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Removal of adult cyathostomins alters faecal microbiota and promotes an inflammatory phenotype in horses

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ABSTRACT

The interactions between parasitic helminths and gut microbiota are considered to be an important, although as yet incompletely understood, factor in the regulation of immunity, inflammation and a range of diseases. Infection with intestinal helminths is ubiquitous in grazing horses, with cyathostomins (about 50 species of which are recorded) predominating. Consequences of infection include both chronic effects, and an acute inflammatory syndrome, acute larval cyathostominosis, which sometimes follows removal of adult helminths by administration of anthelmintic drugs. The presence of cyathostomins as a resident helminth population of the equine gut (the "helminthome") provides an opportunity to investigate the effect helminth infection, and its perturbation, has on both the immune system and bacterial microbiome of the gut, as well as to determine the specific mechanisms of pathophysiology involved in equine acute larval cyathostominosis. We studied changes in the faecal microbiota of two groups of horses following treatment with anthelmintics (fenbendazole or moxidectin). We found decreases in both alpha diversity and beta diversity of the faecal microbiota at Day 7 post-treatment, which were reversed by Day 14. These changes were accompanied by increases in inflammatory biomarkers. The general pattern of faecal microbiota detected was similar to that seen in the relatively few equine gut microbiome studies reported to date. We conclude that interplay between resident cyathostomin populations and the bacterial microbiota of the equine large intestine is important in maintaining homeostasis and that disturbance of this ecology can lead to gut dysbiosis and play a role in the aetiology of inflammatory conditions in the horse, including acute larval cyathostominosis.

1. Introduction

In the western world there has been a marked rise in immune-mediated diseases in human populations (Weinstock et al., 2002). One paradigm invoked to explain this is the hygiene hypothesis, which infers that the rise in allergic and autoimmune diseases is due to an increase in sanitation that has altered the microbiome and reduced exposure to parasitic helminths and other "old friends". There is also growing evidence to support the therapeutic potential of helminth immunoregulation (McKay, 2009).

Infection with intestinal helminths is ubiquitous in grazing horses, with cyathostomins (approximately 50 species of which are recorded) predominating, and reported prevalence of infection of 89–100% (Ogbourne, 1976; Mfitilodze and Hutchinson, 1990; Collobert-Laugier et al., 2002; Morariu et al., 2016). Cyathostomins are also recognised as the most pathogenic intestinal endoparasites in the horse (Boxell et al., 2004; Hinney et al., 2011; Morariu et al., 2016), and horses are frequently given anthelmintics directed at these parasites. The life-cycle of the cyathostomins often involves a period of encystment in the walls of the caecal and colonic mucosa. Larvae can become inhibited, allowing them to exist in the walls for up to 3 years or more (Gibson, 1953). Therefore, large burdens can accumulate, leading to an increased susceptibility to clinical disease, which can manifest both chronically in the form of ill-thrift and weight loss (Murphy et al., 1997), and acutely as a severe local and systemic inflammatory syndrome – acute larval cyathostominosis (ALC), which can be fatal (Giles et al., 1985; Peregrine et al., 2006). In addition, it has been shown that high burdens of encysted cyathostomins contribute to dysmotility of the equine colon (Bueno et al., 1979) and increase the risk of colic (Uhlinger, 1990).

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The presence of cyathostomins as a resident helminth population of the equine gut (which we term here the "helminthome") provides an opportunity to investigate the effect that helminth infection, and its perturbation, has on both the immune system and bacterial microbiome of the gut, as well as to determine the specific mechanisms of pathophysiology involved in equine ALC.

Risk factors associated with ALC include age, time of year and anthelmintic routine (Reid and Hillyers, 1995). One of the citied triggers for ALC is removal of adult cyathostomins in the gut lumen, prompting the emergence and development of encysted larvae. This can occur in late winter, potentially associated with the turnover of the previous season's adult worms. Instead of a decrease in faecal egg counts (FECs) at this time, there is often an increase, attributed to the emergence and maturation of excysted larvae (Ogbourne, 1975, 1976). Similarly, emergence can be attributed to the removal of adult worms after anthelmintic treatment (Reid and Hillyers, 1995; Love et al., 1999). However, there are horses who develop ALC without these associated risk factors (Mair, 1993; Van Loon et al., 1995) and often with herd mates unaffected (Mair, 1993; Murphy and Love, 1997). Therefore, as yet unknown factors influencing individual susceptibility are likely to be involved (Murphy and Love, 1997; Murphy et al., 1997).

Helminths have been shown to exert immunoregulatory effects on their hosts, generally involving a combination of Th2 and Treg responses (reviewed by Maizels and Yazdanbakhsh, 2003). Although there are relatively few specific studies on equine immune responses to cyathostomins, there is evidence that the usual paradigm of Th2/Treg responses applies (Davidson et al., 2005). In other hosts, including humans, helminth-mediated immunoregulation has been shown to have a bystander effect, that is, to modulate responses to other infectious agents/immunological stimuli present at the same time (Yap and Gause, 2018). Furthermore, the removal of helminth infection, and hence its immunoregulatory effect, may influence the dynamics of inflammatory responses (Furze et al., 2006; Pedersen et al., 2013; Ezenwa and Jolles, 2015; Ohnmacht et al., 2015). In the horse, the effects of anthelmintic treatment, i.e. removal of helminths, have been reported to include a significant inflammatory response (Steinbach et al., 2006; Betancourt et al., 2015), although other studies report only mild responses (Nielsen et al., 2013,2015). Therefore, the abrupt removal of adult cyathostomins could lead to a removal of this helminth-mediated "immunoregulatory brake", allowing an overzealous immune reaction to excysting larvae in some individuals and this may be a precipitating factor for ALC.

Another factor determining individual susceptibility to ALC may be the intestinal ecology of the host. There is increasing evidence that helminths may interact with the gut bacterial microbiome and ultimately affect immunological and inflammatory responses (Reynolds et al., 2014; Fricke et al., 2015; Cattadori et al., 2016). Disruption of the gut bacterial microbiota is associated with other equine intestinal tract diseases such as colitis (Costa et al., 2012). We hypothesised that dysbiosis of the equine gut microbiota could be induced by anthelmintic treatment. A recent study of the relationship between equine cyathostomins, the gut bacterial microbiome and inflammation demonstrated a trend toward increase in alpha diversity of the bacterial microbiota associated with cyathostomin infection diagnosed through high FECs (Peachey et al., 2018).

We set out to investigate if anthelmintic treatment in horses changes the faecal microbiota, and if any changes are accompanied by biomarkers of inflammation. Here, using 16S rRNA sequencing, we document changes in the faecal microbiota of two groups of horses on Days 7 and 14 post-treatment with anthelmintics, and concomitant variation in inflammatory markers.

2. Materials and methods

2.1. Animals

This was a cross-sectional prospective study performed on 31 client-owned animals recruited from two different properties (an equine rescue charity and an equestrian centre), carried out between July and September 2017.

Group 1 constituted a group of 20 mixed-breed yearling horses (14 colts and six fillies) at the rescue centre. The yearlings were kept on pasture on the property for the duration of the study, with a move to fresh grazing 1 week after the study was initiated. This group had not received larvicidal treatment, directed at immature cyathostomins, prior to the study but they had been treated with ivermectin, at 0.02 mg/kg orally, 8 weeks previously. They had not received any antibiotic or anti-inflammatory treatment in the previous 2 months.

Group 2 was a group of 11 mixed-breed horses aged from 1 to 7 years (seven geldings and four mares). All were housed at the same equestrian yard. The horses were stabled but had access to grazing daily for short periods. They had a variable anthelmintic history but had not received larvicidal treatment in the 6 months prior to the study nor antibiotic or anti-inflammatory treatment in the previous 2 months.

As this was a longitudinal study using client-owned, rather than experimental, animals, the numbers available were constrained. As we planned to examine parameters related both to clinical biochemistry and gut microbiota in individual animals before and after treatment with anthelmintics, we chose to use an anticipated change in serum fibrinogen as a clinically significant parameter for initial estimation of required group size. We estimated that a for a study with Power = 90%, a group size of $n = 7$, we could reliably measure a change in serum fibrinogen of 0.5 g/L, and that the S.D. of the difference would be no more than 0.4 g/L. Hence, S.D. units for the study would be 1.25, and required group size $n = 7$ (http://www.rcsi.ie/files/research/docs/20160811111051_Sample%20size%202016.pdf). Sample size calculations for equine 16S rRNA microbiome studies are not readily available. We judged our sample size to be within the range of that used by Tyma et al. (2019) who judged that a sample size of $n = 6$ would be sufficient to detect a 25% change in Operational Taxonomic Units (OTU) ($\alpha = 0.05$, Power 80%).

Inclusion criteria comprised a positive ELISA for encysted cyathostomins, no overt clinical signs of gastrointestinal disease, and a body condition score (BCS) of at least 2/5 (Carroll and Huntington, 1988).

2.2. Ethical considerations

This study was approved by the University College Dublin, Ireland Animal Research Ethics Committee (Approval number E-18-39). Informed consent was obtained from owners of all horses involved in the study.

2.3. Clinical examination

Clinical examinations were performed on all horses prior to commencement of treatment and again at each sampling point. A BCS was assigned to each horse and a weight tape measurement taken prior to treatment and at each sampling point.

2.4. Treatment

Groups 1 and 2 were each randomly divided into two sub-groups blocked for gender. Sub-groups 1MOX and 2MOX received

moxidectin (MOX) paste at 0.4 mg/kg orally (Equest ® Zoetis Ireland, Loughlinstown, Dublin, Ireland). Subgroups 1FBZ and 2FBZ received fenbendazole (FBZ) liquid at 10 mg/kg orally (Panacur ® Intervet Ireland Ltd. Citywest Road, Dublin, Ireland) once a day for five successive days. Day 0 was considered as the final day of treatment with FBZ and the day of treatment with MOX. Dosages were calculated using weight tape measurements.

2.5. Faecal and blood sampling

Faecal and blood samples were collected on three separate occasions between 2 and 8 days pre-treatment, and then 7 days and 14 days post-treatment (Table 1).

Naturally-voided faecal samples were obtained from each individual horse. Samples of at least four faecal balls were collected in labelled rectal gloves from which all air was evacuated. The samples were stored at 4 °C within 8 h of collection.

A 3 g sample was taken at random from the overall faecal sample and used for parasitological analysis. A 100 g sample was used for the faecal blood test (FBT) (SUCCEED, Freedom Health LLC, USA). A 3 g subsample was taken at random from this and was stored at −80 °C within 18 h of collection for subsequent microbiome analysis.

Blood samples were taken by jugular venepuncture into sodium citrate, lithium heparin and plain 10 ml vacutainer tubes. These samples were stored at 4 °C within 8 h of collection and analysed within 18 h of collection at the veterinary clinical pathology laboratory of UCD Veterinary Hospital.

2.6. Clinical pathology

Serum fibrinogen concentration was calculated by the Clauss method using a semi-automated coagulation analyser (KC4 Delta Amelung, Trinity Biotech, Ireland). Total protein and albumin levels were obtained by the Biuret method using a clinical chemistry analyser (Rx Imola™ – Randox, Northern Ireland) and globulin levels were derived using these values.

The SUCCEED FBT (Freedom Health LLC, USA) was used to estimate faecal albumin levels, according to the manufacturer's instructions.

2.7. FEc

The Modified McMaster technique (Sloss et al., 1994) (minimum detection level: 50 eggs/g faeces (epg)) was used to enumerate typical strongyle eggs in faecal samples.

Table 1
Experimental design, treatment and sampling timelines, which were identical for Group 1 and Group 2. Both groups of horses were infected with cyathostomins as judged by the presence of strongyle eggs at the start of the experimental period, and sub-groups were then treated with moxidectin or fenbendazole.

Sampling timeline			
Pre-treatment	Day 0	Day 7	Day 14
Fibrinogen	TREATMENT	Fibrinogen	Fibrinogen
Total protein		Total protein	Total protein
Globulin		Globulin	Globulin
Albumin		Albumin	Albumin
FEC		FEC	FEC
Faecal microbiome		Faecal microbiome	Faecal microbiome
FBT		FBT	FBT
ELISA			ELISA

FEC, faecal egg count; FBT, faecal blood test.

2.8. ELISA for encysted cyathostomins

Serum samples were left to coagulate at room temperature for 24 h prior to centrifugation at 1000 g/min for 10 min. An encysted cyathostomin-specific ELISA (a gift from Prof. J.B. Matthews, Moredun Research Institute, Edinburgh, Scotland) was performed as previously described (Mitchell et al., 2016).

2.9. Faecal sample preparation and 16S rRNA gene sequence analysis

Faecal samples for microbiome analysis were homogenized and processed using mechanical and chemical lysis. DNA was extracted using a QIAamp® Fast DNA Stool Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions, with some modifications. DNA concentration was normalized and 16S metagenomic libraries were prepared using primers to amplify the V3-V4 region of the bacterial 16S rRNA gene, with Illumina adaptors incorporated as described in the Illumina 16 s Metagenomic Library Preparation guide. Following index PCR and purification, the products were quantified using the Qubit high sensitivity DNA kit (Life Technologies) and pooled equimolarly. The pooled libraries were assessed using an Agilent high sensitivity DNA kit and examined by quantitative PCR (qPCR) using the Kapa Quantification kit for Illumina (Kapa Biosystems, USA) according to the manufacturer's guidelines. Libraries were then diluted and denatured following Illumina guidelines and sequenced (2 × 300 bp) on the Illumina MiSeq platform.

The sequences obtained were filtered on the basis of quality (removal of low-quality nucleotides at the 3′ end) and length (removal of sequences with less than 200 bp with prinseq (Schmieder and Edwards, 2011), and the paired-end reads with a minimum overlap of 20 bp were joined using Fastq-join (Aronesty, 2013). The sequences were cleaned of replicates, and unique sequences and chimeras were checked against the GOLD database (https://gold.jgi.doe.gov) using the closed reference Usearch v7.0 algorithm (Edgar, 2010). The resulting sequences were matched at operational taxonomic unit level (OTU; with 97% identity level). The taxonomic assignment of these OTUs was matched to results in the Ribosomal Database Project (Cole et al., 2011). Alpha and Beta diversities were determined using QIIME (Caporaso et al., 2010), and additional analyses were performed with the R package phyloseq (McMurdie and Holmes, 2013).

2.10. Statistical analysis

Concentrations of serum fibrinogen, as well as the Albumin: Globulin ratio were compared within Groups 1 and 2 over the three sampling periods using paired t-tests. Data which did not follow a normal distribution (FECs) was assessed using Kruskal-Wallis tests, and categorical variables (positive or negative for faecal albumin) by Chi-square analysis. With the exception of bacterial microbiota measurements, SPSS was used throughout for statistical analysis and visualisation of data.

Downstream analyses were performed and graphical outputs of 16S rRNA gene high throughput sequencing data were generated with various packages in R (R Development Core Team 2016). For beta diversity, the data were statistically analyzed by the Adonis function from the R Vegan package (http://www.worldagroforestry.org/publication/vegan-community-ecology-package-r-package-vegan-vers-22-1).

ANOVA and t-tests were used to calculate differences in the analysis of the alpha diversity index. Statistical differences between multiple samples at Phylum, Family and Genera levels were determined by Kruskal-Wallis or Mann–Whitney U-tests, adjusting for multiple testing according to the method of Benjamini and Hochberg (1995) with the R statistical package

(https://www.r-project.org/). Statistical significance was established at $P < 0.05$.

3. Results

3.1. Sampling

In Group 1, pre-treatment faecal samples were not obtained from three horses due to a lack of availability of voided faeces. On day 14, faecal samples were not obtained from six horses, and blood samples were not collected from four horses due to difficulty in obtaining a jugular blood sample for sampling.

3.2. Clinical parameters

There was no significant change in body weight or BCS in either group over the experimental period. No horse showed overt clinical signs of ALC or any other gastrointestinal related disease.

3.3. Parasitological measurements

In Group 1, the mean strongyle FEC prior to treatment was 419 epg (range 0–1200 epg), and post-treatment the FECs were reduced by 98.66% following MOX treatment and by 94.12 % following FBZ treatment. In Group 2, the mean FEC was 755 epg (range 0–2050 epg), and post- treatment FECs were reduced by 98.8% for both MOX and FBZ sub-groups (Fig. 1A, B).

All animals in both groups were positive by ELISA for encysted cyathostomins at levels ranging from 25-141 percent positivity both prior to treatment and at Day 14 post-treatment, without any consistent change between the two timepoints (data not shown).

3.4. Clinical pathology

Concentrations of serum fibrinogen remained elevated above the reference range in Group 1 throughout the experimental period (Fig. 1C). In Group 2, serum fibrinogen significantly increased on Day 7 post-treatment (reference range 0–2 g/L), ($P < 0.005$, MOX, and $P < 0.05$, FBZ), and then decreased to concentrations comparable with those at Day 0 by Day 14 (Fig. 1D).

Serum Albumin:Globulin (A:G) ratios increased in both Group 1 and Group 2 over the sampling period (Fig. 1E, F), for both FBZ- and MOX-treated groups.

The proportion of animals in Group 1 (Fig. 1E) testing positive for faecal albumin increased between Day 0 and Day 7 ($P = 0.013$, Chi-Square), with FBZ and MOX animals considered together (Fig. 1G). Overall 62% of horses in this Group were positive for faecal albumin (an indicator of hindgut inflammation) on Day 7 (Fig. 1G) and numbers remained elevated. A comparable proportion of animals in Group 2 were positive for faecal albumin prior to treatment, and this number had declined, although not significantly, by Day 14 post-treatment (Fig. 1F).

3.5. Microbiota analyses

After quality filtration and length trimming, an average of 108,178.1 (±23,658.46 S.D.) high quality 16S rRNA sequences were generated per sample. A total of 2201 (±274.84 S.D.) OTUs were detected.

Estimates of species richness and diversity changed significantly over time. In Group 1, alpha diversity was significantly decreased for both sub-groups on Day 7 compared with both pre-treatment and DAY 14 ($P < 0.005$, two-way ANOVA) as assessed using the Simpson and Shannon index (Fig. 2A). In Group 2 (Fig. 2B) there was a significant decrease in diversity and richness, again in both sub-groups, on Day 7 ($P < 0.005$, two-way ANOVA) in both the Shannon and Simpson index, with the latter also showing a significant increase in alpha diversity on Day 14 relative to Day 7 (Fig. 2B). There were no significant differences in alpha diversity between MOX or FBZ sub- groups at any point.

Principal co-ordinate analysis (PCoA) showed that, regardless of anthelmintic used, there was a decrease in beta diversity in both groups at Day 7 post-treatment compared with both Day 0 and Day 14 ($P = 0.001$) (Fig. 3A-C ($P = 0.001$). This analysis also demonstrated the clear separation of the faecal microbiota populations between Groups 1 and 2.

The major Phyla detected were similar in all sub-groups (Fig. 4A) and in keeping with the prior literature relating to the equine faecal microbiota (Shepherd et al., 2012; Dougal et al., 2013; O'Donnell et al., 2013; Moreau et al., 2014; Costa et al., 2015; Peachey et al., 2018). The Phyla Firmicutes and Bacteroidetes, together with Proteobacteria, were the most abundant, followed by Spirochaetes and Fibrobacteres. At Day 7, in both Groups regardless of treatment sub-group, there were significant changes seen in relative abundance at Phylum, Family and Genus level. Specifically, at phylum level overall there was an increase in Proteobacteria and a decrease in Bacteroidetes. The increase in Proteobacteria was seen in Group 1 at Day 7 post-treatment relative to the other time-points, regardless of treatment used 1 (FBZ1: pre-treatment $P < 0.05$, Day 14$P < 0.01$; MOX1: pre-treatment $P < 0.01$, day 14$P < 0.01$). In group 2 a similar significant increase was seen with FBZ (FBZ2: pre-treatment $P < 0.05$, Day 14$P < 0.05$) but in the sub-group MOX2 the increase reached significance only when compared with Day 14 ($P < 0.05$). A significant decrease in Bacteroidetes on Day 7 post-treatment with MOX was seen in both Groups (MOX1: $P < 0.01$; MOX2: $P < 0.05$), although for FBZ this was only significant in sub-group FBZ2 ($P < 0.05$).

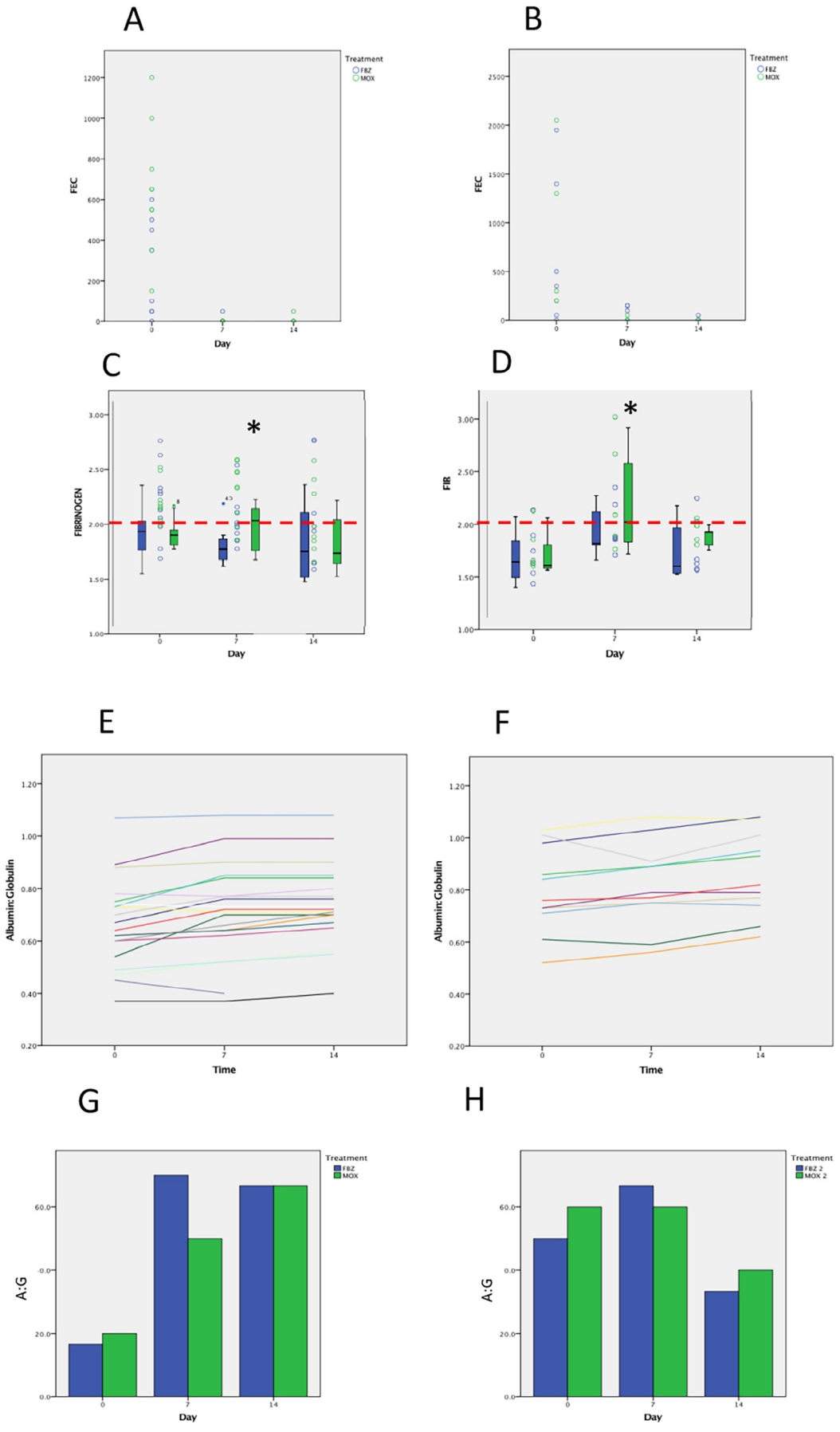
There were a number of changes seen at Day 7 in family groups (Fig. 4B) and genera (Fig. 4C). Most notably, there was a significant increase in the genus *Acinetobacter* in both groups at Day 7 (FBZ1 and MOX1 – $P < 0.01$ compared with pre-treatment and Day 14; FBZ2 $P < 0.01$ compared with pre-treatment, $P < 0.05$ compared with Day 14; MOX2 $P < 0.05$ compared with Day 0.

In general, the magnitude of changes observed was greater in Group 1 than Group 2, although changes were similar qualitatively. The most variable microbiota across samples from each Group at Phylum, Family and Genus levels overall are shown in Table 2.

4. Discussion

Two types of intervention which have received attention due to their significant therapeutic potential to alleviate a range of intractable gut diseases include manipulation of the gut bacterial microbiota (Sartor, 2004), and the use of helminths or their products to diminish inflammatory responses (Matisz et al., 2015).

Horses may provide a fruitful testing ground for hypothesis-based research around these themes for several reasons. They are hindgut fermenters, relying in large part on populations of bacteria in the caecum and colon to render nutrients accessible for host metabolism. In addition, horses have a resident population of helminths in the large intestine, comprising various species of cyathostomins, both as adults and encysted larvae in the mucosa, as well as the less common large strongyles and occasional other species. The very high prevalence of cyathostomins in horses with access to grazing justifies the description of a resident "helminthome". Finally, horses are susceptible to a range of acute and chronic inflammatory conditions, of uncertain aetiology and pathogenesis, involving the large intestine. Studies involving the


A
Treatment
FBZ
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Treatment
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MOX
FEC
Day
C
FIBRINOGEN
Day
D
FIR
Day
E
Albumin:Globulin
Time
F
Albumin:Globulin
Time
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Treatment
FBZ
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MOX 2
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Day

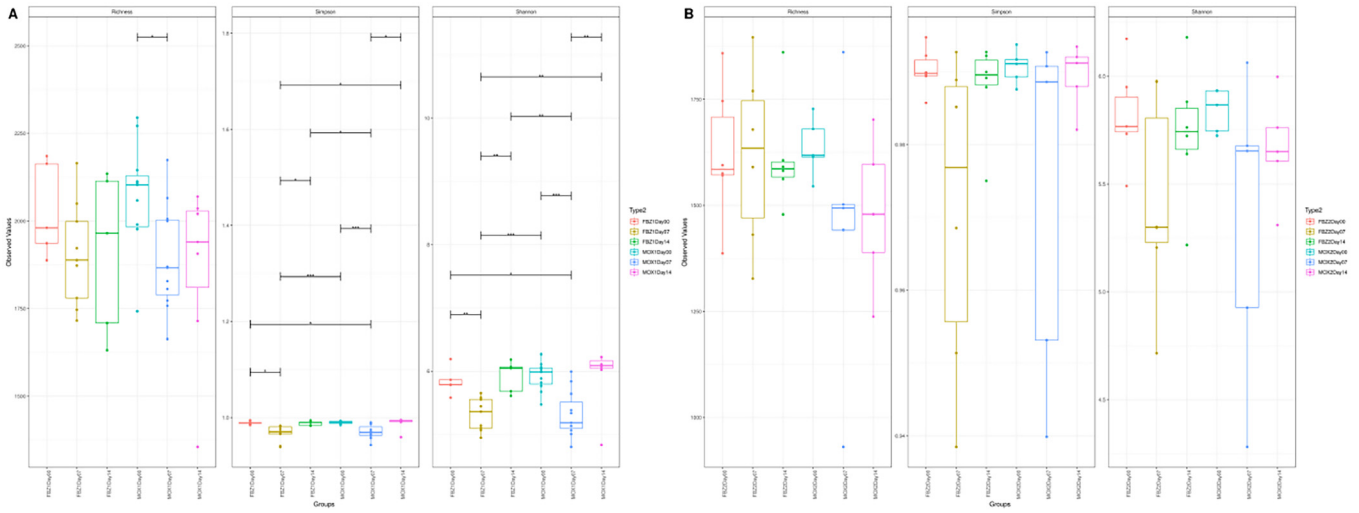

Fig. 2. Two separate groups of horses with cyathostomin infection were divided into two sub-groups and treated with either fenbendazole or moxidectin. Faecal microbiota were assessed and compared at Days 0.7 and 14. Alpha-diversity measurements of faecal microbiota by Richness, Simpson and Shannon indices for Group 1 (A) and Group 2 (B). Significant differences are indicated: * $P < 0.05$ ** $P < 0.01$, *** $P < 0.001$. Within each individual panel the sub-groups are ordered as fenbendazole Day 0, fenbendazole Day 7, fenbendazole Day 14, moxidection Day 0, moxidection Day 7, moxidection Day 14.

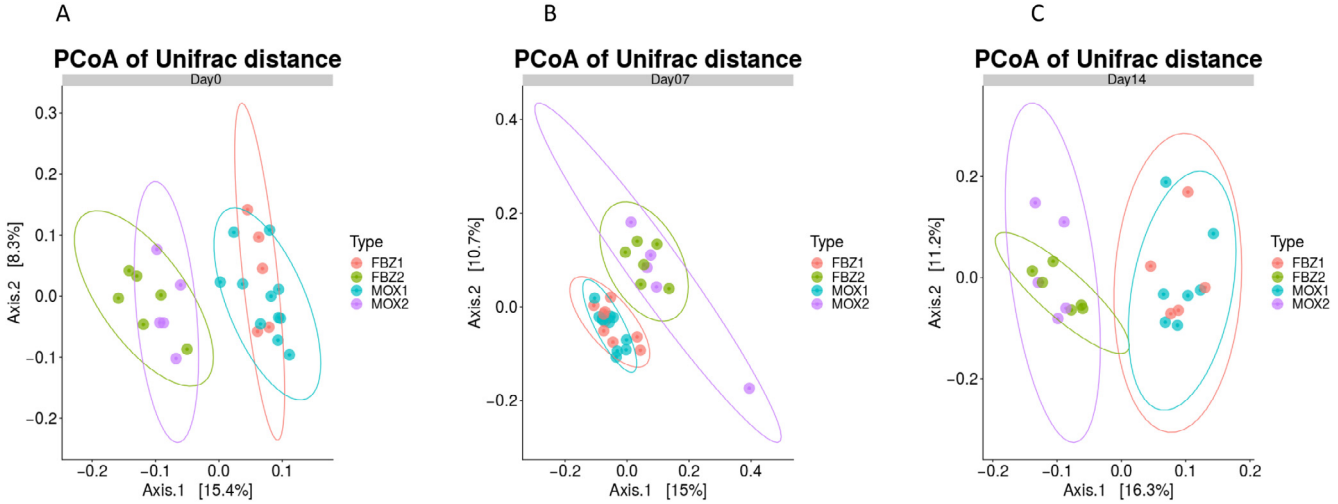

Fig. 3. Two separate groups of horses with cyathostomin infection were divided into two sub-groups and treated with either fenbendazole or moxidectin. Faecal microbiota were assessed and compared at Days 0.7 and 14. Beta-diversity measurements for both Groups at Days 0 (A), 7 (B) and 14 (C) shown as Principal Co-ordinate plots. Groups 1 and 2 clearly segregate, reflecting differences in age and environment, at all three timepoints. In both Groups, decreased measures of beta diversity were observed at Day 7. Type of treatment (fenbendazole or moxidectin) did not affect beta diversity.

bacterial gut microbiota and its interaction with the helminthome may, therefore, provide improved understanding and potential control strategies for such conditions in horses, and also more generally in a One Health context. We acknowledge, of course, that mechanistic studies in large animals are challenging and lack some of the elegant tools available to those working on rodent models. Nevertheless, the ready availability of helminth-infected horses and cyathostomins does provide scope for the creative development of ex-vivo and in-vitro studies aimed at elucidating pathways involved in gut helminth-microbiota interactions.

In this study we sought to establish if there was a relationship between the resident cyathostomin population, the faecal

Fig. 1. Two separate groups of horses with cyathostomin infection were divided into two sub-groups and treated with either fenbendazole or moxidection. Parasitological and clinico-pathological variables from Groups 1 and 2 pre-treatment (0) and at 7 and 14 days post-treatment. Strongyle faecal egg counts were reduced by >94% in both Group 1 (A) and Group 2 (B) after administration of either, indicating the efficacy of both drugs in removing adult strongyles. Individual values are shown. Serum fibrinogen levels remained at or slightly above the reference range (upper level indicated by the horizontal dashed line) in Group 1 (C) throughout the experimental period, but were significantly greater in the moxidection sub-group at Day 7 compared with pre-treatment levels (paired T-tests, $P < 0.05$). In Group 2, (D) mean levels were within the reference range before treatment, rose above this range at Day 7, and declined again at Day 14. Day 7 levels were significantly greater than both Day 0 and Day 14 ($P < 0.005$) for the fenbendazole sub-group and significantly greater in the moxidection sub-group at Day 7 compared with Day 14 ($P < 0.05$). The proportion of animals in Group 1 (E) testing positive for faecal albumin increased between Day 0 and Day 7 (significant, $P = 0.013$, Chi-Square, with fenbendazole and moxidection animals considered together). In Group 2 (F), the proportion of animals was high at the start of the experiment, but declined at Day 14 post-treatment. Serum Albumin:Globulin ratios (A:G) gradually increased in both Groups after treatment, with individual values shown in G, H. Paired T-tests showed significant increases ($P < 0.002$) between Day 0 and Day 7, and Day 7 and Day 14, for both fenbendazole- and moxidection-treated animals.

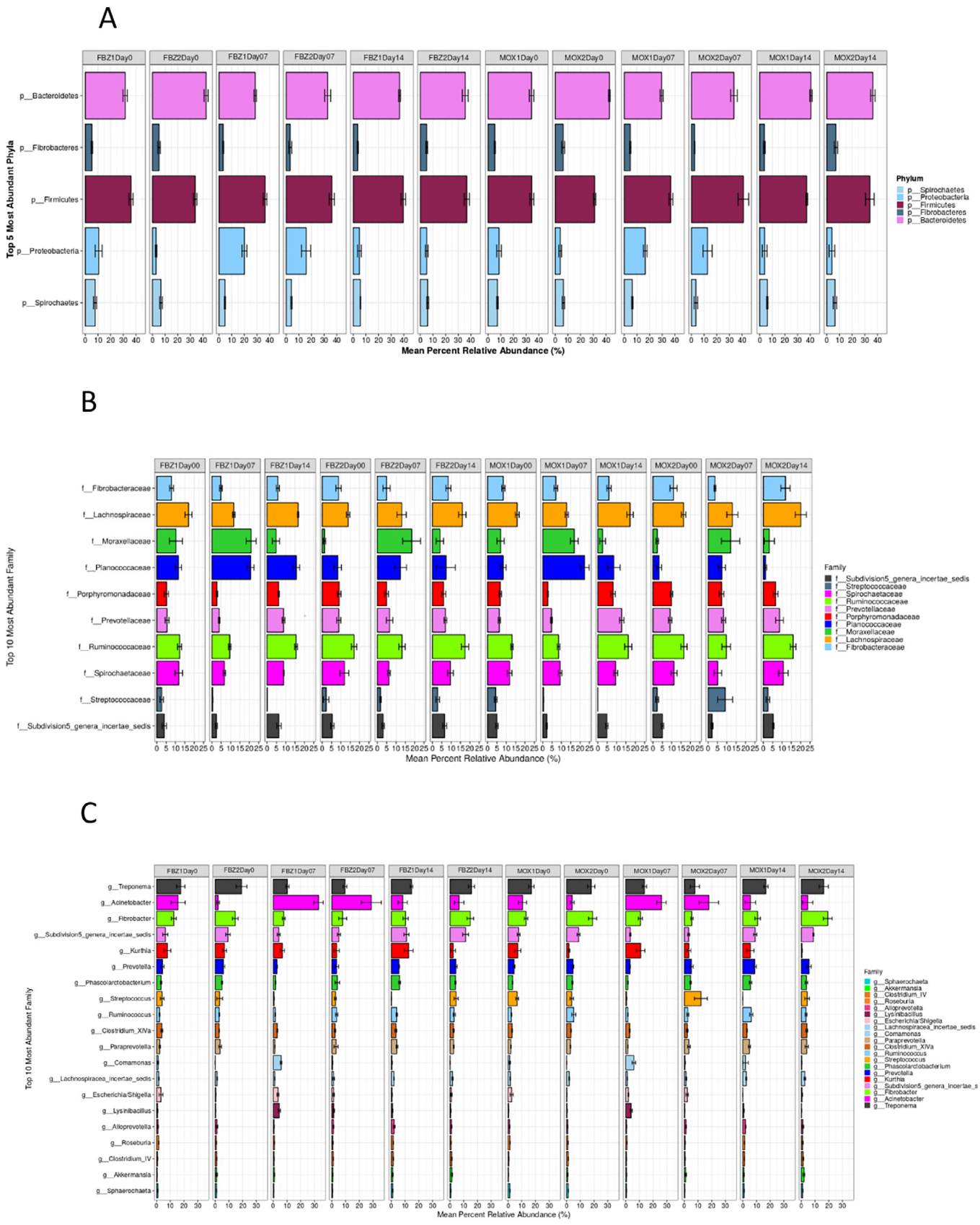

Fig. 4. Two separate groups of horses with cyathostomin infection were divided into two sub-groups and treated with either fenbendazole or moxidectin. Faecal microbiota were assessed and compared at Days 0, 7 and 14. Relative abundance of faecal microbiota, showing the five most abundant Phyla (A), 10 most abundant Families (B) and 20 most abundant Genera (C) across Groups, Treatments and Days. From left to right, each plot shows sub-groups FBZ1, pre-treatment. FBZ2, pre-treatment, FBZ1 Day 7, FBZ2, Day 7, FBZ1 Day 14, FBZ2, Day 14. MOX1, Pre-treatment, MOX2, Pre-treatment, MOX1 Day 7, MOX2, Day 7, MOX1 Day 14, MOX2, Day 14.

Table 2
Top eight most variable results following 16S RNA analysis of faecal samples, at Phylum, Family and Genus level, seen in in Groups 1 and 2 horses, infected with cyathostomins and then treated with either moxidectin or fenbendazole.

Phylum	*P* value	Adj P BH
Group 1		
Bacteroidetes	2.53052E-05	0.000455395
Proteobacteria	3.57949E-05	0.000644112
Chlamydiae	0.000247711	0.004449425
Tenericutes	0.000708885	0.012683328
Fusobacteria	0.002584918	0.045520167
Verrucomicrobia	0.003900176	0.06792354
Spirochaetes	0.006546549	0.111504134
Lentisphaerae	0.011507184	0.188061172
Group 2		
Tenericutes	0.002803368	0.049276009
Bacteroidetes	0.005155678	0.088845009
Proteobacteria	0.007581506	0.128018476
Verrucomicrobia	0.010265291	0.169502331
Lentisphaerae	0.019268318	0.29546318
Actinobacteria	0.020371246	0.309589426
Fibrobacteres	0.030361783	0.425916539
Synergistetes	0.042641691	0.543604666
Family		
Group 1		
Streptococcaceae	2.42984E-07	3.88766E-05
unclassified_*Streptococcaceae*	3.16327E-06	0.000505996
Carnobacteriaceae	4.71535E-06	0.000754174
Acidaminococcaceae	1.33823E-05	0.002138893
Dietziaceae	1.3699E-05	0.002189455
unclassified_*Planococcaceae*	1.76029E-05	0.002812531
Sutterellaceae	2.13183E-05	0.003405157
unclassified_*Micrococcineae*	2.16441E-05	0.003457102
Group 2		
Pseudonocardiaceae	0.003027455	0.384381265
Victivallaceae	0.004635633	0.524517789
Anaeroplasmataceae	0.005513196	0.587100877
Moraxellaceae	0.009963267	0.798530501
Thermoactinomycetaceae 1	0.010145221	0.804369087
unclassified_*Moraxellaceae*	0.013435735	0.885168549
unclassified_*Enterobacteriaceae*	0.013549829	0.887273919
unclassified_*Micrococcineae*	0.014373033	0.901369246
Genus		
Group 1		
Streptococcus	2.06494E-07	4.68731E-05
Desemzia	2.52439E-07	5.7302E-05
Lysinibacillus	8.40451E-06	0.001906014
Phascolarctobacterium	1.43474E-05	0.003251589
Dietzia	2.9127E-05	0.006590121
Paraprevotella	3.72932E-05	0.008429979
Williamsia	5.25586E-05	0.011860219
Acinetobacter	5.25707E-05	0.01186293
Group 2		
Megasphaera	0.000624628	0.132235476
Saccharopolyspora	0.001875961	0.347042431
Anaeroplasma	0.001913925	0.352655903
Victivallis	0.001964017	0.359989248
Taibaiella	0.00709792	0.801501408
Subdivision5_*genera_incertae_sedis*	0.007880581	0.834034936
Acinetobacter	0.008632661	0.860280909
Diplorickettsia	0.010767406	0.914347354

Adj P BH, adjusted *P* value; method of Benjamini and Hochberg.

microbiota, and inflammatory markers in horses, using anthelmintic treatment to disrupt the equilibrium of this system. Two separate groups of animals, positive for encysted cyathostomins by ELISA, were dosed with two different anthelmintics and changes in strongyle FECs, inflammatory markers, and faecal microbiota composition and diversity were monitored at Days 7 and 14 post-treatment. Overall, the results demonstrate that removal of helminths through anthelmintic treatment induces a change in the faecal microbiota, including decreased alpha and beta diversity at Day 7 post-treatment, which mostly resolved by Day 14. This change coincided with local and systemic inflammatory responses, as indicated by serum fibrinogen and faecal albumin measurements. This is in line with the growing evidence of a tolerogenic relationship between the parasite, microbiome and host immune system in the intestinal ecosystem (Broadhurst et al., 2012; Reynolds et al., 2014; Fricke et al., 2015; Ramanan et al., 2016). The type of anthelmintic used (FBZ or MOX) did not influence the results.

The horses in our study did not show any clinical signs of gastrointestinal disease during the course of the study. However, both Groups 1 and 2 had indications of either systemic or local inflammation on Day 7 post- treatment. Inflammation was shown to be significant in the older group (Group 2) in respect to serum fibrinogen levels at this time point. The younger group of animals (Group 1) had high concentrations of fibrinogen prior to treatment, with a group mean value of 2.19 g/L (reference range 0–2 g/L). Serum fibrinogen as an indicator of inflammation in the horse has a reported sensitivity ranging from 37 to 82% (Borges et al., 2007; Belgrave et al., 2013; Hooijberg et al., 2014) and a specificity ranging from 51 to 97% (Belgrave et al., 2013; Hooijberg et al., 2014). However, serum fibrinogen concentrations do not discriminate between local and systemic inflammation, and values within the reference range do not rule out inflammation (Hooijberg et al., 2014). Thus, the findings of the younger cohort (Group 1) could be due to poor test sensitivity or the high initial levels could have masked a potential rise in inflammation associated with treatment. This group did show an increase in respect to the proportion of animals testing positive for faecal albumin at Day 7 post-treatment, indicative of hindgut inflammation, with numbers declining at Day 14. In both groups there was a significant increase in the Albumin:Globulin ratio by Day 14, potentially reflecting resolving inflammation or improved intestinal barrier function.

Chronic helminth infections are associated with modulation of the host immune system. The regulatory network associated with the immunomodulatory effects of intestinal helminths includes regulatory T cells, manipulation of macrophages and dendritic cells, resulting in the production of interleukins that dampen the inflammatory response such as IL-10 and TGF-β (Maizels and Yazdanbakhsh, 2003). There is growing evidence that the use of helminths is effective in correcting dysfunctional inflammatory responses in the gut (Summers et al., 2003; Broadhurst et al., 2010, 2012; Matisz et al., 2015). In the horse, a baseline level of gut helminths (the "helminthome") may form an essential part of the harmonious ecological intestinal environment, meaning that disruption of this, by anthelmintic treatment, could precipitate an excessive inflammatory cascade, in some cases precipitating ALC (Giles et al., 1985; Mair, 1993; Reid and Hillyers, 1995). Hence, immunoregulatory effects may be involved, directly or indirectly, in the pathogenesis of ALC following anthelmintic treatment.

As stated above, one of the factors thought to be associated with re-emergence of encysted larvae is the removal of adult worms (Reid and Hillyers, 1995). Therefore, the inflammatory response could be in response to the emergence of unaffected encysted larvae. Recently, reported efficacy of FBZ and MOX against early L3s (EL3s) of cyathostomins has been documented as 50.4% and 73.8%, respectively (Bellaw et al., 2017). Similarly with regard to late L3s (LL3s) and L4s, efficacy was reported at 70.8% and 74.6% for FBZ and MOX, respectively (Bellaw et al., 2017). Although in two different studies of inflammatory response to treatment, subtle or minimal inflammatory response was elicited post-treatment (Nielsen et al., 2013,2015), the parasite burden in our protocol was higher than in these previous studies. Moreover, it has previously been shown that there is a strong inflammatory response to dead larvae within the intestinal walls, that produces an initial granulomatous mucosal response followed by an ulcerative colitis

(Steinbach et al., 2006). The immune response to degrading larvae in conjunction with removal of the immunoregulatory effects of cyathostomins could give rise to an acute colitis episode.

As would be expected, the profile of the microbiota within the populations differed between Groups 1 and 2 at all time-points, reflecting differences in the ages of the animals, as well as environmental differences. We found significant changes in the faecal microbiome after either FBZ or MOX treatment, with a decrease in alpha diversity (species richness) on Day 7 compared with Day 0 and Day 14. Significance across all indices was seen in Group 1, and significant difference in both the Shannon and Simpson indices in Group 2. Increased alpha diversity has previously been associated with helminth infection and with increased stability of the intestinal microbial community (Walk et al., 2010; Giacomin et al., 2015). In contrast, a decrease in alpha diversity has been shown to be associated with inflammatory intestinal disease in horses (Costa et al., 2012; Schoster et al., 2017) and changes preceding colic in broodmares (Weese et al., 2015). Decreases in alpha diversity are also generally indicative of dysbiosis (Belizário and Napolitano, 2015).

We observed a significant difference in beta diversity between time points, evident in both groups as outlined by PCoA. Beta-diversity reflects the diversity across samples within a population, and a clustering effect in both groups on Day 7 outlines the similar changes in microbiome composition after anthelmintic treatment.

Specific changes in the microbiota associated with anthelmintic treatment in our study included an increase in Proteobacteria and relative decrease in Bacteroidetes, and similar changes have been shown to be an indicator of dysbiosis associated with inflammatory intestinal disease in humans (Packey and Sartor, 2009; Morgan et al., 2012). Specifically, an increase in Proteobacteria has been associated with microbiome instability and inflammation (Shin et al., 2015). An increase in faecal Proteobacteria in horses has previously been shown to be associated with equine colic (Weese et al., 2015) and a corresponding simultaneous decrease in Bacteroidetes is seen in horses with colitis (Costa et al., 2012). The greater magnitude of changes seen in Group 1 compared with Group 2 may reflect a greater malleability of the still-developing gut microbiome in the younger horses, and/or greater numbers of animals in the former.

A high abundance of Bacteroidetes is also associated with nematode infection (Rausch et al., 2013). Bacteroidetes survive on the mucus of the host and are involved in plant polysaccharide digestion. Previous studies have shown that cyathostomin infection increased numbers of mucus-producing goblet cells in the large intestine of the horse (Steuer et al., 2018) and higher burden horses were shown to have an increased level of Bacteroidetes in the faecal microbiome (Peachey et al., 2018). Helminth infection can alter mucin production, which in turn alters the nutritional environment for resident bacteria (Li et al., 2012; Fricke et al., 2015). Modulation of mucus production has been shown to ameliorate colitis (Broadhurst et al., 2012) and small intestinal inflammatory dysfunction (Ramanan et al., 2016) through alteration of the mucosal microbiota. Our study, demonstrating a decrease in Bacteroidetes on Day 7 following anthelmintic treatment, is therefore consistent with studies associating this group with nematode infection and potentially also with the quantity and quality of mucus production in the large intestine, although we did not assess this parameter.

A direct effect of anthelmintic drugs on bacterial microbiota cannot be ruled out as an explanation for the changes documented here. The macrocyclic lactones, of which MOX is an example, were first isolated from soil-derived *Streptomyces* bacteria, which are also the source of many antibacterial agents. Although MOX is not reported as having a wide spectrum of antibacterial activity, it is bactericidal against *Mycobacterium tuberculosis* (Lim et al., 2013). Also, in a recent human study microbiome changes have been shown to result from the use of tribendimidine plus ivermectin (Schneeberger et al., 2018). However, we observed similar changes in horses treated with two drugs which differ in their chemical composition and mechanism of action.

The faecal albumin test is a qualitative test allowing for subjective interpretation. The evidence supporting this test is limited. However, interpretation of local intestinal inflammation is crucial to assessment of changes in the gut ecological environment and thus more specific markers of equine gut inflammation such as faecal calprotectin levels should be investigated further.

Although the sample size used in this study was small, it was within the range used by several of the relatively small number of equine gut microbiome studies carried out to date. There are several possible confounding factors in this study. Horses were of different ages, which has been shown to affect the gut microbiota in humans (Yatsunenko et al., 2012) and, as expected, there was a strong association between various age groups and their intestinal microbiome (Mshelia et al., 2018). The horses were not on a uniform diet, and changes in nutrient levels (Hansen et al., 2015), frequency of feeding (Venable et al., 2017) and diet type (Daly et al., 2012) can all affect the microbiome. Group 1 were moved a different pasture during the study, meaning that both change in diet and transport could be a factor (Faubladier et al., 2013). However, there was no statistical difference between pre-treatment measurements and Day 14 post-treatment regardless of change in environment. This supports the argument that changes in the gut microbiome were associated with anthelmintic treatment with no significant difference noted between the use of two different drug types. Changes were seen in both groups at Day 7 post-treatment, and this time point coincides with degradation of worms post-treatment, 4–6 days and 6–14 days post-treatment for BZ and MOX, respectively (Steinbach et al., 2006).

Faecal samples were used in this study, raising the possibility that the changes observed do not fully reflect changes in the colonic microbiota. However, previous reports have compared the equine colonic and faecal microbiota (Dougal et al., 2013; Sadet-Bourgeteau et al., 2014; Costa et al., 2015; Ericsson et al., 2016). Although differences have been seen between the microbiota of the proximal and distal hind gut (Costa et al., 2015; Ericsson et al., 2016), generally, previous reports support the premise that the equine faecal microbiota are broadly representative of the colonic microbiota (Schoster et al., 2013; Dougal et al., 2012; Costa et al., 2015).

Other authors have reported that an increase in the relative abundance of the families *Moraxellaceae* and *Planococcaceae* can be associated with improper storage of faecal samples resulting in proliferation of bloom taxa (Beckers et al., 2017). The storage and management of samples in our study was uniform throughout the groups and time periods, making it unlikely that the significant changes observed at Day 7 in both Groups 1 and 2, although to a greater extent in Group 1, were due to such factors.

Overall, our findings provide evidence for an interdependence between cyathostomins, the hindgut microbiota and inflammation in the horse. Such relationships have been observed in other studies where removal of resident gut helminths changed the intestinal ecosystem and bacterial microbiota (Reynolds et al., 2014; Fricke et al., 2015; Ramanan et al., 2016; He et al., 2018). The changes we observed are consistent with those associated with intestinal dysbiosis and associated pathological conditions (Frank et al., 2007; Takaishi et al., 2008) and the growing body of evidence that helminth infection can be used therapeutically to ameliorate inflammation (Summers et al., 2003; Broadhurst et al., 2010, 2012; Reynolds et al., 2014; Giacomin et al., 2015; Ohnmacht et al., 2015; Zaiss et al., 2015; Ramanan et al., 2016). Further studies on the benefits/costs of helminth infection in the horse, and the

role cyathostomins, both as adults and encysted larvae, play in maintaining homeostasis in the gut ecosystem, are warranted. Such studies are likely to inform both helminth control and nutritional recommendations for horses, with the aims of preventing clinical disease and optimising health. In addition, they are also relevant to understanding diseases where microbial dysbiosis is involved, such as inflammatory bowel disease, in other host species.

In summary, we have observed significant indications of changes in the equine gut microbiome associated with anthelmintic treatment. These changes were associated with an inflammatory response and could be an indication of the immunoregulatory effects of cyathostomins either directly or through manipulation of bacterial microbiota.

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