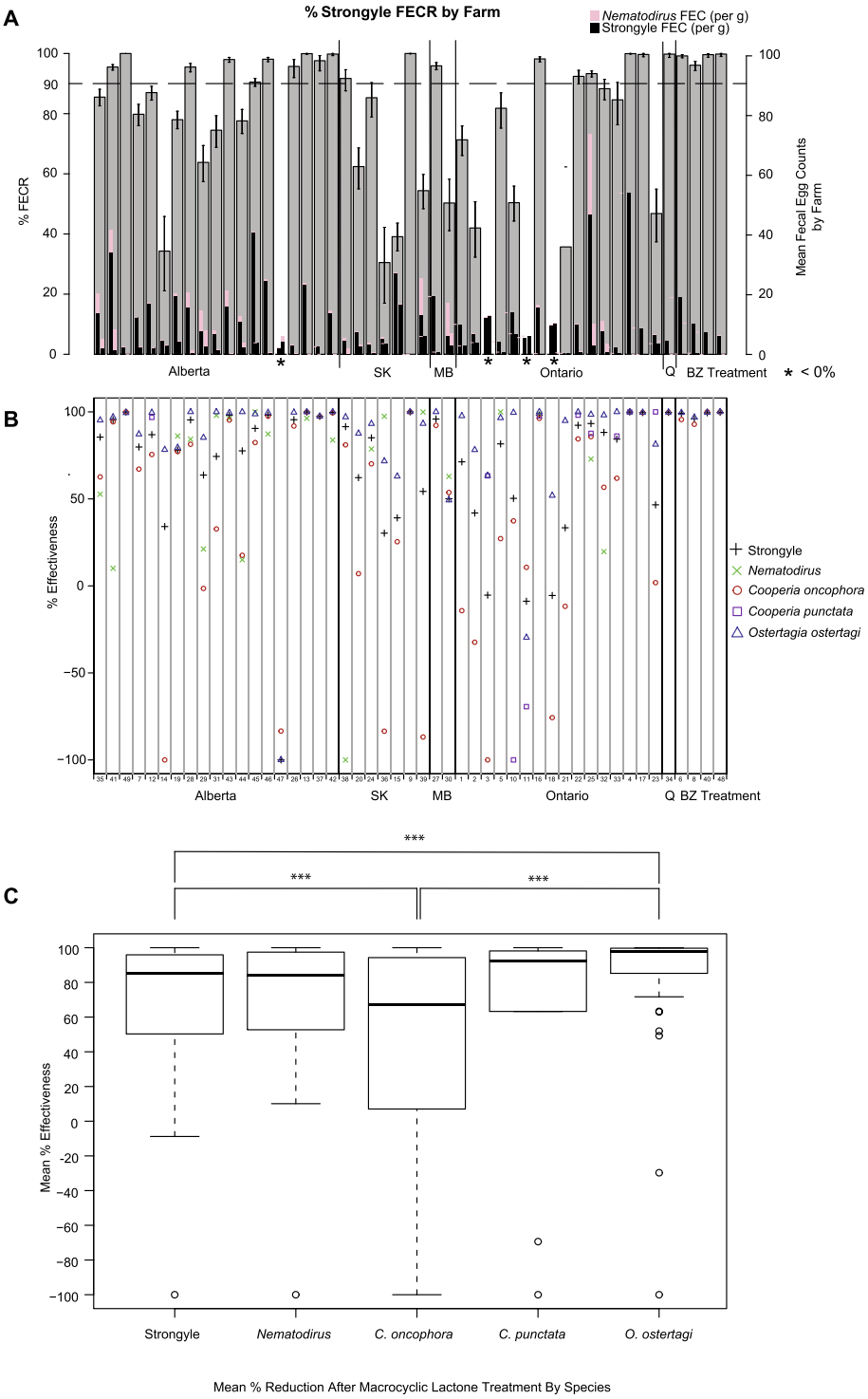

Fig. 2. Effectiveness of anthelmintic treatments on Canadian beef herds. (A) The percentage reduction in arithmetic mean strongyle egg counts from 20 calves from each of 49 beef herds following anthelmintic treatment calculated using the eggCounts R package (Torgerson et al., 2014). The gray bars indicate the percentage reduction in strongyle egg counts with 95% confidence intervals, and were calculated with the eggCounts R package (Torgerson et al., 2014). Macrocyclic lactone treatments were used in all herds except for the last four herds on the chart, which were treated with fenbendazole as indicated (BZ) (further details in Supplementary Table S1). Within each gray bar, the mean fecal egg count (FEC) before treatment (left bar) and after treatment (right bar) is shown for each herd (strongyle egg counts are shown in black, while *Nematodirus* egg counts are shown in pink). Farms with a fecal egg count reduction (FECR) below 0% (indicating an increase in egg counts) are indicated with an *. The gray dashed line indicates 90% effectiveness. (B) The effectiveness of anthelmintic treatments plotted separately for strongyle eggs, *Nematodirus* eggs, *Ostertagia ostertagi*, *Cooperia oncophora* and *Cooperia punctata*. The percentage effectiveness for the individual species was determined using the species proportions as determined by the nemabiome sequence data as described in Section 2. The herd number and province of origin (Saskatchewan (SK), Manitoba (MB), Quebec (Q)) is indicated on the X-axis. If there were fewer than the equivalent of 1 epg per species before treatment, the% effectiveness was not plotted for that species. (C) A boxplot of the overall percentage effectiveness of anthelmintic treatments for each of the major parasite species based on the pooled data from the herds treated with macrocyclic lactones. A repeated measures ANOVA was performed, with a Sidak confidence interval adjustment, to determine if the percentage effectiveness differed significantly between each species ($^{***}P < 0.001$). Both *C. oncophora* and *O. ostertagi* are significantly different from the overall strongyle percent effectiveness ($P < 0.001$), and significantly different from each other ($P < 0.001$). There were too few observations of *C. punctata* to draw statistical significance from the results.

There were significant changes in the species compositions of nematode communities following anthelmintic treatment (Fig. 1B). The average alpha diversity index, as measured by the inverse Simpson index, significantly decreased from 1.826 before to 1.474 after macrocyclic lactone treatment ($P < 0.001$) (Fig. 1C). The *Cooperia* genus as a whole ($P < 0.001$) and *C. oncophora* as a species ($P < 0.001$) significantly increased in abundance after macrocyclic lactone treatment. Although the proportion of *C. punctata* also increased following treatment, this change was not significantly different. The proportion of *O. ostertagi* reduced significantly after treatment ($P < 0.001$).

The percent reduction for each species was calculated by multiplying the total strongyle egg count by the species proportion determined by nemabiome sequencing and plotted for each farm arranged by province (Fig. 2B, C). The average percent effectiveness of macrocyclic lactone treatment for both *C. oncophora* (Mean = 41.6%; Median = 67.1%) and *O. ostertagi* (Mean = 84.4%; Median = 97.7%) differ significantly from each other and the average overall strongyle (Mean = 69.3%; Median = 85.1%) percent effectiveness ($P < 0.001$) using a repeated measures ANOVA. There were too few data points for *Nematodirus* spp. (Mean = 64.8%; Median = 84.3%) and *C. punctata* (Mean = 56.0%; Median = 92.3%) for a repeated measures ANOVA of significance, as all species must be present in the same sample to make a valid comparison. There were no significant differences in percentage reductions between any of the provinces at the species level.

3.3. Comparison of the species composition of GIN communities in beef cattle from Canada, the mid/southern US and Sao Paulo State, Brazil

We used nemabiome sequencing to determine the species composition of GIN communities in beef cattle from three US states as well as Sao Paulo State in Brazil. We assessed the parasite communities in calves derived from 38 beef herds from the mid/southern US (Arkansas, Oklahoma and Nebraska) (Fig. 3A) and from 26 herds in Sao Paulo State in Brazil (Fig. 3B). Compared with the Canadian samples, a greater number of species were present in the US and Brazilian samples (Fig. 3C). *Cooperia punctata* was the most prevalent species in both the US and Brazilian samples, with *H. placei* being the next most prevalent in both cases. Overall, the alpha species diversity (inverse-Simpson index) was significantly lower ($P < 0.001$) for Canadian nematode populations (1.830), compared with the US (2.518) and Brazilian (2.767) populations (Fig. 3D). Although there was a higher overall species diversity in the Brazilian samples than those from the US, this was not statistically significant (Fig. 3D). Beta-diversity was assessed at the population level (weighted UniFrac). The results are as follows: Canada versus US = 0.903308; Canada versus Brazil = 0.909951; US versus Brazil = 0.456751. This indicates that Canadian parasite populations shared little population structure with either the US or Brazilian populations. We can also observe that while the diversity is similar between the US and Brazil, the community structure is quite different. This indicates that different species are present in differing proportions between the two regional communities. Beta-diversity assessments were also used to identify differences in the abundance of each individual parasite species (Table 1) and genera (Table 2). Overall, *O. ostertagi* was significantly less prevalent in the US and Brazilian populations compared with Canada. In addition, *H. placei* was significantly more prevalent in the United States and Brazil compared with Canada. Overall, *C. oncophora* was significantly more abundant in Canada compared with the US ($P < 0.001$), and absent from Brazil. However, at the genus level, there were no significant differences in abundances observed for *Cooperia*.

4. Discussion

Deep amplicon next-generation sequencing approaches have revolutionized the study of complex microbial communities in humans and animals (Morgan and Huttenhower, 2012; McCann et al., 2014; Achberger et al., 2016). The use of these approaches to study the microbiome is revolutionizing biomedical research and changing our understanding of how microorganisms are involved in many physiological processes in previously unsuspected ways (Zmora et al., 2016). The basic profiling of microbial species diversity has predominantly been based on deep amplicon sequencing of the variable regions of the bacterial 16S rDNA locus (de la Cuesta-Zuluaga and Escobar, 2016). More recently, these approaches have also been used to study both microbial and invertebrate communities in environmental ecosystems where the term metabarcoding is commonly used (Taberlet et al., 2012; Yu et al., 2012; Kartzinel and Pringle, 2015). These approaches have huge potential as research and diagnostic tools for protozoan and helminth parasites of both humans and animals. PCR amplification and Sanger sequencing of different regions of the rDNA cistron have been used for many years in the molecular identification of helminth species. However, next-generation sequencing approaches have yet to be widely applied in the profiling of helminth communities. We recently reported the development a deep amplicon sequencing approach to determine the species composition of parasitic nematode larvae isolated from cattle fecal samples (Avramenko et al., 2015). In this case, we used the ITS-2 rDNA locus as the target locus since it provides the appropriate level of inter-species versus intra-species sequence variation for the most important cattle GIN species, most of which belong to the superfamily Trichostrongyloidea. L3s were selected as the stage from which to make DNA because they are a definitive developmental end point, which can ensure consistency between samples. Other sources of DNA template could potentially be used such as eggs or L1s. However, differential development of these stages could introduce additional sources of variance between samples. Any difference in shipping or storage conditions between samples could lead to differences in the developmental stages present, leading to differences in DNA content. This could result in biases in the apparent proportional representation of species based on the nemabiome sequencing assay. More work is needed to explore these issues in order to adapt the technique for use with eggs or L1s.

The nemabiome method provides a large number of advantages over previous approaches such as conventional PCR, real-time PCR and multiplex-tandem (MT)-PCR, in the identification of parasite species present in livestock fecal samples (Roeber et al., 2012, 2017; Höglund et al., 2013). First, this method requires no prior assumptions regarding which parasite species may be present in a sample. Both real-time and MT-PCR rely on species-specific primers to be designed for each species targeted, which can miss species entirely as well as create PCR biases if the primers have differing reaction efficiencies. In the case of nemabiome sequencing, a single pair of PCR primers (NC1 and NC2) are anchored in the 5.8S and 28S rDNA coding regions, flanking the ITS-2 region; these primer sites are conserved across Clade V of the strongylid nematode group (Gasser et al., 1993; Avramenko et al., 2015). Consequently, unanticipated or novel species can be detected, and the same assay can be applied in different climatic and geographical regions without methodological changes or re-optimization. Secondly, since the output is ITS-2 rDNA sequence data, the approach can distinguish between even very closely related strongylid nematode species, as all the major cattle GIN species have at least several species-specific single nucleotide polymorphisms (SNPs) at this locus (eg. *C. oncophora* versus *C. punctata* or *H. contortus* versus

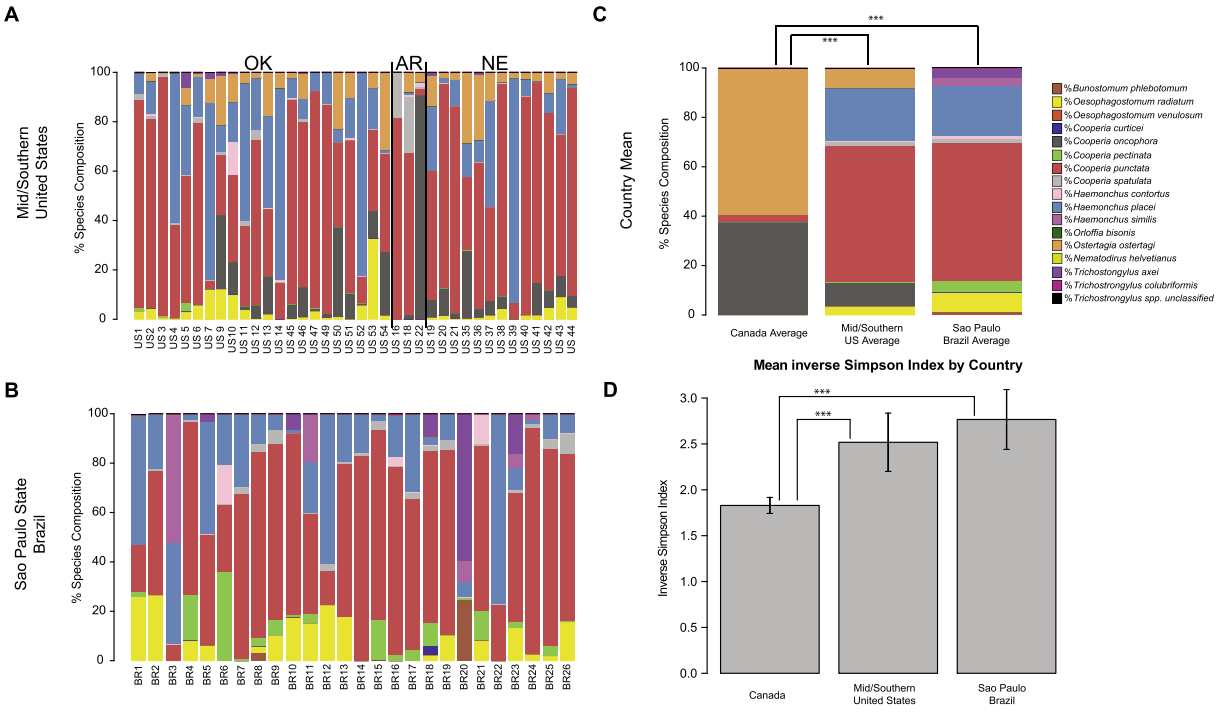

Fig. 3. Species composition of gastro-intestinal parasitic nematode communities in calves from mid/southern US and Sao Paulo state, Brazil. (A) The proportions of the different parasite species determined by nemabiome sequencing of ~1000 L3s extracted from cultured feces collected from 38 individual calves entering feedlot operations from pasture at different locations in the mid/southern US (Oklahoma (OK), Arkansas (AR) and Nebraska (NE)). Each sample was assessed twice with the sequencing assay, (from the same worm lysate) and the mean is displayed. (B) The proportions of the different parasite species determined by nemabiome sequencing of ~1000 L3s extracted from cultured feces collected from 26 herds throughout Sao Paulo State in Brazil. For each herd, fecal samples from three calves were pooled together for fecal cultures. The pooled samples were assessed twice with the sequencing assay, (from the same worm lysate) and the mean is displayed. (C) Comparison of parasite species proportions in the pooled data from all samples from Canada, US and Brazil (excluding the post-treatment samples from Canada). (D) Comparison of alpha diversity of the gastrointestinal parasitic nematode communities from Canada, US and Brazil ($^{***}P < 0.001$). The mean inverse Simpson index for all herds from each country was calculated and the result is displayed. Error bars correspond to two-times the SE for each sample. The mean diversity index is as follows: Canada (Mean = 1.830, SD = 0.308, SE = 0.044), United States (Mean = 2.518, SD = 0.979, SE = 0.159), Brazil (Mean = 2.767, SD = 0.832, SE = 0.163). A one-way ANOVA was calculated assuming non-equal variances, and using a Games-Howell post-hoc test (Canada-US: $P < 0.001$, Canada-Brazil: $P < 0.001$, US-Brazil: $P = 0.522$). We can observe that the diversity of Canadian nematode population is significantly lower when compared with either the US or Brazil. The average nematode population diversity is lower in the US than Brazil; however, this result is not statistically significant at the sample size used.

Table 1
Comparing beta-diversity (MetaStats) significance for individual parasite species in samples from Canada, US and Brazil.

Species	Mean (%) ± SE (Canada)	Mean (%) ± SE (US)	Mean (%) ± SE (Brazil)	*P* Value (Canada-US)	*P* Value (Canada-Brazil)	*P* Value (US-Brazil)
Bunostomum phlebotomum	0.00 ± 0.00	0.00 ± 0.00	1.07 ± 0.95	1.000	0.001	0.007
Oesophagostomum radiatum	0.05 ± 0.02	3.68 ± 0.95	8.08 ± 1.72	0.001	0.001	0.011
Oesophagostomum venulosum	0.03 ± 0.02	0.00 ± 0.00	0.00 ± 0.00	<0.001	<0.001	1.000
Cooperia curticei	0.00 ± 0.00	0.00 ± 0.00	0.15 ± 0.15	1.000	0.001	0.001
Cooperia oncophora	37.64 ± 2.50	9.46 ± 4.08	0.00 ± 0.00	0.001	0.001	0.001
Cooperia pectinata	0.00 ± 0.00	0.15 ± 0.10	4.70 ± 1.60	0.003	0.001	0.001
Cooperia punctata	3.05 ± 1.05	55.26 ± 4.56	55.52 ± 5.04	0.001	0.001	0.562
Cooperia spatulata	0.00 ± 0.00	1.55 ± 0.75	1.92 ± 0.40	0.001	0.001	0.752
Haemonchus contortus	0.02 ± 0.01	0.51 ± 0.35	1.25 ± 0.75	0.034	0.159	0.382
Haemonchus placei	0.05 ± 0.04	21.33 ± 4.10	20.17 ± 3.95	0.001	0.001	0.943
Haemonchus similis	0.00 ± 0.00	0.00 ± 0.00	3.43 ± 2.10	1.000	0.001	0.001
Orloffia bisonis	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.009	0.054	1.000
Ostertagia ostertagi	59.12 ± 2.68	7.65 ± 1.36	0.00 ± 0.00	0.001	0.001	0.001
Trichostrongylus axei	0.00 ± 0.00	0.39 ± 0.18	3.69 ± 2.34	0.038	0.001	0.095
Trichostrongylus colubriformis	0.00 ± 0.00	0.00 ± 0.00	0.02 ± 0.02	0.000	0.880	0.519

H. placei). As other platforms utilize species-specific primers, it can be difficult or impossible to differentiate between closely related species, and those can thus only provide identification to the genus level (Roeber et al., 2012, 2017; Höglund et al., 2013). Third, the method has been validated and optimized using mock parasite communities for each of the most important major cattle strongyle GIN species and provides accurate quantitation of relative species proportions (Avramenko et al., 2015). Fourth, the method is highly sensitive and can detect minor species in samples; the current protocol and analysis pipeline can detect species present at as little as 0.1% of the population in a sample but in principal this could be modified to further improve sensitivity if required. This is potentially more sensitive than traditional morphological identification, as 1000 larvae would need to be visually identified. Fifth, the

Table 2
Comparing beta-diversity (MetaStats) significance for the different parasite genera in samples from Canada, US and Brazil.

Genus	Mean (%) ± SE (Canada)	Mean (%) ± SE (US)	Mean (%) ± SE (Brazil)	*P* Value (Canada-US)	*P* Value (Canada-Brazil)	*P* Value (US-Brazil)
Cooperia	40.69 ± 2.70	66.43 ± 4.36	62.3 ± 5.50	0.672	0.117	0.285
Ostertagia	59.12 ± 2.68	7.65 ± 1.36	0.00 ± 0.00	0.001	0.001	0.001
Haemonchus	0.07 ± 0.04	21.8 ± 4.10	24.85 ± 4.70	0.001	0.001	0.442
Oesophagostomum	0.07 ± 0.04	3.68 ± 0.95	8.08 ± 1.72	0.001	0.001	0.030
Trichostrongylus	0.04 ± 0.02	0.40 ± 0.18	3.72 ± 2.34	0.559	0.163	0.087
Bunostomum	0.00 ± 0.00	0.00 ± 0.00	1.07 ± 0.95	1.000	0.001	0.012
Orloffia	0.01 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	0.009	0.054	1.000

method is also scalable. For the data presented in this paper up to 384 amplicons were pooled on a single Illumina MiSeq run (www.nemabiome.ca). This allows for a large number of samples to be tested in a cost effective manner. Sixth, it generates sequence data that can be further analyzed to confirm species identity, investigate potential cryptic species or undertake population genetic and molecular epidemiology studies. Although the ITS-2 rDNA locus does not generally contain sufficient intra-species variation to be used in population genetic studies other, more variable, loci could be targeted using the nemabiome deep-amplicon sequencing approach. For example, *cox*1, or other mitochondrial genes, could be used to investigate geographical sub-structuring or patterns of transmission of parasites. Genes encoding drug targets could be used for molecular epidemiological investigations of anthelmintic resistance. Markers such as these have been previously used with conventional Sanger DNA sequencing (Blouin et al., 1995; Chaudhry et al., 2015; Redman et al., 2015), but deep amplicon sequencing will potentially allow much larger scale studies to be performed with much reduced labor and cost.

The application of nemabiome sequencing to investigate the GIN parasite community structure in beef cattle presented here illustrates many of the above attributes and power of the approach. There is a paucity of contemporary information on GIN parasite prevalence in Canadian beef cattle with only two recent surveys from Alberta and Saskatchewan and a number of older studies from over 30 years ago (Polley and Bickis, 1987; Kennedy et al., 1989; Kennedy, 1990; Beck et al., 2015; Jelinski et al., 2016). This data is largely confined to FECs, and thus provides little information on the prevalence or infection intensities of individual parasite species. This has led producers and veterinarians to rely on information that is either anecdotal, often from commercial drug trials, or extrapolated from studies in the US or elsewhere. In this study, we sampled 20 beef calves leaving pasture and entering feedlots from each of 50 Canadian beef herds in 2012 and applied nemabiome ITS-2 rDNA sequencing to ~1000 larvae pooled from each herd. Although the data presented in Fig. 1A was generated from a single MiSeq run, it provides the most comprehensive view of the GIN species composition in Canadian beef cattle yet produced. In almost all herds, the two most predominant species by far were *O. ostertagi* and *C. oncophora*. This in itself is not surprising as they are known to be well adapted to more temperate climates (Smith and Grenfell, 1985; Li and Gasbarre, 2009). However, there are a number of interesting features to the data. The GIN parasite species diversity in Canadian beef cattle is strikingly low, with the parasite communities in many herds being comprised almost exclusively of these two predominant species, *O. ostertagi* and *C. oncophora*. The prevalence of *Trichostrongylus* spp. was extremely low, with the highest percentage composition in a single herd of all *Trichostrongylus* spp. combined at just 0.75% and this is consistent with a recent survey of US beef cattle (Stromberg et al., 2015). The presence of *C. punctata* as a major component of the parasite community in several herds in Ontario was unexpected as it is generally considered better adapted to warmer climates. This is a highly pathogenic parasite associated with significant production loss, so its emergence in eastern Canada is a major concern (Stromberg et al., 2012). This parasite species is also associated with macrocyclic lactone resistance and it is possible that its emergence could be a consequence of selection by long-term macrocyclic lactone use.

Ostertagia ostertagi was the most prevalent parasite species overall, comprising 59.1% of the parasite community across the 50 Canadian cow-calf herds compared with 37.6% for *C. oncophora*. This differs from the results reported in the most recent GIN prevalence survey in US cow-calf beef cattle herds by the NAHMS Survey of United States for 2007–2008 (Stromberg et al., 2015). In that study, *Cooperia* spp. were the most prevalent overall, based on a genus-specific PCR assay for larvae harvested from fecal cultures, with a prevalence of a 91% for *Cooperia* compared with 79% for *Ostertagia* (Stromberg et al., 2015). This US result fits with the suggestion that *Cooperia* spp. have displaced *O. ostertagi* as the most predominant species in US cattle as a result of the selection applied by several decades of macrocyclic lactone use (Stromberg et al., 2012). There are two possible reasons for the difference in the US survey and the results from the Canadian study presented here. It is possible that *O. ostertagi* is more predominant in Canada due to it being better adapted to cooler climates (Pandey, 1972). Alternatively, the difference may reflect the two different methodologies used. In the NAHMS survey, a simple genus-specific PCR assay was used to determine the presence of each parasite genus in each herd and did not provide any information on the relative proportions of the different species in each individual herd. In contrast the nemabiome approach used here determines the relative proportions of each species in each herd and so provides much more comprehensive information on parasite community composition. Whatever the situation in the US, the finding of a predominance of *O. ostertagi* in Canadian cow-calf beef herds is of concern as this is the most pathogenic of the cattle GIN species (Smith and Grenfell, 1985). It is also the least fecund and so makes monitoring of parasite burdens and the assessment of production loss using FEC data even more challenging for Canadian beef cattle than for regions where more fecund parasite species predominate.

The nemabiome results also revealed that the GIN species diversity in US beef calves entering feedlots from Arkansas, Nebraska and Oklahoma was much higher than that found in Canada. These mid/southern US parasite communities were dominated by *C. punctata* in most herds with *H. placei* also being a major component in some cases. It is striking how little of the parasite communities in these regions are comprised of *O. ostertagi* and *C. oncophora*, and this also illustrates the value of the information provided by nemabiome sequencing. A simple genus-specific PCR assay would be expected to detect these two parasites in most of these herds and this could lead to an overestimate of their importance in these regions. Moving further south, it is noteworthy that *O. ostertagi* and *C. oncophora* were almost completely absent from the samples collected from beef calves from the Sao Paulo State, Brazil. The striking geographical differences in GIN parasite species community structure between Canada, central/southern US and Sao Paulo State, Brazil, serves to emphasize that it is inappropriate to extrapolate information between geographical regions. For example, a FEC of 40 epg in a Canadian beef herd, where *O. ostertagi* predom-

inates, is likely to reflect a different production impact compared with a similar FEC in beef herds in regions where more fecund and less pathogenic *Cooperia* spp. predominate.

We also examined the effectiveness of macrocyclic lactone pour-on treatments applied to calves on feedlot entry, and used nemabiome sequencing to assess the impact of these treatments on GIN parasite communities in 45 of the Canadian cow-calf beef herds. The effectiveness of the anthelmintic treatments was less than 95% in most cases (69.3% overall) based on a comparison between pre-treatment and 2 week post-treatment FECs. It should be emphasized that this survey was an assessment of the effectiveness of anthelmintic treatments applied by producers, and thus poor treatment effectiveness may be due to improper drug application as well as anthelmintic resistance. Nevertheless, these results are very similar to those reported from similar studies conducted in other regions such as the US, Europe and New Zealand where anthelmintic resistance has been confirmed by subsequent detailed FEC reduction tests (Waghorn et al., 2006; Gasbarre et al., 2009; McMahon et al., 2013; Geurden et al., 2015).

The nemabiome sequencing data revealed that there was a significant increase in *Cooperia* spp. and a significant decrease in *O. ostertagi* following macrocyclic lactone treatments. When the nemabiome sequencing data was used to apportion the overall FECs to each individual species, there was a significantly lower reduction in FEC for *C. oncophora* (Mean = 41.6%, Median = 67.1%) compared with *O. ostertagi* (Mean = 84.4%, Median = 97.7%). This indicates that the observed lack of effectiveness of macrocyclic lactone treatments was predominantly due to *C. oncophora* rather than survival of *O. ostertagi*. This is consistent with previous studies from other regions since *C. oncophora* is known to be the dose limiting species for macrocyclic lactones and is the species for which macrocyclic lactone resistance has been most commonly reported to date (Gasbarre et al., 2009; Geurden et al., 2015). However, a particular concern is the increasing number of confirmed reports of macrocyclic lactone resistance in *O. ostertagi*, the most pathogenic of the cattle GIN parasite species (Edmonds et al., 2010; Waghorn et al., 2006). This would be a major problem for the beef cattle industry if it were to become widespread. Consequently, monitoring for the emergence of macrocyclic lactone- and benzimidazole-resistant *O. ostertagi* should be an important priority over the next few years. However, as can be seen from the data in Fig. 2, such monitoring is extremely challenging if surveillance is limited to FEC reduction data and lacking information on individual parasite species. The results presented here illustrate the potential role for nemabiome sequencing in monitoring the emergence of resistance in specific parasite species. There were several herds in this study where the nemabiome sequencing data revealed that the percentage reduction in *O. ostertagi* fecal counts was similar to or less than that of *C. oncophora* in the same herd (herds 11, 30 and 47 in Fig. 2B). Such herds may warrant further investigation for the presence of macrocyclic lactone-resistant *O. ostertagi*.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) (Grant number RGPIN/371529-2209), the University of Calgary, Canada, NSERC-CREATE Host Parasite Interactions (HPI) graduate training program and the Alberta Livestock and Meat Agency (ALMA), Canada (Grant numbers 2011R024R and 2014R027R). The funders provided support in the form of salaries for RWA and EMR and research materials, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. We would also like to acknowledge Brenda Handsaeme from BioCheck Veterinary Diagnostics and Technologies, Lethbridge, Alberta, Canada, for completing fecal egg counts on the samples described in this paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2017.06.006.

References

Achberger, A.M., Christner, B.C., Michaud, A.B., Priscu, J.C., Skidmore, M.L., Vick-Majors, T.J., Adkins, W., Anandakrishnan, S., Barbante, C., Barcheck, G., Beem, L., Behar, A., Beitch, M., Bolsey, R., Branecky, C., Carter, S., Christianson, K., Edwards, R., Fisher, A., Fricker, H., Foley, N., Guthrie, B., Hodson, T., Jacobel, R., Kelley, S., Mankoff, K., McBryan, E., Mikucki, J., Mitchell, A., Powell, R., Purcell, A., Sampson, D., Scherer, R., Sherve, J., Siegfried, M., Tulaczyk, S., 2016. Microbial community structure of subglacial Lake Whillans, West Antarctica. Front. Microbiol. 7, 1–13. http://dx.doi.org/10.3389/fmicb.2016.01457.

Avramenko, R.W., Redman, E.M., Lewis, R., Yazwinski, T.A., Wasmuth, J.D., Gilleard, J. S., 2015. Exploring the gastrointestinal "nemabiome": Deep amplicon sequencing to quantify the species composition of parasitic nematode communities. PLoS One 10, 1–18. http://dx.doi.org/10.1371/journal.pone.0143559.

Beck, M.A., Colwell, D.D., Goater, C.P., Kienzle, S.W., 2015. Where's the risk? Landscape epidemiology of gastrointestinal parasitism in Alberta beef cattle. Parasites Vectors 8, 434. http://dx.doi.org/10.1186/s13071-015-1040-x.

Blouin, M.S., Yowell, C.A., Courtney, C.H., Dame, J.B., 1995. Host movement and the genetic structure of populations of parasitic nematodes. Genetics 141, 1007–1014.

Bray, J.R., Curtis, J.T., 1957. An ordination of the upland forest communities of southern wisconsin. Ecol. Monogr. 27, 325–349. http://dx.doi.org/10.2307/1942268.

Chaudhry, U., Redman, E.M., Raman, M., Gilleard, J.S., 2015. Genetic evidence for the spread of a benzimidazole resistance mutation across southern India from a single origin in the parasitic nematode *Haemonchus contortus*. Int. J. Parasitol. 45, 721–728. http://dx.doi.org/10.1016/j.ijpara.2015.04.007.

Ciordia, H., 1975. Occurrence of gastrointestinal parasites in Georgia cattle. Am. J. Vet. Res. 36, 457–461.

de la Cuesta-Zuluaga, J., Escobar, J.S., 2016. Considerations for optimizing microbiome analysis using a marker gene. Front. Nutr. 3, 1–12. http://dx.doi.org/10.3389/fnut.2016.00026.

Edmonds, M.D., Johnson, E.G., Edmonds, J.D., 2010. Anthelmintic resistance of *Ostertagia ostertagi* and Cooperia *oncophora* to macrocyclic lactones in cattle from the western United States. Vet. Parasitol. 170, 224–229. http://dx.doi.org/10.1016/j.vetpar.2010.02.036.

Gasbarre, L.C., Ballweber, L.R., Stromberg, B.E., Dargatz, D.A., Rodriguez, J.M., Kopral, C.A., Zarlenga, D.S., 2015. Effectiveness of current anthelmintic treatment programs on reducing fecal egg counts in United States cow-calf operations. Can. J. Vet. Res. 79, 296–302.

Gasbarre, L.C., Smith, L.L., Lichtenfels, J.R., Pilitt, P.A., 2009. The identification of cattle nematode parasites resistant to multiple classes of anthelmintics in a commercial cattle population in the US. Vet. Parasitol. 166, 281–285. http://dx.doi.org/10.1016/j.vetpar.2009.08.018.

Gasser, R.B., Chilton, N.B., Hoste, H., Beveridge, I., 1993. Rapid sequencing of rDNA from single worms and eggs of parasitic helminths. Nucleic Acids Res. 21, 2525–2526. http://dx.doi.org/10.1093/nar/21.10.2525.

Geurden, T., Chartier, C., Fanke, J., di Regalbono, A.F., Traversa, D., von Samson-Himmelstjerna, G., Demeler, J., Vanimisetti, H.B., Bartram, D.J., Denwood, M.J., 2015. Anthelmintic resistance to ivermectin and moxidectin in gastrointestinal nematodes of cattle in Europe. Int. J. Parasitol. Drugs Drug Resist. 5, 163–171. http://dx.doi.org/10.1016/j.ijpddr.2015.08.001.

Grisi, L., Leite, R.C., de Martins, J.R., Barros, A.T.M., de Andreotti, R., Cançado, P.H.D., León, A.A.P., de Pereira, J.B., Villela, H.S., 2014. Reassessment of the potential economic impact of cattle parasites in Brazil. Rev. Bras. Parasitol. Veterinária 23, 150–156. http://dx.doi.org/10.1590/S1984-29612014042.

Hitchcock, D.J., 1956. A survey of gastrointestinal parasites of cattle of South Carolina. J. Am. Vet. Med. Assoc. 129, 34–35.

Höglund, J., Engström, A., von Samson-Himmelstjerna, G., Demeler, J., Tydén, E., 2013. Real-time PCR detection for quantification of infection levels with *Ostertagia ostertagi* and *Cooperia oncophora* in cattle faeces. Vet. Parasitol. 197, 251–257. http://dx.doi.org/10.1016/j.vetpar.2013.04.044.

Ito, S., 1980. Modified Wisconsin sugar centrifugal flotation technique for nematode eggs in bovine feces. J. Jpn. Vet. Med. Assoc. 33, 424–429.

Jelinski, M., Lanigan, E., Gilleard, J., Waldner, C., Royan, G., 2016. Survey of gastrointestinal nematode parasites in Saskatchewan beef herds. Can. Vet. J. 57, 160–163.

Kaplan, R.M., Vidyashankar, A.N., 2012. An inconvenient truth: Global worming and anthelmintic resistance. Vet. Parasitol. 186, 70–78. http://dx.doi.org/10.1016/j.vetpar.2011.11.048.

Kartzinel, T.R., Pringle, R.M., 2015. Molecular detection of invertebrate prey in vertebrate diets: Trophic ecology of Caribbean island lizards. Mol. Ecol. Resour. 15, 903–914. http://dx.doi.org/10.1111/1755-0998.12366.

Kennedy, M.J., 1990. Effect of ivermectin on weight gain of calves on pasture in Alberta. Can. Vet. J. 31, 589–590.
Kennedy, M.J., Zobell, D.R., Goonewardene, L., 1989. Effect of ivermectin on weight gains on yearling steers on pasture in central Alberta. Can. Vet. J. = La Rev. Vet. Can. 30, 346–347.
Li, R.W., Gasbarre, L.C., 2009. A temporal shift in regulatory networks and pathways in the bovine small intestine during *Cooperia oncophora* infection. Int. J. Parasitol. 39, 813–824. http://dx.doi.org/10.1016/j.ijpara.2008.11.007.
Lozupone, C., Knight, R., 2005. UniFrac : a new phylogenetic method for comparing microbial communities unifrac : a new phylogenetic method for comparing microbial communities. Appl. Environ. Microbiol. 71, 8228–8235. http://dx.doi.org/10.1128/AEM.71.12.8228.
Mccann, J.C., Wickersham, T.A., Loor, J.J., 2014. High-throughput methods redefine the rumen microbiome and its relationship with nutrition and metabolism. Bioinform. Biol. Insights. 8, 109–125. http://dx.doi.org/10.4137/BBI.S15389.
McMahon, C., Bartley, D.J., Edgar, H.W.J., Ellison, S.E., Barley, J.P., Malone, F.E., Hanna, R.E.B., Brennan, G.P., Fairweather, I., 2013. Anthelmintic resistance in Northern Ireland (I): Prevalence of resistance in ovine gastrointestinal nematodes, as determined through faecal egg count reduction testing. Vet. Parasitol. 195, 122–130. http://dx.doi.org/10.1016/j.vetpar.2013.01.006.
Morgan, X.C., Huttenhower, C., 2012. Chapter 12: human microbiome analysis. PLoS Comput. Biol. 8. http://dx.doi.org/10.1371/journal.pcbi.1002808.
Pandey, V.S., 1972. Effect of temperature on survival of the free-living stages of *Ostertagia ostertagi*. J. Parasitol. 58, 1042–1046.
Polley, L., Bickis, M.G., 1987. Gastrointestinal nematode parasites in Saskatchewan cattle: egg count distributions in beef animals. Can. J. Vet. Res. 51, 465–469.
Redman, E., Whitelaw, F., Tait, A., Burgess, C., Bartley, Y., Skuce, P.J., Jackson, F., Gilleard, J.S., 2015. The emergence of resistance to the benzimidazole anthlemintics in parasitic nematodes of livestock is characterised by multiple independent hard and soft selective sweeps. PLoS Negl. Trop. Dis. 9, 1–24. http://dx.doi.org/10.1371/journal.pntd.0003494.
Roberts, F., O'Sullivan, P., 1950. Methods for egg counts and larval cultures for strongyles infesting the gastro-intestinal tract of cattle. Aust. J. Agric. Res. 1, 99. http://dx.doi.org/10.1071/AR9500099.
Roeber, F., Hassan, E.B., Skuce, P., Morrison, A., Claerebout, E., Casaert, S., Homer, D. R., Firestone, S., Stevenson, M., Smith, L., Larsen, J., 2017. An automated, multiplex-tandem PCR platform for the diagnosis of gastrointestinal nematode infections in cattle: An Australian-European validation study. Vet. Parasitol. 239, 62–75. http://dx.doi.org/10.1016/j.vetpar.2017.04.011.
Roeber, F., Jex, A.R., Campbell, A.J.D., Nielsen, R., Anderson, G.A., Stanley, K.K., Gasser, R.B., 2012. Establishment of a robotic, high-throughput platform for the specific diagnosis of gastrointestinal nematode infections in sheep. Int. J. Parasitol. 42, 1151–1158. http://dx.doi.org/10.1016/j.ijpara.2012.10.005.
Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, Van Horn, D.J., Weber, C.F., 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75, 7537–7541. http://dx.doi.org/10.1128/AEM.01541-09.
Simpson, E.H., 1949. Measurement of diversity. Nature 163, 688.
Smith, G., Grenfell, B.T., 1985. The population biology of *Ostertagia ostertagi*. Parasitol. Today 1, 76–81. http://dx.doi.org/10.1016/0169-4758(85)90047-X.
Stromberg, B.E., Gasbarre, L.C., 2006. Gastrointestinal Nematode Control Programs with an Emphasis on Cattle. Vet. Clin. North Am. – Food Anim. Pract. 22, 543–565. http://dx.doi.org/10.1016/j.cvfa.2006.08.003.
Stromberg, B.E., Gasbarre, L.C., Ballweber, L.R., Dargatz, D.A., Rodriguez, J.M., Kopral, C.A., Zarlenga, D.S., 2015. Prevalence of internal parasites in beef cows in the United States: Results of the national animal health monitoring system's (NAHMS) beef study, 2007–2008. Can. J. Vet. Res. 79, 290–295.
Stromberg, B.E., Gasbarre, L.C., Waite, A., Bechtol, D.T., Brown, M.S., Robinson, N.A., Olson, E.J., Newcomb, H., 2012. *Cooperia punctata:* Effect on cattle productivity? Vet. Parasitol. 183, 284–291. http://dx.doi.org/10.1016/j.vetpar.2011.07.030.
Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., Willerslev, E., 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. Mol. Ecol. 21, 2045–2050. http://dx.doi.org/10.1111/j.1365-294X.2012.05470.x.
Taylor, M.A., Coop, R.L., Wall, R.L., 2015. Veterinary Parasitology. Wiley-Blackwell, United Kingdom.
Torgerson, P.R., Paul, M., Furrer, R., 2014. Evaluating faecal egg count reduction using a specifically designed package "eggCounts" in R and a user friendly web interface. Int. J. Parasitol. 44, 299–303. http://dx.doi.org/10.1016/j.ijpara.2014.01.005.
Waghorn, T., Leathwick, D., Rhodes, A., Lawrence, K., Jackson, R., Pomroy, W., West, D., Moffat, J., 2006. Prevalence of anthelmintic resistance on 62 beef cattle farms in the North Island of New Zealand. N. Z. Vet. J. 54, 278–282. http://dx.doi.org/10.1080/00480169.2006.36711.
White, J.R., Nagarajan, N., Pop, M., 2009. Statistical methods for detecting differentially abundant features in clinical metagenomic samples. PLoS Comput. Biol. 5. http://dx.doi.org/10.1371/journal.pcbi.1000352.
Yu, D.W., Ji, Y., Emerson, B.C., Wang, X., Ye, C., Yang, C., Ding, Z., 2012. Biodiversity soup: Metabarcoding of arthropods for rapid biodiversity assessment and biomonitoring. Methods Ecol. Evol. 3, 613–623. http://dx.doi.org/10.1111/j.2041-210X.2012.00198.x.
Zarlenga, D.S., Barry Chute, M., Gasbarre, L.C., Boyd, P.C., 2001. A multiplex PCR assay for differentiating economically important gastrointestinal nematodes of cattle. Vet. Parasitol. 97, 199–209.
Zimmerman, W.J., Hubbard, E.D., 1961. Gastrointestinal parasitism in Iowa cattle. J. Am. Vet. Med. Assoc. 139, 555–559.
Zmora, N., Zeevi, D., Korem, T., Segal, E., Elinav, E., 2016. Taking it personally: personalized utilization of the human microbiome in health and disease. Cell Host Microbe 19, 12–20. http://dx.doi.org/10.1016/j.chom.2015.12.016.