




Long-term impact of tylosin on fecal microbiota and fecal bile acids of healthy dogs

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Abstract

Background: Tylosin is commonly prescribed to dogs with diarrhea. Orally administered antibiotics may alter the intestinal microbiota, which is responsible for crucial key bile acid (BA) biotransformation reactions.

Objectives: To prospectively evaluate the impact of tylosin administration on fecal microbiota and unconjugated bile acids (UBAs) over time.

Animals: Sixteen healthy adult dogs.

Methods: Prospective, randomized controlled clinical trial. Dogs were randomized to receive 20 mg/kg of tylosin or a placebo capsule PO q12h for 7 days while undergoing daily fecal scoring. Fecal samples were collected on days 0, 7, 21, and 63. The microbiota was assessed using quantitative PCR and 16S rRNA gene sequencing. Unconjugated BAs were assessed using gas chromatography-mass spectrometry (GC-MS).

Results: Fecal scores were unchanged during placebo and tylosin administration. In the placebo group, no significant changes were observed in fecal microbiota or UBA concentrations. Day 7 samples from tylosin-exposed dogs exhibited decreased bacterial diversity (observed species, Chao1, Shannon, $P < .001$) characterized by decreases in anaerobes *Fusobacteriaceae* (linear discriminant analysis [LDA] score, 5.03) and *Veillonellaceae* (LDA score, 4.85). Primary UBA concentrations were increased at day 21 (median, [range]; 7.42, [0.67-18.77] $\mu\text{g}/\text{kg}$; $P = .04$) and day 63 (3.49 [0-28.43] $\mu\text{g}/\text{kg}$; $P = .02$) compared to day 0 (.14 [0.03-1.19] $\mu\text{g}/\text{kg}$) in dogs receiving tylosin. At day 63, bacterial taxa were not significantly different compared to day 0, but the extent of microbial recovery was individualized.

Conclusions and Clinical Importance: Tylosin causes fecal dysbiosis in healthy dogs with corresponding shifts in fecal UBAs. Changes did not uniformly resolve after discontinuation of tylosin.

KEYWORDS

dysbiosis, antimicrobials, *Clostridium hiranonis*, chronic enteropathy, diarrhea

Abbreviations: ANOSIM, analysis of similarities; BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; DI, dysbiosis index; GC-MS, gas chromatography-mass spectrometry; IBS, irritable bowel syndrome; IFA, immunofluorescence antibody; LCA, lithocholic acid; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; PCoA, principal coordinate analysis; qPCR, quantitative PCR; UBA, unconjugated bile acid; UDCA, ursodeoxycholic acid.

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1 | INTRODUCTION

Tylosin is a macrolide antibiotic commonly prescribed as symptomatic treatment for acute or chronic diarrhea in dogs.^{1,2} Why fecal consistency improves is unknown but is thought to involve modulation of the intestinal microbiota. In the past, it was proposed that dogs with so-called tylosin-responsive diarrhea shared a common enteropathogen. However, this hypothesis has not been substantiated.^{3,4} Other hypotheses about tylosin's mode of action include promotion of beneficial commensal bacteria, decrease in total bacterial load in the small intestine, and suppression of aberrant mucosal immune responses.^{5,6}

Various studies have attempted to characterize tylosin's impact on the intestinal microbiota. In 5 healthy, fistulated, research Beagle dogs treated with tylosin PO q24h for 14 days, 16S rRNA sequencing of jejunal brush samples showed significant increases of *Enterococcus*-like organisms during treatment.⁷ Concurrently, all samples exhibited decreased bacterial diversity. Certain taxa remained significantly altered 14 days after stopping tylosin. Another study found that *Enterococcus* spp. and lactic acid bacteria became more prevalent in fecal samples from 11 client-owned dogs with diarrhea that resolved during 7 days of PO tylosin.⁸ Studies evaluating the impact of tylosin on the fecal microbiota of client-owned, healthy dogs utilizing culture-independent techniques are lacking.

Orally administered antibiotics are known to markedly disrupt the fecal microbiota and metabolome.⁹⁻¹¹ Bile acids (BAs) are a class of metabolites that link host health and microbiota composition. Bile acids within the intestinal lumen regulate the microbiota both directly and indirectly.¹² Selected bacterial species such as *Clostridium hiranonis*, *C scindens*, and *C sordellii* perform 7 α -dehydroxylation required to convert primary to secondary BAs.^{12,13} Because of bacterial biotransformation, secondary BAs predominate in the feces of healthy individuals.¹⁴ Thus, changes in the microbiota can result in altered fecal BA profiles.¹⁵ It is unknown if tylosin treatment impacts BA homeostasis.

The purpose of our study was to analyze bacterial communities and selected unconjugated BAs (UBAs) in a time series of fecal samples obtained from healthy dogs before, during, and after 7 days of tylosin PO q12h. A 7-day duration was chosen because previous evidence suggested that PO antibiotics impact the fecal microbiota

within 2 to 3 days.⁹ We also wanted to maximize compliance and minimize adverse outcomes. In humans, longer courses of antibiotics result in greater selection pressure for resistant strains and increased risk of enteropathogenic infection with *Clostridium difficile*.¹⁶ Fecal samples of dogs given placebo PO q12h for 7 days were evaluated longitudinally for comparison.

2 | MATERIALS AND METHODS

2.1 | Animals

The study was approved by the Institutional Animal Care and Use Committee at Colorado State University (protocol number 15-6292). Apparently healthy, client-owned dogs >10 months of age living in home environments were recruited from the Colorado State University Veterinary Teaching Hospital community. Eligible dogs included those without ongoing medical conditions related to the gastrointestinal or hepatobiliary systems, with no PO antibiotic or probiotic exposure in the previous 4 months. Written, informed client consent was obtained before enrollment of all dogs. Exclusion criteria included current or recent (within the 4 months leading up to enrollment) clinical signs referable to gastrointestinal illness such as hyporexia, vomiting, diarrhea, constipation, or some combination of these. Dogs initially were screened by physical examination and laboratory assessment consisting of a CBC, serum biochemistry profile, urine specific gravity, and fecal parasitology analysis including direct smear, flotation with centrifugation, and *Giardia* and *Cryptosporidium* immunofluorescence antibody (IFA) testing. A canine chronic enteropathy clinical activity index (CCECAI) also was calculated at enrollment with participation of the owner.

2.2 | Study period

A randomized, double-masked, placebo-controlled prospective observational study was performed (Figure 1). No changes in diet or medication (including probiotics) were permitted during the 2-month study period. Dogs already on medications chronically were allowed to continue those medications provided there had been no changes in the month before enrollment (day 0).



FIGURE 1 . Schematic illustrating study intervention, duration, observations, and sampling. The study spanned 64 days. On day 0, client-owned, apparently healthy dogs were screened with a minimum database and a canine chronic enteropathy clinical activity index was calculated. Dogs deemed clinically healthy were randomized to receive either a placebo or tylosin PO q12h for 7 days. Starting on day 0, fecal scores (black circles) were recorded daily by the owner (masked to treatment group). Fecal samples were collected at home by the owner (arrows) before, during, and after study drug administration and stored at 4°C until submission to the Texas A&M Gastrointestinal Laboratory. Each sample was kept at refrigerator temperature for a maximum of 7 days before being frozen at -80°C. Chem, serum chemistry panel, USG, urine specific gravity

Dogs were randomized by the Colorado State University pharmacy staff using a random number generator to receive approximately 20 mg/kg tylosin (Tylan Soluble Powder, Elanco Animal Health, Indianapolis, Indiana) PO q12h (median dose, 17.5 mg/kg; minimum-maximum dose, 16.8-20.0 mg/kg) or a cornstarch placebo with an identical appearance for 7 days. Clear, gelatin capsules (Coni-Snap, Medisca, Las Vegas, Nevada) were used and compounded to be the same size as if the dog were being prescribed 20 mg/kg of tylosin at the Colorado State University pharmacy.

A fecal sample, naturally passed within 12 hours of the screening and enrollment appointment (day 0) was collected by the owner and scored using a previously published pictorial fecal scoring system (Fecal Scoring System, Nestle Purina, St. Louis, Missouri) by the primary investigator (A.C.M.). The owner was made aware of this score and provided with the pictorial scoring key, as well as a blank log to record a fecal score on each day of drug administration. If a dog had >1 bowel movement per day, the highest score was taken as representative of that day. In addition to fecal consistency, owners also monitored for development of gastrointestinal signs (change in appetite, vomiting, flatulence or evidence of bloating). The primary investigator's email address was provided as a point of contact if any clinical signs were to develop. The day 7 fecal sample was collected in the dog's home environment within 12 hours of the last dose of study drug. Owners were instructed to store fecal samples in a refrigerator until delivery, along with the completed fecal scoring sheet, to the clinic, within 48 hours of passage. Subsequently, the owner was asked to collect fecal samples on days 21 (2 weeks after stopping the drug) and 63 (2 months after stopping the drug) and bring them to the clinic as previously described. Samples were refrigerated for up to 7 days before being frozen for storage at -80°C . Samples were batch-shipped on dry ice to the Texas A&M Gastrointestinal Laboratory for analysis.

2.3 | DNA extraction

All samples underwent 1 freeze-thaw cycle before DNA extraction. Fecal DNA was extracted from a 100 mg aliquot of feces using the DNeasy PowerSoil Kit (QIAGEN Inc, Germantown, Maryland) according to the manufacturer's instructions.

2.4 | Quantitative PCR

To quantify the abundance of selected bacterial groups (Universal, *Blautia* spp., *C. hiranonis*, *E. coli*, *Faecalibacterium* spp., *Fusobacterium* spp., *Streptococcus* spp., *Turicibacter* spp.), quantitative PCR (qPCR) was performed as previously described¹⁷ on all fecal samples collected ($n = 81$). Results were expressed as the log amount of DNA (fg) for each particular bacterial group per 10 ng of total isolated DNA. A mathematical algorithm was employed to convert these abundances into a single descriptive numeric value, the dysbiosis index (DI).¹⁷ *Clostridium perfringens* DNA also was quantified using qPCR as previously described.¹⁸ Quantitative PCR analysis of the samples was completed on 6 separate instances over the course of 12 months.

2.5 | 16S rRNA sequencing

A subset of samples, including all provided from days 0, 7, and 63, was submitted for sequencing of the 16S rRNA gene. Amplification and sequencing of the V4 variable region was performed en masse as previously described¹⁸ with a few alterations. Raw sequence data were processed and analyzed using QIIME pipeline version 1.9.1.¹⁹ Sequences were filtered for chimeras using USEARCH61²⁰ and assigned to operational taxonomic units using an open-reference picking protocol in QIIME against the 97% clustered representative sequences from the Greengenes v 13.8 database.²¹ Overall changes in microbiota community structure (β -diversity) were evaluated with phylogeny-based unweighted and weighted UniFrac distance metrics and visualized using principal coordinate analysis (PCoA) plots. Alpha diversity was measured by the number of observed species, Chao1, and Shannon index metrics. Linear discriminant analysis (LDA) effect size (LEfSe)²² highlighted differentially abundant bacterial taxa within the placebo- and tylosin-treated dogs over time. The LEfSe was completed in Calypso, an open-access online platform,²³ and the LEfSe threshold was set to 4.0. Sequence data were uploaded into the Sequence Read Archive of the National Centre for Biotechnology Information, GenBank database, under submission number SRP119755.

2.6 | Analysis of fecal unconjugated BAs

Selected UBAs (i.e., cholic acid [CA], chenodeoxycholic acid [CDCA], lithocholic acid [LCA], deoxycholic acid [DCA], ursodeoxycholic acid [UDCA]) were measured in lyophilized feces using a dilution gas chromatography-mass spectrometry (GC-MS) method as previously reported²⁴ in all samples collected. Primary UBAs (CA and CDCA) were combined to represent primary UBAs measured. Secondary UBAs (DCA, LCA, and UDCA) were combined to represent secondary UBAs measured. Results were reported as micrograms per milligram of lyophilized feces and percentage of total UBA pool. Fecal UBAs were analyzed over the course of 5 distinct runs on the GC-MS machine within a 12-month period.

2.7 | Statistical analysis

Descriptive statistics were calculated for each variable. Data were assessed for normality using the Shapiro-Wilk test and expressed as mean and standard deviation or median and range (minimum-maximum).

Within each treatment group, fecal scores during the 7-day period of administration were compared to the day 0 fecal score using Friedman's test and adjusted for multiple comparisons using Dunn's post-test.

An unstructured, repeated-measures mixed effects model was used to detect differences in qPCR bacterial abundances and DI values in samples from days 0, 7, 21, and 63. Fixed effects included treatment, time, and treatment-by-time interaction. Post hoc analysis using Tukey-Kramer highlighted time points when bacterial abundances were significantly different from day 0 within each treatment.

Analysis of similarities (ANOSIM) was performed using the Primer 6 (PRIMER-E Ltd, Ivybridge, UK) software package on unweighted and weighted UniFrac distances matrices to compare among sample differences in days 0, 7, and 63 samples within each treatment. An unstructured, repeated-measures, mixed effects model was used to evaluate change in alpha diversity metrics within and among treatment groups over time. Fixed effects included treatment, time, and treatment-by-time interaction. Adjustment for multiple comparisons was completed using Tukey-Kramer testing.

Relative abundances of the main (median relative abundance $\geq 0.1\%$) phyla, classes, orders, families, and genera present in fecal samples of either treatment group at any time point were calculated. Data did not meet assumptions of normality, and there were many ties in terms of relative abundance among samples. For this reason, change in relative abundances at the phylum, class, order, and family level was codified as 1 of 2 outcomes: decrease or increase/no change. Fisher's exact test was used to identify differences in occurrence of proportional decreases versus increase/no change between treatment groups compared to baseline (days 0 versus 7, days 0 versus 63). *P* values were adjusted (*q* values) for multiple comparisons using the Benjamini-Hochberg method (false discovery rate [FDR] set to 5%).

The number of dogs with $\leq 10\%$ and $>10\%$ primary fecal UBA was compared between treatment groups at days 0, 7, 21, and 63 using

TABLE 1 Dog characteristics

	Placebo (n = 8)	Tylosin (n = 8)
Median age in years (range)	3 (1.5-6)	7 (3-11)
Sex	2 FS, 6 MC	5 FS, 3 MC
Median weight in kg (range)	19 (8.5-48)	24 (20-39)
Previous antibiotics	4 of 8	5 of 8
Median day 0 CCECAI (range)	0 (0-2)	0 (0-2)
Median day 0 fecal score (range)	2 (2-2)	2 (2-3)

Summary characteristics of clinically healthy, client-owned dogs at day 0 randomly assigned to receive tylosin (approximately 17.5 mg/kg) or identical cornstarch placebo capsule PO q12h for 7 consecutive days. Abbreviations: CCECAI, canine chronic enteropathy clinical activity index; FS, female spayed; MC, male castrated.

Fisher's exact test. Total, primary and secondary UBA concentrations were compared over time using a mixed effects model with an AR(1) covariance structure, using the aforementioned fixed effects and Tukey-Kramer post hoc analysis.

Significance was set at $P < .05$. Statistical analyses were performed using commercially available software (Prism 8 for macOS version 8.1.1, Graph Pad Software; JMP Pro 14.1.0: SAS Institute Inc, Cary, North Carolina).

3 | RESULTS

3.1 | Animal population

A total of 18 dogs initially were enrolled. Dog 2 initially tested positive for hookworms; it was treated with fenbendazole (50 mg/kg PO q24h for 5 days) and enrolled 8 months after initial screening when a subsequent fecal screen was negative. Two dogs (12 and 13) had to be excluded because the owners only provided samples from 1 and 2 time points, respectively.

Baseline characteristics of the 16 dogs completing the study are presented in Table 1 with additional details provided in Table S1. Seven dogs (2 in the placebo group, 5 in the tylosin group) had previous medical diagnoses that were not expected to interfere with the study. Three of the 16 dogs were on long-term daily medications: famotidine at 1.1 mg/kg PO q24h (dog 4), levothyroxine (dog 6), and deracoxib, gabapentin, and tramadol (dog 5). The dose and frequency of these medications were kept constant during the study period. Four of the 8 dogs in the placebo group and 5 of the 8 dogs in the tylosin group had documented PO antibiotic treatment >4 months before study enrollment. All dogs had CCECAI indicative of non-clinically relevant disease.²⁵

Minimum database identified at least 1 biochemical abnormality in most of the dogs, detailed in Table S1. Dog 19, in the placebo group, had a positive result for the *Giardia* spp. IFA. It had normal appearing feces, a negative fecal direct smear and flotation with centrifugation, and no historical gastrointestinal signs and was included without treatment.

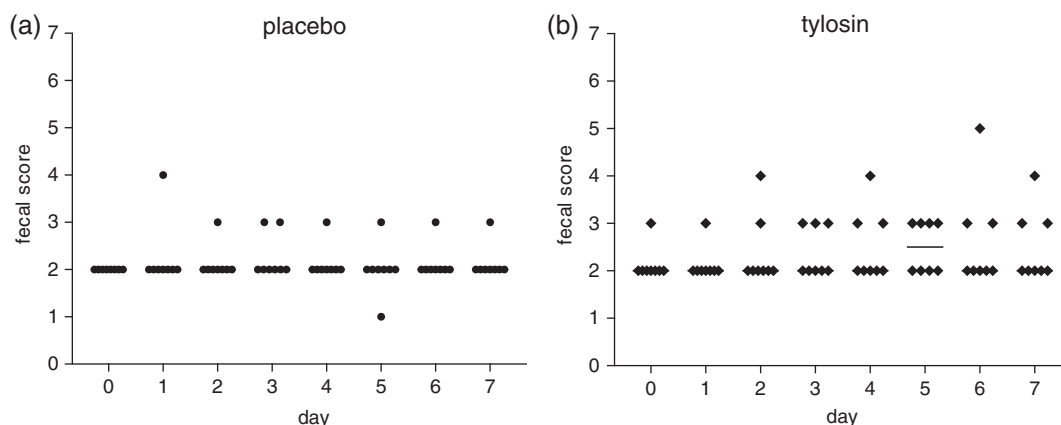


FIGURE 2 Scatter plot of fecal scores from client-owned, clinically healthy dogs before (day 0) and during (days 1-7) PO q12h administration of (A) placebo or (B) tylosin. Horizontal bars represent median values

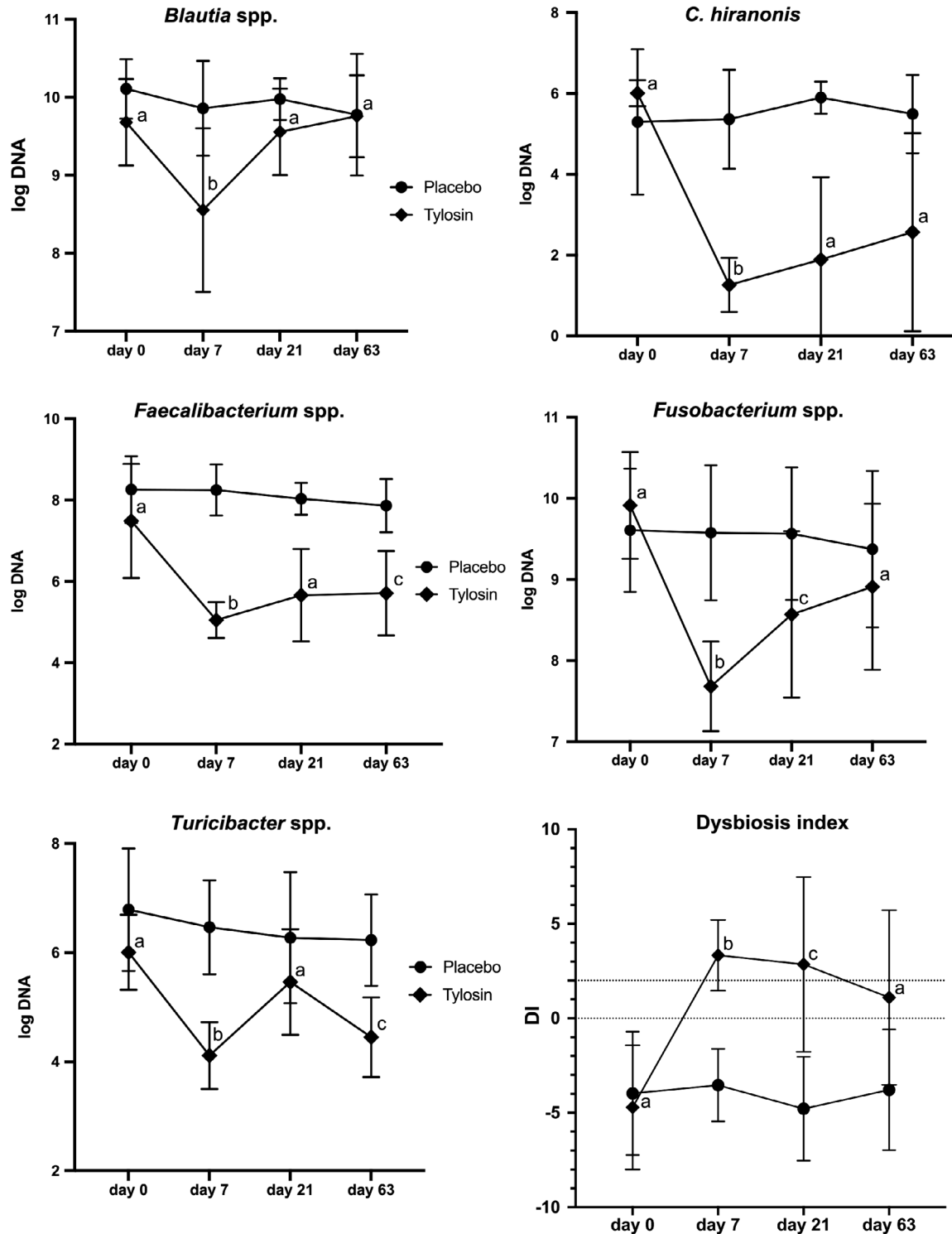


FIGURE 3 Symbols represent mean log bacterial abundances (vertical bars errors representing standard deviation) in fecal samples from healthy dogs before (day 0), during (day 7), and after (days 21 and 63) receiving placebo (circles) or tylosin (approximately 17.5 mg/kg, diamonds) PO q12h on days 1 to 7. For the DI, values <0 are considered consistent with eubiosis, while DI values >2 are indicative of dysbiosis. Mean values summarize results from 8 dogs in each group apart from day 21 (n = 7 placebo, n = 6 tylosin) and day 63 (n = 6 tylosin) because of missing fecal samples. There were no differences in abundance between any of the time points for the placebo group. For the tylosin group, data points labeled with different lowercase letters were significantly different based on the mixed model post hoc analysis. DI, dysbiosis index

3.2 | Study period

Owners did not express concerns regarding adverse effects nor did they report occurrence of diarrhea in their dogs during study drug

administration. All owners provided completed fecal scoring sheets when submitting the day 7 sample. Empty pill vials were not collected. In both the placebo and tylosin groups, day 0 fecal scores were not

TABLE 2 Beta diversity metrics

Comparison	Unweighted		Weighted	
	R statistic	P value	R statistic	P value
Placebo day 0 versus day 7	-0.104	.88	-0.112	.95
Placebo day 0 versus day 63	-0.178	>.99	-0.127	.99
Tylosin day 0 versus day 7	.929	.001	.475	.001
Tylosin day 0 versus day 63	.350	.02	.189	.07

Analysis of similarities of UniFrac distances, unweighted and weighted, in fecal samples from clinically healthy, client-owned dogs randomly assigned to receive PO tylosin (approximately 17.5 mg/kg) or identical cornstarch placebo capsule q12h for 7 consecutive days (days 1-7). Results are displayed from 8 dogs in each group at each time point apart from day 63 in the tylosin group (n = 6) because of missing fecal samples.

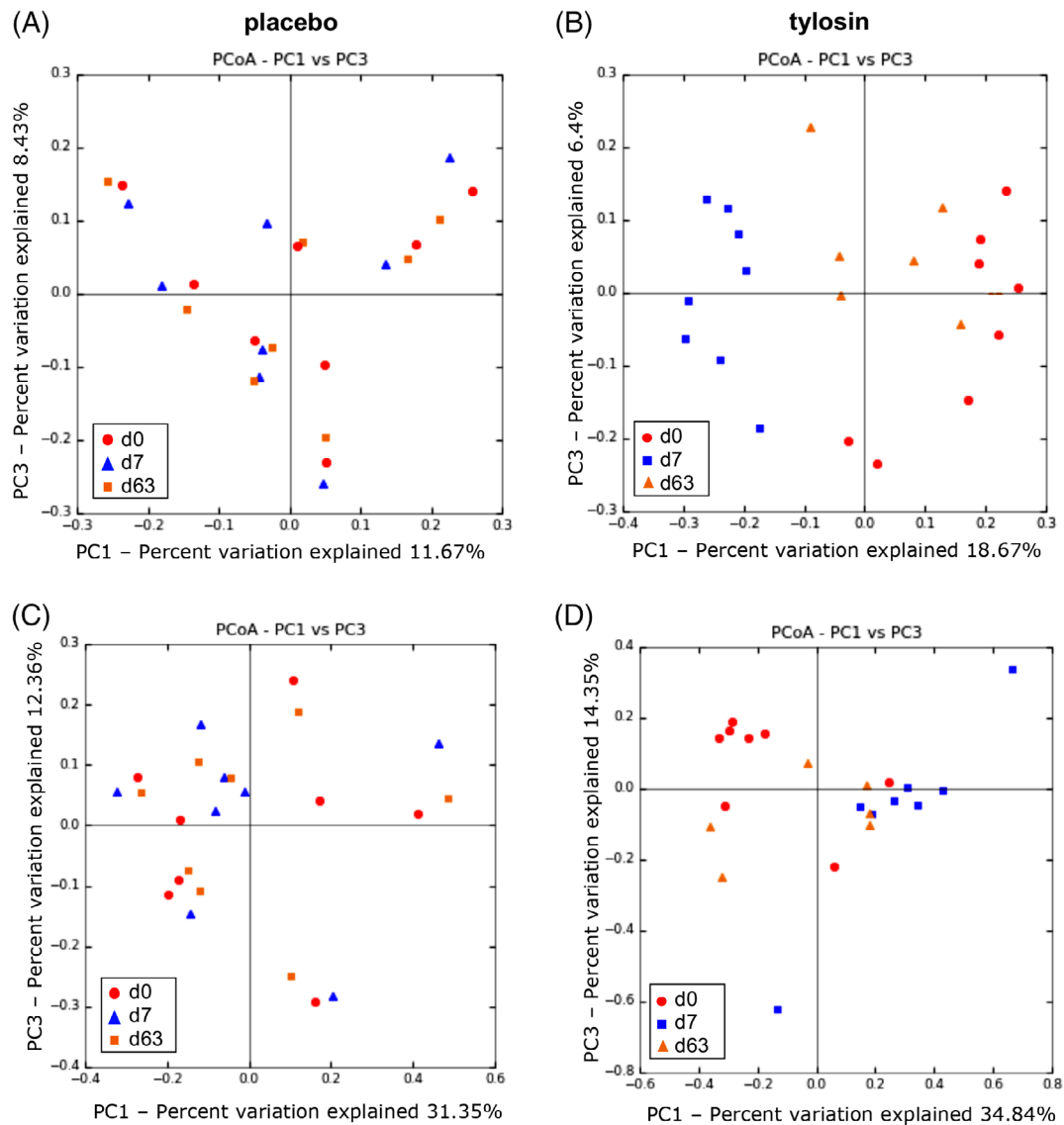


FIGURE 4 Principal coordinate analysis (PCoA) plots based on unweighted (A and B) and weighted (C and D) UniFrac distance metrics of 16S sequencing of rRNA genes from fecal samples from individual healthy dogs before (day 0, red), during (day 7, blue), and after (day 63, orange) administration of placebo or tylosin (approximately 17.5 mg/kg) PO q12h for 7 days. The spatial arrangement of symbols denotes the influence of treatment (placebo, A and C; tylosin, B and D) on beta diversity of microbial communities over time. Results from 8 dogs are displayed for each time point in each treatment group apart from tylosin day 63, where results were available from 6 dogs. For pairwise ANOSIM R statistics and associated P values, see Table 2

TABLE 3 Alpha diversity metrics

Metric	Day 0		Day 7		Day 63		Mixed model analysis, P values			
	Placebo	Tylosin	Placebo	Tylosin	Placebo	Tylosin	Treatment-by-time	Treatment	Time	Tukey's post hoc
Observed species	1243 ± 221	1059* ± 283	1229 ± 322	672* ± 129	1186 ± 254	900 ± 161	.0003	<.001	<.001	*<.001
Chao1	2533 ± 341	2066* ± 523	2433 ± 512	1284* ± 234	2327 ± 440	1855 ± 346	.001	.002	.001	*<.001
Shannon index	5.63 ± .70	5.23* ± .80	5.56 ± .90	3.53* ± .63	5.41 ± .78	4.31 ± .86	<.001	.005	<.001	*<.001

Alpha diversity (mean ± standard deviation) metrics in fecal samples from clinically healthy, client-owned dogs randomly assigned to receive PO tylosin (approximately 17.5 mg/kg) or identical cornstarch placebo capsule q12h for 7 consecutive days (days 1-7). * indicates significantly different time points compared to day 0 after Tukey's HSD test. Upon post hoc analysis, differences were identified exclusively in the tylosin group. Results reflect means from 8 dogs in each group at each time point apart from day 63 in the tylosin group (n = 6) because of missing fecal samples.

significantly different from any time point during the 7-day treatment period (Figure 2). The median day 7 fecal score in both groups was 2.

Owners provided 81 samples from the 16 dogs (day 0, n = 16; day 7, n = 16; day 21, n = 13; day 63, n = 14), with samples from all 4 requested time points from 12 of 16 dogs. In the other 4 dogs, samples were missing from either 1 (n = 3) or 2 (n = 1) time points.

3.3 | qPCR analysis of the fecal microbiota

Log bacterial abundances of the selected bacterial taxa are presented in Figure 3. The interaction of treatment and time significantly influenced log bacterial abundances of *C hiranonis* (F ratio, 163.52; P = .01), *Faecalibacterium* (F ratio, 8.01; P = .006), *Fusobacterium* (F ratio, 15.67; P < .001), and *Turicibacter* (F ratio, 5.39; P = .02; Table S2). Treatment and time independently influenced *Blautia* (F ratio, 5.63; P = .03, and F ratio, 3.95; P = .04, respectively), but the effect of their interaction did not reach significance. Adjustment for multiple comparisons indicated significant differences in log bacterial abundance between days 0 and 7 samples of the tylosin-exposed dogs. *Blautia* (P = .03), *C hiranonis* (P = .01), *Faecalibacterium* (P < .001), *Fusobacterium* (P < .001), and *Turicibacter* (P = .002) were significantly decreased at day 7 compared to day 0, as shown in Figure 3. *Fusobacterium* were significantly decreased at day 21 (P = .04), and *Faecalibacterium* (P = .02) and *Turicibacter* (P = .04) were significantly decreased at day 63 compared to day 0 (Figure 3). Time was identified as a significant effect for universal bacteria (F ratio, 5.56; P = .02), but no significant differences were apparent with post hoc analysis. In the placebo group, the mixed model identified no significant effects of treatment, time, or their interaction on log bacterial abundances. Neither treatment and time, nor their interaction influenced fecal abundances of *E coli*, *Streptococcus*, or *C perfringens*. The interaction of treatment and time significantly influenced the DI (F ratio, 6.74; P = .01). Specifically, this was characterized by an increase in the DI in the tylosin group at days 7 (P = .001) and 21 (P = .02) compared to day 0.

3.4 | 16S rRNA sequencing analysis

The sequencing analysis yielded 3 197 181 quality sequences with an average of 56 091 sequences per sample (range, 38 393-147 807). To

TABLE 4 LEfSe of tylosin-exposed group

Family	Day	LDA score
Day 0 versus day 7		
Fusobacteriaceae	0	5.03
Veillonellaceae	0	4.85
Bacteroidaceae	0	4.66
Ruminococcaceae	0	4.12
Coriobacteriaceae	0	4.09
Alcaligenaceae	0	4.06
Enterococcaceae	7	5.03
Peptostreptococcaceae	7	4.92
Corynebacteriaceae	7	4.73
Planococcaceae	7	4.54
Carnobacteriaceae	7	4.16
Day 0 versus day 63		
Clostridiaceae	0	4.90
Fusobacteriaceae	0	4.84
Turicibacteriaceae	0	4.75
Paraprevotellaceae	0	4.31
Lachnospiraceae	63	4.75

Linear discriminant analysis (LDA) effect size (LEfSe) of Illumina sequencing data sets at the family level based on 16S rRNA gene sequences in fecal samples from 8 clinically healthy, client-owned dogs randomly assigned to receive PO tylosin (approximately 17.5 mg/kg) q12h for 7 consecutive days (days 1-7). Results reflect analysis of samples from 8 dogs at each time point apart from day 63 in the tylosin group (n = 6) because of missing fecal samples. No features were found to be significantly different based on LEfSe analysis in the 8 healthy dogs randomly assigned to receive PO placebo.

account for unequal sequencing depth, subsequent analysis was performed on a randomly selected subset of samples at 38 390 sequences per sample.

3.5 | Beta diversity

The ANOSIM of unweighted and weighted UniFrac distances within the placebo group (Table 2), displayed in PCoA plots (Figure 4), showed no significant differences in β-diversity among fecal bacterial communities in dogs given placebo over time. Conversely, significant

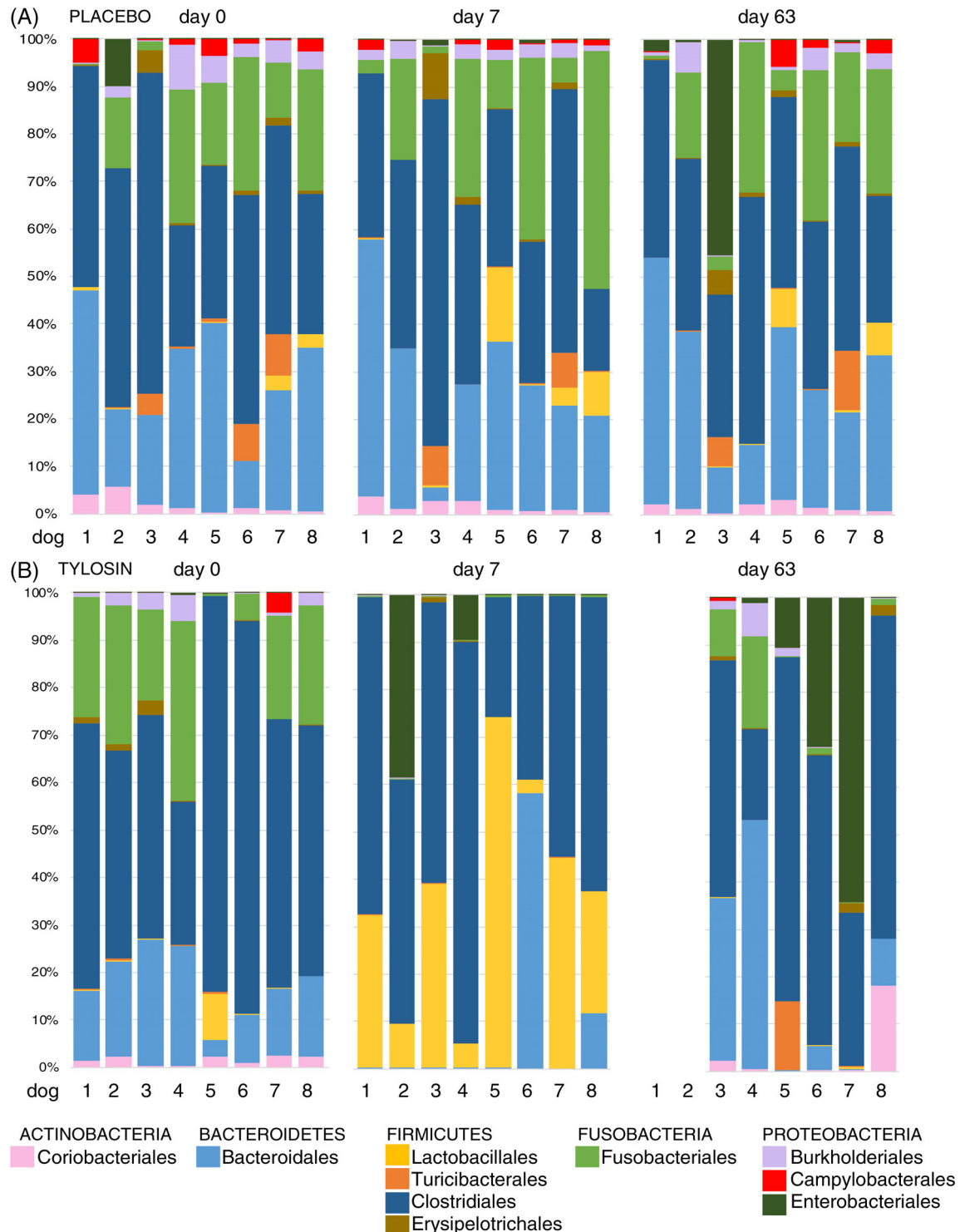


FIGURE 5 Median relative abundances of main bacterial orders (>0.1% of total in either group at any time point) based on 16S sequencing analysis of fecal samples from individual clinically healthy, client-owned dogs randomly assigned to receive (A) cornstarch placebo capsules or (B) tylosin (approximately 17.5 mg/kg) PO q12h for 7 consecutive days (day 1-7). Predominant phyla represented are in uppercase letters above the individual orders alongside their corresponding color. Results are displayed from 8 dogs in each group, apart from day 63 in the tylosin group, where fecal samples were not provided from dogs 1 and 2

differences in β -diversity were identified between days 0 and 7 fecal bacterial communities in dogs given tylosin. At day 63, communities were significantly different based on unweighted analysis but not on weighted analysis.

3.6 | Alpha diversity

The interaction between treatment and time influenced alpha diversity (Table 3). The post hoc analysis indicated significant decreases in

TABLE 5 Unconjugated bile acid concentrations and percentages

	Day 0		Day 7		Day 21		Day 63		Mixed model analysis, P values		
	Placebo	Tylosin	Placebo	Tylosin	Placebo	Tylosin	Placebo	Tylosin	Treatment-by-time	Time	Tukey's post hoc
	µg/mg	µg/mg	µg/mg	µg/mg	µg/mg	µg/mg	µg/mg	µg/mg			
Total UBAs	5.75 (1.18-14.81)	3.02 (1.08-13.92)	3.38 (1.57-7.96)	3.93 (1.26-12.29)	9.63 (1.57-7.96)	7.42* (.67-18.77)	3.97 (.99-8.26)	6.32 (2.73-29.18)	.07	.13	.08
Primary UBAs	.34 (0-13.61)	.14* (.03-1.19)	2.1 (0-73.2)	85.5 (44.1-90.6)	2.3 (3-21.1)	90.0 (5.5-97.2)	6.6 (2-39.6)	3.49* (0-28.43)	.001	<.001	.17
	4.8 (0-91.9)	6.1 (2-22.7)	2.81 (98-13.88)	2.81 (51-1.27)	3.91 (1.22-11.97)	78 (26-11.54)	3.65 (.81-8.10)	77.1 (0-97.4)			*.044, .025
Secondary UBAs	3.09 (1.18-12.23)	2.81 (98-13.88)	3.28 (58-8.06)	72 (51-1.27)	3.91 (1.22-11.97)	78 (26-11.54)	3.65 (.81-8.10)	93 (27-6.63)	.73	.07	.24
	95.2 (8.1-100)	93.9 (77.3-99.8)	97.9 (26.8-100)	14.5 (9.4-55.9)	97.7 (78.9-99.7)	10.0 (2.8-94.5)	93.4 (60.4-99.8)	22.9 (2.6-98.6)			

Total, primary (CA + CDCA), and secondary (DCA, LCA, UDCA) UBA concentrations and percentages (italicized) of selected UBAs in fecal samples from clinically healthy, client-owned dogs randomly assigned to receive PO tylosin (approximately 17.5 mg/kg) or identical cornstarch placebo capsule q12h for 7 consecutive days (days 1-7). Results displayed as median (range). Results reflective of samples from 8 dogs in each group at each time point apart from day 21 (n = 7 placebo, n = 6 tylosin) and day 63 in the tylosin group (n = 6) because of missing fecal samples. * or ^ indicated significantly different fecal UBA concentrations between time points after Tukey's HSD test for multiple comparisons. Significant differences were identified within the tylosin group exclusively. Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; LCA, lithocholic acid; UBAs, unconjugated bile acids; UDCA, ursodeoxycholic acid.

observed species, Chao1, and Shannon index values in the tylosin group comparing day 0 to day 7 samples ($P < .001$ for all 3 metrics). By day 63, alpha diversity was not significantly different compared to day 0. In the placebo group, no significant differences were identified in alpha diversity measures over time.

3.7 | Linear discriminant analysis effect size

Within the placebo group, LEfSe identified zero features that were significantly different across days 0, 7, and 63 samples. Conversely, dogs receiving tylosin exhibited higher proportions of *Enterococcaceae* and decreased proportions of anaerobic taxa such as *Fusobacteriaceae*, *Veillonellaceae*, and *Bacteroidaceae* at day 7 compared to day 0 (Table 4). Comparing day 0 to day 63, the most distinguishing features were decreases in relative abundances of *Clostridiaceae* and *Fusobacteriaceae*.

3.8 | Univariate analysis

Summary statistics of the main bacterial taxa identified in the 16S rRNA gene analysis are presented in Table S3. In both groups at day 0, >90% of sequences belonged to the Firmicutes, Fusobacteria, and Bacteroidetes phyla. The main bacterial orders comprising the fecal microbiota of individual dogs over time are presented in Figure 5. Comparison of day 0 to day 7 samples at this taxonomic level identified different relative abundance shifts (decrease versus increase/no change) in *Fusobacteriales* ($P = .03$). However, after adjustment for multiple comparisons, this difference lost significance. At the family level, *Coriobacteriaceae*, *Paraprevotellaceae*, *Peptostreptococcaceae*, and *Veillonellaceae* abundances changed differently between the 2 groups. After adjustment for multiple comparisons, the only taxa that changed differently were *Paraprevotellaceae* ($q = .020$) and *Peptostreptococcaceae* ($q = .049$). *Paraprevotellaceae* were decreased more frequently, and *Peptostreptococcaceae* were increased more frequently or not changed at day 7 compared to day 0 in dogs given tylosin. Comparing relative abundance shifts between day 0 and day 63 samples, no taxa changed differently between the placebo- and tylosin-exposed dogs.

3.9 | Fecal unconjugated BAs

The UBA profiles were composed of >90% secondary UBAs (DCA and LCA) in day 0 fecal samples from 5 of the 8 dogs in the placebo group and 7 of the 8 dogs in the tylosin group (Table 5). Tylosin administration (day 7) was associated with >10% primary UBAs ($P = .007$). At day 21, a higher proportion of dogs in the tylosin group (5/6) had >10% primary UBAs compared to the placebo group (1/7, $P = .03$), but at day 63, proportions of dogs with >10% primary UBAs were not different between groups ($P = .12$).

Primary UBA concentrations (Figure 6) were influenced by the interaction between treatment and time (F ratio, 4.20; $P = .001$; Table 5). Concentrations of primary UBAs were significantly higher at day 21 ($P = .04$, Figure 6) and day 63 ($P = .02$) compared to day 0 in the tylosin group. No significant changes were identified in primary

normal DI at day 0 and available day 63 samples, only 2 (dogs 3 and 8) returned to a normal DI by day 63. Our observations mimic the unpredictable post-antibiotic period documented in the jejunal microbiota of healthy Beagles after 14 days of tylosin,⁷ as well in healthy humans after antibiotic exposure.^{10,11,45} Based on the individualized changes observed in the dogs of our study, we were not able to draw any broad conclusions regarding tylosin's expected duration of impact across a population. We therefore conclude that 2 months after PO tylosin exposure, reestablishment of the native microbiota is possible but not guaranteed. These observations are relevant for interpretation of past and future microbiome studies in dogs, which almost always involve subjects with historical antibiotic exposure.

Tylosin has been suggested as a treatment for diarrhea attributed to *Clostridium perfringens*.^{46,47} However, tylosin administration was not associated with a consistent decrease in the prevalence of fecal *C perfringens*. This was reported previously in the jejunal microbiota of healthy Beagles,⁷ and might be attributed to a disrupted intestinal microenvironment where commensal enterotoxigenic *C perfringens* are encouraged to sporulate.⁴⁸ It is also plausible that macrolide-resistant *C perfringens* were present.⁴⁶ Further investigations into the relationships between tylosin and enteropathogenic bacteria, particularly in dogs with diarrhea, are warranted.

Tylosin administration was associated with increases in primary UBA concentrations (Figure 6) and proportions in the feces (Table 5). Changes in fecal UBAs were evident during and up to 2 months after tylosin administration. This observation is relevant to the potential long-term impact of a brief course of tylosin. Changes in both concentrations and proportions of BAs can have meaningful consequences for host health. In various experimental models, increased concentrations of CDCA, a primary BA, have been shown to increase intestinal motility,⁴⁹ permeability,⁵⁰ and secretory activity,⁵¹ as well as to induce apoptosis in colonic epithelial cells.⁵² In people with irritable bowel syndrome (IBS), >10% primary fecal BAs were 91% and 87% specific for increased fecal weight and increased colonic motility, respectively.⁵³ The absence of diarrhea in our tylosin-exposed dogs, all of which had >10% primary UBAs at day 7, suggests that this factor in isolation is not sufficient to cause diarrhea. A probable explanation for this finding is that the microbiota and metabolome, as well as the host immune response, must be altered to a certain extent (not achieved in our dogs) before clinical signs ensue. The fecal UBA dysmetabolism documented in these healthy dogs with tylosin administration is a novel finding, and future, longitudinal, hypothesis-driven studies are needed to evaluate its prevalence and associated consequences.

Our study had a number of limitations. First, we tested many dependent variables in each sample, thus increasing the chance of false discovery, particularly for the 16S rRNA gene results. For this reason, we were conservative in our interpretation of the univariate data. This cautious approach, combined with the small sample size used, would have increased the type II error. Compounding our small sample size, samples from certain time points were not available. Therefore, our study lacks power to make definitive conclusions regarding the time needed for recovery of fecal microbiota and UBA profile. Lastly, as with any 16S gene sequencing microbiota analysis, it is important to bear in

mind that results are compositional in nature, and an increase in proportion does not necessarily translate into an increase in absolute abundance of bacteria.⁵⁴ Hopefully, these preliminary findings will lead to future hypothesis-driven research into the long-term effects of tylosin administration in dogs, especially those with chronic enteropathy.

This cohort included dogs receiving medications chronically. Because these were given consistently before and during the study period, it is unlikely that differences observed between treatments and time points were considerably confounded by these medications. We excluded dogs with PO antibiotic exposure in the preceding 4 months, but other exposures could be relevant. In humans, repeated antibiotic courses may result in a microbiota that is less able to recover from future perturbations.¹⁰ Finally, the cohort included some dogs with historical gastrointestinal signs. It is possible that some had occult gastrointestinal disease during the study period. Ultimately, our study provides useful information because (i) all dogs met criteria of clinical health at enrollment, (ii) the baseline microbiota across groups was comparable to previous descriptions of healthy fecal microbiota in dogs,⁴³ and (iii) attributes such as chronic medications and previous illnesses would be expected to make the group more rather than less representative of dogs that would actually be prescribed tylosin.

Although fecal sample handling was not entirely uniform, we do not believe this confounded microbiota results. Fecal microbiota populations based on 16S rRNA gene sequencing are stable during storage at room temperature for 24 hours.⁵⁵ Data from our laboratory suggest that the fecal microbiota is stable at 4°C for at least 7 days (JSS, unpublished data). Samples were stored at 4°C to avoid freeze-thaw cycles that could impact fecal metabolite yield,⁵⁶ because fecal UBAs may be more sensitive to storage conditions than bacterial DNA. Bile acid analysis was limited to a subset of unconjugated BAs. Increases in conjugated and sulfated fecal BAs have been documented in humans with IBS^{15,57} and could have implications for host health. Lastly, 1 aliquot from 1 bowel movement may not be representative of the luminal BA milieu given variation in BA excretion.^{56,58} Diagnosis of excess fecal BAs in human patients involves quantification of BAs in a 48-hour fecal collection during ingestion of a high fat diet.⁵⁹ To our knowledge, this type of protocol has not been completed in dogs. Future investigations should measure a higher number of BAs in serial bowel movements from consecutive days.

Oral tylosin induced marked changes in fecal microbiota and UBA populations of all 8 healthy dogs, which was not associated with appreciable clinical signs. Although the observational design of our study of healthy dogs precludes formation of any conclusions regarding tylosin's therapeutic mechanism of action, these results argue against the hypothesis that tylosin improves clinical signs in a subset of dogs by normalization of a dysbiotic microbiota. Future studies including observations before, during, and after tylosin administration are needed to determine if the changes observed here would be replicated in dogs with naturally occurring intestinal disease.

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CONFLICT OF INTEREST DECLARATION

The authors Alison C. Manchester, Amanda B. Blake, Fatima Sarwar, Jonathan A. Lidbury, Jörg M. Steiner and Jan S. Suchodolski are affiliated with the Gastrointestinal Laboratory, Texas A&M University, which offers gastrointestinal assays on a fee-for-service basis.

OFF-LABEL ANTIMICROBIAL DECLARATION

Tylosin is not FDA approved for usage in dogs, thus, for the purpose of this study, tylosin was administered off-label.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The study was reviewed and approved by the IACUC at Colorado State University (protocol number 15-6292).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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