American Journal of Animal and Veterinary Sciences

Original Research Paper

Anthelmintic Treatment in Growing Cattle: A Preliminary Study on the Effect of Ricobendazole on the Fecal Microbiome

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Article history
Received: 13-09-2021
Revised: 01-02-2022
Accepted: 02-03-2022

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Abstract: There is limited information on the impact that anthelmintic treatments exert on the fecal microbiome of growing cattle. This study characterizes the composition and metabolic function of the cattle gut microbiome associated with different levels of parasite Fecal Egg Counts (FEC) and characterizes the fecal microbiome before and after ricobendazole-treatment. The hypothesis was that FEC levels and ricobendazole treatment (8 mL/animal RICOVERM 15 g) alter the fecal microbiome structure and predicted functionality. Fecal samples from Angus yearling steers (live weight: 249±19 kg) with Low-FEC (<100 epg) and High-FEC (840±207 epg) were collected at three-time points: Pre-Treatment (PRE-T) and 3- and 10-days post-treatment (POST-T3 and POST-T10, respectively). After DNA extraction, high-throughput sequencing of bacterial 16S rRNA amplicons and associated bioinformatics analyses were performed. Microbiome analysis revealed evidence of beta-diversity association with time of sampling. Samples collected post-treatment were characterized by higher microbial richness (alpha diversity) and increased abundance (P<0.05) of *Alistipes* (POST-T3) and *Ruminococcaceae*_UCG-010 (POST-T10) compared with samples PRE-T. On the other hand, *Ruminococcaceae*_UCG-014 and *Christensenellaceae*_R-7_group genera were increased in samples from PRE-T compared with post-treatment. Predicted metagenome analysis revealed that biosynthesis of nucleotides, nucleosides, vitamins and amino acids were the metabolic pathways most affected due to ricobendazole-treatment. On the other hand, no significant differences in microbial diversity, microbiome composition and predicted metabolic pathways were observed pre-treatment between Low-FEC and High-FEC cattle. In general, these findings revealed that ricobendazole-administration altered the microbial diversity and composition in the feces of cattle, which led to changes in the metabolic pathways of the microbiome. This study provides a first known insight into the relationship between the microbiome and the exposure to ricobendazole in grazing cattle and sets a basis for the development of future studies comprising a larger number of animals and different anthelmintic drugs.

Keywords: Cattle, Microbiome, Anthelmintic, Parasites

Introduction

The intestine of cattle is inhabited by multiple organisms that are exposed to changes in the gut environment. There is currently limited understanding of the dynamic relationship between parasites and the microbial community (microbiome) within the gastrointestinal tract of cattle although recent studies suggested that the co-existence of these two communities has established significant interactions (Lee *et al*., 2014; Hogan *et al*., 2019). Most studies of parasite-microbiome interactions have been conducted in humans, rodents and horses (McKenney *et al*., 2015; Peachey *et al*., 2018; Easton *et al*., 2019), while limited data is currently available on the effect of parasite burden on the cattle microbiome.

The relationship between parasites and the host gut microbiome is potentially perturbed by anthelmintic treatments (Daniels *et al*., 2020), which remain an important part of parasite control in grazing animals. (Mederos *et al*., 2018; Kelleher *et al*., 2020). At present, the major classes of anthelmintics available for cattle

belong to the families of the imidazothiazoles, benzimidazoles and macrocyclic lactones (Mederos *et al*., 2018). Irrespectively of the class, anthelmintics are usually administered to all animals within a cattle herd ("mass" treatment), although only a few of them may present high levels of parasite infection. This practice, coupled with the lack of anthelmintic drugs with new active ingredients, is associated with the emergence of resistant parasites (Kaplan and Vidyashankar, 2012) and potential collateral effects on the host's microbiome. Peachey *et al*. (2018), identified differences in bacterial community profiles between horses categorized as having high and low Fecal Egg Counts (FEC) for cyathostomins and the effect of treatment with ivermectin. In sheep, Moon *et al*. (2021) reported that administration of albendazole + abamectin or moxidectin resulted in relatively modest differences in the compositions of the ruminal microbial communities compared to the control group. On the other hand, no changes to the microbiome diversity and the relative abundances of bacterial species were detected in dogs as a result of using commercial anthelmintic treatments (Fujishiro *et al*., 2020).

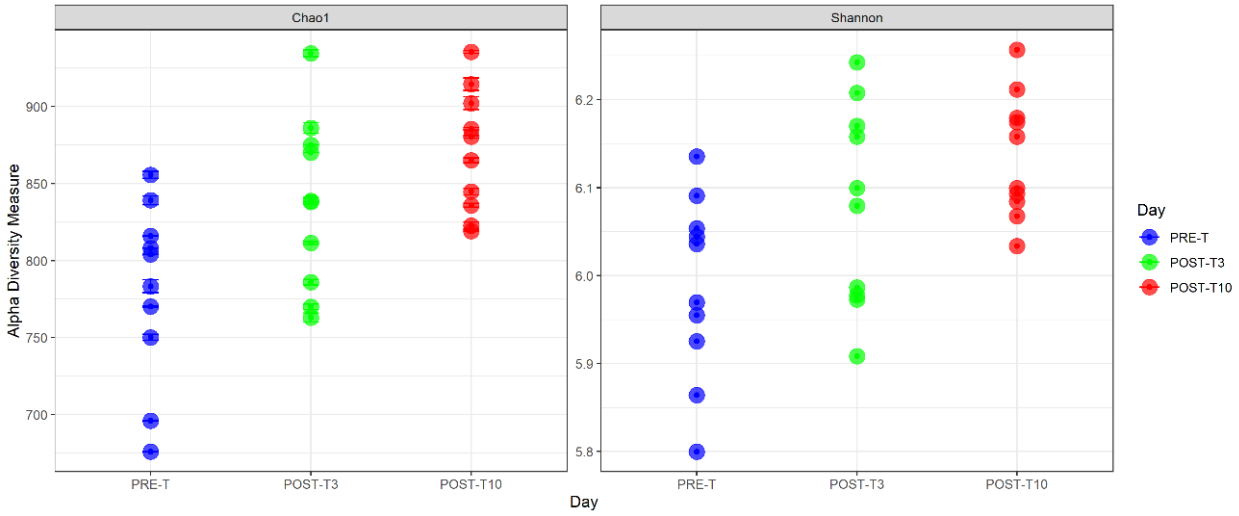

Fig. 1: Comparison of fecal microbiome alpha diversity measures (Chao1 and Shannon indexes) at Amplicon Sequence Variants (ASVs) level separated by days since treatment (8 ml per animal of RICOVERM 15 g). PRE-T: pre-treatment; POST-T3: 3 days post-treat POST-T10: 10 Days post-treatment.

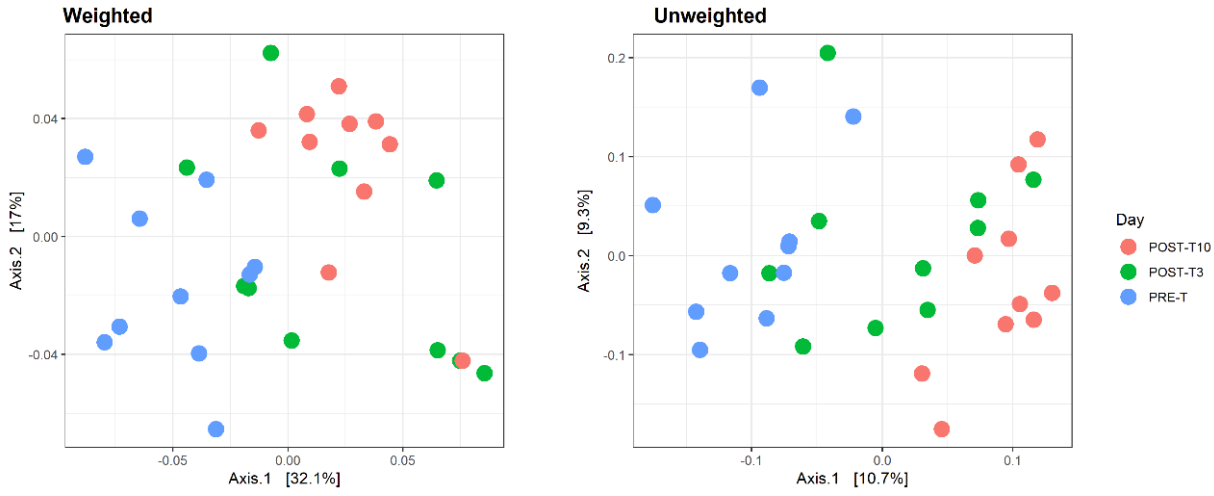

Fig: 2. The principal coordinate plot of beta diversity measured by weighted and unweighted UniFrac at Amplicon Sequence Variants (ASVs) level separated by days since treatment (8 mL per animal of RICOVERM 15 g). PRE-T: pre-treatment; POST-T3: 3 Days post-treatment; POST-T10: 10 Days post-treatment

DOI: 10.3844/ajavssp.2022.77.88

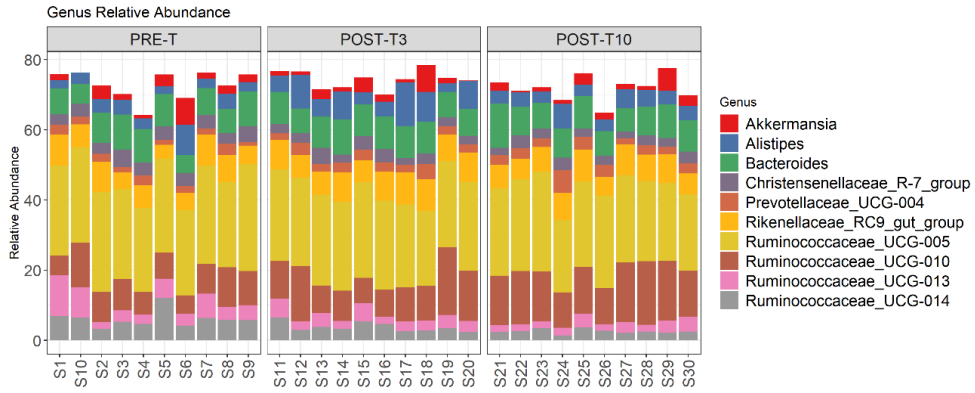

Fig. 3: Relative abundance (%) of top-ten bacterial genera in individual cattle Samples (S) Pre-Treatment (PRE-T) and at days 3 (POST-T3) and 10 (POST-T10) post-treatment (8 mL per animal of RICOVERM 15 g)

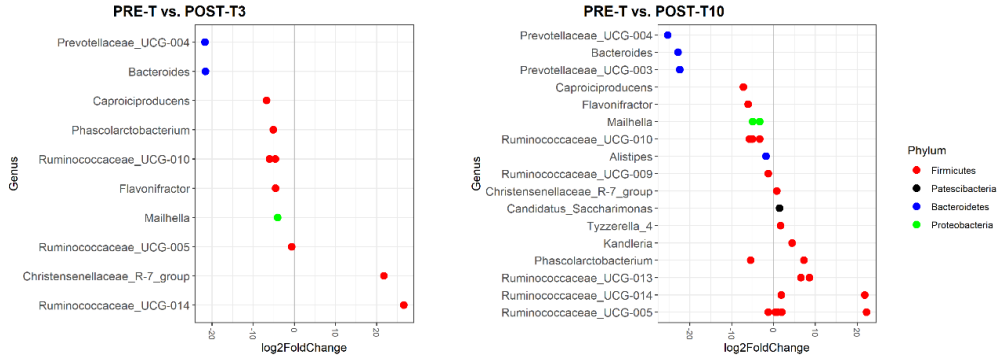

Fig. 4: Significant (P<0,05) differential Amplicon Sequence Variants (ASVs) in cattle feces microbiome between pre- and Post-Treatment (8 mL per animal of RICOVERM 15 g). PRE-T: Pre-Treatment; POST-T3: 3 Days post-treatment; POST-T10: 10 Days post-treatment. The ASVs are grouped by genus (Y-axis) and color-coded according to the phyla they belong to. Negative and positive log_2fold values are enrichments in Post-Treatment (POST-T3 or POST-T10) and pre-treatment, respectively

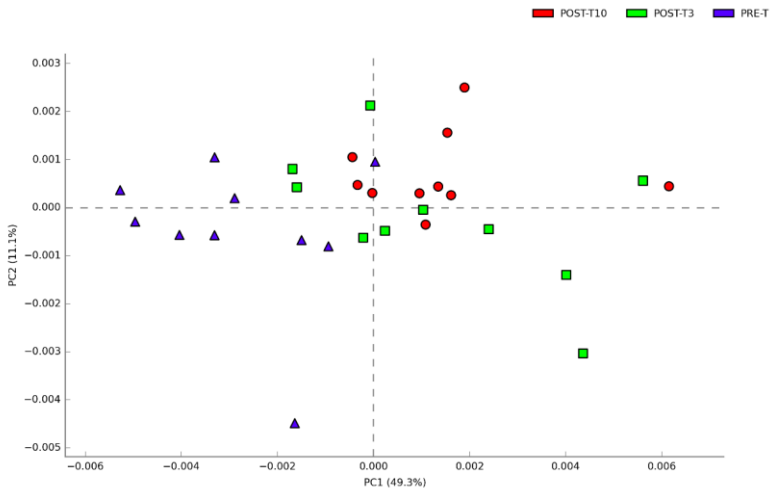

Fig. 5: Principal coordinate analysis plot of Bray-Curtis distances for predicted KEGG level 3 pathways clustered by days of treatment. Cattle were sampled Pre-Treatment (PRE-T) prior to administration of 8 mL per animal of RICOVERM 15 g and then 3 and 10 days after treatment (POST-T3 and POST-T10, respectively). Samples taken PRE-T were significantly separated (P<0.05) from those collected Pos-Treatment (POST-T3 and POST-T10)

The aims of this study were (1) to characterize the feces microbiome of steers with low and high FEC and (2) to explore changes in the microbiome profiles following ricobendazole administration. We hypothesize that FEC levels and ricobendazole treatment alter the fecal microbiome structure and predicted functionality based on a 16S rRNA gene high throughput sequencing approach.

Materials and Methods

Experimental Design and Sample Collection

Individual fecal samples (25 g) were collected from the rectum of 50 Aberdeen Angus yearling steers (249±19 kg of live weight) grazing native grasslands. Samples were immediately transported to the laboratory for FEC conducted on all individual samples by using the McMaster technique to estimate the number of eggs per gram of feces (epg). As a result of FEC results, 10 animals were selected for the present study: 5 Animals with high FEC (H-FEC, 840±207 epg) and 5 animals with low (undetectable) levels of FEC (L-FEC, <100 epg).

Fecal samples from H-FEC and L-FEC steers were collected at three-time points: Pre-treatment (PRE-T) and 3- and 10-days post-treatment (POST-T3 and POST-T10, respectively), resulting in a total of 30 samples. On day 0, all steers were treated with a single dose (8 mL/animal) of RICOVERM 15 g [active ingredient: Ricobendazole] (König, Argentina) administered via subcutaneous injection on the neck region. On each sampling point, fecal samples were immediately transported to the laboratory where one portion (5 g/sample) was destined for FEC analysis and the rest of the material (20 g/sample) was stored at -20°C freezer and remained frozen until further processing. Fecal egg counts examined POST-T3 and POST-T10 confirmed that all animals from both pre-treatment groups (L-FEC and H-FEC) remained free of quantifiable helminth infection (<100 epg) after ricobendazole treatment.

Dna Extraction, Amplification and Sequencing

All samples were transported to the Microbiology Sector of the Laboratorio Tecnológico del Uruguay (LATU, Montevideo) for DNA extraction using the QIAmp DNA Mini kit (QIAGEN) according to manufacturer's instructions. Sample DNA concentration (32,2±,7 ng/uL) was quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific). Final eluted DNA (70 µL aliquots) from all 30 samples were sent to the Macrogen Inc. Laboratory (Seoul, South Korea) for library preparation and amplicon sequencing. An amplicon library was constructed using a 341f-805r primer set to target the V3-V4 region of the 16S rRNA gene. Sequencing was performed on an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) generating paired-end 2 × 150 bp reads.

Data Processing and Sequencing Analysis

All 16S rRNA gene amplicons were processed in R (version 4.0.5) using DADA2 version 1.12.1 (Callahan *et al*., 2016). After trimming primer sequences and low-quality reads, a step was performed to learn the error model of sequencing data. Identical sequences were combined using the dereplication function followed by the merging of reads (forward, reverse) and removal of chimeric sequences. This generated 39.668±2.616 (mean ± s.d.) sequences per sample (min. 34.342, max. 45.905) available for downstream analysis. An Amplicon Sequence Variant (ASVs) table was constructed after taxonomic assignment of sequences against the SILVA reference database version 132 (Quast *et al*., 2013). Sample metadata, sequence taxonomy, ASVs and a phylogenetic tree constructed using the phangorn package (Schliep, 2011), were combined into an object using phyloseq version 1.22.3 for further analysis (McMurdie and Holmes, 2013).

Feces Microbial Community Analysis

Microbiome diversity, composition and function were compared between L-FEC and H-FEC groups and among the three sampling times (PRE-T, POST-T3 and POST-T10). Alpha diversity metrics Chao1 (a metric for ASVs richness) and Shannon (a metric that incorporates both ASVs richness and evenness) were computed using the estimate richness function of the phyloseq package (McMurdie and Holmes, 2013). The statistical significance (P<0,05) of differences in alpha diversity metrics was calculated by pairwise comparisons using the Wilcoxon rank-sum test. Beta diversity, a metric of differences between samples, was calculated using weighted and unweighted Uni Frac distances (Lozupone *et al*., 2014) and visualized by principal coordinates analysis. Adonis, a permutational multivariate analysis of variance in the vegan package version 2.5-6 (Oksanen *et al*., 2018), was performed to test for beta diversity differences (P < 0,05). Differential abundance (P<0,05) of individual taxa members at ASVs and genus level was evaluated using DESeq 2 (Love *et al*., 2014. Genus detected at ≥0.1% relative abundance in ≥50% of samples were considered members of the core microbiome. The potential function of microbiomes was predicted by PICRUSt2 (Douglas *et al*., 2020). The statistical differences (P<0,05) between groups were determined using the two-sided Welch's test and Benjamini-Hochberg FDR was used to correct for multiple tests in the STAMP software (Parks and Beiko, 2010).

Results

Ricobendazole-Treatment Associates with Altered Microbiome Alpha and Beta Diversity in Feces of Beef Cattle

To determine if pre-treatment parasite burden or the anthelmintic treatment influence microbiome alpha diversity, we calculated Chao1 and Shannon values. Both indexes were higher (P<0.05) in animals 10 days after treatment compared to pre-treatment levels (Fig. 1). At ASVs level, mean ± s.d. of Chao1 was 791±58 (PRE-T) and 870±40 (POST-T10) (P<0.05). Similarly, the Shannon index of POST-T10 was higher (P<0.05) than that of PRE-T (6.14±0.07 vs. 5.98±0.10, respectively). Alpha diversity measures in POST-T3 were similar (P>0.05) than those registered PRE-T and POST-T10. Microbial community alpha diversity measures were not affected (P>0.05) by the level of FEC pre-treatment, averaging 768±54 (L-FEC) and 791±67 (H-FEC) for Chao 1 and 5.97±0.07 and 6.00±0.14 for Shannon (L-FEC and H-FEC, respectively).

Microbial beta diversity was assessed by weighted and unweighted UniFrac measure at ASVs level. A significant separation was observed between microbial composition and anthelmintic treatment (P<0.05) (Fig. 2). Permutational Multivariate Analysis of Variance (PERMANOVA) indicated that microbial beta diversity of the 3 groups were different (P<0.05) and that fecal microbiome of ricobendazole-treated cattle remained disturbed for the duration of the study in comparison with PRE-T animals. On the other hand, a multivariate analysis of samples with L-FEC and H-FEC pre-anthelminthic treatment revealed no significant differences (P>0.05) between the fecal microbiome beta diversity of these two groups (Supplementary material, Fig. S1).

Microbiome Composition and Differential Abundance of Taxa Associated with Ricobendazole-Treatment

Overall, 165 genera of bacteria were identified. The microbial community was dominated by genera belonging to the phyla Firmicutes (*Ruminococcaceae, Christensenellaceae*), Bacteroidetes (*Bacteroides, Rikenellaceae RC9, Alistipes, Prevotellaceae*) and Verrucomicrobia (*Akkermansia*) (Fig. 3). To identified genera whose abundance were influenced by anthelmintic treatment we used DESeq 2 which test for differential expression based on a model using the negative binomial distribution. Difference in abundance of individual taxa at the genus level were detected between PRE-T and post-treatment samples in 20 out of 165 genera (12%). Considering the most abundant genera listed in Fig. 3, samples collected post-treatment were characterized by an increased abundance (P<0.05) of *Alistipes* (POST-T3) and *Ruminococcaceae*_UCG-010 (POST-T10) compared with samples PRE-T. In addition, *Agathobacter, Mahiella, Phascolarctobacterium, Ruminiclostridium* and *Treponema*_2 were low-abundant genera with at least 0,1% relative abundance (ra) significantly increased (P<0,05) POST-T10 compared to PRE-T. On the other hand, top-ten *Ruminococcaceae*_UCG-013, *Ruminococcaceae*_UCG-014 and *Christensenellaceae*_R-7_group genera were consistently increased (P<0.05) in samples from PRE-T compared with post-treatment. Low-abundant genera (ra >0,1%) *Paeniclostridium, Ruminiclostridium*_1, *Ruminococcus*_2 and *Tyzzerella*_4 in the PRE-T group were higher than those in the POST-T10 group.

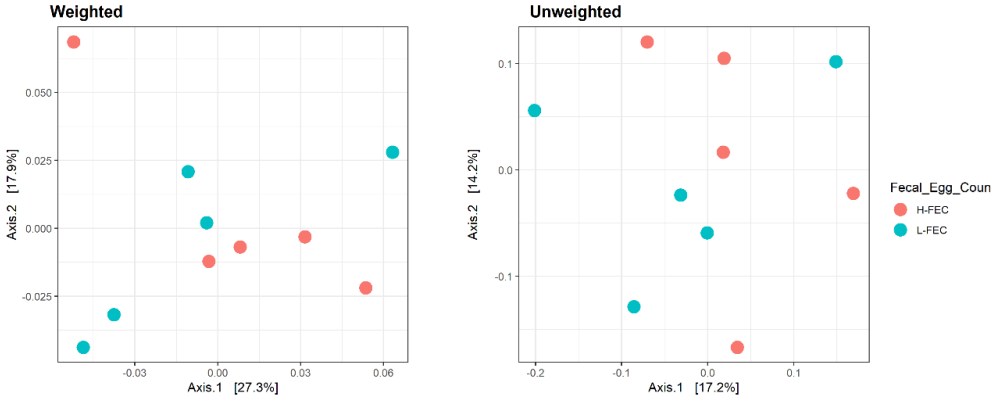

Fig: S1: The principal coordinate plot of beta diversity measured by weighted and unweighted UniFrac at amplicon sequence variants (ASVs) level pre-treatment (8 mL per animal of RICOVERM 15 g) separated by parasite burden (P>0.05). L-FEC: Low fecal egg counts; H-FEC: High fecal egg counts.

At the highest level of taxonomic resolution, we also determined which ASVs were differentially abundant between each anthelmintic treatment group and the control PRE-T group. Compared with animals' PRE-T, anthelmintic treatment was associated with a significant decrease in 2 (POST-T3) and 16 (POST-T10) ASVs, whereas 14 and 24 ASVs were significantly increased in the ricobendazole-treated animals POST-T3 and POST-T10, respectively. Figure 4 shows significantly affected ASVs classified at genus and phylum level (does not include ASVs classified as "NA" at genus level). Overall, a subset of 39 ASVs belonging to 4 phyla and 17 genera were affected. Almost half (41%) of these ASVs corresponded to genera in the Ruminococcaceae family (genera UCG-005, UCG-010, UCG-013, UCG-014) in the phylum Firmicutes. Investigation of the differentially abundant ASVs between the PRE-T and POST-T10 group revealed differences in the abundances of specific ASVs within the same genus. Some ASVs within the genus Ruminococcaceae UCG-005 were highly abundant in the PRE-T group while others were highly abundant in POST-T10 group. Within phylum Bacteroidetes, there were 3 ASVs that were exceptionally enriched (log_2fold >-20) in post-treatment samples corresponding to *Prevotellaceae* (UCG-003 and UCG-004) and *Bacteroides*. Only 3 ASVs differed (P<0.05) in abundance between POST-T3 and POST-T10 (data not shown). Two of them were enriched POST-T10 (genera *Prevotellaceae* UCG-003 and *Christensenellaceae* R7), while the remaining ASV (genus *Ruminococcaceae* UCG-005) was more abundant in POST-T3.

There was no significant difference (P>0,05) in abundance of genera between the FEC groups. When compared at the ASV level, 5 ASVs were differentially abundant (P<0,05) between the L-FEC and H-FEC groups which suggests a lower variation in fecal microbiome compared to the anthelmintic effect. Three of these ASVs (genera *Alistipes*, *Ruminococcaceae* UCG-005 and *Ruminococcaceae* NK4A214) were more abundant in feces from L-FEC cattle, whereas 2 ASVs (genera *Akkermansia* and *Ruminococcaceae* UCG-013) were more abundant in feces from H-FEC cattle.

Predicted Functional Potential of Feces Microbiome

To analyze the functional changes of the fecal microbiome as a consequence of anthelmintic treatment, metagenomes potential between the three groups (PRE-T, POST-T3 and POST-T10) were predicted by PICRUSt2. The results revealed that high abundance of bacterial metagenome in the three groups was mainly associated with "amino acid biosynthesis" (mean ± s.d.: 20,2±0,3% of reads), "nucleotide and nucleoside biosynthesis" (19,1±0,2%), "cofactor, electron carrier and vitamin biosynthesis" (13,4±0,1%), "fatty acid and lipid biosynthesis" (7,6±0,1%) and "fermentation" (5,4±0,1%) in KEGG level 2. At the highest level of resolution, the analyses resulted in 370 KEGG level 3 pathways, with a mean ± s.d. of 322±10 pathways per sample (min. 306; max. 351). Figure 5 shows that samples taken prior to anthelmintic treatment (PRE-T) were clustered apart (P<0.05) from those collected after treatment (POST-T3 and POST-T10). There were 130 KEGG level 3 pathways out of 370 (35%) that were differentially abundant between PRE-T and POST-T10 samples (P<0.05). A total of 59 and 71 pathways were identified as statistically (P<0.05) higher and lower in POST-T10, respectively, compared to PRE-T levels, but not differential abundance was identified between POST-T3 and POST-T10 pathways (P>0.05).

Table S1 (Supplementary Material) shows microbial PICRUSt-predicted KEGG functions greater than 0,01% relative abundance and significantly (P<0.05) affected by ricobendazole (8 ml per animal of RICOVERM 15 g) 10 days after treatment (POST-T10) compared to PRE-T. Nucleoside/nucleotide biosynthesis and cofactor/vitamin biosynthesis were the categories with most individual pathways affected (16 and 15, respectively).

Table. S1: Metabolic KEGG pathways significantly affected (P < 0,05) by anthelmintic treatment (8 ml per animal of RICOVERM 15 g) . PRE-T: pre-treatment; POST-T10: 10 days after treatment.

		Mean relative frequency (%)		Difference between means	
KEGG Pathway Level 2	Level 3	PRE-T	POST-T10	Decreased POST-T10	Increased POST-T10
Amine and Polyamine Biosynthesis	superpathway of polyamine biosynthesis II	0.02	0.04		-0.02
Amine and Polyamine Degradation	aromatic biogenic amine degradation (bacteria)	0.01	0.00	0.01	
Amino Acid Biosynthesis	L-glutamate and L-glutamine biosynthesis	0.47	0.43	0.04	
	L-lysine biosynthesis I	0.81	0.77	0.04	
	L-arginine biosynthesis II (acetyl cycle)	0.79	0.75	0.03	
	L-arginine biosynthesis I (via L-ornithine)	0.78	0.75	0.03	
	L-arginine biosynthesis IV (archaebacteria)	0.78	0.75	0.03	
	superpathway of aromatic amino acid biosynthesis	0.98	0.95	0.03	
	superpathway of L-aspartate and L-asparagine biosynthesis	0.82	0.79	0.03	
	L-lysine biosynthesis II	0.06	0.05	0.01	
	L-arginine biosynthesis III (via N-acetyl-L-citrulline)	0.31	0.36		-0.05
Amino Acid Degradation	L-leucine degradation I	0.03	0.04		-0.01
Aminoacyl-tRNA Charging	tRNA charging	0.91	0.90	0.02	
Aromatic Compound Biosynthesis	chorismate biosynthesis I	0.94	0.91	0.03	
	chorismate biosynthesis from 3-dehydroquinate	0.90	0.88	0.02	
C1 Compound Utilization and Assimilation	formaldehyde assimilation II (assimilatory RuMP Cycle)	0.02	0.01	0.01	
	formaldehyde oxidation I	0.01	0.01	0.00	
	incomplete reductive TCA cycle	0.55	0.59		-0.05
Carbohydrate Biosynthesis	UDP-N-acetyl-D-glucosamine biosynthesis I	0.70	0.66	0.04	
	dTDP-L-rhamnose biosynthesis	0.80	0.77	0.03	
	CMP-3-deoxy-D-manno-octulosonate biosynthesis	0.23	0.28		-0.05
	colanic acid building blocks biosynthesis	0.38	0.42		-0.04

Table. S1: Continue

	superpathway of GDP-mannose-derived O-antigen building blocks biosynthesis	0.36	0.41		-0.05
Carbohydrate Degradation	sucrose degradation III (sucrose invertase)	0.24	0.20	0.05	
	frucose degradation	0.07	0.05	0.02	
Cell Structure Biosynthesis	peptidoglycan maturation (meso-diaminopimelate containing)		0.84	0.73	0.10
	UDP-N-acetylmuramoyl-pentapeptide biosynthesis II (lysine-containing)		0.94	0.91	0.02
	peptidoglycan biosynthesis III (mycobacteria)	0.91	0.90	0.02	
	peptidoglycan biosynthesis I (meso-diaminopimelate\ containing)		0.92	0.90	0.02
	Kdo transfer to lipid IVA III (Chlamydia)	0.22	0.27		-0.05
	lipid IVA biosynthesis	0.24	0.29		-0.05
	peptidoglycan biosynthesis IV (Enterococcus faecium)	0.26	0.30		-0.04
Cofactor, Prosthetic Group,	thiazole biosynthesis II (aerobic bacteria)	0.18	0.11	0.07	
Electron Carrier and	thiamine salvage II	0.70	0.64	0.06	
Vitamin Biosynthesis	superpathway of thiamine diphosphate biosynthesis II	0.34	0.29	0.05	
	thiazole biosynthesis I (facultative anaerobic bacteria)	0.53	0.48	0.05	
	adenosylcobalamin biosynthesis from adenosylcobinamide-GDP I	0.69	0.65	0.04	
	superpathway of adenosylcobalamin salvage from cobinamide II		0.70	0.65	0.04
	superpathway of adenosylcobalamin salvage from cobinamide I		0.71	0.67	0.04
	cob(II)yrinate a,c-diamide biosynthesis I (early cobalt insertion)		0.08	0.05	0.03
	coenzyme A biosynthesis I (prokaryotic)	0.87	0.85	0.02	
	N10-formyl-tetrahydrofolate biosynthesis	0.72	0.71	0.01	
	flavin biosynthesis I (bacteria and plants)	0.57	0.59		-0.02
	polyisoprenoid biosynthesis (E. coli)	0.50	0.55		-0.05
	pyridoxal 5'-phosphate biosynthesis I	0.16	0.21		-0.05
	superpathway of pyridoxal 5'-phosphate biosynthesis and salvage	0.21	0.27		-0.06
	superpathway of thiamine diphosphate biosynthesis I	0.45	0.48	-0.03	
Fatty Acid and Lipid Biosynthesis	phosphatidylglycerol biosynthesis I (plastidic)	0.87	0.83	0.04	
	phosphatidylglycerol biosynthesis II (non-plastidic)	0.87	0.83	0.04	
	superpathway of phospholipid biosynthesis I (bacteria)	0.92	0.88	0.04	
	CDP-diacylglycerol biosynthesis I	0.99	0.97	0.03	
	CDP-diacylglycerol biosynthesis II	0.99	0.97	0.03	
	fatty acid elongation -- saturated	0.54	0.61		-0.07
Fermentation	acetylene degradation	0.54	0.46	0.09	
	pyruvate fermentation to isobutanol (engineered)	1.05	1.00	0.04	
	glycerol degradation to butanol	0.06	0.03	0.03	
	pyruvate fermentation to acetate and lactate II	0.95	0.92	0.03	
	Bifidobacterium shunt	0.17	0.20		-0.03
	homolactic fermentation	0.46	0.51		-0.05
	pyruvate fermentation to propanoate I	0.37	0.42		-0.05
Glycan Biosynthesis	glycogen biosynthesis I (from ADP-D-Glucose)	0.96	0.92	0.04	
Glycolysis	glycolysis III (from glucose)	0.98	0.96	0.01	
	glycolysis II (from fructose 6-phosphate)	0.40	0.46		-0.06
Inorganic Nutrient Metabolism	nitrate reduction VI (assimilatory)	0.02	0.01	0.01	
	superpathway of sulfur oxidation (Acidianus ambivalens)	0.02	0.02		-0.01
	urea cycle	0.02	0.03		-0.01
Nucleic Acid Processing	queuosine biosynthesis		0.35	0.39	-0.04
Nucleoside and Nucleotide Biosynthesis	adenine and adenosine salvage III	0.91	0.88	0.03	
	adenosine ribonucleotides de novo biosynthesis	1.05	1.03	0.02	
	UMP biosynthesis I		0.98	0.97	0.02
	5-aminoimidazole ribonucleotide biosynthesis II	0.94	0.93	0.01	
	superpathway of 5-aminoimidazole ribonucleotide biosynthesis	0.94	0.93	0.01	
	pyrimidine deoxyribonucleotide phosphorylation	0.35	0.40		-0.05
	pyrimidine deoxyribonucleotides de novo biosynthesis I	0.36	0.41		-0.05
	pyrimidine deoxyribonucleotides de novo biosynthesis II	0.47	0.50		-0.04
	superpathway of guanosine nucleotides de novo biosynthesis I	0.45	0.50		-0.06
	superpathway of guanosine nucleotides de novo biosynthesis II	0.48	0.54		-0.06
	superpathway of purine nucleotides de novo biosynthesis I	0.60	0.65		-0.05
	superpathway of purine nucleotides de novo biosynthesis II	0.61	0.66		-0.05
	superpathway of pyrimidine deoxyribonucleoside salvage	0.40	0.43		-0.04
	superpathway of pyrimidine deoxyribonucleotides de novo biosynthesis (E. coli)	0.47	0.50		-0.03
	superpathway of pyrimidine ribonucleosides salvage	0.51	0.57		-0.07
	superpathway of pyrimidine ribonucleotides de novo biosynthesis	0.58	0.64		-0.06
Nucleoside and Nucleotide Degradation	superpathway of pyrimidine deoxyribonucleosides degradation	0.46	0.39	0.07	
	superpathway of purine deoxyribonucleosides degradation	0.41	0.35	0.06	
	purine ribonucleosides degradation	0.41	0.35	0.06	
	adenosine nucleotides degradation II	0.23	0.26		-0.03
	guanosine nucleotides degradation III	0.25	0.29		-0.03
	inosine 5'-phosphate degradation	0.64	0.69		-0.05
Secondary Metabolite Biosynthesis	methylerythritol phosphate pathway I	0.89	0.87	0.02	
	methylerythritol phosphate pathway II	0.89	0.87	0.02	
	superpathway of geranylgeranyl diphosphate biosynthesis II (via MEP)	0.88	0.86	0.02	
	taxadiene biosynthesis (engineered)	0.12	0.17		-0.05
Secondary Metabolite Degradation	superpathway of hexitol degradation (bacteria)	0.03	0.02	0.01	
	anhydromuropeptides recycling I	0.31	0.37		-0.06
	D-galacturonate degradation I	0.22	0.25		-0.03
TCA cycle	TCA cycle I (prokaryotic)	0.45	0.48		-0.04
	TCA cycle V (2-oxoglutarate:ferredoxin oxidoreductase)	0.53	0.57		-0.04
	TCA cycle VII (acetate-producers)	0.04	0.07		-0.03

In general, pyrimidine and purine biosynthesis increased POST-T10, whereas adenosine and aminoimidazole decreased POST-T10. Within the cofactor/vitamin category, pathways related to thiamine

(vitamin B_1) and adenosyl cobalamin (vitamin B_{12}) biosynthesis decreased POST-T10, while flavin, polyisoprenoid and pyridoxal (vitamin B_6) biosynthesis increased POST-T10. In addition, ricobendazole administration decreased amino acid and chorismate biosynthesis, sucrose and fucose degradation, fatty acid biosynthesis (phosphatidylglycerol and CDP-diacylglycerol). On the other hand, pathways related to TCA cycle and secondary metabolite degradation were enriched after ricobendazole administration.

Discussion

The recent advances and understanding of the microbiome-helminth association (Zaiss and Harris, 2016) have raised questions regarding anthelmintic use and the unintended effects on the cattle microbiome. In the present study, we determined the effect that a single subcutaneous injection of ricobendazole has on the fecal microbiome of growing steers. Anthelmintic treatment had a significant effect on the fecal microbial community, with the greatest effect observed 10 days after treatment (POST-T10) based on microbial richness (Fig. 1), principal coordinate analysis (Fig. 2), the differential abundance of individual taxa (Fig. 4) and predicted metagenomes function (Fig. 5). This was unexpected given that POST-T3 was the most immediate sampling time following administration and likely when ricobendazole concentrations were higher. However, considering that ricobendazole dissolves in very low pH, the introduction of the drug in a subcutaneous area with a pH close to neutrality could favor precipitation of the drug, its slow absorption and sustained plasma concentrations over time (Formentini *et al*., 2001). The Permutational Analysis of Variance (PERMANOVA) suggested that changes in microbial profiles were still occurring up to 10 days after treatment, but we do not know whether a new equilibrium was reached at POST-T10 or the shift in microbial profile continued to occur. Further study with the observation of fecal microbiomes for more than 10 days post-treatment would help to answer this question. We speculate that any microbial population affected by anthelmintic treatment might be expected to return to the pre-treatment state following clearance of the drug and a period of microbiome recomposition.

The alteration to the microbial community associated with ricobendazole administration, a member of the benzimidazoles group (Kopel *et al*., 2015), is usually referred to as "dysbiosis" and attributed to systemic immunological effects and suppression of helminth infection (Cooper *et al*., 2008; Wammes *et al*., 2016; Sharpton *et al*., 2020). However, since the dysbiosis occurred regardless of the parasite burden in L-FEC and H-FEC groups, it is possible that some of the changes observed in this study could be the result of indirect, rather than direct, effects of ricobendazole. For example, Daniels *et al*. (2020) reported a reduction in fecal pH after administration of moxidectin to horses which determined changes in the cellulolytic bacterial population altering the microbiome composition and richness. In the present study, the increased microbial richness after ricobendazole administration may represent one mechanism by which animals enhance their performance after anthelmintic treatment as microbial richness has been associated with mature, stable and healthy gut microbial environments (Mosca *et al*., 2016; Jenkins *et al*., 2018).

Few studies demonstrated compositional changes in the gut microbiome in response to anthelmintic treatment in humans (Yang *et al*., 2017) and horses (Sirois, 2013), while other studies did not find major effects of anthelmintics on the intestinal microbiome of mice (Korte *et al*., 2018) and horses (Crotch-Harvey *et al*., 2018; Kunz *et al*., 2019). Different results from published studies may be attributable to different species, the time of sampling, the stage of parasite infection, different active ingredients, dosages, or route of delivery (Peachey *et al*., 2017; Fujishiro *et al*., 2020). In the present study in cattle, abundant genera such as *Alistipes and Ruminococcaceae* UCG-010 exhibited an increasing trend after anthelmintic treatment, while *Christensenellaceae* R7, *Ruminococcaceae* UCG-013 and *Ruminococcaceae* UCG-014 showed a decreasing trend. The genera in the Ruminococcaceae family and genus *Christensenellaceae* R7 belong to the class Clostridia, which is known to facilitate the host immune responses due to their production of short-chain fatty acids including butyrate with anti-inflammatory properties (Hu *et al*., 2021). In addition, Ruminococcaceae are highly cellulolytic bacteria and thus, an increase following ricobendazole treatment could possibly increase the host's ability to digest plant material and improve its overall performance. *Alistipes* could have a role in the modulation of the animal response after anthelmintic treatment (Hu *et al*., 2021) as it belongs to the Bacteroidetes phylum, commonly associated with anti-inflammatory cytokines and immune suppression. Other members of the phylum Bacteroidetes at the ASV level (Fig. 4) were consistently enriched on ricobendazole-treated animals suggesting a role of Bacteroidetes modulating intestinal and immune functions in the host (Rubel *et al*., 2020).

Using the PICRUSt method to predict metagenomes and functions, we found that microbial metabolism was altered after anthelmintic treatment. This is contrary to a previous study that did not find significant differences in predicted metabolic pathways in horse's fecal microbiome after administration of moxidectin (Daniels *et al*., 2020).

We speculate that ricobendazole-associated changes in taxa abundance were accompanied by changes in the inferred gene abundance of microbial metabolic pathways. In our study, nucleotide and nucleoside biosynthesis and cofactor, electron carrier and vitamin biosynthesis were the two most affected metabolic functions with 16 and 15 KEGG level 3 pathways significantly affected, respectively, after ricobendazole-treatment (Table S1). Within nucleotide and nucleoside biosynthesis, most of the pathways (11 out of 16) increased after ricobendazole-treatment and were mostly related to purine and pyrimidine *de novo* biosynthesis over salvage biosynthesis using bases recovered from the environment (Kumari and Tripathi, 2021). The *de novo* pathway is energy-inefficient compared to the salvage pathway and usually prevails in niches that have low nucleotide availability (Kumari and Tripathi, 2021). This suggests competition for nutrients between members of the fecal microbiome probably associated with increased microbial diversity after treatment with ricobendazole.

Among the metabolic functions related to cofactors and vitamins, the biosynthesis of vitamins B family was the most affected, including B1 and B12, which decreased after ricobendazole-treatment and B6 which increased after anthelmintic administration. B vitamins participate in several metabolic pathways (Putnam and Goodman, 2020), including the maintenance of immune homeostasis (Hosomi and Kunisawa, 2017) and the intestinal microbiome is a key supplier as B vitamins cannot be synthesized by cattle (Yoshii *et al*., 2019). A decrease in metabolic pathways related to amino acid biosynthesis was observed in the ricobendazole-administered feces microbiome, especially in the glutamate family (glutamate, glutamine, arginine). Glutamate and glutamine are both key nitrogen/amino group donors for amino acid synthesis and provide the major entry points of ammonia into bacterial metabolism (Shimizu and Hirasawa, 2006), particularly important for gut bacteria unable to use other nitrogen sources (Fischbach and Sonnenburg, 2011). Although we observed robust PICRUSt changes in response to ricobendazole treatment, we cannot definitively conclude that shifts in microbiome composition resulted in altered metabolic activity without additional supporting data (such as transcriptomics or metabolomics).

The level of parasite burden prior to ricobendazole administration (L-FEC and H-FEC groups) did not cause significant alterations of the fecal microbiome composition, diversity and functionality. In contrast with published literature showing that helminths in people were associated with increased microbial diversity (Kreisinger *et al*., 2015; Lee *et al*., 2014) as a host mechanism to reduce intestinal inflammation (Glendinning *et al*., 2014), cattle in the present study had similar PRE-T microbiome composition, regardless of FEC levels (Fig. S1). When we compared the differential abundance of taxa between L-FEC and H-FEC animals PRE-T, only 5 ASVs out of 2,641 were significantly different, suggesting that the level of parasite infection had a lower impact on the feces microbiome compared to anthelmintic treatment. It is possible that the parasite burden in the H-FEC group (840 eggs per gram) in the present study was not high enough to induce inflammation and changes in microbial diversity (Peachey *et al*., 2017) or that FEC was a poor indicator of parasite burden (Nielsen *et al*., 2010).

To our knowledge, this is the first study to show that ricobendazole cause substantial changes in the fecal microbiome of grazing cattle harboring low and high levels of FEC. The key strengths of this study were the longitudinal design and the mass ricobendazole-treatment which mirrors what happens in commercial farms. However, our findings are limited by the small sample size, the sex of the cattle (only males), the limited follow-up time and the lack of control groups for each sampling time. Nonetheless, determining the impact that anthelmintics exert on the structure and function of the feces microbiome of cattle is of paramount importance, as the gut metabolism of livestock (and consequently their productivity) is greatly dependent on the maintenance of a 'healthy' and diverse commensal flora (Peachey *et al*., 2017). From the research standpoint, previous exposure of animals to anthelminthic products should be considered in the design of experiments since it can be a confounding factor in the interpretation of the results.

Conclusion

This study demonstrated that the composition and function of the feces microbiome of growing steers were significantly altered 3 and 10 days after a single subcutaneous injection of ricobendazole. Advancements in the understanding of the effects of anthelmintics on gut bacteria may lead to new precise treatments to promote healthy gut microbiomes and more productive animals. Future directions of this research include comparative studies by using different anthelmintic agents with larger numbers of animals for a prolonged period to establish more precise associations between anthelmintic use, microbiome composition and phenotypic traits (i.e., average daily gain).

Acknowledgment

Special thanks to Pablo Lorenzo (INIA) for handling the animals and collecting the samples. We

also thank Dr. Fernando Dutra (DILAVE "Miguel C. Rubino", Treinta y Tres) and Ing. Alim. Paula Mussio (LATU, Montevideo) for assistance regarding handling and initial processing of fecal samples. Finally, we would like to thank the Instituto Nacional de Investigación Agropecuaria (INIA Uruguay) for supporting this study.

Funding Information

This study was funded by the Instituto Nacional de Investigación Agropecuaria (INIA Uruguay).

Ethics

The author declares no conflict of interest and declares that this study is an original work containing unpublished material.

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