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The relationships between faecal egg counts and gut microbial composition in UK Thoroughbreds infected by cyathostomins

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A B S T R A C T

A growing body of evidence, particularly in humans and rodents, supports the existence of a complex network of interactions occurring between gastrointestinal (GI) helminth parasites and the gut commensal bacteria, with substantial effects on both host immunity and metabolic potential. However, little is known of the fundamental biology of such interactions in other animal species; nonetheless, given the considerable economic losses associated with GI parasites, particularly in livestock and equines, as well as the global threat of emerging anthelmintic resistance, further explorations of the complexities of host-helminth-microbiota interactions in these species are needed. This study characterises the composition of the equine gut commensal flora associated with the presence, in faecal samples, of low (Clow) and high (Chigh) numbers of eggs of an important group of GI parasites (i.e. the cyathostomins), prior to and following anthelmintic treatment. High-throughput sequencing of bacterial 16S rRNA amplicons and associated bioinformatics and statistical analyses of sequence data revealed strong clustering according to faecal egg counts ($P = 0.003$). A trend towards increased populations of Methanomicrobia (class) and Dehalobacterium (genus) was observed in C low in comparison with C high. Anthelmintic treatment in C high was associated with a significant reduction of the bacterial Phylum TM7 14 days post-ivermectin administration, as well as a transient expansion of Adlercreutzia spp. at 2 days post-treatment. This study provides a first known insight into the discovery of the intimate mechanisms governing host-parasite-microbiota interactions in equines, and sets a basis for the development of novel, biology-based intervention strategies against equine GI helminths based on the manipulation of the commensal gut flora.

1. Introduction

Cyathostomins are amongst the most important intestinal nematodes of horses globally (Love et al., 1999; Matthews, 2011; Stratford et al., 2011) with reported prevalence rates as high as 89–100% in equine herds (Mitilidze and Hutchinson, 1990; Collobert-Laugier et al., 2002; Hinney et al., 2011; Moraniu et al., 2016). Clinical signs of cyathostomin infection range from non-specific weight loss to colic and colitis caused by mass emergence of larvae from the large intestinal wall (= larval cyathostominosis), which may prove fatal (Uhlinger, 1991; Murphy and Love, 1997; Lyons et al., 2000; Peregrine et al., 2006). Young Thoroughbred (TB) stock kept in herds are at high risk of developing serious complications of infection, and hence the implementation of effective strategies of parasite control is a top priority for the TB industry. Control of cyathostomin infections has traditionally relied on the regular administration of chemotherapeutic drugs (i.e. anthelmintics); however, the frequent and uncontrolled use of these compounds has led to the global emergence of resistant populations of parasites (Nielsen et al., 2014; Peregrine et al., 2014). In particular, foci of multi-drug resistance have been recently reported in TB stud farms in the United Kingdom (Relf et al., 2014). This observation, coupled with the lack of novel anthelmintic compounds licenced for use in equids, represents a ‘Damocles’s sword’ for the UK (and global) equine industry. Therefore, alternative strategies for parasite control are urgently needed; in order to support the discovery of such strategies, a deeper understanding of the complex interactions occurring at the host-parasite interface, particularly at the site/s of infection (i.e. the gut), is required.

While a multitude of factors is responsible for the host-parasite interactions which determine infection outcome, increasing attention is being paid to the complex interplay between gastrointestinal
(GI) parasites and the host commensal gut flora (Bancroft et al., 2012; Glendinning et al., 2014). Indeed, recent studies have reported significant fluctuations in the composition of the vertebrate gut microbiota associated with helminth infections, that were accompanied by shifts in both systemic and local immunity (Bancroft et al., 2012; Leung and Loke, 2013; Fricke et al., 2015; Houlden et al., 2015; Cattadori et al., 2016; Gause and Maizels, 2016). However, thus far, knowledge of helminth–microbiota cross-talk relies heavily on studies conducted in humans and/or rodent models of infection and disease (Walk et al., 2010; Rausch et al., 2013; Cantacessi et al., 2014; Lee et al., 2014; Reynolds et al., 2014a; Fricke et al., 2015; Giacomin et al., 2015, 2016; Holm et al., 2015; Houlden et al., 2015; Kreisinger et al., 2015; McKenney et al., 2015; Cattadori et al., 2016). In particular, while only a handful of studies to date have characterised the composition of the gut microbiota of veterinary species infected by GI helminths (Li et al., 2011, 2012, 2016; Wu et al., 2012; Slapeta et al., 2015; Duarte et al., 2016; reviewed by Peachey et al., 2017), no data is currently available on the effects of infections by GI helminths such as cyathostomin on the composition of the equine commensal flora. Acquiring this fundamental knowledge will be key to the development of novel holistic approaches to equid parasite control aimed at improving host responses to infections. In this study, we characterise the gut microbial profiles of a cohort of UK TB broodmares with low and high numbers of cyathostomin eggs in faeces (as determined by faecal egg count (FEC) analysis), and examine the effects that administration of a commonly used anthelmintic, i.e. ivermectin, exerts on the overall composition of the gut microbiota as well as relative abundances of individual microbial species.

2. Materials and methods

2.1. Ethics statement

This study was approved and carried out in strict accordance and compliance with the guidelines of the Institutional Ethical Review Committee, Department of Veterinary Medicine, University of Cambridge, UK (Research Project No. CR190). Written informed consent was obtained from the stud farm from which study samples were collected.

2.2. Sampling and diagnostic procedures

For this study, a cohort of TB broodmares was recruited from a stud farm in eastern England, UK. The stud hosts approximately 130 pregnant broodmares each year, which are kept at pasture in groups of 2–8 across 480 hectares. All broodmares are subjected to targeted anthelmintic treatments (ivermectin and moxidectin), based on FEC measurements at 3 monthly intervals. In addition, praziquantel is administered to each broodmare three times a year for tapeworm control, whilst a single moxidectin treatment is administered in late November for control of encysted cyathostomin larvae. Samples used in this study were collected in September–October 2016; all broodmares had received ivermectin and praziquantel in May and August 2016, respectively. A total of 117 TB pregnant broodmares, between 5 and 8 months of gestation at the time of sampling, were screened for infection by cyathostomins. Briefly, individual faecal samples were collected on three consecutive days over a 7 day period; aliquots of each sample were subjected to (i) FEC analysis using a centrifugal floatation technique sensitive to one egg per gram (e.p.g.) (Christie and Jackson, 1982), and (ii) screening for infections with the common equine cestode Anoplocephala perfoliata using a double sugar flotation technique (Rehbein et al., 2011). Upon observation of strongyle eggs during FEC analysis, the remaining faecal aliquots were subjected to larval culture to allow for subsequent identification of infecting nematode species using an established Reverse Line Blot (RLB) hybridisation method (Traversa et al., 2007; Cwiklinski et al., 2012). Briefly, genomic DNA was extracted from individual L3s harvested from each larval culture, and the intergenic spacer (IGS) region was amplified by nested PCR using conserved biotin labelled primers (Traversa et al., 2007). The PCR products were then incubated with biotinylated membrane-bound specific DNA probes for 21 different cyathostomin species (Cwiklinski et al., 2012), incubated with extravidin peroxidase and visualised using x-ray film. Horses were recruited in our study if they satisfied the following criteria: (i) FEC of ≥100 e.p.g. (= Chig) or ≤10 e.p.g. (Clow) in three consecutive samples collected over a 7 day period; (ii) matched by approximate age and paddock; (iii) negative for co-infections with other GI helminths; (iv) no antibiotic treatment for at least 2 months prior to sampling; and (v) no previous anthelmintic treatment other than praziquantel for at least 4 months prior to sampling. Horses enrolled were kept at pasture for the duration of the study and fed 1 kg of custom concentrate mix daily.

2.3. Anthelmintic treatment

Individual, naturally voided, faecal samples were collected from the centre of the faecal mass from Chig and Clow animals, as well as from three non-pregnant broodmares on day 0 (D0). Then, an anthelmintic treatment (Eqvalan: ivermectin 0.2 mg/kg) was immediately administered to each animal. Sampling was repeated as above at day 2 (D2) and day 14 (D14) post-treatment (p.t.). A 100 g aliquot of each faecal sample was snap frozen, transported to the laboratory and stored at −20 °C within 2 h of collection, prior to genomic DNA extraction and high-throughput sequencing of a hypervariable region of the bacterial 16S rRNA gene (see Section 2.4), while the remainder was kept fresh and subjected to FEC analysis as described above.

2.4. High-throughput 16S rRNA sequencing

Genomic DNA was extracted from individual faecal samples, as well as from five negative ‘blank’ (= no DNA) controls, using the PowerSoil™ DNA Isolation Kit (Qiagen, Carlsbad, CA, USA), according to the manufacturers’ instructions. Microbial communities in each sample were identified via Illumina high-throughput sequencing of the V3-V4 hypervariable region of the bacterial 16S rRNA gene. In particular, the V3-V4 region was PCR-amplified using universal primers (Forward, 5′-TGC TCG GCA GCG TCA GAT GTG TAT AAG AAG CAG CTT ACG GGN GGC WGC AG-3′; Reverse, 5′-GTC TCG TGG GCT CAG AGA TGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3′) (Kilworth et al., 2013), that contained Illumina (San Diego, California, USA) adapter over-hang nucleotide sequences, using the NEBNext® Q5® Hot Start HiFi DNA polymerase (New England Biolabs Inc., Massachusetts, USA). For PCR amplification, the following thermocycling protocol was used: 98 °C/2 min, 20 cycles of 98 °C/15 s, 63 °C/30 s, and 72 °C/30 s, and 72 °C/5 min. Amplicons were purified using AMPure XP PCR Purification beads (Beckman Coulter, Brea, California, USA). The index PCR was performed using the NEBNext hot start high-fidelity DNA polymerase and Nextera XT index primers (Illumina) according to the following thermocycling protocol: 98 °C/30 s, 8 cycles of 98 °C/10 s, 65 °C/75 s and at 65 °C/5 min. The indexed samples were purified using AMPure XP beads and quantified using the Qubit Quanti-ITM dsDNA Broad-Range Assay Kit (Life Technologies, Carlsbad, California, USA). Then, equal quantities from each sample were pooled and the resulting library was quantified using the NEBNext® Library Quant Kit for Illumina® (New England Biolabs Inc.). High-throughput sequencing was per-
formed on an Illumina MiSeq platform using the v3 chemistry (301 bp paired-end reads).

2.5. Bioinformatics analyses

Following trimming of primer sequences using Cutadapt (https://cutadapt.readthedocs.org/en/stable/), raw paired-end Illumina reads were joined using the Quantitative Insights Into Microbial Ecology (QIME) software suite (version 1.9.0) (Caporaso et al., 2010) and quality filtered using the ‘search_qf’ script with default settings. Then, high-quality sequences were clustered into Operational Taxonomic Units (OTUs) on the basis of similarity to bacterial sequences available in the Greengenes database (http://greengenes.secondgenome.com/; 97% sequence similarity). Operational Taxonomic Units (OTUs) were clustered de novo based on pairwise sequence identity (97% sequence identity cut-off) (cf. Duarte et al., 2016). Singleton OTUs and OTUs matched to references in the Greengenes database were subtracted from individual datasets prior to downstream analysis. For normalisation, a subsampled OTU table was generated by random sampling (without replacement) of the input OTU table using an implementation of the Meresse merfield algorithm (http://www.numpy.org). Cumulative-sum scaling (CSS) and log2 transformation were applied to account for the non-normal distribution of taxonomic counts data. Statistical analyses were conducted on the Calypso platform (cgenome.net/calypso/); samples were compared with C high and C low by random sampling (without replacement) of the input OTU table using an implementation of the Mersenne twister algorithm, a total of 8,077,490 high quality sequences (mean = 73,423 ± 3,610) were retained for further bioinformatics analysis (not shown). Rarefaction curves generated following in-silico subtraction of low-quality and contaminant sequences indicated that the vast majority of faecal bacterial communities were represented in the remaining sequence data, thus allowing us to undertake further analyses. These sequences were assigned to 95,286 OTUs and 15 bacterial phyla, respectively. The Phyla Bacteroidetes (39.9%) and Firmicutes (34.0%) were predominant in all samples, followed by the Phyla Verrucomicrobia (12.0%), Spirochaetes (3.9%), Fibrobacteres (2.4%), Cyanobacteria (1%), Proteobacteria (0.9%), Euryarcheota (0.4%), Tenericutes (0.4%), TM7 (0.3%), Actinobacteria (0.3%), Lentisphaerae (0.3%), Synergistetes (0.2%), WPS-2 (0.2%) and Planctomycetes (0.1%) (Fig. 1), while 3.4% of OTUs could not be assigned to any bacterial group. Predominant sub-taxa were Bacteroidia (class), Bacteroidales (order) and Bacteroidales (family) within the Phylum Bacteroidetes, and Clostridia (class), Clostridiales (order) and Ruminococcaceae (family) within the Firmicutes (Fig. 1). Two samples, LVN1 and HS2, differed markedly in the relative proportions of the two most abundant phyla, Bacteroidetes and Firmicutes, when compared with samples from other broodmares (Fig. 1), likely indicating dysbiosis. Therefore, in order to reduce biases due to these potential ‘outliers’, these samples were excluded from further statistical analyses (Fig. 1).

3.3. Differences in microbial composition between Chigh and Clow, and pre- and post-anthelmintic treatment

Microbial community profiles of each sample were grouped by hierarchical clustering and ordinated by supervised CCA. Using these methods, a significant association was observed between microbial composition and FEC (Chigh versus Clow) (P = 0.003), while clustering according to time point pre- and post-anthelmintic treatment (D0 versus D14) did not reach statistical significance (P = 0.686) (Fig. 2A). CCA of C200 versus C0 led to a clear separation according to FEC (P = 0.001), whilst the effect of anthelmintic treatment remained insignificant (P = 0.811) (Fig. 2B).

No significant differences in OTU alpha diversity (Shannon Index) were recorded between Chigh and Clow, or between samples collected at D0, D2 and D14 (Fig. 3A–C). A trend towards increased alpha diversity in Chigh versus Clow at all time-points was observed (P = 0.087) (Fig. 3A). This trend was also observed when C200 samples were compared with C0 at D0, despite smaller group sizes (P = 0.102) (Fig. 3C). No significant differences in beta diversity, as measured by PERMDISP, were observed between groups (Fig. 4).
Differences in abundance of individual taxa at the phylum, class, order, family, genus and species level were detected between \( C_{\text{high}} \) and \( C_{\text{low}} \) samples, as well as between samples collected at D0, and D2 and D14 p.t. (Fig. 5). Samples from \( C_{\text{low}} \) at D0 (pre-treatment) were characterised by an increased abundance of Methanobacteria (class), \textit{Dehalobacterium} (genus) and unclassified \textit{Dehalobacterium} and \textit{Ruminococcus} (species) compared with samples from \( C_{\text{high}} \) (Fig. 5A). The same taxa were increased in \( C_{\text{0}} \) compared with \( C_{\text{200}} \), with the addition of methanogens of the Family Methanocorpusculaceae belonging to Order Methanomicrobiales, Class Methanobacteria, Phylum Euryarchaeota; Order Endomicrobiales, Phylum Elusimicrobia; Rickettsiales (order, family, genus, species); Family Bac-teroidaceae, genus BF311 and species RFN20 (Fig. 5B). The taxa \textit{GMD14H09} (order, family, genus, species) of the Phylum Proteobacteria were increased in samples from \( C_{\text{200}} \) compared with \( C_{\text{0}} \) (Fig. 5B). Anthelmintic treatment in \( C_{\text{high}} \) was accompanied by a decrease in the Phylum TM7 at D14, when compared with pre-treatment samples (Fig. 5C). Additionally, the taxa \textit{Adlercreutzia} and R445B were increased at D2 and D14, respectively, compared with D0 samples (Fig. 5C). In \( C_{\text{low}} \), treatment was also associated with an increase in R445B (family, genus, species) at D14 (Fig. 5D).

4. Discussion

This study is the first known to report a significant association between numbers of cyathostomin eggs in faecal samples from UK Thoroughbreds and the composition of the host gut microbiota. A particularly significant shift in microbial profiles was observed when the faecal bacterial populations of a group of broodmares with FEC of \( \geq 200 \) e.p.g. were compared with those with observed FEC of 0. These data are consistent with observations from published studies in both humans and other veterinary species, including rodent models of infection and disease (Lee et al., 2014; Holm et al., 2015; Houlden et al., 2015; McKenney et al., 2015; Duarte et al., 2016; Li et al., 2016). In addition, the administration of a routinely used anthelmintic (i.e. ivermectin) to both \( C_{\text{high}} \) and \( C_{\text{low}} \) resulted in further progressive changes of the microbial profiling of treated horses. While such changes did not reach statistical significance when analysed using a multivariate model, this trend suggests that parasite-associated modifications in the composition of the host gut microbiota may be transient, and dependent on the presence of live infections, a hypothesis which requires thorough testing.

Overall, the bacterial phyla identified in this study were consistent between groups of animals enrolled; this observation differs
from the results of previous studies that had reported significant variability in faecal microbial profiling between horses, largely related to variations in diet and age, and the presence of underlying diseases (Costa et al., 2012; Daly et al., 2012; Steelman et al., 2012; O’Donnell et al., 2013; Dougal et al., 2014; Fernandes et al., 2014; Weese et al., 2014). Thus, our finding likely indicates that the impact of such confounding factors was successfully minimised by our study design, and that the recorded differences in microbial composition were indeed associated with parasite infections. Bacteria belonging to the Phylum Bacteroidetes were predominant in animals examined in our study; conversely, other investigations had reported Firmicutes as being the most prevalent phylum in the horse gut flora (Costa et al., 2012, 2015a,b; Shepherd et al., 2012; Dougal et al., 2014; Fernandes et al., 2014; Weese et al., 2014; Proudman et al., 2015). Dietary differences between horse cohorts enrolled in this and previous studies are likely to be responsible for this discrepancy (cf. Daly et al., 2012; Fernandes et al., 2014).

Fig. 1. Bar charts depicting the relative abundances of faecal bacterial phyla from broodmares with faecal egg counts (FEC) > 100 eggs per gram (e.p.g.) (= Chigh) and < 10 e.p.g. (= Clow), according to sampling time point (i.e. pre-antihelminthic treatment (Day 0 (D0), and 2 and 14 days post-treatment (D2 and D14, respectively)), and from non-pregnant controls (NPC). Samples from broodmares with FEC > 200 e.p.g. (C200) and 0 (C0) are indicated in red, while remaining samples are indicated in black.

Fig. 2. The microbial composition of faecal samples ordered by supervised Canonical Correspondence Analysis (CCA) from broodmares with (A) faecal egg counts (FEC) > 100 eggs per gram (e.p.g.) (= Chigh) and < 10 e.p.g. (= Clow), pre-antihelminthic treatment (Day 0 (D0)) and at 14 days post-treatment (D14) (B) with FEC > 200 e.p.g. (C200) and 0 (C0) at D0 and D14. OTU, Operational Taxonomic Unit.
Overall, a trend towards increased microbial alpha diversity, i.e. the number of different OTUs in each sample (‘richness’) and their relative abundance (‘evenness’) (Tuomisto, 2010), was observed in samples from C_high compared with those from C_low at D0 (pre-anthelmintic treatment) and in C_200 versus C_0, although these differences did not reach statistical significance. Nevertheless, this observation is supported by the results of a number of previous studies in other host-helminth systems, in which the establishment of parasitic infections was associated with an overall increase in alpha diversity of the gut microbiota (Broadhurst et al., 2012; Lee et al., 2014; Giacomin et al., 2015, 2016). Given that a number of inflammatory GI and/or systemic diseases are accompanied by a
reduced alpha diversity (Manichanh et al., 2006; Sepehri et al., 2007; Abrahamsson et al., 2012, 2014), the increase in GI microbial diversity observed in the presence of helminth infections has been hypothesized to represent one of the possible mechanisms by which parasites suppress host inflammatory responses, thus ensuring their long-term survival in the host gut (Bancroft et al., 2012; Glendinning et al., 2014). Therefore, the trends towards increased alpha diversity observed in the faecal microbiota of horses moderately infected by cyathostomins may also result from an increase in gut homeostasis promoted by the parasites. Future studies evaluating the prevalence and incidence of equine inflammatory diseases (e.g. inflammatory bowel disease and recurrent airway obstruction) in the presence or absence of parasitic infections could represent significant first steps in this area of research.

In addition to global microbial diversity, significant variations in the abundance of specific bacterial taxa were observed between groups. In particular, a higher abundance of microorganisms belonging to the Class Methanomicrobia was observed in C\textsubscript{low} when compared with C\textsubscript{high}. This difference was exacerbated in C\textsubscript{200} versus C\textsubscript{0}, with further significant increases in methanogens belonging to Class Methanomicrobia recorded in C\textsubscript{0}, thus suggesting a negative correlation between methanogen abundance and FEC. Methanomicrobia belong to the Phylum Euryarchaeota, Kingdom Archaea and are phylogenetically distinct from bacteria and eukaryotes, although they retain the prokaryote 16S rRNA gene (Woese and Gupta, 1981; Winker and Woese, 1991). Particularly in ruminants, the role of the Archaeal methanogens in the digestion of fibre has been well documented (St-Pierre et al., 2015). In equids, little is known of the functional diversity of methanogens; however, consistent with our findings, a recent study reported Methanomicrobiales as being predominant in the horse gut (Lwin and Matsui, 2014). The underlying mechanisms by which GI helminths may be promoting a reduction in populations of methanogens are unclear. Similarly to hypotheses formulated for other host-helminth systems, cyathostomins may prevent expansion of methanogens directly, e.g. via their excretory-secretory products, or indirectly via parasite-induced changes in mucosal immunity (reviewed by Peachey et al., 2017). Alternatively, a high abundance of methanogens prior to helminth infections may bias host immune responses against cyathostomins, thus resulting in the observed low (or absent) parasite burdens. Interestingly, some methanogens (i.e. Methanosphaera stadtmanae) have been shown to regulate Th17 responses in mice (Blais Lecours et al., 2011; Bernatchez et al., 2017); in turn, these responses have been linked to the ability of mice to clear experimental infections by Heligmosomoides polygyrus (Reynolds et al., 2014b). Mechanistic studies aiming to evaluate the effects of expanding populations of gut methanogens on host mucosal responses and, in turn, GI helminth establishment, may assist the elucidation of these interactions.

An increased abundance of Methanomicrobia in C\textsubscript{low} and C\textsubscript{0} may also be linked to other environmental factors that are simultaneously responsible for the low FEC observed. An example is represented by horse grazing behaviour; indeed, it is known that some individuals within a herd favours less nutritional swards of grass in order to avoid faecal contamination (Hutchings et al., 2000). In
turn, as animal faeces often act as fertilisers, individuals favouring nutritious grass are exposed to higher numbers of infective larvae. Grazing different swards of grass may also impact on dietary fibre levels, and thus on gut methanogen populations, as observed in ruminants (McAllister et al., 1996). In horses, dietary factors have also been associated with changes in abundance of Methanomicrobia; for example, Methanocorpusculum archaea were observed at a median of 17.7% in horses fed a forage-grain diet, and at a median of 31.9% in horses maintained on pasture (Fernandes et al., 2014). Differences in grazing behaviour between individuals may also be accountable for the increased abundance of bacteria of the Phylum Elusimicrobia in C0 versus C200 as these taxa are primarily a component of termite hind-gut microbiota (Gómez and González-Megías, 2007; van Klink et al., 2015; Mikaelyan et al., 2017). Experimental cyathostomin infections of stabled horses may eliminate the effect of grazing behaviour on gut microbial profiles, although ethical concerns may prevent the execution of such studies in the future.

In contrast to uninfected horses, the faecal microbial profiles of C200 were characterised by an increased abundance of GMD14H09, Phylum Proteobacteria, Class Deltaproteobacteria. Increases in Proteobacteria abundance have repeatedly been reported in association with helminth infections, e.g. in mice infected by Trichuris muris and H. polygyrus, pigs infected by Trichuris suis, and rabbits infected by Trichostrongylus retortaeformis (Li et al., 2012; Holm et al., 2015; Cattadori et al., 2016). Proteobacteria are known to increase in the presence of GI inflammation (Shin et al., 2015); hence, the expansion of populations of Proteobacteria in the faecal microbiota of horses with higher infection burdens may be indicative of an inflammatory status of the intestinal tract of these horses at the time of sampling.

One of the objectives of this study was to assess the impact of anthelmintic treatment on the faecal microbial profiling of cyathostomint-infected horses. In particular, ivermectin administration to C0 was followed by a significant decrease in populations of the Phylum TM7 at D14. Since the relative abundance of TM7 changed following ivermectin administration in Clov, it is tempting to speculate that a mutualistic association may exist between TM7 and cyathostomins, whereby each promote establishment of the other, similar to the mutual relationship described for Lactobacillaceae and H. polygyrus (Reynolds et al., 2014a). Bacteria belonging to the Phylum TM7 are obligate epibionts of Actinomyces spp. (He et al., 2015), and are thus uncultivable. While TM7 have not previously been linked to GI helminth infections, this phylum of bacteria has been associated with mucosal inflammatory disease in humans (Kuebacher et al., 2008). Interestingly, TM7 isolates have been shown to repress the induction of TNF-α production in macrophages infected by Actinomyces odontolyticus, thus suggesting a potential immune suppressive activity (He et al., 2015); hence, TM7 may promote the establishment of cyathostomins by suppressing effective anti-parasite immune responses. Furthermore, an increase in the taxa Adlercreuzia (Phylum Actinobacteria) and R445B (Phylum Lentisphaeraceae) was observed in C0 versus C200, with the emergence of hypoebiotic larval stages of cyathostomins (which is known to occur post-anthelmintic treatment; Lyons et al., 2000), via the suppression of effective mucosal immune responses. This hypothesis requires testing in controlled mechanistic experiments.

FEC are often utilised as proxy of parasite infection burdens; however, several investigations have confounded this practice, as weak correlations have been detected between FEC and parasite burdens in horses with >500 e.p.g. of faeces (Nielsen et al., 2010). While the FEC cut-offs used in this study are indicative of differing infection burdens between groups, any inference on the relationship between number of worms in the horse intestine and gut microbial profiling must be taken with caution. Ethical considerations prevent us from performing post-mortem total worm counts in experimentally infected horses; nevertheless, in the future, it may be possible to establish unequivocal relationships between cyathostomin infection burdens (including encysted larvae) and gut microbial profiling from samples collected in an abattoir.

Clearly, a complex network of host-parasite interactions, as well as environmental factors, contribute to the findings reported in this study, and thus further work is needed to disentangle the causality of these relationships. However, one key question that needs addressing is whether differences in host immunity may be associated with significant changes in gut microbial composition (and vice versa) and, if such is the case, whether the horse gut microbiota could be manipulated to improve resistance to helminth infection. Indeed, previous investigations in cattle and mice have reported that host genes encoding for antimicrobial proteins are up-regulated in the mucosae of animals resistant to helminth infection (D’Elia et al., 2009; Li et al., 2015). In addition, dietary supplementation with both pro- (Bautista-Garfiás et al., 1999, 2001; Martinez-Gomez et al., 2009, 2011; Oliveira-Sequeira et al., 2014; El Temsahy et al., 2015) and pre-biotics (Petkovicus et al., 2003, 2004, 2007; Thomsen et al., 2005; Jensen et al., 2011), has led to significant reductions in worm burdens in murine and swine helminth infection models, thus indicating that alterations of the gut bacterial flora may bias host immune responses against parasites. Further characterisation of equine host mucosal responses and GI microbiota, in the presence or absence of helminth infection and accompanied by total enumeration of infecting parasites, is a key area of future research, as it may lead to the identification of microbial factors linked to host susceptibility.

In conclusion, cyathostomin infection in horses was associated with global shifts in faecal microbial composition and diversity, in accordance with previous studies in other host-helminth systems, as well as significant changes in specific populations of gut bacteria. Such changes predominantly involved ‘minor’ phyla, thus suggesting that the equine ‘core’ gut microbiota remains unaltered in the presence of burdens of cyathostomins such as those observed in this study. Our findings also suggest that the hypothesis that selected bacterial taxa, and/or their metabolites, may play roles in biasing the host immune response either for, e.g. TM7, or against, e.g. Methanomicrobia, cyathostomin infection. These data pave the way for future mechanistic studies aimed at identifying microbial factors linked to host susceptibility, and to manipulate the GI microbiota of horses (e.g. via dietary or probiotic interventions), in order to improve resistance to cyathostomins.

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El Temsayh, M.M., Ibrahim, I.R., Mossallam, S.F., Mahmoud, H., Abdel Bary, A., Abdel Salam, S.A., 2015. Evaluation of newly isolated probiotics in the protection against experimental intestinal trichinellosis. Vet. Parasitol. 214, 303–314.

Fernandez, J.A., Kittelman, C.W., Carney, E.K., Bolwell, C.F., Bermingham, E. N., Thomas, D.G., 2014. Faecal microbiota of forage-fed horses in New Zealand and the population dynamics of microbial communities following dietary change. PLoS One 9, e11284.

Fricke, W.F., Song, Y., Wang, A.J., Smith, A., Grinchuk, V., Mongodin, E., Pet, C., Ma, B., Lu, N., Urban Jr, J.F., Shea-Donohue, T., Zhao, A., 2015. Type 2 immunity-dependent reduction of segmented filamentous bacteria in mice infected with the gut pathogenic parasite Heligmosomoides polygyrus. Microbiome 3, 40.

Gause, W.C., Maizels, R.M., 2016. Macrotissue – helminths as active participants and partners of the microbiota in host intestinal homeostasis. Curr. Opin. Microbiol. 32, 14–18.

Giocomo, P., Zakrzewski, M., Croese, J., Su, X., Sotillo, J., McCann, L., Navarro, S., Mitreva, M., Krause, I., Loukas, A., Cantassisi, C., 2015. Experimental hookworm infection and escalating glutathione challenges are associated with increased microbial richness in celiac subjects. Sci. Rep. 5, 13797.

Giocomo, P., Zakrzewski, M., Jenkins, T.P., Xu, X., Al-Hallaf, R., Croese, J., de Vries, S., Grant, A., Mitreva, M., Loukas, A., Krause, I., Cantassisi, C., 2016. Changes in duodenal tissue-associated microbiota following hookworm infection and consecutive gluten challenges in humans with celiac disease. Sci. Rep. 6, 36797.

Glendinning, L., Nausch, N., Free, A., Taylor, D.W., Mutapi, F., 2014. The microbiota and helminths: sharing the same niche in the human host. Parasitology 141, 1255–1271.

Gómez, J.M., González-Megías, A., 2007. Long-term effects of ungulates on phytophagous insects. Ecol. Entomol. 32, 229–234.

He, X., McLean, J.S., Edlund, A., Youosef, S., Hall, A.P., Liu, S.Y., Dorenstein, P.C., Esquezani, E., Hunter, R.C., Cheng, G., Nelson, K.E., Lux, R., Shi, W., 2011. Cumulation of a humoral response to Toll TM7 phenotype required for both inflammation and epithelial parastatic lifestyle. Proc. Natl. Acad. Sci. U.S.A. 112, 244–249.

Hinney, B., Wirthler, N.C., Kyule, M., Miethe, N., Zessin, K.H., Clausen, P.H., 2011. Prevalence of helminths in horses in the state of Brandenburg Germany. Parasit. Res. 108, 1083–1089.

Holm, J.B., Sorobeeta, D., Kuepperich, P., Ramayo-Caldas, Y., Estelle, J., Ma, T., Madsen, L., Kristiansen, K., Svensson-Frej, M., 2015. Chronic Trichuris muris infection decreases diversity of the intestinal microbiota and concommitantly increases the abundance of Lactobacillus. PLoS One 10, e0125495.

Houln, A., Hayes, K.S., Bancroft, A.J., Worthington, J.J., Wang, P., Gencs, R.K., Roberts, I.S., 2015. Chronic Trichuris muris infection in C57BL/6 mice causes significant changes in gut microbiota and metabolism: Effects reversed by probiotics [P.eugene]. PLoS One 10, e0125945.

Hutchings, M.R., Kyriazakis, I., Papachristou, T.G., Gordon, I.J., Jackson, F., 2000. The herbivores’ dilemma: trade-offs between nutrition and parasitism in foraging decisions. Oecologia 124, 242–251.

Jensen, A.N., Meier, H., Molbak, L., Langkjær, M., Jensen, T.K., Angen, O., Martinussen, T., Klitgaard, B., Baggesen, D.L., Thamsborg, S.M., Roepstorff, A., 2011. The effect of a diet with fructan-rich chicory roots on intestinal helminths and microbiota with special focus on Bifidobacteria and Campylobacter in piglets at weaning. Animal 5, 851–860.

Klimworth, A., Pruesse, E., Schweer, T., Pielsh, J., Quast, C., Horn, M., Glickner, F.O., 2013. Evaluation of general 16s ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nuclear Acids Res. 41, e1.

Kreisinger, J., Bastien, G., Chastel, G., Marchesi, J., Perkins, S.E., 2015. Interactions in the porcine colon microbiota induced by the gastrointestinal nematode Trichinella spiralis. Parasite 8, S226–S228.

Li, R.W., Wu, S., Li, W., Huang, Y., Gasbarre, L.C., 2011. Metagenome plasticity of the gut microbiota following hookworm infection and escalating gluten challenges are associated with increased diversity of the gut microbiota. Nat. Microbiol. 7, 335–336.

Love, S., Murphy, D., Mellor, D., 1999. Pathogenicity of cyathostome infection. Vet. parasitol. 85, 113–121.

Lyons, E.T., Drudge, J.H., Tolliver, S.C., 2000. Larval cyathostomiasis. Vet. Clin. North. Am. Equine Pract. 16, 501–513.

Kuehatcher, R., Rehman, A., Lepage, P., Hellmg, S., Folsch, U.R., Schreiber, S., Ott, S.J., 2008. Intestinal TM7 bacterial phylogenies in active inflammatory bowel disease. J. Med. Microbiol. 57, 1569–1576.

Lee, S.C., Tang, M.S., Lim, Y.A., Choy, S.H., Kurtz, Z.D., Cox, L.M., Gundra, U.M., Cho, I., Bonneau, R., Blaser, M.J., Chua, K.H., Loke, P., 2014. Helminth colonization is associated with increased diversity of the gut microbiota. PLoS Negl. Trop. Dis. 8, e2880.

Leung, J.M., Loke, P., 2013. A role for IL-22 in the relationship between intestinal helminths, gut microbiota and mucosal immunity. Int. J. Parasitol. 43, 253–257.

Li, R.W., Li, W., Sun, J., Yu, P., Baldwin, R.L., Urban, J.F., 2016. The effect of helminth infection on the microbiomal composition and structure of the caprine abomasum microbiome. Sci. Rep. 6, 36797.

Li, R.W., Wu, S., Li, C.J., Schroeder, S.G., 2011. Metagenome plasticity of the porcine colon microbiota following hookworm infection and escalating gluten challenges in humans with celiac disease. Sci. Rep. 5, 13797.

Love, S., Murphy, D., Mellor, D., 1999. Pathogenicity of cyathostome infection. Vet. parasitol. 85, 113–121.
Manichanh, C., Rigottier-Gois, L., Bonnaud, E., Goupy, C., Petersen, E., Grune, L., Nalin, R., Jarrin, C., Chardon, P., Marteau, P., Roca, J., Dore, J., 2006. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut 55, 205–211.

Martinez-Fenez, G., Fuentes-Castro, B.E., Bautista-Garfas, C.R., 2011. The intraperitoneal inoculation of Lactobacillus casei in mice induces total protection against Trichinella spiralis infection at low challenge doses. Parasitol. Res. 109, 1609–1617.

Martinez-Fenez, G., Santiago-Rosales, R., Ramon Bautista-Garfas, C., 2009. Effect of Lactobacillus casei Shira strain intraperitoneal administration in CD1 mice on the establishment of Trichinella spiralis adult worms and on IgA anti-T. spiralis production. Vet. Parasitol. 162, 171–175.

Maruo, T., Sakamoto, M., Ito, C., Toda, T., Benno, Y., 2008. Adlercreutzia equihaeferiens gen. nov., sp. nov, an equal-producing bacterium isolated from human faeces, and enended description of the genus Eggerthella. Int. J. Syst. Evol. Microbiol. 58, 1221–1227.

Matthews, J.B., 2011. Facing the threat of equine parasitic disease. Equine Vet. J. 43, 126–132.

McAllister, T.A., Okine, E.K., Mathison, G.W., Cheng, K.-J., 1996. Dietary, environmental and microbiological aspects of methane production in ruminants. Can. J. Anim. Sci. 76, 231–243.

McKenney, E.A., Williamson, L., Yoder, A.D., Bilbo, S.D., Parker, W., 2015. Alteration of the rat cecal microbiome during colonization with the helminth Hymenolepis diminuta. Gut Microbes 6, 182–193.

Militodze, M.W., Hutchinson, G.W., 1990. Prevalence and abundance of equine strongyles (Nematoda: Strongylidae) in tropical Australia. J. Parasitol. 76, 487–494.

Mikaelyan, A., Thompson, C.L., Meuser, K., Zheng, H., Rani, P., Prille, R., Brune, A., 2017. High-resolution phylogenetic analysis of Endomicrobia reveals multiple acquisitions of endosymbiotic lineages by termite gut flagellates. Environ. Microbiol. Rep. 9, 477–483.

Morariu, S., Mederle, N., Badea, C., Darabus, G., Ferrari, N., Genchi, C., 2016. The prevalence, abundance and distribution of cyathostomins (small strongyles) in horses from Western Romania. Vet. Parasitol. 223, 205–209.

Murphy, D., Love, S. 1997. The pathogenic effects of experimental cyathostome infections in ponies. Vet. Parasitol. 70, 99–110.

Nielsen, M.K., Christensen, K.E., Tolliver, S.C., Collins, S.S., Lyons, E.T., 2010. Analysis of multiyear studies in horses in Kentucky to ascertain whether counts of eggs and larvae per gram of feces are reliable indicators of numbers of strongyles and ascarids present. Vet. Parasitol. 174, 77–84.

Nielsen, M.K., Reinemeyer, C.R., Donecker, J.M., Leatherwick, D.M., Marchiondo, A.A., Kaplan, R.M., 2014. Anthelmintic resistance in equine parasites–current evidence and knowledge gaps. Vet. Parasitol. 204, 55–63.

O'Donnell, M.M., Harris, H.M., Jeffery, I.B., Claesson, M.J., Younge, B., O'Toole, P.W., 2014. The influence of dietary carbohydrates on the hindgut microflora in horses and potential links to chronic laminitis. BMC Vet. Res. 8, 231.

Stratford, C.H., McGregor, B.C., Pickles, K.J., Matthews, J.B., 2011. An update on cyathostomins: anthelmintic resistance and diagnostic tools. Equine Vet. J. Suppl., 133–139.

Thomsen, L.E., Potekhivius, S., Bach Knudsen, K.E., Roepstorff, A., 2005. The influence of dietary carbohydrates on experimental infection with Trichuris suis in pigs. Parasitology 131, 857–865.

Reversa, D., Jorio, R., Klei, T.R., Kharchenko, V.A., Gawor, J., Ortono, D., Sparagan, O.A., 2007. New method for simultaneous species-specific identification of equine strongyles (Nematoda, Strongylida) by reverse line blot hybridization. J. Clin. Microbiol. 45, 2937–2942.

Sepehr, S., Kotolowski, R., Bernstein, C.N., Krause, D.O., 2007. Microbial diversity of infected and noninfected gut biopsy tissues in inflammatory bowel disease. Inflamm. Bowel Dis. 13, 675–683.

Shepherd, M.L., Swecker, J.W., Jensen, R.V., Ponder, M.A., 2012. Characterization of the faecal bacteria communities of forage-fed horses by pyrosequencing of 16S rRNA V4 gene amplicons. FEMS Microbiol. Lett. 326, 62–68.

Shepherd, M.L., Swecker, J.W., Jensen, R.V., Ponder, M.A., 2012. Characterization of the faecal bacteria communities of forage-fed horses by pyrosequencing of 16S rRNA V4 gene amplicons. FEMS Microbiol. Lett. 326, 62–68.

Shin, N.R., Whon, T.W., Bae, J.W., 2015. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol. 33, 496–503.

Slapeta, J., Dowd, S.E., Alain, A.D., Westman, M.E., Brown, G.K., 2015. Differences in the faecal microbiome of non-diarrhoeic clinically healthy dogs and cats associated with Giardia duodenalis infection: impact of hookworms and coccidia. Int. J. Parasitol. 45, 585–594.

St-Pierre, B., Cersosimo, L.M., Ishag, S.L., Wright, A.D., 2015. Toward the identification of metagenomic archaeal groups as targets of methane mitigation in livestock animals. Front. Microbiol. 6, 776.

Steelman, S.M., Chowdhary, B.P., Dowd, S., Suchodolski, J., Janeczka, J.E., 2012. Pyrosequencing of 16S rRNA genes in fecal samples reveals high diversity of hindgut microflora in horses and potential links to chronic laminitis. BMC Vet. Res. 8, 231.

Stratford, C.H., McGregor, B.C., Pickles, K.J., Matthews, J.B., 2011. An update on cyathostomins: anthelmintic resistance and diagnostic tools. Equine Vet. J. Suppl., 133–139.

Thomsen, L.E., Potekhivius, S., Bach Knudsen, K.E., Roepstorff, A., 2005. The influence of dietary carbohydrates on experimental infection with Trichuris suis in pigs. Parasitology 131, 857–865.

Traversa, D., Jorio, R., Klei, T.R., Kharchenko, V.A., Gawor, J., Ortono, D., Sparagan, O.A., 2007. New method for simultaneous species-specific identification of equine strongyles (Nematoda, Strongylida) by reverse line blot hybridization. J. Clin. Microbiol. 45, 2937–2942.

Tuomisto, H., 2010. A consistent terminology for quantifying species diversity? Yes, it does exist. Oecologia 164, 853–860.

Uhinger, C.A., 1991. Equine Small Strongyles - Epidemiology, Pathology, and Control. Comp. Cont. Educ. Pract. 13, 863–869.

van Klink, R., van der Plas, F., van Noordwijk, C.G., WallisDeVries, M.F., Olf, H., 2015. Effects of large herbivores on grassland arthropod diversity. Biol. Rev. Camb. Philos. Soc. 90, 347–366.

Winker, S., Woese, C.R., 1991. A definition of the domains Archaea, Bacteria and Eucarya in terms of small subunit ribosomal RNA characteristics. Syst. Appl. Microbiol. 126–132.