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Anthelmintic Treatment Reveals Sex-Dependent Worm–Gut Microbiota Interactions

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ABSTRACT

Gastrointestinal helminths interact with the gut microbiota in ways that shape microbiota structure and function, but these effects are highly inconsistent across studies. One factor that may help explain variation in parasite–microbiota interactions is host sex since helminths can induce sex-specific changes in feeding behaviour and diet that might cascade to shape gut microbial communities. We tested this idea using an anthelmintic treatment experiment in wild Grant's gazelles (*Nanger granti*). We found that in males, anthelmintic treatment induced short-term shifts in microbial diversity and structure within ~40–70 days, but in females, treatment had effects on microbiota structure that emerged over a longer period of ~500 days. Long-term effects of treatment on the microbiota of females were potentially due to sex-specific changes in feeding behaviour since deworming nearly doubled the time females spent feeding, but did not affect feeding time in males. In support of this idea, anthelmintic treatment eliminated associations between microbial diversity and diet in females, and treated females maintained a more stable abundance of microbial taxa and predicted functions. Together, these findings suggest that accounting for host traits can help uncover mechanisms, such as changes in diet, by which helminths interact with the microbiota.

1 | Introduction

Within the gastrointestinal tract, helminth parasites and commensal bacteria commonly co-occur and can interact via their use of space, release of metabolic products and modulation of host immune responses [1, 2]. In laboratory rodents, for example, helminths have been shown to influence gut microbiota composition in ways that suppress antiworm immunity and promote their own persistence [3–5]. However, our ability to characterise helminth–microbiota interactions in natural settings has been complicated by inconsistencies across studies. For example, the presence of helminth infections has been associated with changes in gut microbial diversity in some species (e.g., wild primates [6]), but not others (e.g., wild mice [7]). These differential effects of helminths on gut microbes are likely influenced by a range of factors including host and helminth species identity [7, 8]. In addition, intraspecific variation among hosts should play a further role in determining how helminths interact with the microbiota. For example, in primates, helminth effects on gut microbial diversity were influenced by host habitat use [6].

Host sex is another factor that drives considerable variation in aspects of host physiology and behaviour that influence the gut microbiota. In particular, males and females often differ in their feeding behaviour and diet [9–11], and diet is one of the strongest drivers of gut microbial composition and function [12, 13]. Indeed, in a range of wild animals studied to date, gut microbiota composition tracks changes in diet across seasons [12, 14–17]. Interestingly, such microbiota–diet relationships may be further modified by helminth infection, which also commonly affects host feeding behaviour, often by reducing feeding rates [18] or changing dietary preferences [19–21]. Given this, host sex represents a promising starting point for examining intraspecific variation in helminth–microbiota interactions – if both host sex and helminth infection influence host feeding behaviour, these factors should interact to shape how the microbiota responds to diet.

In this study, we used an anthelmintic treatment experiment in a wild mammal, Grant's gazelle to test for sex-based differences in helminth–microbiota interactions. Specifically, we examined the effects of de-worming on gut microbiota diversity and composition and host feeding behaviour in male and female gazelles. We asked (i) whether anthelmintic treatment had sex-specific effects on the gut microbiota and (ii) if these effects could be explained by sex-dependent effects of treatment on host feeding behaviour. If so, we predicted that anthelmintic treatment would affect the microbiota's ability to track changes in diet, with functional consequences for the degradation of dietary components.

2 | Materials and Methods

2.1 | Animal Capture and Sampling

Male and female Grant's gazelles (*Nanger granti*) were captured at the Mpala Research Centre, Kenya in June 2011 using helicopter net gunning [22–24]. All animals were tagged for individual identification and randomly assigned to an anthelmintic treatment group based on capture sequence. Treated animals received a single dose of moxidectin (0.05 mL/kg of cydectin long-acting intramuscular injection for sheep, Virbac Animal Health) and control animals received saline. Cydectin provides protection against gastrointestinal nematodes for up to 120 days in sheep [25], and a similar duration of efficacy was reported in Grant's gazelle [22, 23]. After collecting demographic information and biological samples, animals were released back into the wild. In this study, we report data collected from 13 adult females (6 control, 7 anthelmintic-treated) and 11 adult males (4 control, 7 anthelmintic-treated) that were monitored between June 2011 and November 2012. Because male Grant's gazelles transition between nonreproductive (bachelor) and reproductive (territorial) states during adulthood and territorial males are most distinct from females in both behaviour and helminth parasitism [22], we focused only on territorial males.

To examine anthelmintic treatment-associated changes in gazelle feeding behaviour, we collected data on individual feeding behaviour. Past work on this population of gazelles has shown that anthelmintic treatment nearly doubles the amount of time female gazelles spend feeding [23], so here we performed a similar analysis focused on males that were captured at the same time from the same population. To quantify male behaviour, solitary males or male–female groups were located by vehicle and territorial males were selected for focal observation [22]. For each observation, we recorded continuous data on five core behaviours (feeding, vigilance, resting, moving, agonism/dominance) using a hand-held recording device. Feeding was defined as grazing or browsing at any height or actively searching for food [24]. Observations were performed by a single observer and each observation period ranged from 15 to 34 min.

For microbiota and parasitological analysis, a pretreatment faecal sample was collected at capture, and subsequently, posttreatment samples were collected by monitoring free-ranging, individually identifiable animals. In total, 372 faecal samples were collected, with 2–30 samples collected per individual. A subset of each faecal sample was used for parasitological analysis and a second subset was stored at −20°C until DNA extraction for microbiota sequencing.

2.2 | Parasitological Analysis

To assess helminth infection status we focused on strongyle nematodes (Nematoda: Strongylidae), a group of gastrointestinal nematodes found at high prevalence in Grant's gazelle [26]. Strongyle egg output in faeces was quantified using a modification of the McMaster faecal egg counting technique. Briefly, 3 g of each faecal sample was homogenised in water and strained to remove debris, followed by centrifugation and suspension of the pellet in sodium chloride solution (specific gravity 1.2). Aliquots of the resulting suspension were used to fill two chambers of a McMaster slide, and the number of strongyle eggs per gram faeces was calculated from the average egg count across both chambers [26]. In all cases, egg counts were performed on the day of sample collection.

2.3 | Gut Microbiota Sequencing and Processing

Gut microbial communities were assessed via 16S rRNA gene sequencing according to Earth Microbiome Project protocols [27]. Briefly, the V4 region of the 16S rRNA gene was amplified using 515F and 806R PCR primers and sequenced using an Illumina MiSeq. Sequences were uploaded to the QIITA repository, where forward 150 bp reads were clustered into amplicon sequence variants (ASVs) with Deblur v. 2021.09 [28]. We excluded rare ASVs present at an abundance of $< 0.01\%$ of the total dataset, and normalised samples across sequencing depths by rarefying each sample to 5000 ASVs [29]. Study individuals were required to have both a pretreatment sample and at least one posttreatment sample that passed these processing steps to be included in further analyses. A phylogenetic tree relating ASVs was constructed using the fragment insertion method (SATé-enabled phylogenetic placement or SEPP [30]). We assessed microbial alpha diversity by calculating ASV richness and Faith's phylogenetic diversity (PD), which accounts for phylogenetic relationships among taxa. We assessed microbial community structure by calculating Bray–Curtis dissimilarities between samples, which considers both the presence and relative abundance of ASVs, and weighted UniFrac distances, which also accounts for phylogenetic

relationships among taxa [31]. Both alpha diversity and community structure metrics were calculated using QIIME2 v. 2020.11 [32].

For analysis of microbial taxonomic abundance, we assigned taxonomy to ASVs using the GreenGenes2 database (v. 2022.10) and collapsed taxonomy at the genus level. To assess whether anthelmintic treatment affected microbiota functions in addition to taxonomic composition, we predicted the functional capacity of the microbiota using the PICRUSt2 plug-in [33] in QIIME2 v. 2019.7. We used the "custom tree pipeline" with SEPP [30] to compare ASVs to the PICRUSt2 annotated reference genome database, predicted the abundance of gene families with the maximum parsimony method, and mapped counts to MetaCyc pathways using MinPath [34]. We removed rare pathways present at an abundance of $<0.01\%$ of the total data set and removed "engineered" pathways before rarefying samples to 425,000 pathways for analysis.

2.4 | Statistical Analyses

To examine whether effects of anthelmintic treatment on the gut microbiota were sex-dependent, we performed all analyses separately in males and females. This approach allowed us to analyse how microbiota patterns differed by sex without using three-way interaction terms, which require higher sample sizes [35]. First, we examined if anthelmintic treatment had short-term effects on the microbiota. In gazelles, treatment resulted in reduced egg shedding for a period of ~120 days [22, 23]. Within this 120-day drug efficacy period, treated individuals shed fewer parasite eggs than controls, whereas after 120 days, treated and control individual did not differ in the level of parasite shedding [22, 23]. About halfway through the efficacy period (samples collected 40–70 days after treatment, mean: 55 days), treated individuals were shedding zero parasite eggs prior to any worm re-accumulation. We tested for differences in microbiota diversity and structure between pretreatment samples (when worms were present) and samples collected 40–70 days posttreatment and considered these differences as short-term effects. For alpha diversity, we tested for an effect of time point (pre- vs. posttreatment), treatment group (treated vs. control) and an interaction between time point and treatment group using repeated measures analysis of variance (ANOVA) implemented in *rstatix* v. 0.7.2 [36] in R v. 4.3.1. Diversity values were normalised using Box-Cox transformations (Shapiro–Wilk's test: $W>0.937$, $p>0.386$). For structure, we tested for an effect of the interaction between time point and treatment group after accounting for animal ID, using permutational ANOVA (PERMANOVA) implemented in *adonis2* in *vegan* v. 2.6-4 [37]. PERMANOVA models were run on square root-transformed distance matrices with 1000 permutations.

Second, we examined if anthelmintic treatment had effects on long-term trajectories of microbiota structure. To do this, we calculated pairwise differences in microbiota structure between an individual's pretreatment sample and each posttreatment sample spanning the entire duration of the study (~500 days). We tested for an effect of treatment group, the number of days since treatment and the interaction between days since treatment and treatment group on dissimilarity using generalised linear mixed models with a beta distribution implemented in *glmmTMB* v. 1.1.7 [38]. The dispersion of simulated model residuals was assessed using *DHARMa* v. 0.4.6 [39]. Animal ID was included as a random effect in the model and to improve model fit, days since treatment was

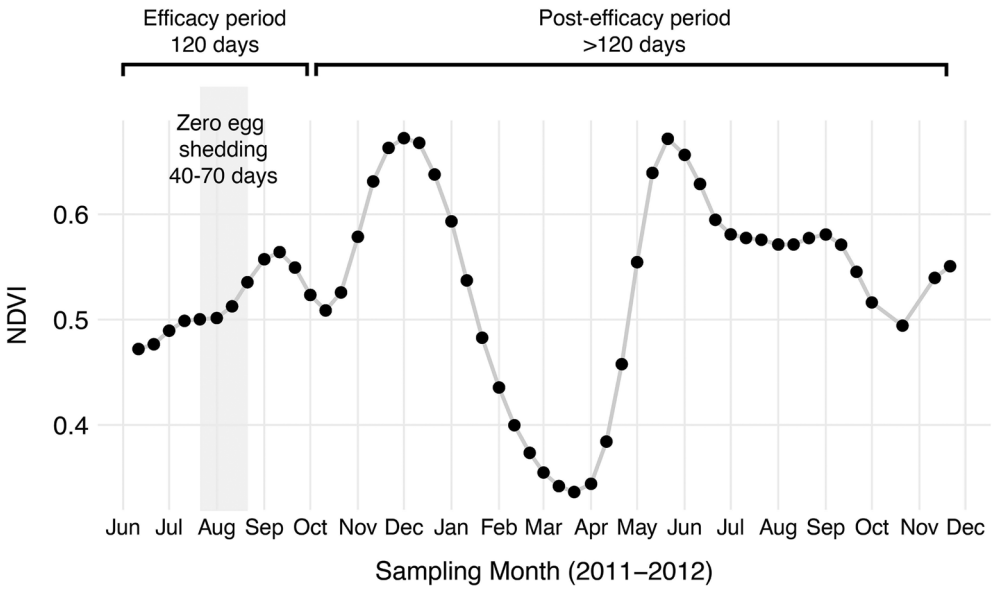

FIGURE 1 | Changes in forage greenness during the study period. Forage greenness was assessed via average normalised difference vegetation index (NDVI) values for Laikipia County, Kenya over 10-day intervals. The study period was divided into several time frames for analysis: the anthelmintic treatment was efficacious for 120 days posttreatment, during which treated gazelles shed significantly fewer parasite eggs than control gazelles. We analysed the short-term effects of treatment by comparing pretreatment samples to samples collected 40–70 days posttreatment, when treated gazelles shed zero parasite eggs prior to any worm re-accumulation. We analysed the long-term effects of treatment using samples collected from the entire study period, which included samples collected after the 120-day treatment efficacy period, when treated and control gazelles did not differ in parasite egg shedding.

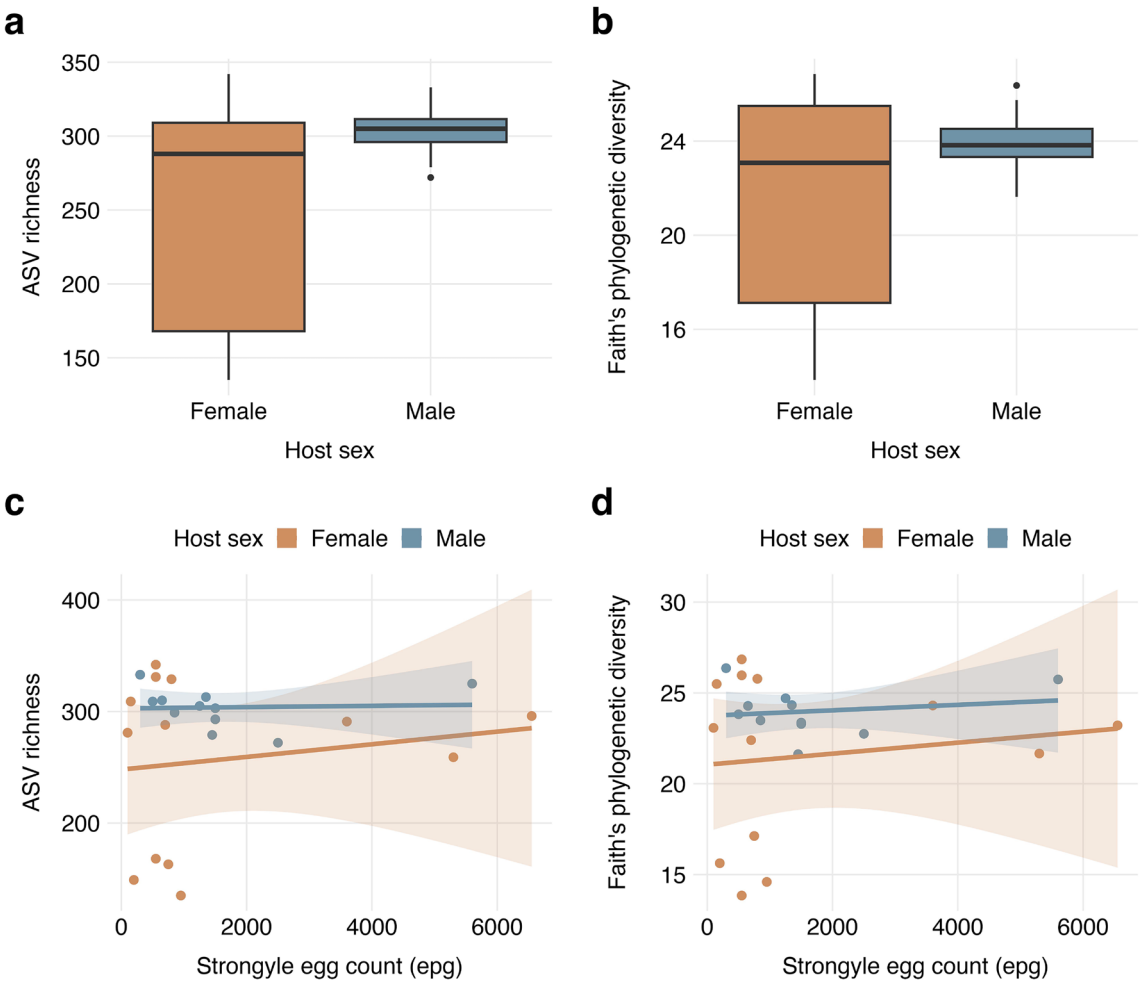

FIGURE 2 | Patterns of gut microbial diversity did not differ between male and female Grant's gazelles prior to anthelmintic treatment. Pretreatment, male ($N = 11$) and female ($N = 13$) gazelles did not differ in microbial alpha diversity as measured by (a) amplicon sequence variant (ASV) richness or (b) Faith's phylogenetic diversity. Pretreatment, (c) ASV richness and (d) Faith's phylogenetic diversity were not correlated with worm egg counts, measured as eggs per gram of faeces (epg), in either male or female gazelles.

scaled without centering for analysis. To explore the potential for nonlinear trajectories in microbiota structure over time, we also fit these models with polynomial relationships. However, polynomial models did not improve model fit compared to linear models ($\Delta AIC > 2$), so we report the linear models. This analysis of dissimilarities in microbiota structure as a function of time allowed us to examine whether microbial communities continuously diverged from pretreatment communities throughout the study (reflected by a positive slope, indicating that dissimilarities from baseline increased over time), did not accumulate changes from pretreatment communities (reflected by a slope of zero, indicating that dissimilarities from baseline remained stable over time), or initially diverged from pretreatment communities, but then converged toward pretreatment communities later in the study (reflected by a negative slope, indicating that dissimilarities from baseline decreased over time). While we recognise that using only one pretreatment sample per individual may have limited our ability to fully characterise baseline (i.e., pretreatment) microbial communities, given our focus here on long-term trajectories at the treatment group level, uncertainty about the exact composition of each individual's baseline community should not significantly bias our results.

Third, we examined if sex-specific effects of anthelmintic treatment on the microbiota could be explained by changes in host feeding behaviour. As a first step, we tested for an effect of treatment on male feeding behaviour as was previously done for the females [23]. Male and female gazelles were sampled from the same population and received anthelmintic treatment at the same time, so we were able to compare if the effects of treatment on feeding behaviour differed by sex. To understand whether treatment changed the behaviour of treated males relative to control males, we used Wilcoxon rank-sum tests to compare the proportion of time spent feeding by control and treated males during the anthelmintic efficacy period (≤ 120 days posttreatment), during which treated individuals maintained significantly lower parasite burdens compared to control individuals [22, 23], as well as after drug efficacy waned (> 120 days posttreatment). This methodology mirrors that used to examine the effects of anthelmintic treatment on feeding behaviour for female gazelles in this population [23]. Next, we looked for evidence of an anthelmintic treatment effect on the relationship between host feeding behaviour and diet. If helminths affect the microbiota via changes in host feeding behaviour, then treatment should disrupt expected relationships between the microbiota and host diet. Therefore, we tested whether anthelmintic treatment affected the ability of the

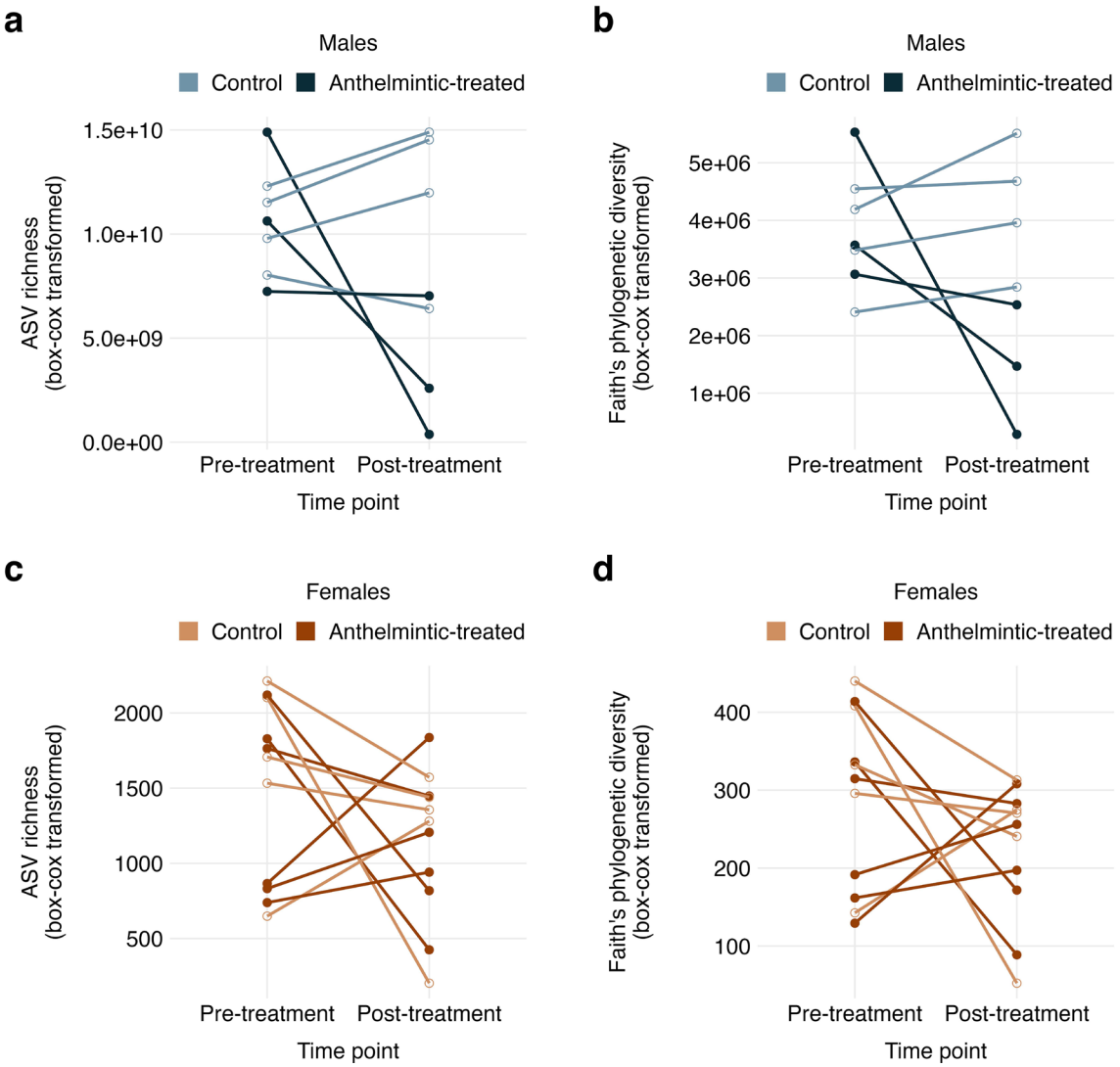

FIGURE 3 | Anthelmintic treatment had short-term effects on gut microbial diversity in male Grant's gazelles. By 40–70 days posttreatment, male gazelles ($N = 7$) showed a marginal decrease in microbial diversity as measured by (a) amplicon sequence variant (ASV) richness, and a significant decrease microbial diversity as measured by (b) Faith's phylogenetic diversity. (c, d) By 40–70 days posttreatment, there was no change in either measure of microbial diversity in female gazelles ($N = 11$; Table S1). Points are connected by animal ID.

microbiota to track changes in diet in males and females. To measure how diet changed over time, we estimated the normalised difference vegetation index (NDVI), a "vegetation greenness" index frequently used as a proxy for diet quality in wild ruminants [40, 41]. NDVI values were obtained from the USGS Early Warning programme (Figure 1). These values represent the average NDVI for our study region over 10-day intervals, and here we assume that all gazelles experienced the same forage greenness at any given point in time. We used linear mixed models (*lme4* v. 1.1-34 [42] and *lmerTest* v. 3.1-3 [43]) to test whether anthelmintic treatment modified the relationship between microbiota diversity and forage greenness. Model predictors included days since treatment, NDVI, treatment group and the interaction between NDVI and treatment group. Animal ID was included as a random effect. Diversity values were normalised using Box-Cox transformations (Shapiro–Wilk's test of model residuals: $W > 0.965$, $p > 1e10^{-4}$). We also assessed how much inter-individual variability contributed to these patterns by estimating the amount of variance explained by the random effect of animal ID. To do so, we subtracted the marginal pseudo-R^2 value (representing the variance explained by the fixed effects) from the conditional pseudo-R^2 value (representing the total variance explained by the model) for each model using *performance* v. 0.12.2 [44].

Finally, for either sex that showed an effect of treatment on the relationship between the microbiota and NDVI, we also tested for specific microbial genera and predicted functions contributing to these effects. First, we tested if treatment modified relationships between microbial genera abundance and forage greenness. To do so, we used Analysis of Compositions of Microbiomes with Bias Correction (*ANCOMBC* v. 2.4.0), which analyses differences in microbial abundance among groups while accounting for uneven sampling across samples [45]. For this analysis, forage greenness values were binned according to whether they fell above (high greenness) or below (low greenness) the median NDVI value for our study period. Then, we compared how microbial genera abundance differed between samples from treated individuals during periods of high greenness and all other groups: treated individuals during low greenness, control individuals during high greenness and control

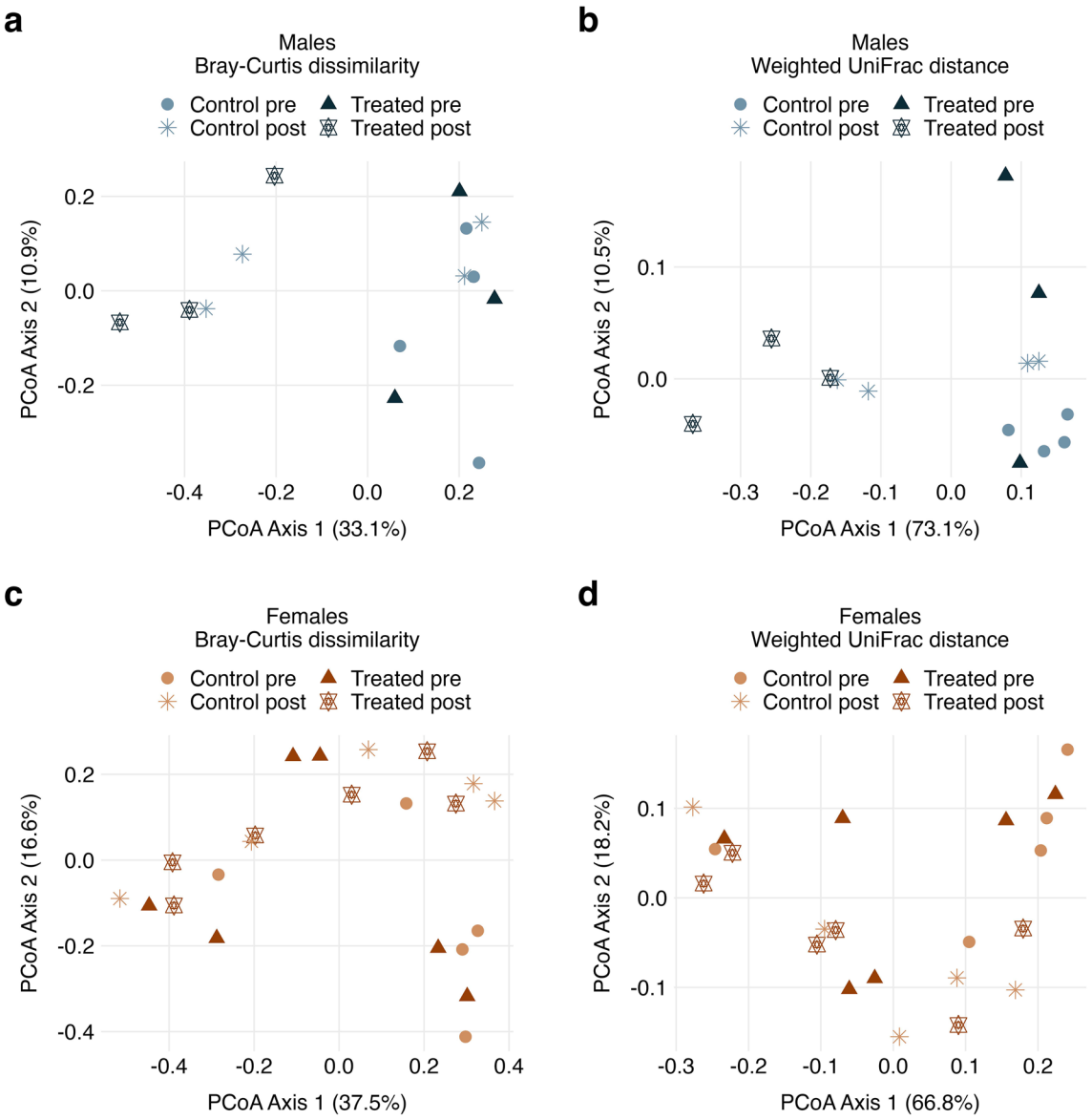

FIGURE 4 | Anthelmintic treatment had short-term effects on gut microbiota structure in male Grant's gazelles. Principal coordinate analysis (PCoA) plots showed that treatment was associated with a shift in gut microbial structure, as measured by Bray–Curtis dissimilarity and weighted UniFrac distance, by 40-70 days posttreatment in (a, b) male ($N = 7$), but not (c, d) female ($N = 11$) gazelles (Table S2).

individuals during low greenness. We corrected *p*-values for multiple comparisons using the Benjamini–Hochberg method.

Second, we tested whether taxonomic patterns translated to microbiota function by identifying how treatment modified relationships between forage greenness and the abundance of predicted functional pathways. To do so, we used a 'songbird' multinomial regression model [46] in QIIME2 v. 2019.7 to identify PICRUSt2-predicted functional pathways that were associated with the interaction between NDVI and treatment group (see the Supporting Information S1). We viewed the output of this model using the Qurro visualisation tool, selected the top 5% of predicted pathways that were most strongly associated with NDVI in control versus anthelmintic-treated individuals, and calculated the log-ratio of their abundances [47]. We then used a linear mixed model to test how the abundance of these pathways changed with NDVI in each treatment group. Model predictors included days since treatment, NDVI, treatment group and the interaction between NDVI and treatment group, with animal ID as a random effect. When models indicated a significant interaction effect, we tested whether slopes for each treatment group differed from zero using *emmeans*, v. 1.8.7 [48] and corrected *p*-values using the Benjamini–Hochberg method.

3 | Results

3.1 | Anthelmintic Treatment Had Short-Term Effects on the Microbiota of Males, but Long-Term Effects on the Microbiota of Females

Before anthelmintic treatment, samples from males and females did not differ in microbial alpha diversity as measured by ASV richness or Faith's PD (Wilcoxon test: W range = 46.5–53.0, $p > 0.156$; Figure 2a,b) nor was microbial diversity correlated with pretreatment worm egg count in males (Spearman

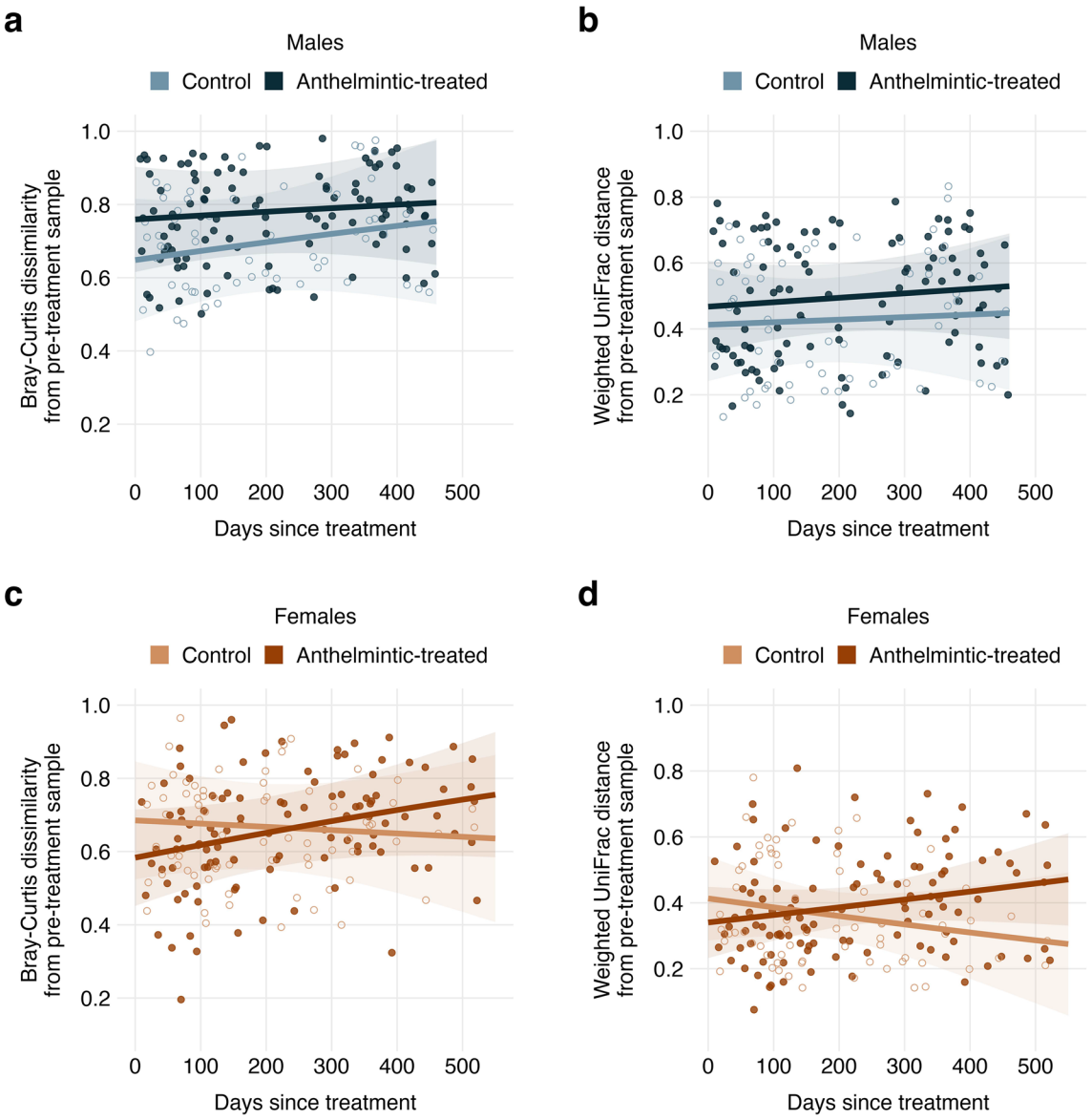

FIGURE 5 | Anthelmintic treatment had long-term effects on gut microbial structure in female Grant's gazelles. Anthelmintic treatment altered long-term trajectories of gut microbial structure, as measured by Bray–Curtis dissimilarity and weighted UniFrac distance, in (c, d) female ($N = 182$), but not (a, b) male ($N = 166$) samples (Tables S3 and S4). Fitted lines are derived from a generalised linear mixed model and shaded areas represent standard errors. Open circles represent control samples and closed circles represent anthelmintic-treated samples.

correlation: ρ range = −0.387 to −0.410, $p > 0.210$) or females (ρ range = −0.072 to −0.083, $p > 0.788$; Figure 2c,d).

Our analysis of short-term microbiota patterns examined how samples collected 40–70 days after treatment (when worm egg shedding was zero) differed from pretreatment samples. In a model testing the effect of treatment, time (pretreatment vs. posttreatment) and the interaction between the two, we found that there was a marginal decline in microbial diversity as measured by ASV richness (repeated measures ANOVA: treatment × time point, $F = 6.155$, $p = 0.056$; Figure 3a), and a significant decline in diversity as measured by Faith's PD ($F = 7.211$, $p = 0.044$; Figure 3b, Table S1) among males. In contrast, there was no effect of treatment on any measure of microbial diversity in females (F range = 0.157–0.254, $p > 0.627$; Figure 3c,d, Table S1). A short-term effect of anthelmintic treatment on the microbiota of males was also apparent in microbiota structure; treatment was associated with a difference in structure between pretreatment and posttreatment samples as measured by both Bray–Curtis dissimilarity (PERMANOVA: treatment × time point, $R^2 = 0.212$, $p = 0.012$; Figure 4a) and weighted UniFrac distance (PERMANOVA: treatment × time point, $R^2 = 0.340$, $p = 0.002$; Figure 4b, Table S2). There was no effect in females (R^2 range = 0.095–0.112, $p > 0.266$; Figure 4c,d, Table S2).

Our analysis of long-term microbiota trajectories examined if and how samples collected throughout the entire study period (~500 days) differed from an individual's baseline sample. In a model testing the effect of treatment, time and the interaction between the two, we found a main effect of treatment for one of two measures of microbiota structure in males (Bray–Curtis dissimilarity GLMM: treatment, estimate ± SE = 0.537 ± 0.220,

$p = 0.015$, Figure 5a; Weighted UniFrac GLMM: treatment, estimate ± SE = 0.223 ± 0.220, $p = 0.311$; Figure 5b, Table S3). Treated males had a higher Bray–Curtis intercept (0.76) than control males (0.65), suggesting more dissimilarity in microbiota structure between pretreatment samples and the first samples collected (within weeks) after treatment. However, there was no interaction effect for either measure of microbiota structure (Bray–Curtis dissimilarity GLMM: treatment × time, estimate ± SE = −0.126 ± 0.190, $p = 0.507$, Figure 5a; Weighted UniFrac GLMM: treatment × time, estimate ± SE = 0.055 ± 0.202, $p = 0.786$, Figure 5b, Table S3), indicating that treatment did not affect trajectories over time.

In females, for Bray–Curtis dissimilarity, there was a significant main effect of treatment (GLMM: treatment, estimate ± SE = −0.441 ± 0.210, $p = 0.035$) and a significant treatment × time interaction effect (treatment × time, estimate ± SE = 0.448 ± 0.170, $p = 0.008$; Table S3), where the slope was positive for treated females and zero for control females (treated: estimate ± SE = 0.350 ± 0.102, $p = 0.001$; control: estimate ± SE = −0.098 ± 0.136, $p = 0.472$; Figure 5c, Table S4). The positive slope in treated females suggests a directional change in microbiota structure over time. For weighted UniFrac distance, there was a significant main effect of time (GLMM: time, estimate ± SE = −0.274 ± 0.137, $p = 0.046$) and a significant treatment × time interaction effect (GLMM: treatment × time, estimate ± SE = 0.516 ± 0.168, $p = 0.002$, Table S3), where the slope was positive for treated females (estimate ± SE = 0.241 ± 0.097, $p = 0.025$; Figure 5d, Table S4), also suggesting a directional change in microbiota structure. However, the slope was negative for control females (estimate ± SE = −0.274 ± 0.137, $p = 0.046$; Figure 5d, Table S4), indicating that microbiota structure initially deviated from baseline samples, but then became more similar to baseline throughout the rest of the study.

3.2 | Treatment Affected Whether Gut Microbial Diversity, Abundance and Predicted Function Tracked Forage Greenness in Females

The lasting effect of anthelmintic treatment on the female gut microbiota may be linked to treatment-associated changes in feeding behaviour. In males, treatment had no effect on time spent feeding (Wilcoxon test: $W = 1210$, $p = 0.564$; Figure 6, Table S5), which contrasts sharply with females, where treatment nearly doubled feeding time [23]. To evaluate whether anthelmintic treatment influenced how the microbiota responded to changes in diet, we tested the effect of treatment, forage greenness and the interaction between the two on microbial diversity measured as ASV richness and Faith's PD. For females, we found a significant interaction between treatment and forage greenness for both ASV richness (LMM: treatment × NDVI, $F = 8.028$, $p = 0.005$; Figure 7c) and Faith's PD (LMM: treatment × NDVI, $F = 5.745$, $p = 0.018$; Figure 7d, Table S6), where control females showed a negative relationship between forage greenness and diversity (ASV richness: estimate ± SE = $-2.84 \times 10^4 \pm 9.34 \times 10^3$, $p = 0.005$; Faith's PD: estimate ± SE = $-3.34 \times 10^3 \pm 1.24 \times 10^3$, $p = 0.015$), and treated females did not (ASV richness: estimate ± SE = $6.74 \times 10^3 \pm 8.23 \times 10^3$, $p = 0.414$; Faith's PD: estimate ± SE = $603.63 \pm 1.09 \times 10^3$, $p = 0.581$; Figure 7c,d, Table S7). In contrast, for males, both control and treated individuals showed negative relationships between forage greenness and diversity as measured by ASV richness (LMM: treatment, $F = 4.381$, $p = 0.038$; control: estimate ± SE = −1790 ± 1201; treated: estimate ± SE = −1400 ± 927; Figure 7a), and Faith's PD (LMM: treatment, $F = 4.364$, $p = 0.038$; control: estimate ± SE = −585 ± 377; treated: estimate ± SE = −416 ± 291; Figure 7b, Table S6). Interestingly, the fixed effects of forage greenness and treatment explained the same amount of variation (5.9%–6.8%) as individual identity (5.9%–6.2%) in males, whereas in females, the pattern was starkly different, with the fixed effects explaining ~3–5 times more variation (18.6%–19.4%) than individual identity (3.4%–5.4%), highlighting the biological importance of the combined effect of forage greenness and treatment in explaining microbiota patterns in females.

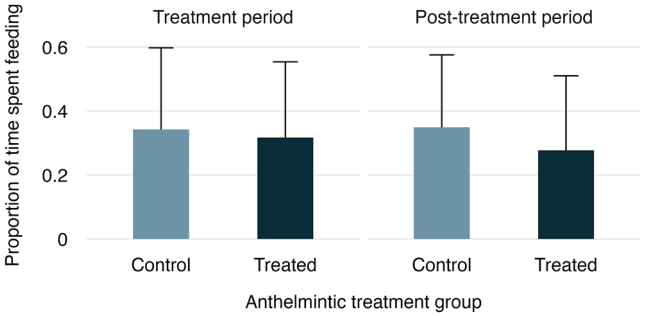

FIGURE 6 | Anthelmintic treatment did not affect the proportion of time that male Grant's gazelles spent feeding. Control and anthelmintic-treated males spent similar amounts of time feeding both during the treatment efficacy period (≤120 days after treatment, $N = 9$ individuals, 97 observations) and following the treatment efficacy period (>120 days after treatment, $N = 8$ individuals, 124 observations; Table S5). Bars represent group means and error bars represent standard deviations.

The disruption of microbiota–diet relationships in treated females also affected the abundance of specific microbial genera. Overall, we identified 159 microbial genera in the gazelle microbiota. Samples from treated females collected during periods of high forage greenness resembled those from treated females during periods of low greenness (difference in 3 genera: W range = 3.464–3.876, $p < 0.028$; Figure 8a, Table S8) and those of control females during periods of high greenness (difference in 1 genus: $W = 4.073$, $p = 0.007$; Figure 8b, Table S8). In contrast, samples from treated females collected during periods of high greenness were most dissimilar to samples from control females during low greenness (difference in 20 genera: W range = −4.270–6.046, $p < 0.048$; Figure 8c, Table S8). While treated-high greenness samples were enriched with genera from the Bacteroidetes and Proteobacteria phyla, control-low greenness samples were enriched with genera within the Firmicutes A, Firmicutes D and Actinobacteriota phyla (Figure 8c, Table S8).

Similar to the microbial abundance results, anthelmintic treatment stabilised the abundance of predicted microbial functional pathways in females. The log-ratio of predicted functional pathways associated with NDVI in control versus treated females was calculated from a "songbird" multinomial regression model (Table S9), and the abundance of these pathways was differentially sensitive to changes in NDVI across treatment groups

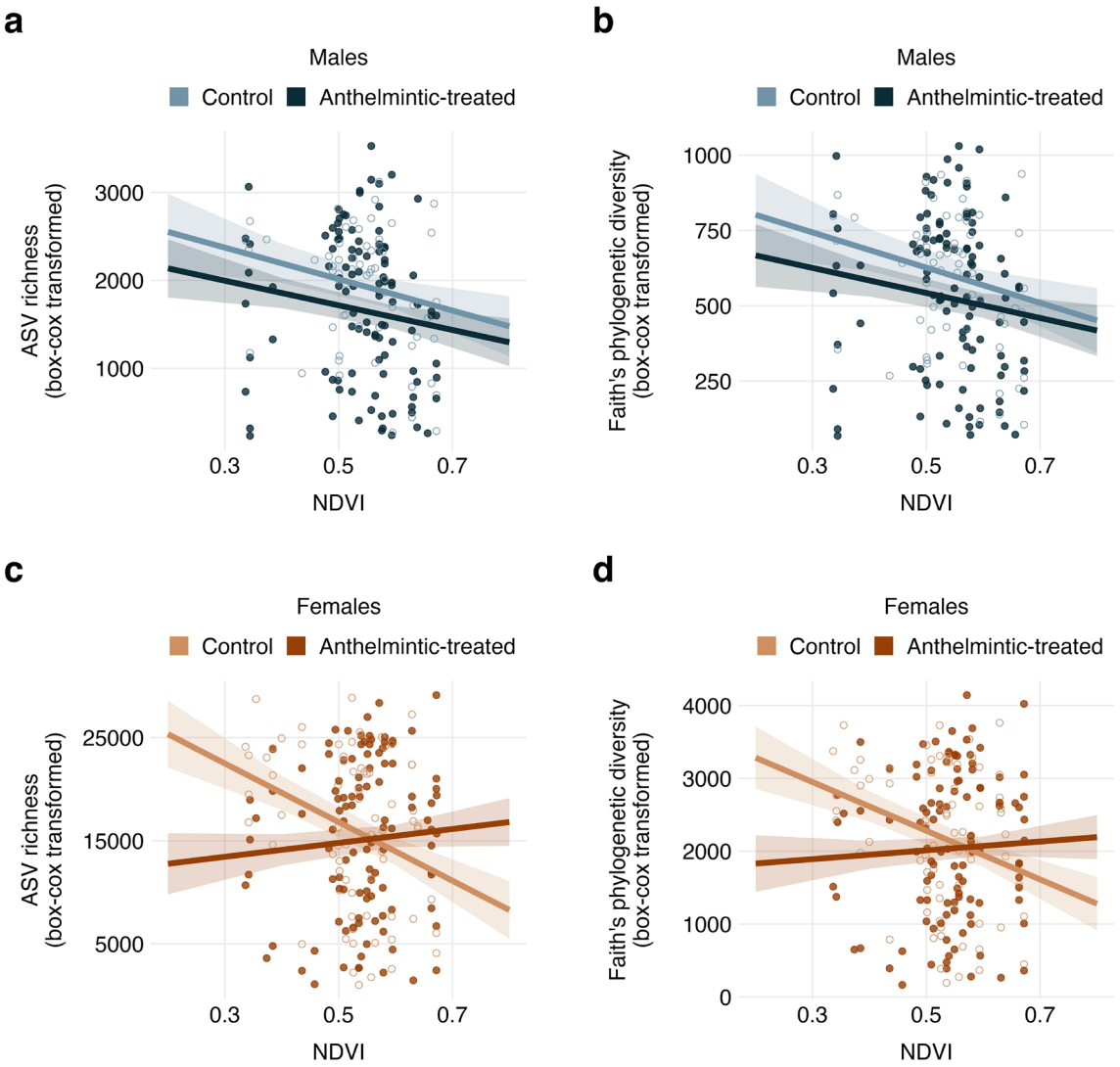

FIGURE 7 | Anthelmintic treatment decoupled relationships between gut microbial diversity and forage greenness in female, but not male, Grant's gazelles. (a, b) Both control and anthelmintic-treated male gazelles ($N = 166$ samples) showed a negative relationship between forage greenness and gut microbial alpha diversity, as measured by (a) amplicon sequence variant (ASV) richness and (b) Faith's phylogenetic diversity (Table S6). (c, d) However, the anthelmintic treatment caused relationships between forage greenness and gut microbial alpha diversity to be absent in female gazelles ($N = 182$ samples; Tables S6 and S7). Forage greenness was assessed via normalised difference vegetation index (NDVI) values for Laikipia County, Kenya. ASV richness values were Box-Cox transformed for analysis.

(LMM: treatment × NDVI, $F = 5.305$, $p = 0.022$; Table S10). Pathway abundance tended toward a negative association with NDVI in control females (estimate ± SE = −2.848 ± 1.268, $p = 0.052$), while there was no association with NDVI in treated females (estimate ± SE = 1.022 ± 1.116, $p = 0.361$; Figure 8d, Table S11). Specifically, increases in NDVI were associated with the enrichment of different pathways involved in the synthesis of B vitamins and amino acids in control (e.g., glutamine) versus treated (e.g., tyrosine) females (Table S9). As forage greenness decreased, samples from control females were also enriched with predicted pathways related to the synthesis of siderophores and cell membrane components, and the degradation of amino acids, nucleotides and sucrose, whereas samples from treated females were enriched with predicted pathways for the synthesis of haeme molecules and degradation of various aromatic compounds (Table S9). Together, these results suggest that treatment interacted with forage greenness in females, resulting in a more stable abundance of microbial taxa with implications for predicted microbial functions.

4 | Discussion

Conflicting effects of gastrointestinal helminths on the gut microbiota are widely described across human and animal hosts [8, 49]. Here, we demonstrate that host sex can help explain some of this variation. By using an anthelmintic treatment experiment in a free-ranging mammal, we show that the magnitude and timing of helminth effects on the gut microbiota are sex-dependent. Specifically, effects of anthelmintic treatment on the microbiota emerged in the short-term (40–70 days) in males and were likely a direct result of the absence of worms, whereas

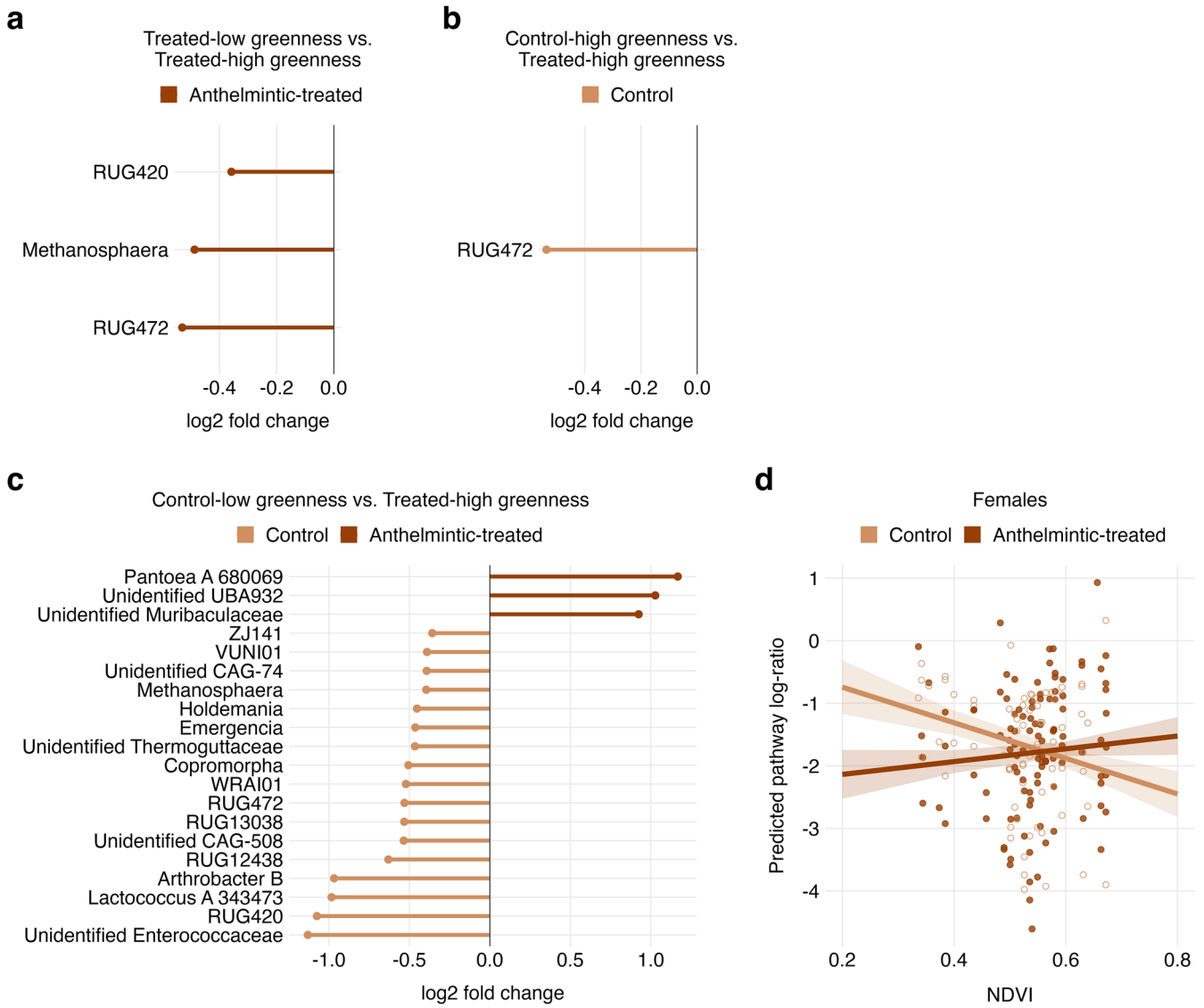

FIGURE 8 | Anthelmintic treatment stabilised changes in gut microbial abundance and predicted function amidst changes in forage greenness in female Grant's gazelles. Results of ANCOM-BC tests for differences in genera abundance in comparison to treated female samples at high forage greenness ($N = 57$; Table S8). While these samples showed only minor differences in abundance compared to samples from (a) control gazelles at high forage greenness ($N = 31$) and (b) treated gazelles at low forage greenness ($N = 52$), they had more differences from (c) control gazelle samples at low forage greenness ($N = 42$). (d) A 'songbird' multinomial regression model used to identify PICRUSt2-predicted functional pathways that were associated with forage greenness in control versus anthelmintic-treated female gazelle samples ($N = 182$). The log-ratio of the top 5% of predicted pathways associated with NDVI in treated females (numerator) versus the top 5% of predicted pathways associated with NDVI in control females (denominator) was calculated to test how pathway abundances varied between treatment groups (Table S9). The abundance of this log-ratio tended to be negatively associated with NDVI in control females but was not associated with NDVI in anthelmintic-treated females (Tables S10 and S11). Fitted lines are derived from a linear mixed model and shaded areas represent standard errors. Open circles represent control samples, and closed circles represent anthelmintic-treated samples.

effects of treatment accumulated over a longer time frame (~500 days) in females. Given that treatment increased feeding behaviour in females but not males, and that the strong diet–microbiota relationships observed in males and control females were absent in treated females, we hypothesise that long-term effects of anthelmintic treatment on the female gazelle microbiota were driven by an effect of worms on host feeding behaviour.

Anthelmintic treatment had transient effects on the microbiota of males that were only present during the period when worm egg shedding was absent. Moreover, treatment had no effect on male feeding behaviour, and for all males (control and treated), there was a strong negative association between microbial diversity and forage greenness (NDVI). Negative relationships between microbial diversity and diet quality have been observed in other ruminant hosts (e.g., African buffalo [50], cattle [51]) and may reflect the dominance of select microbes that best degrade readily metabolisable components available when diet quality is relatively high, versus an expansion of more diverse microbial taxa capable of degrading nutrients that remain as diet quality declines [15, 52–54]. For example, in cattle fed a low protein diet, fermentation of fibre was associated with increases in microbial

diversity compared to starch, likely because complex fibre structures yield a greater diversity of by-products for microbes to degrade [51]. Thus, the consistent change in microbial diversity in response to forage greenness in both control and treated male gazelles implies that the microbiota was responding similarly to environmentally mediated changes in host diet in both groups. Coupled with the absence of a treatment effect on male feeding behaviour, and the presence of a treatment effect on the microbiota that coincided with the absence of worm egg shedding, this result suggests that the observed effects of anthelmintic treatment on the male microbiota were unlikely to be mediated by diet and could be the direct result of worm clearance.

In contrast to males, anthelmintic treatment had longer-term effects on the microbiota of females that were accompanied by strong effects of treatment on diet. Treatment nearly doubled the time that females spent feeding [23]; it was associated with directional changes in microbiota structure over time; and it substantially weakened the negative association between microbial diversity and forage greenness observed in males and control females. This latter result is emphasised by the large amount of variance explained by the interaction between treatment and forage greenness in female compared to male models. Taken together, these results suggest that long-term microbiota changes observed in females could reflect the influence of anthelmintic treatment on host feeding behaviour. For example, if treated females spend more time feeding when forage quality is low, they might maintain higher levels of diet quality and correspondingly lower levels of microbial diversity. Indeed, we found that microbial diversity remained at lower levels in treated compared to control females as forage greenness declined. A similar pattern was observed in wild squirrels and bats, where microbial diversity increased in the spring with increases in the variety of dietary substrates available, but decreased through the summer as feeding rates increased [52, 53]. Interestingly, in populations where helminth prevalence is high, worms have been proposed to help maintain high levels of microbial diversity [7, 55]. Our results extend this hypothesis by suggesting that helminth-induced changes in feeding behaviour and diet could be one mechanism contributing to the maintenance of gut microbiota diversity.

Changes in the feeding behaviour of treated females may also explain effects of anthelmintic treatment on microbial abundance and predicted function. As observed for microbial diversity, both taxa abundance and predicted functional capacity were less sensitive to changes in forage greenness in treated females compared to control females. Specifically, during periods of high forage greenness, the microbiota of treated females maintained a high abundance of microbes in the Bacteroidota phylum that have been associated with cellulose and plant fibre degradation (e.g., Muribaculaceae, UBA932 [56, 57]), whereas at low forage greenness, the microbiota of control females were enriched with a greater number of genera in the Firmicutes phyla, consistent with the potential to metabolise a broader range of substances. These taxonomic differences also translated to patterns of predicted microbial function. As forage greenness declined, the microbiota of control females showed an increased predicted capacity to degrade a variety of substrates, spanning nucleotides, amino acids and sucrose, as well as to synthesise siderophores, compounds released by bacteria to scavenge iron from the environment [58]. In contrast, a majority of the degradation pathways enriched in the microbiota of treated females were for aromatic compounds derived from plant cell walls [59, 60]. This suggests that the functional capacity of the gut microbiota of control females could have been responding to resource limitation, whereas increased feeding rates associated with anthelmintic treatment allowed the gut microbiota of treated females to remain focused on degrading dietary components found in a potentially higher quality diet. Importantly, our functional analysis relied on predicting the functional capacity of the microbiota from taxonomic identifications rather than directly measuring functional metabolites. Since databases available for functional prediction are unlikely to capture the full range of metabolic pathways present in the microbiota of wildlife hosts, these results should be viewed as a starting point for exploring changes in microbial function. Nonetheless, our combined diversity, abundance and predicted function analyses support a link between anthelmintic treatment-induced changes in female feeding behaviour and changes in microbial abundance and metabolism.

Sex-dependent microbiota responses to host helminth infection have been documented in the laboratory [61], and here, we show that sex-dependent microbiota shifts also occur in a natural host–helminth–microbiota system. Our results indicate that studying wild animals can help uncover new mechanisms driving variation in helminth–microbiota interactions. For example, while sex differences in immunity are thought to shape cestode-induced microbiota shifts in laboratory stickleback [61], our study suggests that sex-specific changes in host feeding behaviour and diet may also determine microbiota responses to helminths. By highlighting host-dependent connections among helminths, diet and the microbiota, our findings reveal that factors driving variation in not only host physiology but also behaviour, can contribute to heterogeneity in parasite–microbiota associations.

Author Contributions

V.O.E. designed the anthelmintic treatment study and K.A.S., A.C. and V.O.E. conceptualised the anthelmintic treatment-microbiome project. V.O.E. contributed to data collection; S.J.S. and R.K. contributed to methodology and software resources; and K.A.S. and A.C. performed data analysis. V.O.E. supervised the project. K.A.S. and V.O.E. prepared the original manuscript draft, and all authors participated in manuscript review and editing.

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Disclosure

The authors have nothing to report.

Data Availability Statement

Data for this study are available in the QIITA repository at study ID 10323 (file 131002).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.