Effect of Infection with, and Treatment of, Sensitive and Resistant Strains of *Teladorsagia Circumcincta* on the Ovine Intestinal Microbiota

Craig A. Watkins,[a]# Dave J. Bartley,[a] Burcu Gündüz Ergün,[b]* Büşra Yıldızhan,[b] Tracy Ross-Watt,[b] Alison A. Morrison,[a] Maria J. Rosales Sanmartín,[b] Fiona Strathdee,[b] Leigh Andrews,[a] Andrew Free,[b]

[a]Moredun Research Institute, Edinburgh UK.

[b]School of Biological Sciences, University of Edinburgh, Edinburgh, UK.

Running Head: Microbiota-Helminth Interactions in Ovine Intestine

#Address correspondence to Craig A. Watkins, craig.watkins@moredun.ac.uk.

*Present address: Department of Biotechnology, Middle East Technical University, Ankara, Turkey

C.A.W. and D.B. contributed equally to this work.

ABSTRACT: 246 words

Text: 5292 words

ABSTRACT Nematodes are one of the main impactors on health, welfare and productivity of farmed animals. *Teladorsagia circumcincta* is arguably one of the most globally important nematode species in sheep. Control of these nematode infections is essential and heavily reliant on chemotherapy (anthelmintics), but this has been complicated by the development of anthelmintic resistance. In mammals the composition of the intestinal microbiota has been shown to have a significant effect on overall health. The interactions between host, microbiota and pathogens are complex and influenced by numerous factors. In this study, the interactions between *T. circumcincta* infections and microbial composition and abundance were investigated. In a preliminary study the intra- and inter-individual diversity and composition of the microbiota of grazing sheep was assessed in two distinct regions of the ovine intestinal tract, the terminal ileum (TI) and rectal contents. Additionally, the effects of experimental infection of sheep with two strains of *T. circumcincta* (anthelmintic resistant or sensitive) on the microbiota were assessed with and without anthelmintic (monepantel) administration. The inter-animal variability was greater in the TI compared to the rectal samples. However, the alpha-diversity (species richness) was significantly lower in the TI samples. In the experimental study, clear differences were observed between successfully treated animals and those sheep that were left untreated and/or those carrying resistant nematodes. Differences in microbiota between the four different experimental conditions were observed and potential predictive biomarkers were identified. In particular, a restoration of potentially beneficial *Bifidobacteria* sp. in successfully-treated animals was observed.

IMPORTANCE Roundworms are one of the main impactors on health, welfare and productivity of farmed animals. The roundworm *Teladorsagia circumcincta* is arguably one of the most globally important in sheep. Control of these roundworm infections is essential and heavily reliant on chemotherapy, but this has been complicated by the development of drug resistance. In mammals including humans, the composition of the intestinal microbiota has shown significant effects on health. Interactions between roundworm infections and microbial composition and abundance were investigated. The significance of this current work is that: i) the within- and between-animal variability in microbial composition was assessed. ii) the interaction between roundworm and microbiota of sheep with and without drug administration were evaluated. The use of resistant and sensitive helminth strains was compared. iii) successful removal of pathogenic roundworms results in a higher abundance of specific microflora indicating those organisms that are associated with a healthy gut microflora.

INTRODUCTION

Within the gastrointestinal tract of mammals including humans, micro-organisms form a complex relationship with their hosts, be they commensal symbionts, pathobionts or pathogenic, as a consequence of co-evolution to provide homeostasis in the host intestinal tract (1-3). In healthy animals, the microbiota provides essential nutrients and protection against the colonisation by pathogenic species (4). However, constant regulation is required to prevent breakdown of these essential relationships between microorganisms and mammals. Where commensal bacteria, invading pathogenic bacteria and/or opportunistic pathobionts colonise similar ecological niches, competition for available nutrients occurs (5). Such interactions can lead to perturbations in the microbial communities leading to

dysfunction of the gastro-intestinal tract (6). The response of commensal microbiota therefore needs to be robust to out-compete and deny the incoming, infectious agent from colonising and proliferating (7, 8). However, when the microbiota becomes disrupted, unstable or damaged (for example due to antibiotics, pathogenic infections, physiological and/or environmental stresses), the ability of the commensal microorganisms to maintain a competent resistance to colonisation by pathogenic organisms can be compromised. These invading pathogenic microorganisms have their own arsenal to counteract the defensive mechanisms developed by the commensal microflora and the host, including physiological and immunological responses (5, 9-12). Gastrointestinal parasites provide just the type of disruption that results in breach of physical boundaries, inflammation and/or modulation of the immune response of the host, offering an opportunity for invading microorganisms to colonise (13). A number of factors exist which impact on the helminth-host microbiota interaction including; age, sex, nutrition and immune status (14, 15). Together, the host, its microbial community and the presence of parasites are known to shape both the microbial landscape and host health (3).

The impact of gastro-intestinal parasites on the microbiota has not been extensively studied in mammals, let alone specifically in sheep and other ruminants (3). However, it is clear that the perturbation of the gastrointestinal tract can be pronounced when pathogenic micro-organisms are introduced, either naturally or experimentally (4, 13, 16, 17). *Teladorsagia circumcincta* is a pathogenic parasitic nematode that can cause severe gastroenteritis. It is one of the most common gastrointestinal nematodes in sheep worldwide within the temperate zone (18), causing considerable morbidity and occasionally mortality in heavily infected animals (19). Clinical symptoms are attributed to both excretory-secretory (ES) products

94 derived from the invading nematode and to histopathological tissue damage (20, 21).
95 Control of *T. circumcincta* remains a challenge, with anthelmintics being the
96 mainstay of control although the use of some treatments has become restricted due
97 to increased, and widespread, multi-drug resistance developed by *T. circumcincta*
98 (22, 23).

99 The pathogenic nature of gastro-intestinal round worms has a significant
100 effect on the microbiota of the gut (3). However, quantifying precisely the dynamics
101 of the microbial community has not been possible until the advent of culture-
102 independent 16S rDNA-based sequence analysis and whole genome shotgun high-
103 throughput sequencing (24). In this study, Illumina MiSeq technology was used to
104 sequence amplicons of the V4 region of the 16S rRNA gene, to determine the
105 inherent variability of the gut microbiota between samples obtained from two different
106 areas of the intestinal tract (rectum and terminal ileum) in six identically-treated
107 sheep. The same approach was then used to define the effect of infection with, and
108 treatment of, sensitive and resistant strains of *T. circumcincta* on the ovine intestinal
109 microbiota, via rectal faecal (RF) composition.

110

111 **RESULTS**

112 **Inherent variation of the ileal and rectal microbiotas.** In this initial study,
113 samples from two distinct regions of the gut, the terminal ileal contents (TIC) and
114 rectal faeces (RF), in six identically managed sheep (TIC 1-6 and RF 1-6
115 respectively), were analysed to investigate intra- and inter-sheep variation of the
116 bacterial communities. Good's coverage estimates on sequence data obtained from
117 these 12 samples exceeded 99% for all samples, demonstrating that the sequencing

depth was adequate to capture true diversity (minimum ~119,000 sequences reads per sample; Table S1). Consistent with alpha rarefaction curves of operational taxonomic unit (OTU) richness (Fig. 1A), Shannon diversity (mean ± SD at maximum rarefaction depth) was lower in the TIC samples (5.054 ± 1.595) than the RF samples (7.869 ± 0.252), with a corresponding reduction in Pielou's evenness (0.668 ± 0.262 compared to 0.951 ± 0.048). This shows that there is a significantly lower diversity (Welch's t-test, p = 0.0006) of the microbial communities found in the TIC compared to the rectum (Fig. 1A), with the former dominated by a few abundant OTUs. However, alpha diversity was also more variable in the TIC samples than in the RF samples, as illustrated by the error bars in Fig. 1A. Consistent with this, compositional variability, shown by Principal Coordinates Analysis (PCoA) ordination based on Bray-Curtis similarity (S), was greater between the TIC samples (mean S = 38.3; SEM= 6.21) than between those taken from the rectum (Fig. 1B). The RF samples showed greater similarity in composition (mean S = 60.9; SEM = 1.47) than the TIC samples, a difference shown to be highly significant by PERMDISP analysis of multivariate dispersions (F = 12.89; P = 0.003). As illustrated by the PCoA plot, the tightly-clustered RF samples were also compositionally distinct from the more variable TIC samples, verified by a PERMANOVA test (Pseudo-F = 13.62; P =0.004). Vectors illustrated in Fig. 1B show that the abundances of the major phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were all closely correlated (r > 0.9) with the axes of the plot. Elevated abundances of *Bacteroidetes* were associated with the RF samples, while *Proteobacteria* and *Firmicutes* were associated with the TIC, but in proportions which vary between the individual animals.

Relative abundance plots of bacterial Orders showed the different taxonomic distribution patterns between TIC and RF samples (Fig. S1). Twelve different Orders

of bacteria were identified in the TC and RF samples at a limit of >4% abundance. *Clostridiales* was the dominant order for TIC samples (35-70%) except TIC-5 (18%), where *Enterobacteriales* was the dominant population of bacteria (76% of the total). For RF samples *Clostridiales* (34-43%) and *Bacteroidales* (38-50%) made similar contributions to the bacterial abundance. Average relative abundance (mean ± SD) of *Enterobacteriales* was generally higher in TIC samples (12.5 ± 6.77%, TIC-5 excluded) than RF samples (0.64 ± 0.55%). The relative abundance and distribution of the five most abundant bacterial OTUs in each of the TIC and RF samples was analysed and illustrated using a heatmap (Fig. 2), indicating the very different profiles of microbiota obtained from the two different sampling sites. This analysis suggests that OTUs from the Genus *Ruminococcaceae* UCG-005 (25), various *Bacteroidales* OTUs, and OTUs from the Genera *Campylobacter* and *Akkermansia* were over-represented in the RF samples compared to the ileal samples. In contrast, OTUs corresponding to various Genera from the Order *Clostridiales*, the species *Escherichia coli*, the Genus *Turicibacter*, the Order *Victivallales*, the Family *Bifidobacteriaceae* and the Genus *Methanobrevibacter* were better represented in the TIC samples. A single OTU (denovo19121) assigned to the genus "*Candidatus Hepatincola*" (26) was abundant in one sheep TIC sample (TIC5), but poorly represented in all other samples. Full details of the percentage abundance and taxonomy of the 15 most abundant OTUs in each sample, including those shown in Fig. 2, are presented in Table S2.

These experiments showed that the inherent variability of the microbiota between sheep was much less for RF samples than for TIC samples. Therefore, RF samples were collected to investigate the effect of treatment for sensitive versus resistant *T. circumcincta* on microbiota composition.

Effect of infection with, and treatment of, sensitive and resistant strains of *T. circumcincta*. Sequence data were obtained on the RF samples from four groups of five sheep (n = 20) infected with sensitive or resistant strains of *T. circumcincta* and either treated with monepantel (MPTL) or left untreated (Table 1). Good's coverage estimates were ≥ 97% for all samples, demonstrating that the sequencing depth was adequate to capture true diversity (minimum ~118,000 sequences reads per sample; Table S1). Observed species rarefaction curves for the four groups of infected sheep (susceptible treated, ST; susceptible untreated, SUT; resistant treated, RT; resistant untreated, RUT) showed high diversity within these faecal samples (Fig. 3A), consistent with the faecal samples analysed in the first experiment. However, Shannon diversities (mean ± SD at maximum rarefaction depth) for those sheep left untreated (SUT, RUT) and those not successfully treated (RT) for worm infections were higher (8.694 ± 0.229) than those for samples representing sheep that had been successfully treated (ST) (8.434 ± 0.192), a difference which, though small, is significant at the 5% level (Welch's t-test, p = 0.037). This suggests that there is a slight but detectable increase in microbial species diversity in sheep with active nematode infections in this experiment.

At the Order level of taxonomy, the composition of the RF samples from all four treatment groups were dominated by *Bacteroidales* (30-49%) and *Clostridiales* (27-48%; Fig. S2). There were also significant but smaller abundances of *Spirochaetales* (1-4%) and *Campylobacterales* (0.4-6%). To investigate finer-scale similarity between the four different groups, Bray-Curtis similarity based on OTU abundances was visualised by PCoA ordination (Fig. 3B). This analysis revealed a large degree (72%) of inter-individual variability unrepresented in the first two PCoA axes, which nevertheless suggested some compositional differences associated with

193 the four treatment groups, and showed significant correlations with the abundances
194 of the phyla *Bacteroidetes*, *Firmicutes* and *Proteobacteria* ($P \leq 0.003$) and
195 *Actinobacteria* ($P = 0.023$). Despite the observed variability, those sheep groups with
196 ongoing nematode infections (SUT, RT, RUT) showed a significant difference in
197 composition from those treated successfully (ST; PERMANOVA Pseudo-F = 1.594,
198 $P = 0.016$). No such statistical significance was detected for the factors of drug
199 treatment or *T. circumcincta* strain ($P > 0.18$). To isolate the variation due to the pre-
200 defined treatment groups in the experimental set-up, a Canonical Analysis of
201 Principal coordinates (CAP) ordination plot based on these groups was used. The
202 CAP plot (using the same OTU-level similarity data as in Fig. 3B) showed that there
203 was a clear separation of the successfully-treated group (ST) from the untreated or
204 resistant groups (SUT, RT, RUT) on the primary CAP1 axis, while the three latter
205 groups are separated from each other on the CAP2 axis (Fig. 4A). These treatment
206 group-specific separations are associated with high eigenvalues (0.939 and 0.672 for
207 CAP1 and CAP2 respectively), and a significant first canonical correlation ($P =$
208 0.005) (27). As expected, the vectors in Fig. 4A show that there is a strong
209 correlation ($r = 0.626$; $P = 0.002$) between worm burden and the sample groups as
210 separated on the CAP1 axis. There is also a strong positive correlation between the
211 abundance of OTUs assigned to the Phylum *Actinobacteria* and the ST group ($r =$
212 0.651; $P = 0.001$), while the separation of the other three treatment groups on the
213 CAP2 axis is correlated with the abundance of the Phylum *Proteobacteria* ($r = 0.805$;
214 $P < 0.0001$), although driven mainly by a single RUT sample (Fig. S2).

215 To confirm the effects detected in faecal samples, we analysed microbial DNA
216 present in terminal ileal mucosal scraped (TIMS) samples taken from the same
217 animals at post mortem. As the yield of microbial DNA from these samples was

much lower than from faecal pellets or TIC, lower numbers of sequences per sample were obtained (Table S1). However, rarefaction curves (Fig. S3A) and Good's coverage estimates (>94% for all samples) suggested that the low microbial diversity in these samples was covered adequately at this sequencing depth. OTU richness and Shannon diversity were relatively low in these samples, consistent with our previous analysis of the terminal ileal contents (TIC). However, there was a small but significant (Welch's t-test; p = 0.015) decrease in diversity in those samples from sheep treated with monepantel compared to the untreated sheep: Shannon diversities (mean ± SD) were 5.934 ± 1.039 in treated animals compared to 6.972 ± 0.480 in untreated animals.

Sixteen different Orders of bacteria were identified in the TIMS samples at a limit of >4% abundance (Fig. S3B), including sequences corresponding to the 16S rRNA gene from the remnant chloroplast (apicoplast) of the apicomplexan parasite *Eimeria*. The most abundant Order across the sample set was *Clostridiales* (12-64%). Although large inter-sample variability was observed, similar to the TIC microbiota samples, a PERMANOVA test showed that there was a significant compositional difference between successfully treated and nematode-infected sheep (Pseudo-F = 1.073; P = 0.027). A CAP analysis of Bray-Curtis similarities based on OTU abundances (Fig. 4B) indicated that, as for the faecal samples, the sheep with successfully-treated nematode infections clustered separately from those with untreated or resistant infections (eigenvalues = 0.943 and 0.881; trace statistic P = 0.008). There were also strong correlations between the CAP axes and worm burden (r = 0.696; P = 0.0003), as expected, and the abundance of *Bacteroidetes* (r = 0.753; P < 0.0001) and *Fibrobacteres* (r = 0.696; P = 0.0003), with samples derived from the untreated sheep (SUT and RUT). Weaker but significant correlations were also

observed with the abundance of *Firmicutes* (r = 0.491; P = 0.014) and *Actinobacteria* (r = 0.486; P = 0.015) with ST samples (Fig. 4B).

Identification of biomarkers defining infected and uninfected sheep. To evaluate structural differences in the constituent microbial communities of infected and uninfected sheep, a heatmap illustrating the distribution of the five most abundant OTUs in the RF samples was constructed (Fig. 5). The vast majority of these OTUs were assigned to taxa within the Order *Bacteroidales*, with individual OTUs assigned to the genera *Ruminococcaceae* UCG-005, *Campylobacter*, *Helicobacter* and *Methanocorpusculum* also represented. Full details of the percentage abundance and taxonomy of the 15 most abundant OTUs in each sample, including those shown in Fig. 5, is presented in Table S3. Although some minor differences between individual samples at the OTU level were apparent, differences specific to the four treatment groups were not obvious among this subset of abundant OTUs. The TIMS samples from the same sheep exhibited much more variability in the abundant OTUs between animals, and a preponderance of OTUs from the Phylum *Firmicutes*. Variation in most of these OTUs between the four treatment groups was not readily apparent, but a single *Bifidobacterium* OTU (denovo3328) was largely restricted to the successfully-treated ST group (Fig. S4; Table S4).

LEfSe analyses (28) at the Genus level were therefore employed in order to detect genera elevated specifically in both sets of samples from those animals either infected or uninfected with *T. circumcincta.* In the RF samples (Fig. 6A), three genera were identified as significant biomarkers for infected sheep (groups SUT, RUT and RT), along with the Families *Microbacteriaceae* and *Mycoplasmataceae*, while in the uninfected sheep (group ST), eleven different taxa including three

genera from the family *Ruminococcaceae*, the Order *Myxococcales* and the Genera *Aeriscardovia* and *Bifidobacterium* were significantly elevated (LDA score > 2.5). The p values of these associations varied between 0.001 and 0.05 (Table S5). When the data from the TIMS samples were analysed (Fig. 6B), a larger set of taxa were identified as biomarkers for infected sheep (genera including *Rikenellaceae* RC9, *Treponema* 2, *Prevotella* 1 and *Fibrobacter*, plus the entire Class *Deltaproteobacteria*), while the Genus *Sharpea*, the Family *Bifidobacteriaceae* and the Genus *Acetitomaculum* were identified as biomarkers for the uninfected group (LDA score >3.5). The p values of these associations varied between 0.0002 and 0.05 (Table S5). This suggests that, while the taxa which drive the differences seen at the Genus level between uninfected and infected sheep differ depending on whether RF or TIMS samples are analysed, specific elevation of several biomarkers is seen in uninfected animals at each sampling site, including sequences derived from the *Bifidobacteriaceae* at both sampling sites.

DISCUSSION

Few studies have examined the host-microbiota-helminth relationship in animal hosts. To address this knowledge gap, the primary aim of this study was to investigate the species compositional changes in intestinal microbiota resulting from *T. circumcincta* infection and subsequent anthelmintic administration in sheep. However, it is well documented that factors such as age, diet, environment and sampling site are significant sources of inter-animal variation in microbial composition (29). To investigate this inherent variation between sheep, a preliminary trial was undertaken on the microbiota of the terminal ileum and faeces derived from

292 age-matched lambs that were all grazed under similar conditions. By comparing the
293 inherent variation between RF samples and TIC samples, our results suggest that
294 there was significantly less variability of the microbiota between identically treated
295 sheep in RF than in samples collected from the terminal ileum. Further, results
296 showed that there were significant differences in microbial diversity between the RF
297 and TIC samples with a reduction in alpha diversity in the latter. These findings were
298 consistent with previously published results which showed that bacterial abundance
299 in the large intestine was greater than in the small intestine and that the microbiota
300 within the lumen of the terminal ileum is less diverse than in the rectum (29, 30).
301 Bacterial taxonomic distribution patterns were also different between TIC and RF
302 samples. *Clostridiales* was the dominant Order for all but one sample in the TIC,
303 whereas the *Clostridiales* and *Bacteroidales* shared the dominant position in RF
304 samples (Fig S1).

305 Although faeces are not necessarily the optimal correlate for examining the
306 microbiota within a specific zone in the intestine (29, 31, 32), faecal samples were
307 selected for the main study (to identify compositional differences of microbial
308 communities between treated and untreated sheep, infected with resistant or
309 sensitive *T. circumcincta*) due to the more consistent sample microbiota composition
310 within each treatment group. In this study, the successful treatment of the infection
311 did appear to have a significant impact on the diversity and composition of the faecal
312 microbiota. The diversity of the microbiota of sheep successfully treated (ST) was
313 significantly lower than that of the three groups of sheep that maintained the parasitic
314 infection (SUT, RT, RUT). There are also compositional differences in the faecal
315 microbiota of successfully-treated sheep, which are strongly correlated with a
316 reduction in worm burden (as expected), and an increase in the abundance of the

317 Phylum *Actinobacteria*. However, there was little difference in the diversity and
318 abundance of the commensal microorganisms between the untreated sheep infected
319 with the two different strains (resistant and sensitive) of parasitic nematode in the
320 absence of treatment.

321 As a result of the large inter-sample variation between samples from the TIC
322 (when compared to RF samples), the experimental design was modified to include
323 the TIMS from nematode-infected sheep. This addition to the experimental design
324 expanded the study to include an assessment of epithelial-attached microbiota in
325 comparison to RF microbiota. The results derived from these latter samples should
326 be taken with caution, as they required a different DNA extraction technique and the
327 microbial DNA yield was much lower compared to the other samples in this study.
328 However, the results of the 16S rDNA sequencing suggested that the samples were
329 of low bacterial diversity and of very different composition from the faecal samples,
330 with large inter-sample variation (similar to the TIC samples). This was expected as
331 the resident gut microbes are known to be less abundant than and taxonomically-
332 distinct from those which pass through the gut (29). Interestingly, microbial alpha-
333 diversity of samples derived from the TIMS of the ST and RT groups was
334 significantly decreased compared to those from the SUT and RUT groups,
335 suggesting that in this location, MPTL treatment affects microbial diversity
336 irrespective of whether the parasite infection is successfully treated or not. However,
337 compositional (beta) diversity was affected by whether the infection was maintained,
338 and these differences were strongly correlated to worm burden, a result consistent
339 with that obtained from the RF samples. Unlike in faeces, where worm burden was
340 strongly negatively correlated with the abundance of *Actinobacteria*, the abundances
341 of *Bacteroidetes* and *Fibrobacteres* were positively correlated with worm burden in

342 TIMS samples, while a negative correlation with the abundance of *Firmicutes* was
343 observed.

344 Although different sets of biomarkers for parasite-infected and uninfected
345 sheep were identified between the TIMS and RF datasets, some biomarkers were
346 consistently associated with both sets of data. Species from the physiologically-
347 uncharacterised *Rikenellaceae* RC9 gut group, which are abundant in rumen and
348 faecal samples from other mammals (25, 33), were consistently elevated in both
349 TIMS and RF samples from infected sheep. OTUs mapping to this Genus were also
350 abundant in both sample sets (Figs. 5 and S4). Genera within the *Prevotellaceae*,
351 known to be abundant in the rumen of sheep (34), were also biomarkers for
352 nematode infection in both datasets. In contrast, members of the *Bifidobacteriaceae*
353 were elevated in both TIMS and RF (represented by the genera *Aeriscardovia* and
354 *Bifidobacterium*) of successfully treated sheep (ST). In the ileal epithelium, a single
355 *Bifidobacterium* OTU (denovo3328) is clearly more abundant in the absence of *T.*
356 *circumcincta* infection (Fig. S4). *Bifidobacteria* are abundant members of the
357 gastrointestinal tract in mammals, and are thought to be acquired via maternal
358 transmission in milk (35), although they have not previously been studied in the
359 gastrointestinal tract of sheep. In humans, they are generally thought to be a marker
360 of good gut health (35). It is also noteworthy that *Sharpea* species identified as the
361 most significant biomarker for uninfected sheep in TIMS samples, also comprised
362 one of the most abundant OTUs in these samples (denovo4171; Fig. S4). *Sharpea*
363 has been identified as a Genus enriched in the rumen of low-methane yield sheep,
364 where it is thought to be associated with rapid fermentation (36).

365 Although the direct effects of MPTL on the microbiota of sheep are not well
366 characterised, in this study we were unable to detect differences in the RF samples

367 due to MPTL application *per se*. However, there was a small but significant
368 decrease in diversity of the microbiota in TIMS samples from sheep treated with
369 monepantel compared to the untreated sheep, and a compositional effect additional
370 to that caused by the successful eradication of helminths. This effect may be a
371 direct effect on the microbial community but, as with other anthelmintics, the effect
372 may be immunological and/or physiological (37).

373 There is some inconsistency in the literature regarding the effect of helminth
374 infection on microbial alpha-diversity in the gut. The increase in microbial diversity
375 described here is consistent with a study of helminth-colonised humans in Malaysia
376 (38), whereas controlled studies of *Trichuris muris* infection in mice (39, 40) suggest
377 an association between helminth infection and decreased microbial diversity. There
378 is also divergence between these studies and our results in terms of the beta-
379 diversity changes associated with helminth infection and clearance, which are
380 probably due to the different mammalian hosts, parasite species and conditions
381 (natural versus experimental) studied. In the Malaysian human study, helminth
382 infection was associated with an increase in *Paraprevotellaceae*, especially when
383 *Trichuris* spp. were present (38). In contrast, the experimental mouse studies with
384 controlled and treated *T. muris* infection both detected a reduction in members of the
385 *Prevotellaceae* associated with infection, and in one case suggested transient
386 elevation of *Bifidobacterium*, followed by *Lactobacillus*, in infected animals (39, 40).
387 Treatment and clearance of the parasitic infection with the drug mebendazole
388 enabled partial restoration of microbial alpha- and beta-diversity in infected animals,
389 providing evidence that the parasitic infection was responsible for the induction and
390 maintenance of the altered microbiota (39). While universal changes in richness and
391 taxonomic composition due to helminth infection across multiple hosts and parasite

392 species are unlikely to be found, it is established as a result of these previous
393 studies and our work that these parasites cause changes in the gut microbiota in
394 murine, ovine and human systems, which can be resolved by drug treatment.

395 In conclusion, we find microbiota variation in the ovine gut to be niche
396 specific, as the microbiota sampled from either TIC or TIMS samples not only have
397 reduced richness but are also much more variable between individual sheep when
398 compared to the RF sample. However, in this study biomarkers were identified
399 common to both the TIMS samples and the RF samples distinguishing sheep with
400 helminth infection from those that cleared infection using anthelmintic treatment. In
401 future studies, longitudinal investigation of changes in the microbiota of artificially or
402 naturally infected animals with parasites and/or pathogenic bacteria will help clarify
403 the causal relationship between infection, treatment and microbial composition.
404 However, it is important to be aware that other factors may also play a part in
405 alterations of the microbiota, including the effect of infection on metabolites (41).
406 Further work will consider how the introduction of helminths can also alter the
407 metabolome profiles within the intestine and the effect on the immune system (3,
408 42).

409 Understanding the dynamic mechanisms required to sustain and control the
410 balance between pathogenic, beneficial and commensal microbial communities
411 within the gastrointestinal tract will enable progress in the discovery and
412 development of therapeutic reagents based on beneficial microorganisms and/or
413 their excreted/secreted products to restore dysfunctional microbiotas or prevent the
414 destabilisation of the microbiota by invading pathogens or opportunistic commensal
415 bacteria (43, 44). These novel microbiota-based treatments will be particularly
416 relevant to the problems of multidrug and anthelmintic resistance.

MATERIALS AND METHODS

Inherent variation of the gut microbiota between identically treated sheep from two different sampling areas of the gastrointestinal tract - the terminal ileum and rectum. Six one-year-old parasite-naïve lambs were grazed for four weeks on a paddock infected with a mixed population of ovine nematodes including *T. circumcinta* and *Haemonchus contortus.* Following grazing the lambs were re-housed for four weeks and necropsied as per (45). At post mortem faeces (1-2 g) were taken from the rectum, as well as the TIC. Samples were transferred to ice and stored at -80^{o}C before processing.

Effect of treatment on sensitive and resistant parasitic nematodes on the gut microbiota. The experimental design is outlined in (46). In brief, twenty parasite-naïve lambs of 8-9 months old were raised in containment, free from adventitious nematode contamination. These sheep were divided into two groups of ten animals and experimentally challenged *per os* with 7,000 *T. circumcincta* infective larvae (L_3) of either a monepantel (MPTL)-sensitive (S) parental strain (designated MTci7) or an MPTL-resistant (R) strain that had been artificially derived from the parent strain (designated MTci7-12; Table 1).

Twenty-eight days post-infection the two groups were subdivided into groups of five animals and either left untreated (UT) to act as controls, or weighed and dosed orally by syringe at the manufacturer's recommended dose rate with MPTL (2.5 mg kg^{-1} body weight; T). The four groups MTci7 untreated, MTci7 MPTL-treated MTci7-12 untreated and MTci7-12 MPTL-treated are designated as SUT, ST, RUT and RT respectively (Table 1). All of the animals were necropsied seven days post-

441 treatment. Abomasa were collected and processed for worm burden estimation (45).
442 Total worm burdens were used to confirm treatment outcome (Table 2).

443 RF samples were collected and stored at -80°C before processing. TIMS
444 samples were also collected from these sheep. Briefly, terminal ileum tissue was
445 thawed on ice and opened longitudinally, to reveal the mucosal layer of the lumen.
446 The lumen was washed with PBS to remove debris before the mucosal layer was
447 scraped off using a microscope slide, transferred to a 50 ml tube and vortexed for 30
448 seconds. A volume of 400 µl of scraped sample was added to 4.60 ml of RNAlater®
449 and stored at -80°C.

450 All experimental procedures described were approved by the Moredun
451 Research Institute Experiments and Ethics Committee and performed under the
452 legislation of a UK Home Office License (reference PPL 60/03899), complying with
453 the Animals (Scientific Procedures) Act 1986.

454 **DNA extraction.** Microbial genomic DNA was purified with a MO BIO
455 PowerFecal™ DNA Isolation Kit, according to the manufacturer's protocols. Briefly,
456 0.25 g of faeces or 0.25 ml of TIC were homogenized, using bead beating, to
457 facilitate microbial cell lysis. The total microbial genomic DNA was eluted in DNA
458 elution buffer. Additionally, DNA was extracted from TIMS samples using the Qiagen
459 Tissue/Blood kit following the manufacturer's protocol after bead beating as
460 described above. Genomic DNA was quantified using NanoDrop™
461 spectrophotometry.

462 **Amplification of bacterial 16S rRNA genes.** The bacterial 16S rRNA gene
463 V4 region was amplified for Illumina MiSeq sequencing via a barcoded-adapter
464 based PCR approach (47). PCR reactions for the bacterial 16S rRNA gene V4

region contained 1x *Taq* buffer plus additional $MgCl_2$ (final concentration of 2.5 mM), 0.2 mM of each of the four dNTPs, 0.25 μM of each primer, 0.05 U μl^{-1} *Taq* DNA polymerase, and 1 ng μl^{-1} template DNA in a total volume of 25 μl with PCR grade water, set up under contaminant-free conditions (48). For each amplification reaction the same forward primer (515F) together with a different barcoded reverse primer (806R) was used (the reverse primer sequences differed only at the barcode region (47)). Amplification of the bacterial 16S rRNA gene V4 region was as follows: 94°C for 3 min; followed by 25 cycles at 94°C for 45 sec, 50°C for 60 sec, 72°C for 90 sec; followed by a single cycle of 72°C for 10 min. For TIMS samples, which contained a low amount of target DNA, 35 cycles of amplification via the above protocol were used. Because of this extended amplification protocol, a PCR negative control showed the presence of amplified sequences corresponding to possible contamination: two such OTUs assigned to the genus *Staphylococcus* and the species *Bradyrhizobium elkanii* were later removed from the dataset as a result.

Size and concentrations of PCR amplicons were analysed by agarose gel electrophoresis. The PCR amplicons were gel-purified using a Wizard® Gel and PCR Clean-Up System (Promega, UK) and quantified using a Quant-iT PicoGreen ds DNA Assay Kit (Life Technologies, UK) before pooling in equimolar quantities for Illumina sequencing.

Preparation of PCR amplicons for Illumina sequencing. Pooled PCR amplicons were sequenced at Edinburgh Genomics, University of Edinburgh. Paired-end sequencing (2x250 bp) was run on the Illumina MiSeq platform and ~11M raw read clusters were generated. Three separate sequencing primers were used for sequencing; two used to read sequences from either end: Read 1 primer

(TATGGTAATT GT GTGCCAGCMGCCGCGGTAA) to yield the 5' read; Read 2 primer (AGTCAGTCAG CC GGACTACHVGGGTWTCTAAT) to yield the 3' read and a third as the indexing primer (ATTAGAWACCCBDGTAGTCCGGCTGACTGACT) used to read the barcode sequence (47).

Data processing and filtering. Raw Illumina sequence reads were analysed using the QIIME workflow (49). Demultiplexed forward and reverse sequence reads were paired with a minimum overlap of 200 bp for maximum accuracy (50) before quality filtering with a minimum quality score of 20. As the bacterial 16S rRNA gene V4 region is universally bigger than 250 bp, short reads below this cut-off were removed using the Python script filter_short_reads.py (https://gist.github.com/walterst/7602058). Chimeric sequences were also removed using UCHIME (51) with version 128 of SILVA (52) as the reference database. Total numbers of quality-filtered sequences were ~2.4M for the inherent variation experiment (n = 2x6) and ~4.4M (RF) or ~446K (TIMS) for the nematode infection experiment (n = 4x5). *De novo* OTU picking at 97% similarity and taxonomic assignments against SILVA 128 were performed using Uclust; OTU representative sequences which failed to align with the dataset using PyNast (53) and singleton OTUs were removed before OTU tables were generated. OTU data were summarized by taxonomic ranks and via heat maps, and alpha rarefaction curves for observed species and Shannon diversity were generated using QIIME.

Multivariate statistical analysis. Relative abundance OTU data were imported into Primer 6 Version 6.1.12 (Primer-E, Ivybridge, UK) and used to generate Bray-Curtis similarity matrices. PCoA and CAP ordinations were performed on the Bray-Curtis similarity matrices in Primer 6. Additional multivariate statistical analyses, PERMANOVA and PERMDISP (27) were performed with the

514 PERMANOVA+ add on package for Primer 6. PERMANOVA and PERMDISP were
515 used to test for significant differences in the distribution and dispersion of sample
516 groups based on Bray-Curtis similarities. Linear discriminant analysis effect size
517 (LEfSe) analysis (28) was performed on relative abundance taxonomy tables
518 generated in QIIME using the LEfSe online Galaxy tool
519 (http://huttenhower.sph.harvard.edu/galaxy/).
520 Accession number: Sequencing data and metadata were uploaded to the European
521 Nucleotide Archive (ENA) at the European Bioinformatics Institute (EBI); study
522 accession number PRJEB24185. http://www.ebi.ac.uk/ena/data/view/PRJEB24185
523

524 **SUPPLEMENTAL MATERIAL**

525 Supplemental material for this article is as follows:
526 **TABLE S1**
527 **TABLE S2**
528 **TABLE S3**
529 **TABLE S4**
530 **TABLE S5**
531 **FIG S1**
532 **FIG S2**
533 **FIG S3**
534 **FIG S4**
535

536

ACKNOWLEDGEMENTS

We gratefully acknowledge funding from The Scottish Government's Rural and Environment Science and Analytical Services Division (RESAS) and the M.Sc. Biotechnology programme of the University of Edinburgh. B.Y. was supported by the Erasmus Programme, and M.J.R.S. was supported by the Xunta de Galicia under the Galeuropa programme. We are also grateful to the Bioservices Division, Moredun Research Institute, for expert care and assistance with animals.

REFERENCES

1. Bancroft AJ, Hayes KS, Grencis RK. 2012. Life on the edge: the balance between macrofauna, microflora and host immunity. Trends Parasitol 28:93-8.
2. Glendinning L, Nausch N, Free A, Taylor DW, Mutapi F. 2014. The microbiota and helminths: sharing the same niche in the human host. Parasitology 141:1255-71.
3. Zaiss MM, Harris NL. 2016. Interactions between the intestinal microbiome and helminth parasites. Parasite Immunol 38:5-11.
4. Li RW, Wu S, Li W, Huang Y, Gasbarre LC. 2011. Metagenome plasticity of the bovine abomasal microbiota in immune animals in response to *Ostertagia ostertagi* infection. PLoS One 6:e24417.
5. Kamada N, Chen GY, Inohara N, Nunez G. 2013. Control of pathogens and pathobionts by the gut microbiota. Nat Immunol 14:685-90.
6. Buffie CG, Pamer EG. 2013. Microbiota-mediated colonization resistance against intestinal pathogens. Nat Rev Immunol 13:790-801.

7. Hooper LV, Macpherson AJ. 2010. Immune adaptations that maintain homeostasis with the intestinal microbiota. Nat Rev Immunol 10:159-69.

8. Hooper LV, Littman DR, Macpherson AJ. 2012. Interactions between the microbiota and the immune system. Science 336:1268-73.

9. Giel JL, Sorg JA, Sonenshein AL, Zhu J. 2010. Metabolism of bile salts in mice influences spore germination in *Clostridium difficile*. PLoS One 5:e8740.

10. Le Bouguenec C, Schouler C. 2011. Sugar metabolism, an additional virulence factor in enterobacteria. Int J Med Microbiol 301:1-6.

11. Crosa JH, Walsh CT. 2002. Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. Microbiol Mol Biol Rev 66:223-49.

12. Kamada N, Kim YG, Sham HP, Vallance BA, Puente JL, Martens EC, Nunez G. 2012. Regulated virulence controls the ability of a pathogen to compete with the gut microbiota. Science 336:1325-9.

13. Li RW, Li W, Sun J, Yu P, Baldwin RL, Urban JF. 2016. The effect of helminth infection on the microbial composition and structure of the caprine abomasal microbiome. Sci Rep 6:20606.

14. Stephenson LS, Latham MC, Ottesen EA. 2000. Malnutrition and parasitic helminth infections. Parasitology 121 Suppl:S23-38.

15. Lwanga F, Kirunda BE, Orach CG. 2012. Intestinal helminth infections and nutritional status of children attending primary schools in Wakiso District, Central Uganda. Int J Environ Res Public Health 9:2910-21.

16. Jolles AE, Ezenwa VO. 2015. Ungulates as model systems for the study of disease processes in natural populations. Journal of Mammalogy 96:4-15.

17. Kaevska M, Videnska P, Sedlar K, Bartejsova I, Kralova A, Slana I. 2016. Faecal bacterial composition in dairy cows shedding *Mycobacterium avium*

586 subsp. *paratuberculosis* in faeces in comparison with nonshedding cows. Can
587 J Microbiol 62:538-41.
588 18. Burgess CG, Bartley Y, Redman E, Skuce PJ, Nath M, Whitelaw F, Tait A,
589 Gilleard JS, Jackson F. 2012. A survey of the trichostrongylid nematode
590 species present on UK sheep farms and associated anthelmintic control
591 practices. Vet Parasitol 189:299-307.
592 19. McKellar QA. 1993. Interactions of *Ostertagia* species with their bovine and
593 ovine hosts. Int J Parasitol 23:451-62.
594 20. McNeilly TN, Rocchi M, Bartley Y, Brown JK, Frew D, Longhi C, McLean L,
595 McIntyre J, Nisbet AJ, Wattegedera S, Huntley JF, Matthews JB. 2013.
596 Suppression of ovine lymphocyte activation by *Teladorsagia circumcincta* larval
597 excretory-secretory products. Vet Res 44:70.
598 21. Simpson HV. 2000. Pathophysiology of abomasal parasitism: is the host or
599 parasite responsible? Vet J 160:177-91.
600 22. Kaplan RM, Vidyashankar AN. 2012. An inconvenient truth: global worming and
601 anthelmintic resistance. Vet Parasitol 186:70-8.
602 23. Bartley DJ, Donnan AA, Jackson E, Sargison N, Mitchell GB, Jackson F. 2006.
603 A small scale survey of ivermectin resistance in sheep nematodes using the
604 faecal egg count reduction test on samples collected from Scottish sheep. Vet
605 Parasitol 137:112-8.
606 24. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA,
607 Turnbaugh PJ, Fierer N, Knight R. 2011. Global patterns of 16S rRNA diversity
608 at a depth of millions of sequences per sample. Proceedings of the National
609 Academy of Sciences of the United States of America 108:4516-4522.

610 25. Li JG, Wang CD, Tang ZH, Guo YQ, Zheng TC, Li YZ, You ZQ. 2017. The Gut
611 Bacterial Community Composition of Wild *Cervus albirostris* (White-Lipped
612 Deer) Detected by the 16S Ribosomal RNA Gene Sequencing. Curr Microbiol
613 doi:10.1007/s00284-017-1288-9.
614 26. Wang Y, Stingl U, Anton-Erxleben F, Zimmer M, Brune A. 2004. *'Candidatus*
615 *Hepatincola porcellionum*' gen. nov., sp. nov., a new, stalk-forming lineage of
616 *Rickettsiales* colonizing the midgut glands of a terrestrial isopod. Arch Microbiol
617 181:299-304.
618 27. Anderson MJ, Gorely RN, KR C. 2008. PERMANOVA+ PRIMER: Guide to
619 Software and Statistical Methods. Primer-E, Plymouth, UK.
620 28. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS,
621 Huttenhower C. 2011. Metagenomic biomarker discovery and explanation.
622 Genome Biol 12:R60.
623 29. Moore RJ, Stanley D. 2016. Experimental design considerations in
624 microbiota/inflammation studies. Clin Transl Immunology 5:e92.
625 30. Douglas JL, Worgan HJ, Easton GL, Poret L, Wolf BT, Edwards A, Davies E,
626 Ross D, McEwan NR. 2016. Microbial diversity in the digestive tract of two
627 different breeds of sheep. J Appl Microbiol 120:1382-9.
628 31. Donaldson GP, Lee SM, Mazmanian SK. 2016. Gut biogeography of the
629 bacterial microbiota. Nat Rev Microbiol 14:20-32.
630 32. Pang W, Vogensen FK, Nielsen DS, Hansen AK. 2012. Faecal and caecal
631 microbiota profiles of mice do not cluster in the same way. Lab Anim 46:231-6.
632 33. Zened A, Combes S, Cauquil L, Mariette J, Klopp C, Bouchez O, Troegeler-
633 Meynadier A, Enjalbert F. 2013. Microbial ecology of the rumen evaluated by

454 GS FLX pyrosequencing is affected by starch and oil supplementation of diets. FEMS Microbiol Ecol 83:504-14.

34. Stiverson J, Morrison M, Yu Z. 2011. Populations of select cultured and uncultured bacteria in the rumen of sheep and the effect of diets and ruminal fractions. Int J Microbiol 2011:750613.

35. Turroni F, van Sinderen D, Ventura M. 2011. Genomics and ecological overview of the genus *Bifidobacterium*. Int J Food Microbiol 149:37-44.

36. Kamke J, Kittelmann S, Soni P, Li Y, Tavendale M, Ganesh S, Janssen PH, Shi W, Froula J, Rubin EM, Attwood GT. 2016. Rumen metagenome and metatranscriptome analyses of low methane yield sheep reveals a *Sharpea*-enriched microbiome characterised by lactic acid formation and utilisation. Microbiome 4:56.

37. Mulcahy G, Quinn PJ. 1986. A review of immunomodulators and their application in veterinary medicine. J Vet Pharmacol Ther 9:119-39.

38. Lee SC, Tang MS, Lim YA, Choy SH, Kurtz ZD, Cox LM, Gundra UM, Cho I, Bonneau R, Blaser MJ, Chua KH, Loke P. 2014. Helminth colonization is associated with increased diversity of the gut microbiota. PLoS Negl Trop Dis 8:e2880.

39. Houlden A, Hayes KS, Bancroft AJ, Worthington JJ, Wang P, Grencis RK, Roberts IS. 2015. Chronic *Trichuris muris* Infection in C57BL/6 Mice Causes Significant Changes in Host Microbiota and Metabolome: Effects Reversed by Pathogen Clearance. PLoS One 10:e0125945.

40. Holm JB, Sorobetea D, Kiilerich 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 Concomitantly Increases the Abundance of Lactobacilli. PLoS One 10:e0125495.

41. Preidis GA, Hotez PJ. 2015. The newest "omics"--metagenomics and metabolomics--enter the battle against the neglected tropical diseases. PLoS Negl Trop Dis 9:e0003382.

42. Brestoff JR, Artis D. 2013. Commensal bacteria at the interface of host metabolism and the immune system. Nat Immunol 14:676-84.

43. Broadhurst MJ, Ardeshir A, Kanwar B, Mirpuri J, Gundra UM, Leung JM, Wiens KE, Vujkovic-Cvijin I, Kim CC, Yarovinsky F, Lerche NW, McCune JM, Loke P. 2012. Therapeutic helminth infection of macaques with idiopathic chronic diarrhea alters the inflammatory signature and mucosal microbiota of the colon. PLoS Pathog 8:e1003000.

44. Ubeda C, Djukovic A, Isaac S. 2017. Roles of the intestinal microbiota in pathogen protection. Clin Transl Immunology 6:e128.

45. Patterson DM, Jackson F, Huntley JF, Stevenson LM, Jones DG, Jackson E, Russel AJ. 1996. The response of breeding doses to nematodiasis: segregation into "responders" and "non-responders". Int J Parasitol 26:1295-303.

46. Bartley DJ, Devin L, Nath M, Morrison AA. 2015. Selection and characterisation of monepantel resistance in *Teladorsagia circumcincta* isolates. Int J Parasitol Drugs Drug Resist 5:69-76.

47. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. Isme J 6:1621-4.

682 48. Padua RA, Parrado A, Larghero J, Chomienne C. 1999. UV and clean air result
683 in contamination-free PCR. Leukemia 13:1898-1899.
684 49. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello
685 EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights
686 D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M,
687 Reeder J, Sevinsky JR, Tumbaugh PJ, Walters WA, Widmann J, Yatsunenko
688 T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput
689 community sequencing data. Nature Methods 7:335-336.
690 50. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013.
691 Development of a dual-index sequencing strategy and curation pipeline for
692 analyzing amplicon sequence data on the MiSeq Illumina sequencing platform.
693 Appl Environ Microbiol 79:5112-20.
694 51. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. 2011. UCHIME
695 improves sensitivity and speed of chimera detection. Bioinformatics 27:2194-
696 200.
697 52. Yilmaz P, Parfrey LW, Yarza P, Gerken J, Pruesse E, Quast C, Schweer T,
698 Peplies J, Ludwig W, Glöckner FO. 2014. The SILVA and “All-species Living
699 Tree Project (LTP)” taxonomic frameworks. Nucleic Acids Research 42:D643-
700 D648.
701 53. Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R.
702 2010. PyNAST: a flexible tool for aligning sequences to a template alignment.
703 Bioinformatics 26:266-7.

704

705

706 **FIGURE LEGENDS**

707

708 **FIG 1** (A) Alpha rarefaction curves for OTUs in terminal ileal content (TIC) and rectal
709 faecal (RF) samples (n=6 for each sample type). Error bars represent standard
710 deviations. (B) PCoA ordination based on Bray-Curtis similarity of OTU distributions
711 for TIC and RF samples. Vectors show Pearson correlations between the
712 abundances of major Phyla and the PCoA axes.

713

714 **FIG 2** Heat map representing the abundances of individual OTUs in the TIC and RF
715 samples. The five most abundant OTUs in each sample were included in the
716 analysis. The most accurate levels of taxonomic classification for each OTU is
717 shown next to the OTU identifier, and Phylum membership is indicated with coloured
718 bars.

719

720 **FIG 3** (A) Alpha rarefaction curves for OTUs in RF samples from monepantel-
721 treated and untreated sheep infected with sensitive or resistant *T. circumcincta* (n=5
722 for each treatment). Error bars represent standard deviations. (B) PCoA ordination
723 based on Bray-Curtis similarity of OTUs in RF samples from monepantel treated and
724 untreated sheep infected with sensitive or resistant *T. circumcincta*. Vectors show
725 Pearson correlations between the abundances of major Phyla and the PCoA axes.

726

727 **FIG 4** CAP ordination based on Bray-Curtis similarity of OTUs in RF samples (A)
728 and TIMS (B) from monepantel-treated and untreated sheep, infected with sensitive
729 or resistant *T. circumcincta* (n=5 for each treatment). Vectors show Pearson
730 correlations between the abundances of major Phyla or the worm burden and the

CAP axes. Shaded data points represent anthelmintic treatment, while squares and circles indicate the sensitive and resistant *T. circumcincta* strains respectively.

FIG 5 Heat map of abundances of individual OTUs in RF samples from monepantel-treated and untreated sheep, infected with sensitive or resistant *T. circumcincta* (n=5 for each treatment). The five most abundant OTUs in each sample were included in the analysis. The most accurate levels of taxonomic classification for each OTU is shown next to the OTU identifier, and Phylum membership is indicated with coloured bars.

FIG 6 Bar graph representation of taxonomic biomarkers identified in RF samples (A) and TIMS (A) from worm-free sheep (open bars) or worm-infected sheep (solid bars). LDA scores of >2.5 in panel A and >3.5 panel B are shown.

FIG S1 Percentage abundance plot of taxonomic orders in TIC and RF samples. Orders with abundances <4% in all samples were grouped as “Others”.

FIG S2 Percentage abundance plot of taxonomic orders in RF samples from monepantel-treated and untreated sheep, infected with sensitive or resistant *T. circumcincta* (n=5 for each treatment). Orders with abundances <4% in all samples are groups as “Others”.

FIG S3 (A) Alpha rarefaction curves for OTUs in TIMS from monepantel-treated and untreated sheep infected with sensitive or resistant *T. circumcincta* (n=5 for each treatment). Error bars represent standard deviations. (B) Percentage abundance

plot of taxonomic orders in TIMS from monepantel-treated and untreated sheep, infected with sensitive or resistant *T. circumcincta* (n=5 for each treatment). Orders with abundances <4% in all samples are grouped as "Others".

FIG S4 Heat map of abundances of individual OTUs in TIMS samples from monepantel-treated and untreated sheep, infected with sensitive or resistant *T. circumcincta* (n=5 for each treatment). The 5 most abundant OTUs in each sample were included in the analysis. The most accurate levels of taxonomic classification for each OTU is shown next to the OTU identifier, and Phylum membership is indicated with coloured bars. Full details of the percentage abundance and taxonomy of these OTUs in each sample is presented in Table S4.

768 **TABLE 1** *Teladorsagia circumcincta* isolate designations pre- and post-selection for
769 monepantel resistance

Original isolate designation	Anthelmintic sensitivity[a]				Anthelmintic administration (dose rate; mg kg^{-1} body weight)	Designation during microbiota characterisation
	BZ	LV	ML	MP		
MTci7	R	R	R	S	None	SUT
					Monepantel - Zolvix® (2.5)	ST
MTci7-12	R	R	R	R	None	RUT
					Monepantel - Zolvix® (2.5)	RT

770 [a]BZ, benzimidazole; LV, levamisole; ML, macrocyclic lactone (avermectin and
771 milbemycin); MP, monepantel; S, sensitive to anthelmintic class; R, resistant to
772 anthelmintic class

773

TABLE 2 Sheep infection and treatment groups

Sheep ID	Group ID	Nematode Isolate	Treatment	Faecal egg count (eggs g^{-1} faeces)
OV1	ST	Sensitive	MPTL	0
OV2	ST	Sensitive	MPTL	1
OV3	ST	Sensitive	MPTL	0
OV4	ST	Sensitive	MPTL	0
OV5	ST	Sensitive	MPTL	0
OV6	SuT	Sensitive	Untreated	360
OV7	SuT	Sensitive	Untreated	45
OV8	SuT	Sensitive	Untreated	33
OV9	SuT	Sensitive	Untreated	63
OV10	SuT	Sensitive	Untreated	360
OV16	RT	Resistant	MPTL	333
OV17	RT	Resistant	MPTL	333
OV18	RT	Resistant	MPTL	333
OV19	RT	Resistant	MPTL	270
OV20	RT	Resistant	MPTL	1962
OV11	RuT	Resistant	Untreated	972
OV12	RuT	Resistant	Untreated	351
OV13	RuT	Resistant	Untreated	792
OV14	RuT	Resistant	Untreated	306
OV15	RuT	Resistant	Untreated	387

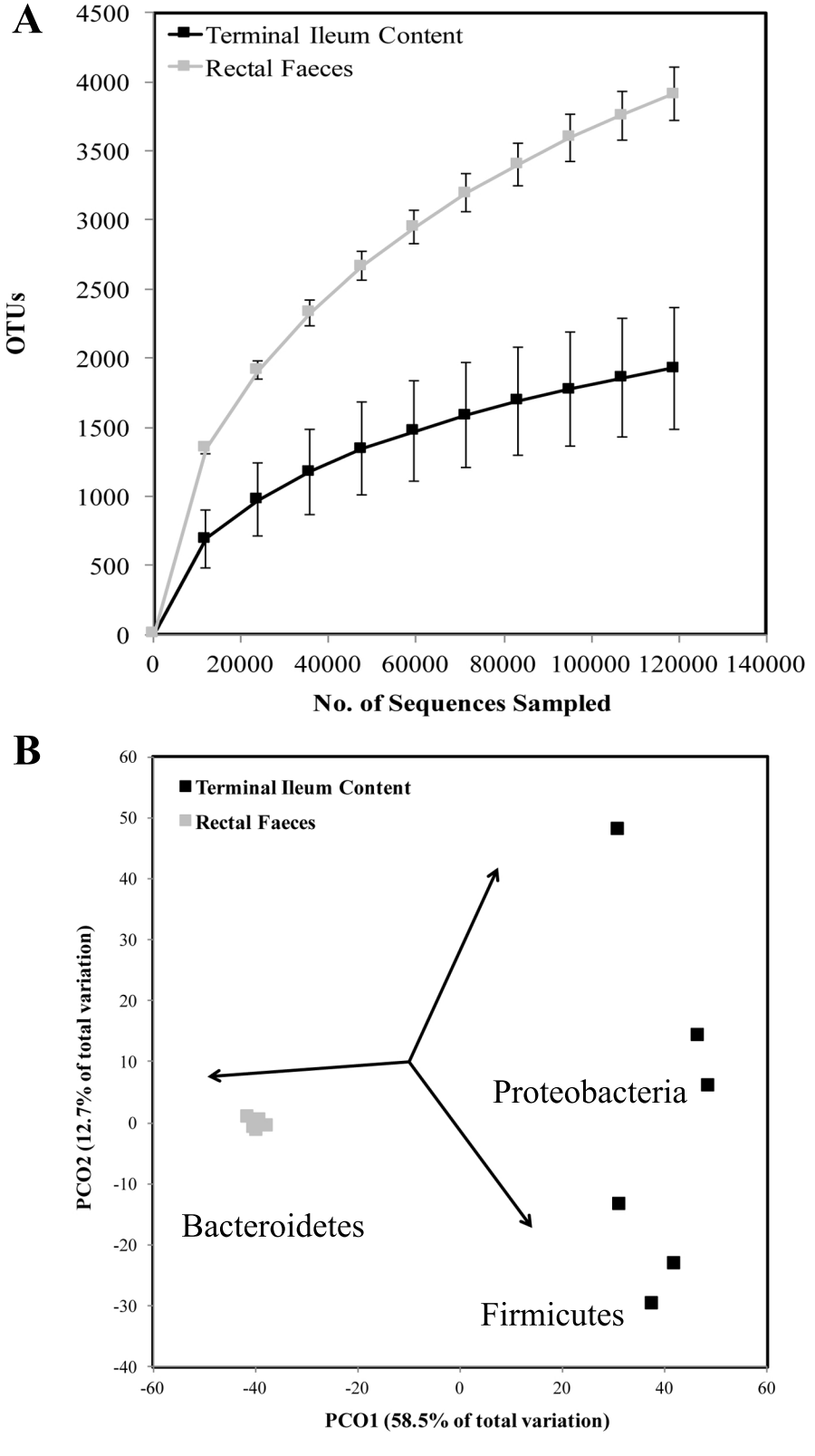

FIG 1 (A) Alpha rarefaction curves for OTUs in terminal ileal content (TIC) and rectal faecal (RF) samples (n=6 for each sample type). Error bars represent standard deviations.
(B) PCoA ordination based on Bray-Curtis similarity of OTU distributions for TIC and RF samples. Vectors show Pearson correlations between the abundances of major Phyla and the PCoA axes.

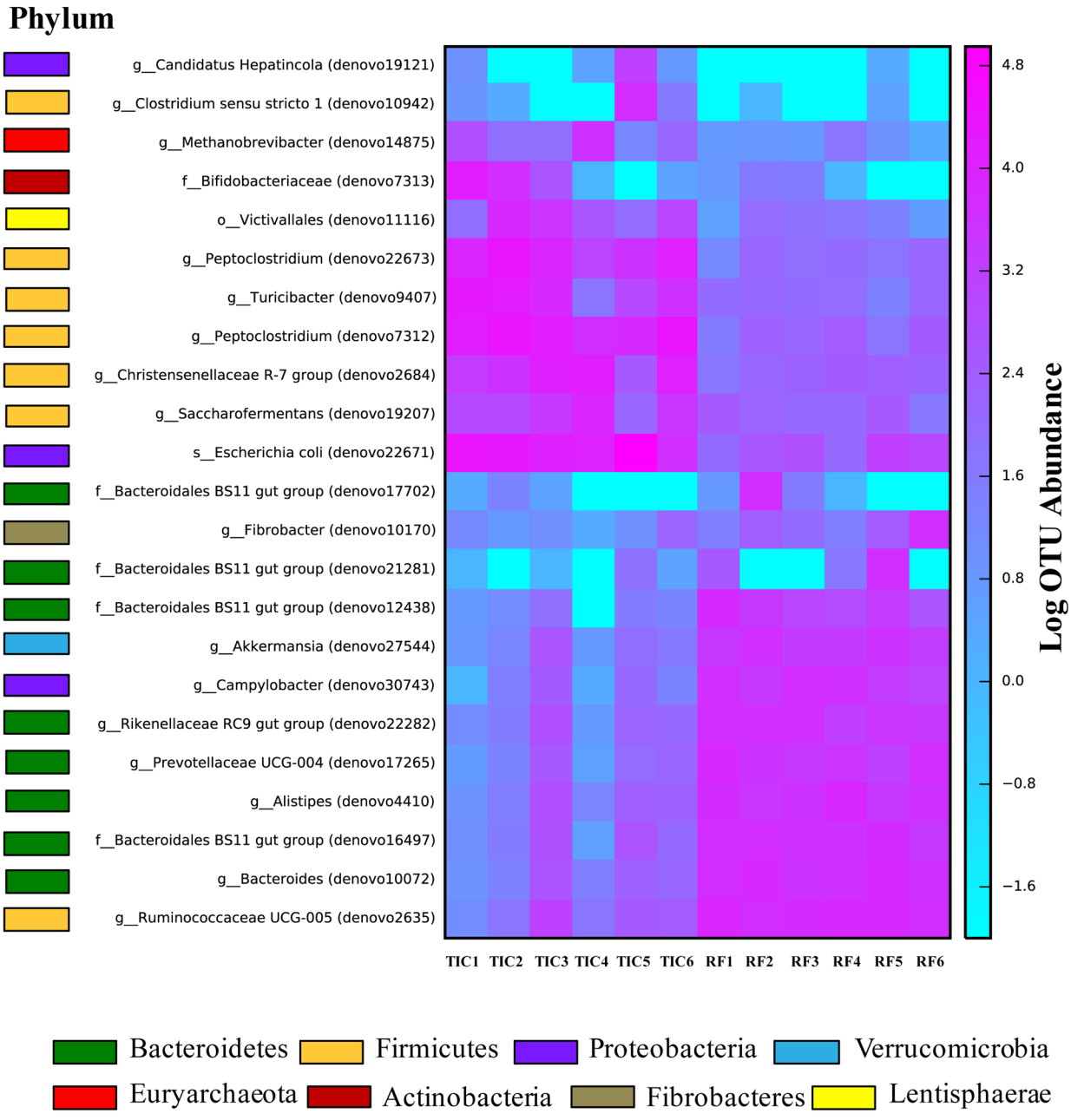

FIG 2 Heat map representing the abundances of individual OTUs in the TIC and RF samples. The five most abundant OTUs in each sample were included in the analysis. The most accurate levels of taxonomic classification for each OTU is shown next to the OTU identifier, and Phylum membership is indicated with coloured bars.

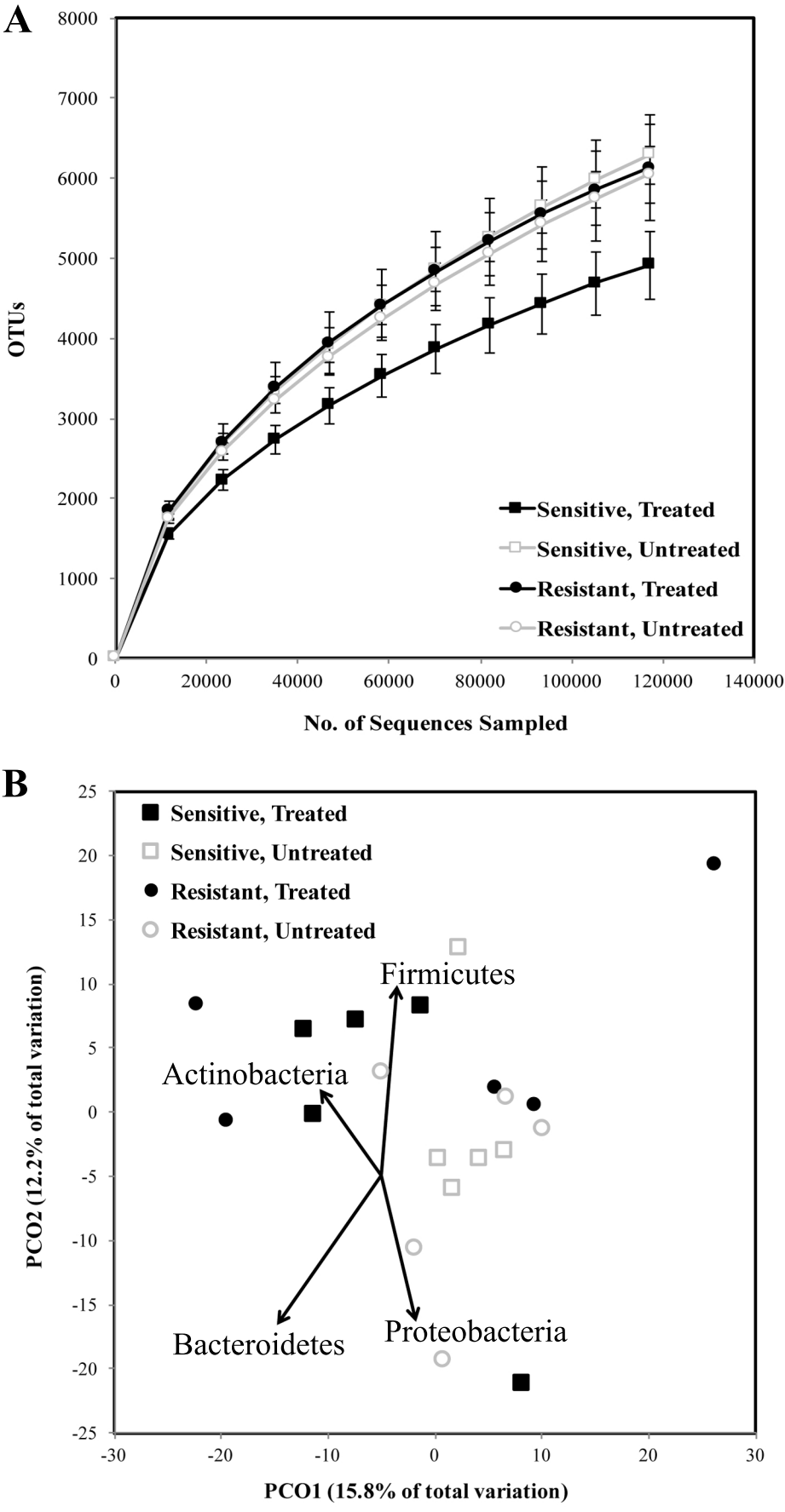

FIG 3 (A) Alpha rarefaction curves for OTUs in RF samples from monepantel-treated and untreated sheep infected with sensitive or resistant *T. circumcincta* (n–5 for each treatment). Error bars represent standard deviations. (B) PCoA ordination based on Bray-Curtis similarity of OTUs in RF samples from monepantel treated and untreated sheep infected with sensitive or resistant *T. circumcincta*. Vectors show Pearson correlations between the abundances of major Phyla and the PCoA axes.

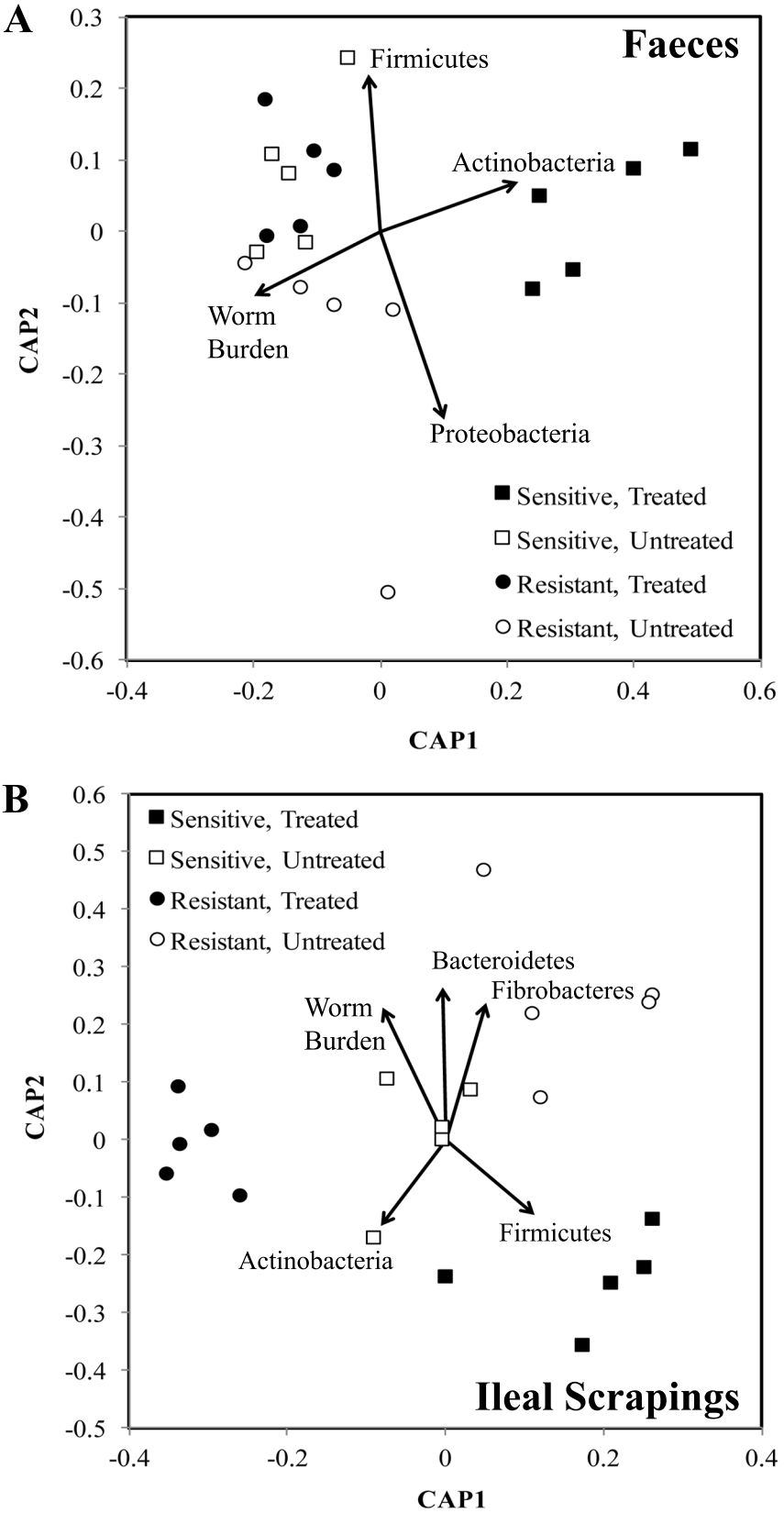

FIG 4 CAP ordination based on Bray-Curtis similarity of OTUs in RF samples (A) and TIMS (B) from monepantel-treated and untreated sheep, infected with sensitive or resistant *T. circumcincta* (n=5 for each treatment). Vectors show Pearson correlations between the abundances of major Phyla or the worm burden and the CAP axes. Shaded data points represent anthelmintic treatment, while squares and circles indicate the sensitive and resistant *T. circumcincta* strains respectively.

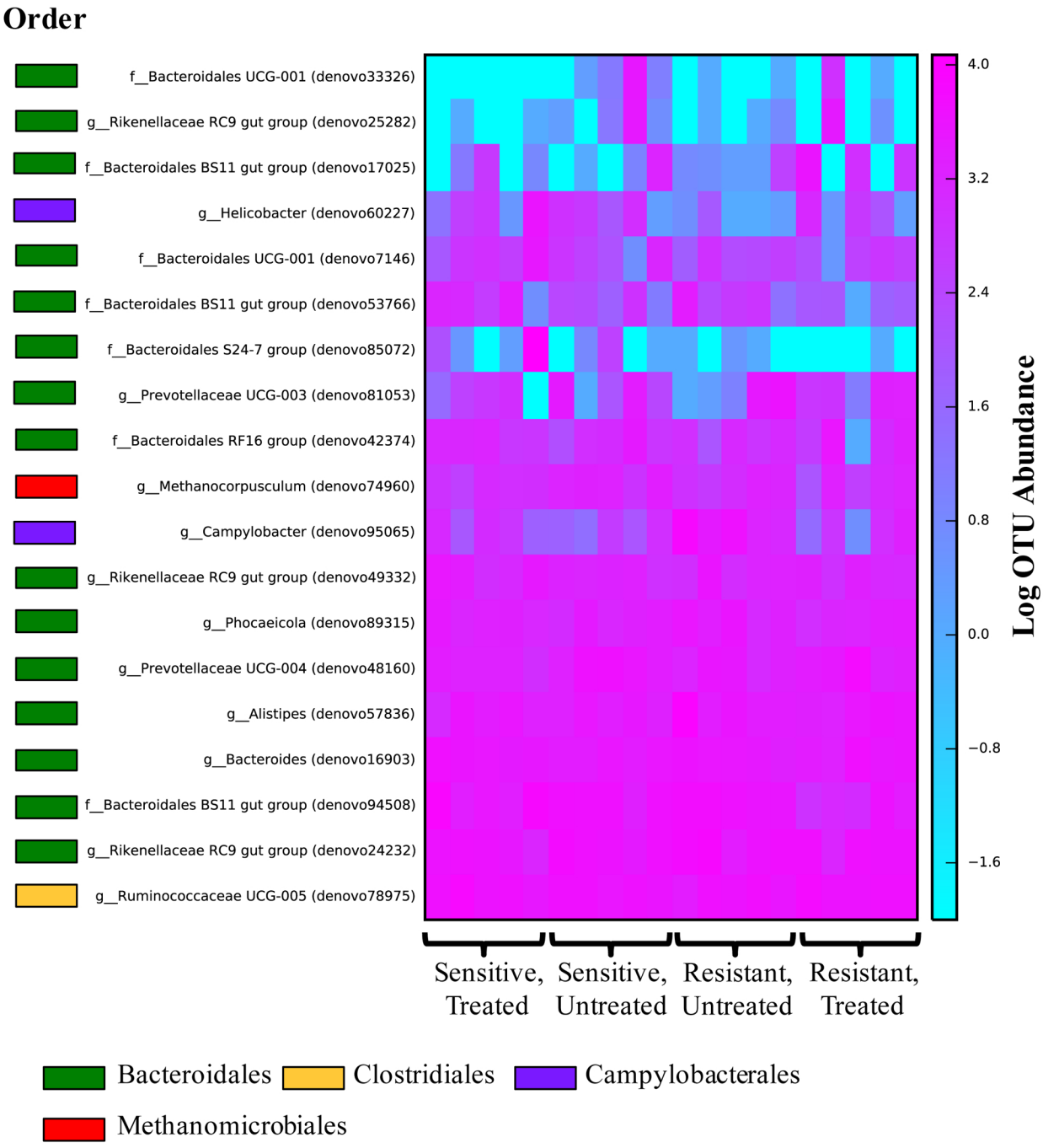

FIG 5 Heat map of abundances of individual OTUs in RF samples from monepantel-treated and untreated sheep, infected with sensitive or resistant *T. circumcincta* (n=5 for each treatment). The five most abundant OTUs in each sample were included in the analysis. The most accurate levels of taxonomic classification for each OTU is shown next to the OTU identifier, and Phylum membership is indicated with coloured bars.