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STANDARD ARTICLE

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Effects of orally administered clioquinol on the fecal microbiome of horses

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Abstract

Background: Whereas restoration of fecal consistency after treatment with clioquinol for chronic diarrhea and free fecal water syndrome has been attributed to its antiprotozoal properties, actions of clioquinol on the colonic bacterial microbiota have not been investigated.

Objectives: Characterize the dynamics of fecal microbial diversity before, during, and after PO administration of clioquinol to healthy horses.

Study Design: Experimental prospective cohort study using a single horse group.

Methods: Eight healthy adult horses received PO clioquinol (10 g, daily) for 7 days. Feces were obtained daily for 7 days before, during, and after conclusion of treatment, and again 3 months later. Libraries of 16S rRNA V4 region amplicons generated from fecal DNA were sequenced using the Illumina sequencing platform. Bioinformatic analysis was undertaken with QIIME2 and statistical analyses included analysis of variance (ANOVA) and permutational multivariate ANOVA (PERMANOVA).

Results: The richness and composition of the fecal microbiome was altered after administration of clioquinol, reaching a maximum effect by the fifth day of administration. Changes included a 90% decrease in richness, and compensatory expansion of facultative anaerobes including *Streptococcaceae*, *Enterococcaceae*, and *Enterobacteriaceae*. Multiple horses had *Salmonella* cultured from feces.

Main Limitations: Limitations including lack of control group and modest sample size are obviated by robust longitudinal study design and strong effect size associated with drug exposure.

Conclusions: Clioquinol has broad-spectrum antibacterial effects on the fecal microbiome of horses, but spares certain bacterial families including several pathogens and pathobionts. Clioquinol should be used with caution in horses, in an environment free of contamination with fecal pathogens.

Abbreviations: ANOVA, analysis of variance; ASV, amplicon sequence variant; CNS, central nervous system; FC, fold-change; FFWS, free fecal water syndrome; GIT, gastrointestinal tract; PCoA, principal coordinate analysis; PERMANOVA, permutational multivariate analysis of variance; RA, relative abundance; SCFA, short-chain fatty acid; SMON, subacute myelo-optic neuropathy; USDA, United States Department of Agriculture.

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KEYWORDS
antimicrobial, clioquinol, equine, iodochlorhydroxyquin, microbiota

1 | INTRODUCTION

Clioquinol (Clioquinol, iodochlorhydroxyquin, 5-chloro-7-iodo-quinolin-8-ol) is a halogenated 8-hydroxyquinoline with antimicrobial activity that was originally introduced as a topical antiseptic and an orally administered intestinal amebicide in 1934. The drug (5-10 g PO q24h) has been advocated for the treatment of chronic nonspecific colitis or idiopathic colonic dysfunction in horses for many years.[1-5] As is currently still the case, establishment of a satisfactory etiological explanation for diarrheal syndromes in horses has long been challenging, even after a necropsy examination.[5-7]

The concept that chronic diarrheal syndromes in horses are sometimes associated with protozoal dysbiosis (referred to as protozoal diarrhea) has been discussed for many years; various implicated protozoal pathogens have been identified based on their microscopic appearance and the ease with which they are identified in wet preparation for microscopy including *Trichomonas* spp, *Giardia* spp, and *Balantidium* spp.[1-3] Early observations that excessive numbers of motile trichomonads often were seen in the feces from horses affected with diarrhea led to the use of clioquinol in those cases. Moreover, clioquinol treatment led to disappearance of the trichomonads and normalization of fecal consistency. The original concept that chronic diarrhea was a result of protozoal infection has been replaced by the concept that protozoal dysbiosis, as identified by the presence of large numbers of motile protozoa evident on fecal microscopy, is more likely a secondary phenomenon.[2,8]

Free fecal water syndrome (FFWS) or "free fecal liquid" is a novel condition of horses, characterized by a 2-phase separation of feces, 1 solid and 1 liquid phase voided together or separately, and is being increasingly recognized.[9-11] Unpublished observations in our hospital have suggested that motile protozoa often are observed in feces from FFWS-affected horses and that treatment with anti-protozoal drugs, including clioquinol, commonly results in both normalization of fecal consistency and disappearance of the protozoa. These observations have led to increasing use of clioquinol in these horses.

Anecdotal reports suggest that treatment with clioquinol, sometimes in combination with nasogastric administration of fecal extracts from healthy horses, results in restoration of normal fecal consistency, although watery feces may recur upon discontinuation of treatment.[1,3,4,12] Although studies to affirm its efficacy for the management of diarrhea in horses are lacking, modification of the colonic microbiota and possible antiprotozoal activity have been suggested to play roles.[7] In 1 in vitro study, clioquinol was shown to mildly decrease gas and volatile fatty acid production in feces of horses, suggesting that this drug might restore fecal consistency by effects on microbial fermentation.[12]

Antimicrobial administration to horses is associated with risk of potentially severe colitis (antimicrobial-associated diarrhea) and increased fecal shedding of commensal enteropathogens, such as *Clostridioides difficile*, *Clostridium perfringens*, and *Salmonella* spp.[13-17] Several recent studies have described the impact of certain antimicrobials (doxycycline, penicillin, ceftiofur, trimethoprim-sulfa, erythromycin, and metronidazole) on the colonic bacterial microbiota of horses using amplicon sequencing (ie, metabarcoding) approaches.[13,18-23] Results of these studies have demonstrated that some antimicrobial treatments cause significant alterations to the intestinal microbiota, provoking a dysbiotic state and potentially increasing the risk of inflammation and diarrhea.[23]

Although it has been proposed that clioquinol is able to restore fecal consistency by its antimicrobial actions on the colonic microbiota, much of the advocacy for its effectiveness in chronic diarrhea and FFWS has hitherto been based on the notion that it is principally regulating a protozoal dysbiosis, likely a secondary phenomenon. To date, the effect of clioquinol on the bacterial microbiota of healthy adult horses has not been reported. Our aim was to describe changes in the fecal bacterial microbiota during and after a 7-day PO clioquinol treatment.

2 | METHODS

2.1 | Horses

Healthy adult horses (n = 8) from the University of Missouri (MU) teaching herd were transported from pasture at Middlebush Farm approximately 9.3 miles (15 km) to the MU Veterinary Health Center (VHC) in 2 separate cohorts of 4 horses each on July 6 and July 31, 2023. Table S1 shows basic subject demographics. Each group of horses then remained in the MU VHC, a climate-controlled environment, for 21 days, receiving Timothy grass hay twice per day and ad libitum fresh water. The horses were bedded on cedar wood chips. Physical examinations including vital data (rectal temperature, heart rate, and respiratory rate) were obtained daily. Specific observations for evidence of gastrointestinal disease included monitoring of appetite, voluntary water consumption, borborygmi, signs of colic, and fecal consistency.

2.2 | Experimental design and fecal sample collection

Beginning on the day after transport to the VHC, freshly evacuated fecal samples were collected every morning between the hours of 7:00 and 9:00 am, for 21 consecutive days. Beginning on Day 8, horses received 10 g of clioquinol as a paste (30 mL, compounded at Wedgwood Pharmacy, Swedesboro, New Jersey), administered PO

via dose syringe once daily for 7 days, followed by a 7-day washout period after discontinuation of the drug. For collection of fecal samples, recently cleaned stalls were surveyed for fresh fecal material each morning. Fecal samples were manually collected from the ground and brushed free of any bedding material, before placing a portion weighing several grams into a sterile 1 mL specimen container. Containers were promptly sealed, labeled, and placed in a −80°C freezer until transport to the MU Metagenomics Center for further processing.

2.3 | Salmonella biosecurity surveillance, culture, and analysis

As per VHC biosecurity surveillance policy, fecal samples from each horse were submitted to the MU Veterinary Medical Diagnostic Laboratory (VMDL) for bacteriological culturing for Salmonella identification on admission into the VHC and subsequently on all Mondays and Wednesdays. For this purpose, freshly collected fecal samples (approximately 10 g) are routinely incubated in a selective enrichment broth (tetrathionate with 2% iodine solution) at 35°C for 24 hours. Salmonella-positive colonies subsequently are identified after inoculation onto selective agar (Brilliant Green and XLT-4) by a specific colorimetric reaction. Fecal samples that test negative for Salmonella on primary enrichment are subjected to secondary enrichment, employing a 1 mL aliquot of the originally inoculated tetrathionate, placed into a second 10 mL of enrichment broth (tetrathionate with 2% iodine solution) and incubated at room temperature for 5 days before being streaked on selective agar. Isolated Salmonella cultivars were submitted to the United States Department of Agriculture (USDA) National Veterinary Services Laboratories for serotyping.

2.4 | DNA extraction

Fecal and Salmonella isolate DNA was extracted using QIAamp PowerFecal Pro DNA kits (Qiagen) according to the manufacturer instructions, with the exception that samples were homogenized in the provided bead tubes using a TissueLyser II (Qiagen, Venlo, Netherlands) for 10 minutes at 30/second, rather than performing the initial homogenization of samples using the vortex adapter described in the protocol, before proceeding according to the protocol and eluting in 100 µL of elution buffer (Qiagen). The DNA yields were quantified by fluorometry (Qubit 2.0, Invitrogen, Carlsbad, California) using quant-iT BR dsDNA reagent kits (Invitrogen) and normalized to a uniform concentration and volume.

2.5 | 16S rRNA library preparation and sequencing

Library preparation and sequencing were performed at the University of Missouri (MU) Genomics Technology Core. Bacterial 16S rRNA amplicons were constructed by amplification of the V4 region of the 16S rRNA gene with universal primers (U515F/806R), flanked by Illumina standard adapter sequences.[24,25] Dual-indexed forward and reverse primers were used in all reactions. Polymerase chain reaction was performed in 50 µL reactions containing 100 ng metagenomic DNA, primers (0.2 µM each), dNTPs (200 µM each), and Phusion high-fidelity DNA polymerase (1U, Thermo Fisher). Amplification parameters were $98°C^{(3\ min)} + [98°C^{(15\ sec)} + 50°C^{(30\ sec)} + 72°C^{(30\ sec)}] \times 25\ cycles + 72°C^{(7\ min)}$. Amplicon pools (5 µL/reaction) were combined, thoroughly mixed, and then purified by addition of Axygen Axyprep MagPCR clean-up beads to an equal volume of 50 µL of amplicons and incubated for 15 minutes at room temperature. Products then were washed multiple times with 80% ethanol and the dried pellet was resuspended in 32.5 µL EB buffer (Qiagen), incubated for 2 minutes at room temperature, and then placed on a magnetic stand for 5 minutes. The final amplicon pool was evaluated using the Advanced Analytical Fragment Analyzer automated electrophoresis system, quantified using quant-iT HS dsDNA reagent kits, and diluted according to Illumina's standard protocol for sequencing as 2 × 250 base pair (bp) paired-end reads on the MiSeq instrument.

2.6 | Bioinformatics

The 16S rRNA sequences were processed using Quantitative Insights Into Microbial Ecology 2 (QIIME2) v2021.8.[26] Illumina adapters and primers were trimmed from forward and reverse reads using cutadapt.[27] Reads then were truncated to 150 bp, then denoised into unique amplicon sequence variants (ASVs) using DADA2.[28] Unique sequences then were assigned taxonomy using an a sklearn algorithm and the QIIME2-provided 99% non-redundant SILVA[29] v138 reference database trimmed to the 515F/806R[25] region of the 16S rRNA gene. Alpha diversity metrics (Chao-1 and Simpson indices) were determined using the *microbiome*[30] and *vegan*[31] libraries. Differences in beta diversity were visualized with principal coordinate analysis (PCoA) using Bray-Curtis (weighted) and Jaccard (unweighted) distances. Briefly, a distance matrix was generated with the *vegdist* function from the *vegan* library using a quarter-root transformed feature table. Principal coordinate analysis was performed using the *ape*[32] library with a Calliez correction. The cladogram was generated using Graphlan[33] v1.1.4. The Core microbiome analysis was performed at the phylogenetic level of family using MicrobiomeAnalyst.[34]

2.7 | Statistics

Univariate data (reported as mean ± SE) first were tested for normality using the Shapiro-Wilk method, followed by the appropriate parametric or non-parametric test. Whenever possible and practical, multifactor tests (eg, 2-way analysis of variance [ANOVA]) were used, with day and horse as factors. Differences in multivariate data were tested using permutational multivariate ANOVA (PERMANOVA) and visualized using PCoA. The PERMANOVAs were performed with

9999 permutations. Both PERMANOVA and PCoA were performed using unweighted (Jaccard) and weighted (Bray-Curtis) distances.

3 | RESULTS

3.1 | Clinical observations

Throughout the treatment period, vital signs remained normal, and clinically relevant evidence of gastrointestinal disturbance was not observed in a majority of the horses. Horse 1A developed mild colic signs on post-treatment Days 2 and 3. Signs included increased recumbency and decreased passage of feces and responded quickly to treatment with electrolytes mixed with mineral oil (500 mL via nasogastric tube) and flunixin meglumine (0.5 mg/kg of body weight, IV). Horse 2A developed severe, acute colitis on post-treatment Day 19 and was euthanized (acute colitis was confirmed at necropsy). Horse 2B passed soft feces on treatment Days 4, 5, and 6 and post-treatment Days 1 and 2; fecal consistency had normalized by post-treatment Day 3. Transient signs of colic developed in Horse 2C on treatment Day 6. Horse 2D developed acute cellulitis, necessitating antimicrobial treatment, on treatment Day 6, and was withdrawn from the study.

3.2 | Salmonella isolation

Feces collected at the time of entry into the VHC from all horse in both cohorts were negative for Salmonella during routine pathogen surveillance. Salmonella was never isolated from any of the horses in Cohort 1. *Salmonella enterica* ser. Anatum was positively identified in feces from Horses 2A (post-treatment Days 1 and 3), 2B (post-treatment Days 3 and 7), and 2D (treatment Day 3). *Salmonella enterica* ser. Agbeni was positively identified in feces from Horse 2C on treatment Day 3.

Notably, the first Salmonella-positive fecal sample in Cohort 2 occurred 7 days after a mare and foal had been accommodated in the VHC for treatment and from both of which *Salmonella enterica* ser. Anatum was identified in their feces at time of admission.

3.3 | Microbiome analysis

With the exception of 1 sample that was removed because of poor sequencing, mean (±SD) depth was 111 228 ± 16 057 reads per sample (range, 51 854 to 147 829). Considering the range in depth, data from all samples were rarefied by random subsampling to 51 853 reads per sample. Based on the rarefied dataset, the mean observed richness over the first 8 days ranged from 1102 to 1364 unique amplicon sequence variants (ASVs) per horse ($P = .05$, $F = 2.2$), and the observed richness did not change significantly over the course of the first 7 days ($P = .3$, $F = 1.0$). Visualization of beta-diversity among horses at baseline using unweighted (Jaccard; Figure 1A) or weighted (Bray-Curtis; Figure 1B) dissimilarities identified substantial inter-subject dissimilarity, and no significant change over time during the first 8 days. A family-level core microbiome analysis found 22 bacterial families present at >0.01% relative abundance (RA) in at least 25% of subjects (Figure 1C).

After the initial dose of clioquinol, a significant decrease in richness was observed within 24 hours ($P < .001$, $t = 6.8$ compared to the preceding day) that reached a lower threshold within 3 days of treatment (Figure 2A). Microbial richness began to increase slowly after discontinuation of treatment but was still <25% of its pre-treatment value after 1 week of recovery. After 3 months back on pasture, fecal richness had returned to its pretreatment level. We next assessed the change over time in dominant members of the core microbiome, including those families represented by >1000 read counts per sample, on average, before treatment (Figure 2B). Family *Lachnospiraceae* was less affected than the other dominant families, all of which returned to pre-treatment levels with the exception of *Fibrobacteraceae*. In contrast, 7 families became dominant members of the microbiome (represented by >1000 reads per sample, on average) after initiation of treatment (Figure 2C). In particular, members of families *Streptococcaceae*, *Enterobacteriaceae*, and *Enterococcaceae* increased in relative abundance (RA) during exposure to clioquinol. Similarly, other families of facultative anaerobes commonly found in the upper gastrointestinal tract (GIT) of horses including *Lactobacillaceae*, *Bifidobacteriaceae*, and *Veillonellaceae* also increased in RA immediately after start of treatment. We also noted an unusual, delayed increase in the RA of *Bacteroidaceae* that did not occur until the fifth day of treatment before stabilizing at approximately 3 orders of magnitude higher RA than pretreatment. The abundance of these families remained higher than baseline during the immediate recovery period but returned to pre-treatment levels by Day 111.

Viewed collectively, *Streptococcaceae* clearly became the dominant family within 24 hours of the initial treatment, and comprised >50% of fecal DNA within 48 hours (Figure 3A). The relative increase in *Streptococcaceae* represented 236 amplicon sequence variants (ASVs) annotated as various *Streptococcus* spp., including *S. caballi*, *S. dentasini*, *S. devriesei*, *S. equi*, *S. henryi*, *S. orisasini*, *S. ovis*, *S. porci*, and *S. tangierensis*, and 1 ASV annotated as *Lactococcus garviae* (Figure S1). The relative increase in family *Enterobacteriaceae* reflected ASVs that could be resolved to *Escherichia-Shigella* or unclassified (UC) *Enterobacteriaceae* (Figure S1). Manual curation of amplicon sequences using NCBI BLAST detected 100% identities between several of these ASVs and *Citrobacter*, *Klebsiella*, *Enterobacter*, and *Salmonella* spp. Principal coordinate analysis using unweighted dissimilarities to visualize beta-diversity showed the expected tight clustering of all pre-treatment samples, and movement along PCo1, PCo2 (Figure 3B), and PCo3 (Figure 3C) after treatment. Ordination using weighted similarities yielded a similar pattern (Figure S2). After discontinuation of the drug, sample composition assumed a completely different trajectory along the first 3 principal coordinates, toward the baseline composition. Samples collected at Day 111 partially, but not completely, overlapped with the baseline sample cluster (Figure 3B,C). Permutational multivariate analysis of variance confirmed a significant

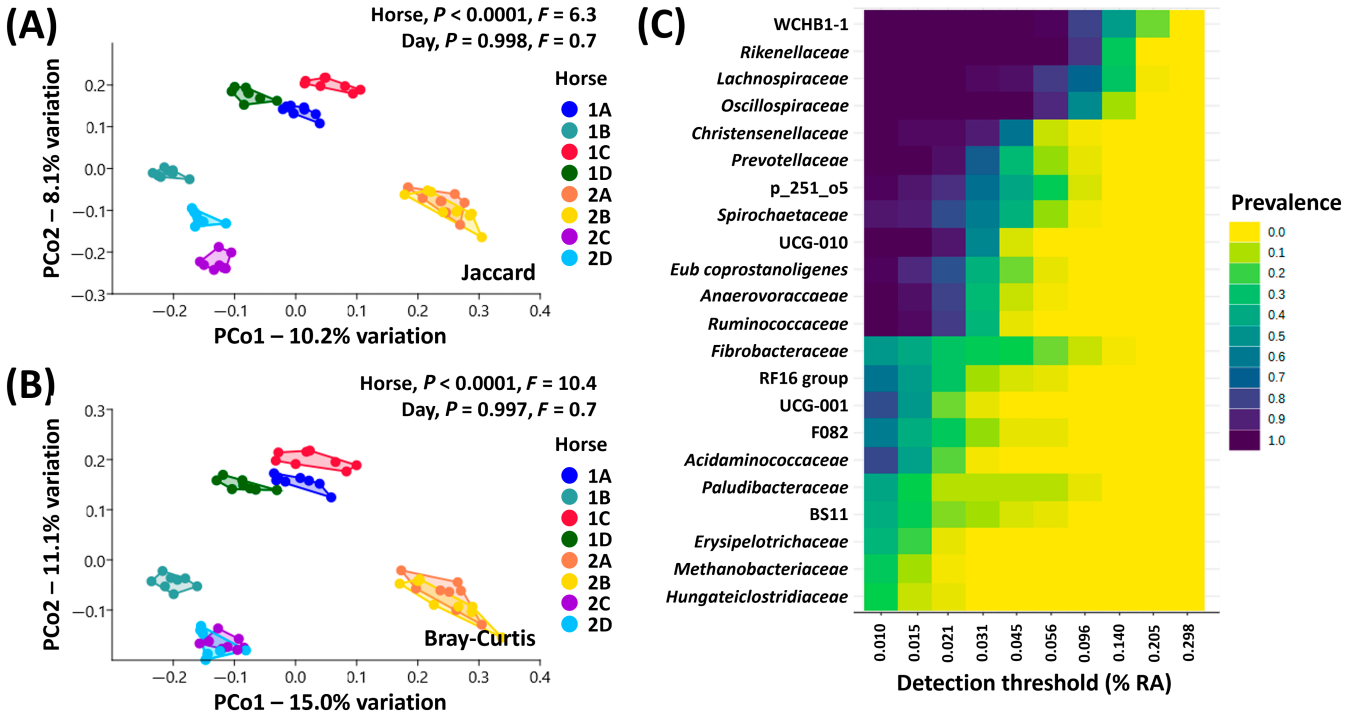

FIGURE 1 Principal coordinate analysis plots show significant inter-subject Jaccard (A) and Bray-Curtis (B) dissimilarity, and intra-subject stability at baseline, of healthy horses (n = 8) before administration of clioquinol. Subject key on the right, results of PERMANOVA at upper right. (C) Heatmap showing the family-level core microbiome of healthy horses at baseline, including all bacterial families present at a minimum relative abundance (RA) of 0.01% and prevalence of 25%. The color key at right indicates prevalence at increasing RA thresholds.

overall effect of day using Bray-Curtis dissimilarities ($P < .0001$, $F = 3.4$). Pairwise comparisons among all days (Table S2) confirmed the stable composition at baseline, and an abrupt effect of the drug that plateaus by Day 13 (5 days of administration) and persists after discontinuation of the drug. Pairwise comparisons indicated that the fecal microbiome had not fully recovered to its pre-treatment composition at Day 111 ($P = .0002$, $F = 2.1$ compared to Day 8).

Principal coordinate analysis performed using only samples collected at baseline and Day 111 reflected the lasting effect of the drug on unweighted (Figure 4A) and weighted (Figure S3) beta diversity. To determine if a relationship existed between baseline RA and long-term effects of clioquinol, the $\log_2$-transformed fold change (FC) in families between baseline (mean of all pre-treatment days) and Day 111 was plotted against the $\log_{10}$-transformed baseline RA of each family (Figure 4B). Among dominant families, some increased whereas others decreased in RA. A prominent example is *Fibrobacteraceae*, which experienced a 9-fold decrease in RA at Day 11, compared with baseline. Other families that were decreased in RA at Day 111 were low- to medium-RA before treatment. Interestingly, these families included many of the families that proliferated during treatment, including *Streptococcaceae*, *Enterobacteriaceae*, and *Enterococcaceae*. Several families at very low RA pre-treatment were enriched at Day 111. Although most of these families still were detected at a modest number (<35) of reads per sample, the *Spirochaetota* family MVP-15 experienced a >200-FC increase to a mean count of 941 reads per sample (using subsampled data at a uniform read count). Table S3 provides baseline and Day 111 abundance, and FC between timepoints, of all families. Collectively, these data show the rapid, marked, and lasting effects of PO clioquinol on the fecal microbiome of healthy horses.

4 | DISCUSSION

Unexpectedly, our data indicate that PO administration of clioquinol to healthy horses results in rapid and comprehensive depletion of the resident gut bacterial microbiome, with greater efficacy than several recently evaluated antibiotics in terms of its effects on microbial richness. For example, twice daily PO administration of doxycycline for 5 days induces a slightly >50% decrease in richness,[13] whereas direct administration of metronidazole via cecal cannula for 3 days results in an approximately 30% decrease in richness.[19] Similarly, daily IV administration of enrofloxacin, ceftiofur, and oxytetracycline for 5 consecutive days induces a nadir in richness at Day 3 representing an approximately 25%-33% decrease.[21] In contrast, our data indicate an approximately 90% decrease in richness, which was surprising given the limited frequency of administration (once daily) and considerable volume and motility of the GIT of horses. Given that clioquinol apparently spares facultative anaerobes within families that include several pathogens, its utility as a PO antibacterial treatment seems questionable. Regarding the bacterial DNA that is enriched after exposure to clioquinol, we speculate that the loss of resident bacteria

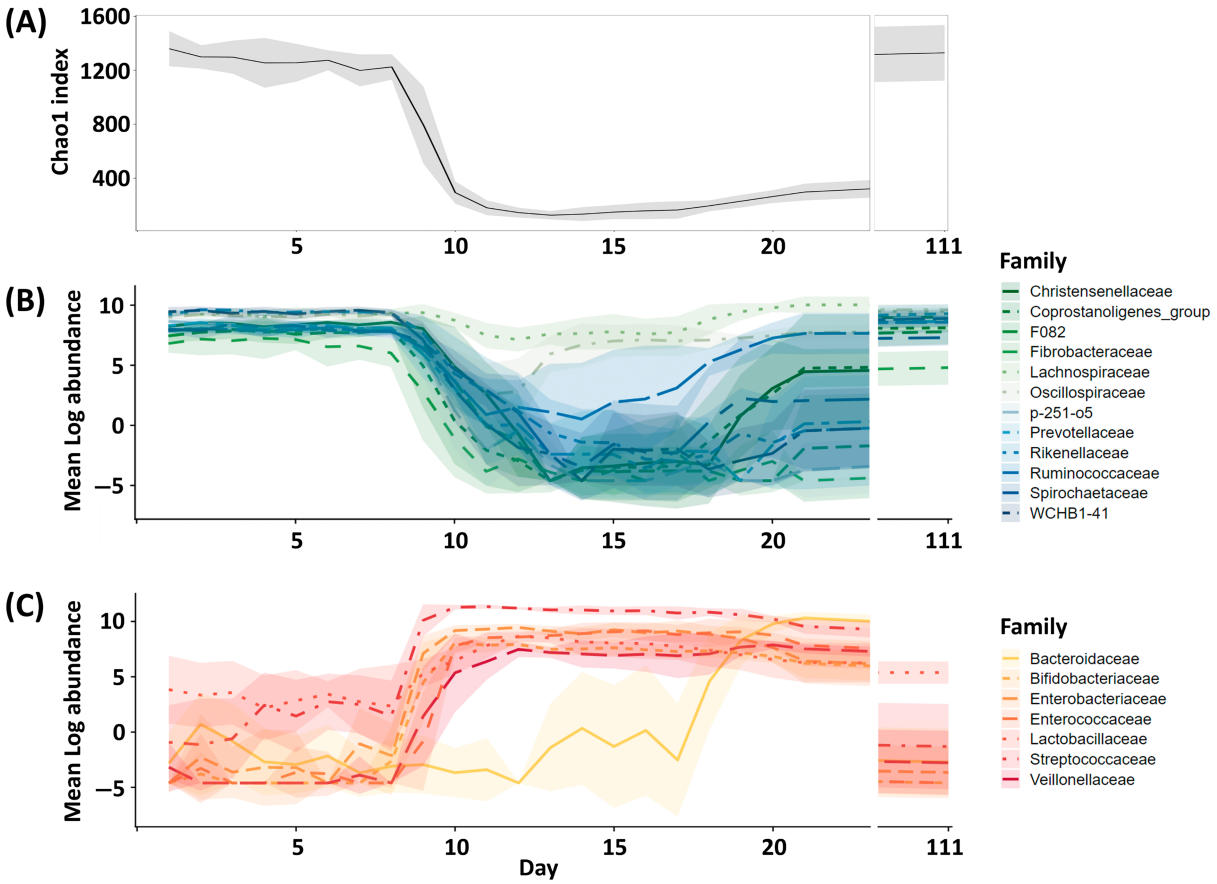

FIGURE 2 (A) Line graph showing daily mean Chao1 index from Day 1 to Day 21 and again 90 days later (Day 111), in healthy horses (n = 8) receiving oral clioquinol beginning from Day 8 to Day 14. (B, C) Line graphs showing the Log-scale read counts of the 14 families detected at greater than 1×10^3 sequences per sample before treatment (B) and 7 families detected at greater than 1×10^3 sequences per sample during or after treatment (C). Shaded areas in all plots represent 95% confidence intervals.

allows expansion of those few taxa capable of surviving in the presence of clioquinol. This hypothesis is supported by the positive culture of *Salmonella* spp. from clioquinol-treated horses. We cannot however rule out that a portion of the fecal DNA detected after clioquinol treatment represents DNA from bacteria present in the upper digestive tract.

The mechanisms underlying clioquinol's antibacterial activity and the apparent resistance of selected families of bacteria are unclear. Potent anti-protozoal[35,36] and anti-fungal[37-39] activities both have been reported, whereas its effects on bacteria have received less attention than have other quinoline derivatives.[40] Clioquinol is a strong zinc and copper chelator,[41] providing an explanation for its antimicrobial activity.[42,43] As in eukaryotes, zinc (Zn^{2+}) is also an essential micronutrient for bacteria, serving as a metalloenzyme cofactor for 5%-6% of the bacterial proteome.[44] Similarly, numerous cuproproteins are widely distributed among bacteria including cytochrome *c* oxidases,[45,46] superoxide dismutase, and enzymes involved in nitrogen reduction.[47] As such, chelation of these metals may starve bacteria of essential cofactors and lead to bacteriostatic and eventually bactericidal effects. *Enterobacteriaceae* are particularly versatile and capable of anaerobic respiration with different terminal electron acceptors (TEAs). Thus, if respiration via nitrogen reduction is disrupted, perhaps genera within this family are better equipped to rapidly adapt to a new TEA. The proliferation of *Enterobacteriaceae* also may be secondary to loss of resident microbes involved in production of short-chain fatty acids (SCFAs) used by colonocytes as an energy source and for regulation of gene transcription. Taxa within *Enterobacteriaceae* often expand in the context of impaired epithelial health, potentially because of their metabolic versatility and aerotolerance.[48-50]

The development of clinical signs in several of the horses, including colic, soft feces, and overt colitis, are likely attributable to the observed antibacterial effects of the drug, rather than any direct toxicity. The fact that other Salmonella-positive horses were present in the VHC during the second half of the study (with Cohort 2) raised questions regarding their contribution to study outcomes. It is possible that other patients shedding *Salmonella* contributed to the dramatic increases in *Enterobacteriaceae* observed in clioquinol-treated horses with no protective resident microbiome. Sequencing data could not resolve the clinical isolates from other patients and ASVs present in the fecal microbiome of study horses. The fact that 1 of the isolates cultured from a study horse was distinct from the other

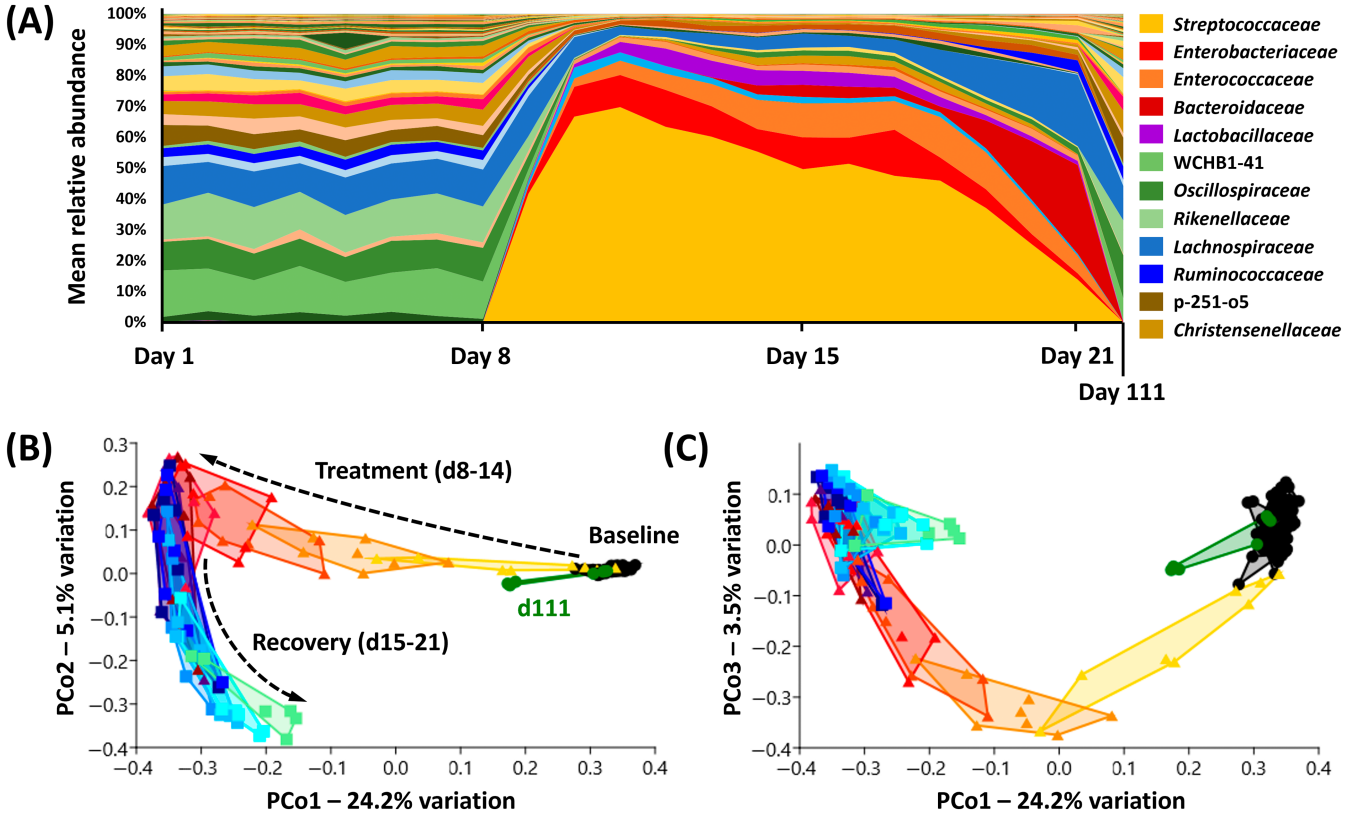

FIGURE 3 (A) Area plot showing the mean relative abundance of bacterial families detected at each timepoint of the study. The color key at right showing identity of 12 most abundant families. (B, C) Principal coordinate analysis plots using unweighted (Jaccard) dissimilarities showing immediate change in fecal beta-diversity along principal coordinate 1 (PCo1), PCo2 (B), and PCo3 (C) following treatment of healthy horses (n = 8) with oral clioquinol. Samples from Day 1 up to and including Day 8 (Baseline) are colored black. Samples collected during treatment are shown in warm colors from Day 9 (gold) to Day 14 (dark red); samples collected during recovery are shown in cool colors from Day 15 (dark purple) to Day 21 (pale green) and Day 111 (dark green). Dotted lines indicate a general shift in beta-diversity over the course of the study.

clinical isolates suggests that at least some of the clioquinol-induced *Salmonellae* proliferated independently of other horses in the VHC. Moreover, the rapid expansion of *Enterobacteriaceae* also was observed in the first cohort of horses, when no other known *Salmonella*-positive horses were present.

Although the lack of a control group could be considered a limitation in the experimental design, the rapid activity and effect size of clioquinol obviate the need for an untreated control group. Additionally, we examined the effects of clioquinol on the taxonomic composition of the fecal microbiome, but did not directly assess microbial function using metagenomic or metabolomic platforms. A previous study using fermentation chambers inoculated with feces from horses indicated a detrimental effect of clioquinol on butyrate production.[12] This finding aligns with the changes observed in our study. We also did not examine protozoal or fungal populations, both of which conceivably could interact with the bacterial microbiome. Lastly, our data represent RA data after PCR-based library preparation. As such, apparent increases or decreases in RA actually may reflect substantial changes in other taxa, and no data on absolute abundance are provided.

Originally introduced in the human medical field as a topical antiseptic and an orally administered intestinal amebicide in 1934, clioquinol also was adopted, with some success, for treatment of nonspecific (idiopathic) chronic diarrheal conditions in people.[41] The drug was introduced into equine veterinary clinical practice in the 1960s for the treatment of nonspecific chronic diarrheal conditions.[1-4] At that time, the ease with which various motile protozoa could be identified in diarrheal feces led to the supposition that they must be important from an etiological perspective, and the condition was sometimes referred to as equine protozoal diarrhea.[1-3] Treatment with clioquinol often was successful, sometimes being undertaken in parallel with fecal microbiota transfer (ie, transfaunation).[1,2] Clioquinol eventually was withdrawn from the human medical market in the 1970s because it appeared to be associated with development of subacute myelo-optic neuropathy (SMON), a painful demyelinating disease of the central nervous system (CNS) and peripheral nerves.[51] The association between clioquinol and SMON is controversial because SMON is extremely rare outside Japan and there is increasing interest in the use of this drug (and its modern derivatives) for the treatment of important neurodegenerative diseases in humans including Alzheimer and Parkinson disease. The potential value of clioquinol for management of neurodegenerative diseases in humans is based on its metal chelating properties.[52] An emerging role for the accretion of elemental metals, especially iron, in the CNS is increasingly recognized

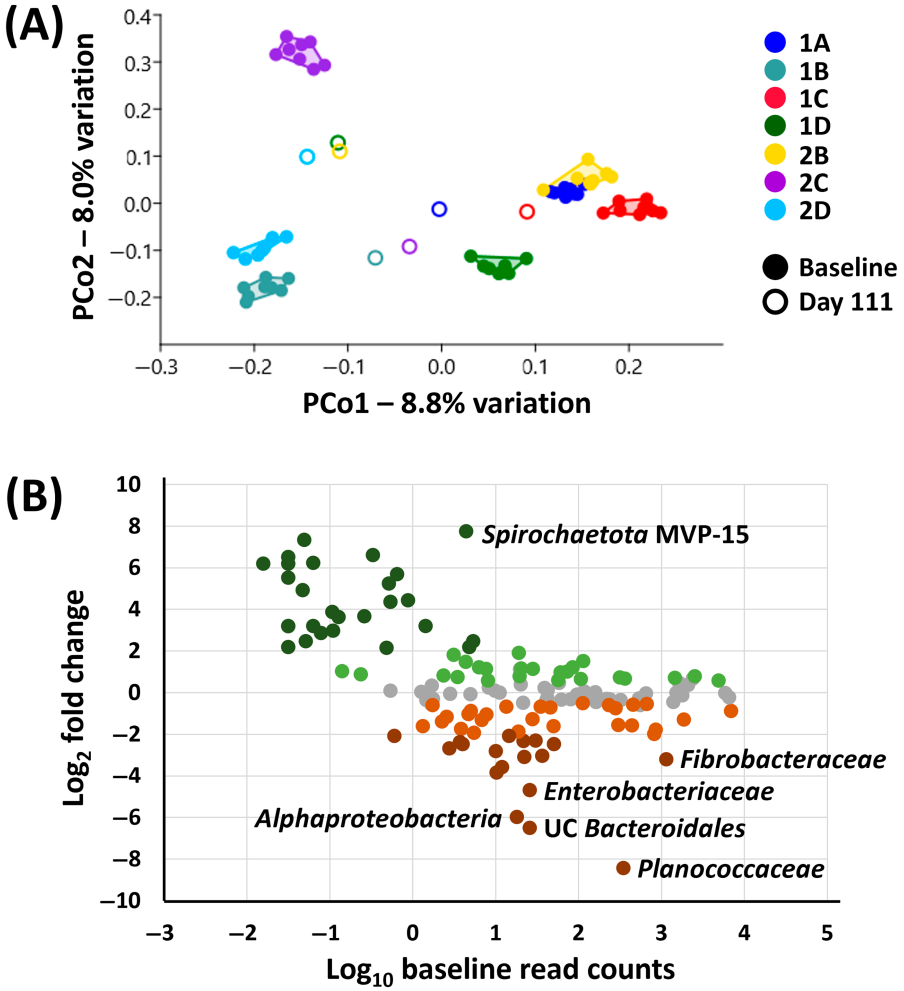

FIGURE 4 (A) Principal coordinate analysis plot showing inter-subject Jaccard dissimilarities between samples collected from healthy horses (n = 7) before administration of clioquinol (closed circles) and at Day 111 (open circles). Subject key at right. (B) Dot plot showing the Log2-transformed fold change (FC) in read count abundance from baseline to Day 111, plotted against the baseline mean abundance, of families in the fecal microbiome. Light and dark brown symbols indicate Log2FC values of −0.5 to −2, and less than −2, respectively. Similarly, light and dark green indicate Log2FC values of 0.5 to 2, and greater than 2, respectively.

as pathophysiologically important in neurodegenerative conditions of humans, including Alzheimer and Parkinson disease.[53] To our knowledge, clioquinol has not been reported to cause neurological conditions similar to SMON in treated horses.

The extent to which clioquinol continues to be used by veterinarians for the treatment of diarrheal conditions is unknown. However, in more recent years, clioquinol has been effective for the management of horses affected with FFWS. Although anecdotal reports suggest that clioquinol is usually effective for restoration of normal fecal consistency in FFWS-affected horses, a satisfactory explanation for both the etiology of FFWS and the mechanism by which clioquinol normalizes fecal consistency is presently lacking.

Whereas, anecdotally, the treatment of horses with clioquinol has been broadly regarded as safe, our results indicate the need for a higher level of concern when using this drug. Upon consideration of the extent to which it causes colonic microbial disruption, it is surprising that clioquinol has not been reported more frequently in the context of inducing diarrhea (namely antimicrobial associated diarrhea). Some of the horses in our second group that were receiving treatment with clioquinol developed clinical disease that may have resulted from exposure to nosocomial Salmonella pathogens. In 1 of those cases, the Salmonella typhlocolitis was very severe and necessitated euthanasia. It could be argued that Salmonella would have affected the experimental principals, regardless of whether they were being treated with clioquinol or not. However, upon consideration of the extent to which clioquinol affected the colonic microbiome, it is reasonable to conclude that those horses had been specifically predisposed to opportunistic pathogen incursion. Therefore, it is appropriate to consider the potential for clioquinol to predispose to enteropathogen infection in treated horses and to avoid using this drug when risk of exposure to Salmonella or other enteropathogens is high (eg, in the presence of potential Salmonella shedders, during stressful events, during interactions with other horses). Logically, clioquinol should not be used for the treatment of acute colitis and it might be recommended that a patient's feces be tested for enteropathogens (including Salmonella) before initiating clioquinol treatment.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

The study examined the effect of the off-label use of PO clioquinol (iodochlorhydroxyquin) in horses, a common treatment for free fecal water syndrome or suspected protozoal diarrhea.

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

Approved by the MU IACUC, protocol #42682.

HUMAN ETHICS APPROVAL DECLARATION

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

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SUPPORTING INFORMATION

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

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