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Equine Gastroenterology

Longitudinal Changes in Fecal Microbiota During Hospitalization in Horses With Different Types of Colic

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ABSTRACT

Background: Research on fecal microbiota changes during hospitalization of horses with colic is emerging.

Objectives: Describe changes of the fecal microbiota during hospitalization of horses with colic caused by inflammatory (INFL), simple (SIMPLE), and strangulated (STR) obstructions, and investigate associations with survival.

Animals: Twenty-three horses with colic: 9 in INFL, 5 in STR, and 9 in SIMPLE groups. Seventeen horses survived, and 6 were euthanized.

Methods: Prospective observational study. Fecal samples were collected on admission (D1), on days 3 (D3) and 5 (D5). Bacterial taxonomy profiling was obtained by V1V3 16S amplicon sequencing. Data were compared using a 2-way permutational analysis of variance (PERMANOVA). Linear discriminant analysis Effect Size (LEfSE) analysis identified significant bacterial population differences, with significance set at $p < 0.05$ and a linear discriminant analysis (LDA) cut-off > 3.0.

Results: Alpha diversity indices remained stable during hospitalization within each colic group. However, at D5, the INFL group had significantly higher richness ($p < 0.01$) and diversity (Shannon, $p < 0.001$ and Simpson, $p < 0.05$) than other colic types. Beta diversity (Jaccard membership and Bray-Curtis indices) was significantly different in the INFL compared to SIMPLE and STR groups (both $p < 0.001$) but not between SIMPLE and STR. Beta diversity membership analysis by analysis of molecular variance (AMOVA) indicated a significant difference between survivors and non-survivors within the INFL group ($p < 0.01$). Increased relative abundances of *Bacilliculturomica* and *Saccharofermentans* were associated with survival.

Conclusions: Microbiota showed no significant variation over 5 days of hospitalization. Colic type influenced fecal microbiota more than hospitalization duration. Specific bacterial populations may differ between survival and non-survival groups.

Abbreviations: AMOVA, analysis of molecular variance; D1, admission; D3, days 3; D5, days 5; HOMOVA, homogeneity of molecular variance; INFL, inflammatory pathologies; LDA, linear discriminant analysis; LEfSe, Linear discriminant analysis Effect Size; LI, large intestine; NS, non-survivors; PCoA, principal coordinate analysis; PERMANOVA, permutational analysis of variance.; RDP, ribosomal Database Project; S, survivors; SCFA, short chain fatty acids; SIMPLE, simple obstruction; STR, strangulated obstruction.

Clémence Loublier and Marcio Costa contributed equally as first authors. Carla Cesarini and Hélène Amory contributed equally as last authors.

1 | Introduction

Colic caused by visceral abdominal pain remains the leading cause of morbidity and mortality in horses [1]. This condition is symptomatic of various gastrointestinal disorders, including obstructions and severe inflammatory processes such as typhlocolitis [2]. The gastrointestinal microbiota of horses, a complex ecosystem of bacteria, viruses, archaea, protozoa, and fungi, plays a crucial role in maintaining intestinal homeostasis [3]. Bacteria are particularly important, facilitating nutrient breakdown, optimizing polysaccharide fermentation, and producing short-chain fatty acids (SCFAs) such as butyrate, propionate, and acetate, which are primary energy sources for horses [4]. Dysbiosis, or severe alteration in the microbiota, is linked to diseases in both humans and horses, leading to the proliferation of pathogenic bacteria, toxin production, mucosal inflammation, and disruptions in SCFA metabolism [5–7].

The interplay between colic and the gut microbiota remains an area of ongoing exploration. Longitudinal studies have shown microbial composition changes associated with colic. For example, post-partum mares before episodes of colic exhibited an increase in Proteobacteria and a decrease in Firmicutes, but findings on community structure, diversity, and evenness are inconsistent [8]. A previous study found no significant differences in alpha and beta diversity of fecal microbiota between colic and control horses at clinical admission [9]. Conversely, another study reported significantly lower richness and diversity of fecal microbiota in horses admitted for colic compared with those admitted for an elective procedure [10]. In the same cohort, a decrease in species richness was observed over the first 3 days of hospitalization, influenced by the duration of signs and lesion location [11].

These studies vary widely in results because of factors such as sample size and treatments administered, including fasting, PO laxatives, anesthesia, surgery, and antimicrobial administration, which are known to influence fecal microbiota [12–15]. The resilience of fecal microbiota to these interventions varies among individuals, complicating the understanding of microbiota dynamics [16]. Current literature suggests certain gut microbiota bacterial species as potential outcome markers in horses with colitis, but comprehensive data on this subject remain sparse [17].

Given these gaps, new research is needed to elucidate fecal microbiota dynamics during colic hospitalization and its potential association with survival outcomes. We aimed to evaluate fecal microbiota dynamics in horses with different types of colic during hospitalization and investigate possible associations between these microbiota profiles and survival outcomes. We hypothesized that fecal microbiota may differ between colic groups, change during hospitalization, and that a specific fecal microbiota profile is associated with better survival rates. Our objectives were to monitor fecal microbiota changes during hospitalization and identify microbial patterns that correlate with survival, providing insights to inform better therapeutic strategies for managing colic in horses.

2 | Materials and Methods

2.1 | Horses and Clinical Management

Horses (≥ 1 year-old) admitted to the Equine Teaching Hospital of Liège (Belgium) between September 2022 and January 2023 with signs of colic, and that remained hospitalized for at least 5 days, were considered for inclusion in our prospective longitudinal observational study. Based on the literature, the term "colic" was defined as acute abdominal pain originating from indistinct causative factors [2]. Exclusion criteria in our study included all horses < 1 year of age, ponies, donkeys, horses with proximal enteritis or a ruptured intestinal tract, horses with ambiguous diagnoses that were impossible to classify, horses with ≥ 2 concomitant gastrointestinal disorders (e.g., obstructive simple and strangulated disorders), and horses that were euthanized or discharged from the clinic before 5 days of hospitalization. Additionally, horses treated with antimicrobial drugs or receiving PO treatments in the month before to admission were excluded from the study. However, horses that had received nonsteroidal anti-inflammatory drugs (NSAIDs) before admission for the current episode of colitis were included.

Horses were classified into 3 groups according to diagnosis (type of colic): simple obstruction (SIMPLE), strangulated obstruction (STR), and inflammatory (INFL).

The diagnosis was made based on clinical history, complete physical examination, rectal palpation, nasogastric intubation, and results of ancillary diagnostic tests (hematology, serum biochemistry, venous blood gas analysis, abdominal ultrasonography, and, if indicated, peritoneal fluid analysis). When performed, findings of exploratory laparotomy also were used for the group classification.

The SIMPLE group included horses with non-strangulating, non-inflammatory disorders of the gastrointestinal tract and without signs of intestinal devitalization (e.g., colon displacements and impactions). The STR group included horses with strangulating intestinal lesions, such as intestinal volvulus or torsion, epiploic foramen entrapment, inguinal hernias, and intussusceptions. The INFL group included horses diagnosed with acute inflammation of the large intestine (colitis, typhlitis) based on a combination of clinical signs and diagnostic criteria. The diagnosis of INFL was horses with acute onset of diarrhea (< 24 h before admission or within the first 24 h of hospitalization) and at least 3 of the following criteria: a history of toxic plant ingestion, hypoproteinemia or hypoalbuminemia, electrolyte imbalances such as hyponatremia and hypochloremia, leukopenia, and ultrasound evidence of cecal or colonic wall thickening (> 4 mm).

Horses were further classified according to outcome in 2 groups: survivors (S) and non-survivors (NS). The S group was defined as horses that responded positively to treatment with resolution of the primary complaint and that were discharged from the hospital after a minimum of 5 days of hospitalization. Conversely, the NS group consisted of horses that were euthanized during hospitalization (after a minimum of 5 days of hospitalization) based on worsening of disease with a poor prognosis. Horses that left

the hospital before 5 days of hospitalization or that were euthanized for economic constraints were excluded from the study. All horses were supervised by equine internal medicine diplomates (European College of Equine Internal Medicine or American College of Veterinary Internal Medicine) and surgery diplomates (European College of Veterinary Surgeons) during hospitalization.

Information about age, sex, breed, and treatments was obtained from the history and medical records.

2.2 | Fecal Samples

Fecal samples were taken during rectal palpation or from freshly evacuated feces. Within 1 h of sampling, they were subdivided using a clean non-sterile forceps into approximately 2 g aliquots from the core of a fecal ball to avoid external bacterial contamination. The aliquots were stored individually in clean non-sterile plastic bags at 45°C until analysis. Samples were collected upon admission (D1) and on days 3 (D3) and 5 (D5) of hospitalization.

3 | Microbiota Analysis

3.1 | Bacterial DNA Extraction and Amplicon Sequencing

Total bacterial DNA extraction was performed with the PSP Spin Stool DNA Plus Kit 00310 (Invitek, Berlin, Germany), following the manufacturer's recommendations. Polymerase chain reaction (PCR) amplification of the V1–V3 hypervariable region of the 16S rRNA gene and library preparation was performed with the following primers: forward (5′-GAGAGTTTGATYMTGGCTCAG-3′) and reverse (5′-ACCGCGGCTGCTGGCAC-3′). Each PCR product was purified with the Agencourt AMPure XP beads kit (Beckman Coulter, Pasadena, CA, USA) and a second PCR using the Nextera XT was performed for indexing. Positive controls using DNA from 20 defined bacteria and a negative control were included in the sequencing run.

3.2 | Bioinformatics

Bioinformatic analysis was performed using the software Mothur following the standard operating procedure previously described for sequence cleaning, including the VSEARCH algorithm for chimera detection [18, 19]. The clustering of sequencing reads was set to a 0.03 distance cut off and sequences were aligned with the SILVA database. Taxonomic classification was obtained from the Ribosomal Database Project (RDP) using the 2020 update. Reads then were clustered together if classified as the same genus (phylotype approach).

The number of genera per sample (observed species richness: Sobs) and the Chao index were used as measures of richness and the Simpson's and Shannon indices to study diversity (the number of species in a community as well as their evenness). For characterization of beta-diversity (similarity of taxonomic composition among samples), the Jaccard index and the Bray Curtis index were used respectively to evaluate community membership (that considers presence or absence of each taxon) and structure (that considers also how often each bacterium appeared in the analysis). A 2-dimensional principal coordinate analysis (PCoA) plot was generated to visualize the similarity among samples. Community structure was further visualized using bar charts representing the relative abundance of the main genera in each colic group.

3.3 | Statistical Analysis

Richness and diversity (alpha diversity) indices were compared among the colic groups (INFL, SIMPLE, STR) and among hospitalization days (D1, D3, D5) using a 2-way repeated measures analysis of variance.

Beta diversity was compared using permutational analysis of variance (PERMANOVA), considering the type of colic, hospitalization days, and outcome as variables. The test was run in R! with the command adonis2 from the vegan package.

Homogeneity of molecular variance (HOMOVA) was used to determine the significance of clustering among types of colic and within each group in community membership and structure (beta diversity) and *p*-values were adjusted using the Bonferroni test for multiple comparisons correction.

Linear discriminant analysis effect size (LEfSe) was used to detect bacteria with significant differences in relative abundances associated with type of colic (INFL, SIMPLE and STR), day of hospitalization (D1, D3, D5) or survival (survivors and non-survivors) [20]. Samples originating from the same horse were recorded to account for repeated measures. A $p < 0.05$ and a linear discriminant analysis cut-off > 3.0 were used to determine significance.

Data collection and graphical presentation were performed using commercially available computer software (Microsoft Excel 2403 Version; GraphPad Prism version 10.2.2).

4 | Results

4.1 | Population

A total of 23 horses aged between 1 and 27 years (median, 17 years) were included in the study. Among all horses, 11 (48%) were geldings, 11 (48%) mares, and 1 (4%) was a stallion. Horse breeds were as follows: Arabian ($n = 3$), French saddle ($n = 3$), Standardbred ($n = 3$), Belgian Warmblood ($n = 2$), Oldenburg ($n = 2$), English thoroughbred ($n = 1$), Fell ($n = 1$), Fjord ($n = 1$), Irish Cob ($n = 1$), Dutch Warmblood ($n = 1$), Lusitanian ($n = 1$), and saddle horses of unknown breed ($n = 4$).

The distribution of the population regarding type of colic, treatment, and outcome is shown in Table 1. Nine horses (39%) were included in the INFL group, 5 (22%) horses in the STR group, and 9 (39%) in the SIMPLE group. The 9 horses from the INFL group included 3 (33%) geldings and 6 (67%) mares. Among these 9 horses, 6 (67%) had colitis of unknown origin, 2 (22%) had colitis secondary to oak intoxication, and 1 had colitis secondary to

larval cyathostominosis (11%). Among these 9 horses, 5 (56%) survived, and 4 (44%) were euthanized.

The 5 horses from the STR group included 3 (60%) geldings and 2 (40%) mares, all of which underwent surgical laparotomy. Among these 5 horses, 2 (40%) had a strangulated pathology of the large intestine (LI), 2 (40%) had a strangulated pathology of the small intestine (SI) 1 lipoma, and 1 volvulus, and 1 horse had a strangulated obstruction of both the SI and LI (torsion of the LI and volvulus of SI). All horses from the STR group survived.

The 9 horses from the SIMPLE group had displacement of the large colon. Among them, 2 (22%) underwent surgery (1 survived, 1 did not), 2 (22%) were euthanized, and 7 (78%) survived.

From all colic groups, only the horses that underwent surgery received antibiotics (22 000 UI/kg penicillin IM q12h and 6.6 mg/kg gentamicin IV q24h) for 5 days during hospitalization (therefore simultaneously with the D3 and D5 samples; Table S1).

TABLE 1 | Distribution of the horse population included in the study, regarding the diagnosis of the intestinal disease, treatment, and outcome. INFL: Inflammatory pathology; SIMPLE: Simple obstruction; STR: Strangulated obstruction.

Horses included in the study ($n=23$)				
Diagnostic groups		**INFL $n=9$**	**SIMPLE $n=9$**	**STR $n=5$**
Treatment	Medical ($n=16$)	9	7	0
	Surgical ($n=7$)	0	2	5
Outcome	Survivors ($n=17$)	5	7	5
	Non-survivors ($n=6$)	4	2	0

Fourteen horses were fasted at the time of sampling upon admission, which included all horses in the SIMPLE (9 horses) and STR (5 horses) groups. However, by D3, only 2 horses in the STR group and 4 horses in the SIMPLE group were still fasting. All horses that were fasted at D3 were also receiving laxatives.

4.2 | Microbiota Analysis

An average of 8 288 077 reads were obtained, of which 3 708 922 reads remained after cleaning and chimera removal with a median read length of 415 base pairs (bp) and a mean sampling good's coverage of 99%.

4.3 | Alpha Diversity: Richness and Diversity

4.3.1 | Type of Colic

On D5, the number of genera, the Shannon index, and Simpson index were significantly higher in the INFL group compared with the SIMPLE group (number of genera [Sobs] $p=0.01$, Shannon index $p=0.0001$ and Simpson index $p=0.002$, respectively). Moreover, the Shannon index was significantly higher in the INFL group compared with STR at D5 ($p=0.02$). The Chao index did not differ statistically among types of colic ($p=0.08$; Figure 1).

4.3.2 | Day of Hospitalization

Concerning statistical comparison among days of hospitalization, neither the richness (Sobs and Chao index) nor the diversity (Shannon index and Simpson index) significantly differed considering each group (INFL, SIMPLE, STR) individually ($p=0.56$, $p=0.29$, $p=0.74$, and $p=0.43$, respectively).

4.4 | Beta Diversity: Community Composition

Beta diversity (membership and structure) was compared among types of colic and days of hospitalization (D1, D3, D5) using PERMANOVA and HOMOVA.

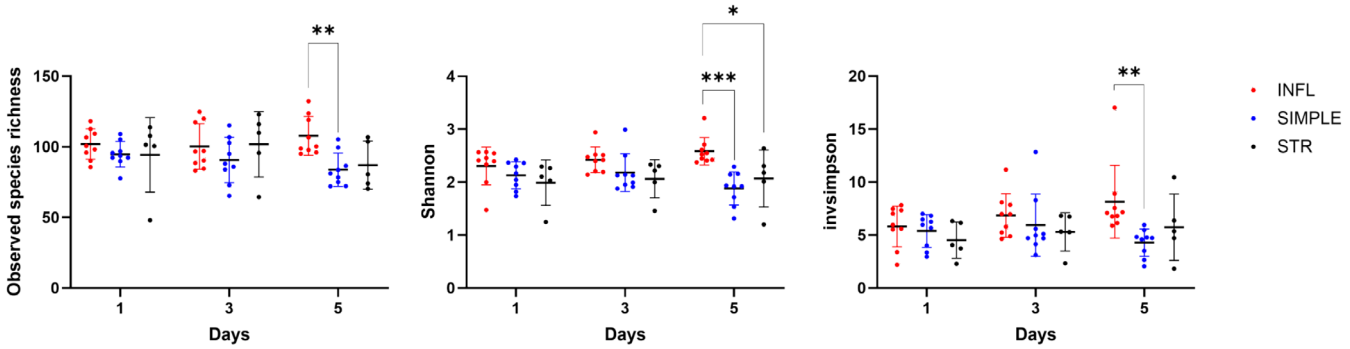

FIGURE 1 | Interleaved scatter plot depicting observed species richness, Shannon diversity index, and Simpson diversity index in the feces of horses with different types of colic: Inflammatory (INFL), simple obstruction (SIMPLE), and strangulated obstruction (STR) at different days of hospitalization (Days 1, 3, 5). Statistical analysis was performed using 2-way ANOVA. Bars represent means and standard deviations. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

4.4.1 | Type of Colic

In terms of membership (genera present or absent, not considering their abundances), group clustering testing among types of colic indicated significant differences between INFL and SIMPLE and between INFL and STR (both $p < 0.001$), but not between SIMPLE and STR ($p = 0.51$). Concerning community structure (presence of each genus and their relative abundances), group clustering testing between groups of colic indicated significant differences between INFL and SIMPLE and between INFL and STR (both $p < 0.001$), but not between SIMPLE and STR ($p = 0.45$; Figure 2). The HOMOVA identified non-significant differences ($p = 0.59$), suggesting a homogeneous distribution of samples within each group.

4.4.2 | Day of Hospitalization

According to PERMANOVA results, the day of hospitalization had no impact on community membership ($p = 0.73$) or structure ($p = 0.76$). The HOMOVA indicated non-significant differences ($p = 0.95$), suggesting homogeneity of samples in the first 5 days of hospitalization.

4.5 | Relative Abundances: Most Abundant Bacterial Populations

Nine main phyla (with respective average) were identified in all diagnostic groups: Firmicutes (57.68%), Bacteroidetes (23.37%), Verrucomicrobia (9.42%), unclassified Bacteria (5.00%), Proteobacteria (1.36%), Fusobacteria (0.86%), Fibrobacteres (0.92%), Spirochaetes (0.54%) and Actinobacteria (0.44%). The remainder of the phyla accounted for < 1% of relative abundance (RA; Figure S1).

Twenty-nine main genera were identified for all groups of colic (Figure 3). From the 3 most abundant genera, 2 were unclassified from the families *Lachnospiraceae* and *Ruminococcaceae*, and 1 was *Akkermansia*.

4.5.1 | Type of Colic

Linear discriminant analysis effect size indicated differences in the relative abundances of 5 taxa among types of colic (INFL, SIMPLE, and STR; Figure 4). The main discriminants between the colic types were *Treponema*, which was more abundant in the INFL group and unclassified *Acidaminococcaceae* in the SIMPLE group.

4.5.2 | Day of Hospitalization

The LEfSe analysis indicated 9 taxa statistically different between days of hospitalization (D1, D3, D5) in horses in the STR group. Within the SIMPLE group, 14 taxa were statistically different between days (D1, D3 and D5) of hospitalization. Within the INFL group, only *Pyramidobacter* was statistically different between days (D1, D3, and D5) of hospitalization (Figures S2–S4).

4.5.3 | Survival

From the 6 horses that did not survive (all euthanized for prognostic reasons) 4 were from the INFL group and 2 from the SIMPLE group. None of the horses in the STR group died. Therefore, because of the uneven distribution between survivors and non-survivors in the other groups, further analysis involving outcomes was conducted exclusively within the INFL group using a post hoc analysis of molecular variance (AMOVA) in mothur.

Beta diversity membership and structure showed no statistical difference (pairwise error rate Bonferroni correction defined at $p = 0.02$) between survivors and non-survivors (respectively, $p = 0.05$ and $p = 0.32$). However, analysis after post hoc exclusion of a horse deemed as an outlier based on PCoA plots identified a statistical difference between survivors and non-survivors in community membership ($p = 0.001$), but not in structure ($p = 0.08$; Figure 5).

The LEfSe analysis (linear discriminant analysis scores > 3) between samples from survivors and non-survivors within the INFL group indicated significant differences in the relative abundances of 8 genera (Figure 6). Of those, increased relative abundances of *Bacilliculturomica* and *Saccharofermentans* were associated with survival, and increased abundances of *Oscillibacter* and *Ruthenibacterium* were associated with non-survival of horses with colic.

5 | Discussion

We evaluated the fecal microbiota of horses referred for colic, identifying differences in microbiota patterns according to the type of colic, in contrast to the stability observed over the first 5 days of hospitalization. Additionally, some bacterial genera showed a potential association with survival in horses suffering from intestinal inflammatory disease despite nonrobust statistical significance, suggesting that additional studies are needed to confirm these observations.

The fecal microbiota differed among the different types of colic. Group clustering analysis identified significant variations in microbial composition (membership and structure) between the INFL and obstructive groups (SIMPLE and STR). No difference in community membership and structure was identified between SIMPLE and STR groups, suggesting that obstruction similarly affected the intestinal microbiota regardless of the cause of the colic.

The LEfSe analysis identified an unclassified member of the *Acidaminococcaceae* family as significantly more abundant in the SIMPLE group compared with the STR and INFL groups. Relative abundance varied from < 0.05% to 0.25%, differing largely among individual horses. This family consists of Gram-negative, butyric acid-producing species within the phylum Firmicutes [21]. It has been associated with stress in piglets, but its role in the equine gut remains unclear [22]. Furthermore, *Treponema* had significantly higher abundance in the INFL group compared with SIMPLE and STR. *Treponema* is associated with the digestion of pectin, soluble starches, and

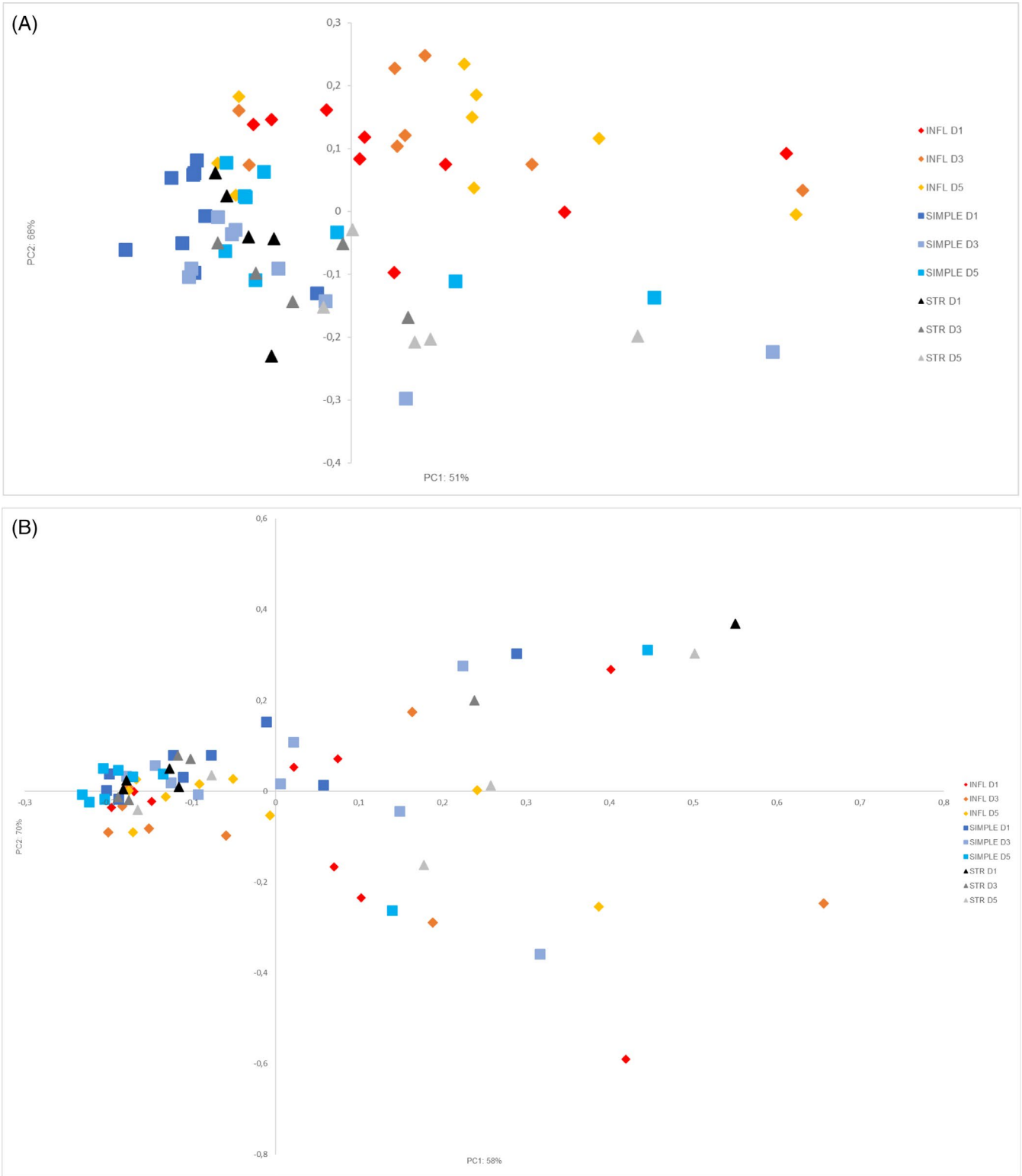

FIGURE 2 | Bidimensional representation of the principal coordinate analysis (PCoA) of bacterial communities in the feces of horses with different diagnostic groups of intestinal disease in terms of community membership (A, Jaccard index) and structure (B, Bray-Curtis index). Inflammatory (INFL, diamonds with color gradation: The lightest: D1, medium: D3, dark: D5), simple obstruction (SIMPLE, squares with color gradation: The lightest: D1, medium: D3, dark: D5) and strangulated obstruction (STR, triangles with color gradation: The lightest: D1, medium: D3, dark: D5) groups.

hemicellulose, and thus with plant fiber digestion [23]. The increased abundance of *Treponema* in the INFL group could relate to the continuous dietary fiber intake of patients with colitis compared with fasting in the other groups. Notably, a previous study described a decreased abundance of this genus in fecal samples from the first 3 days of hospitalization compared

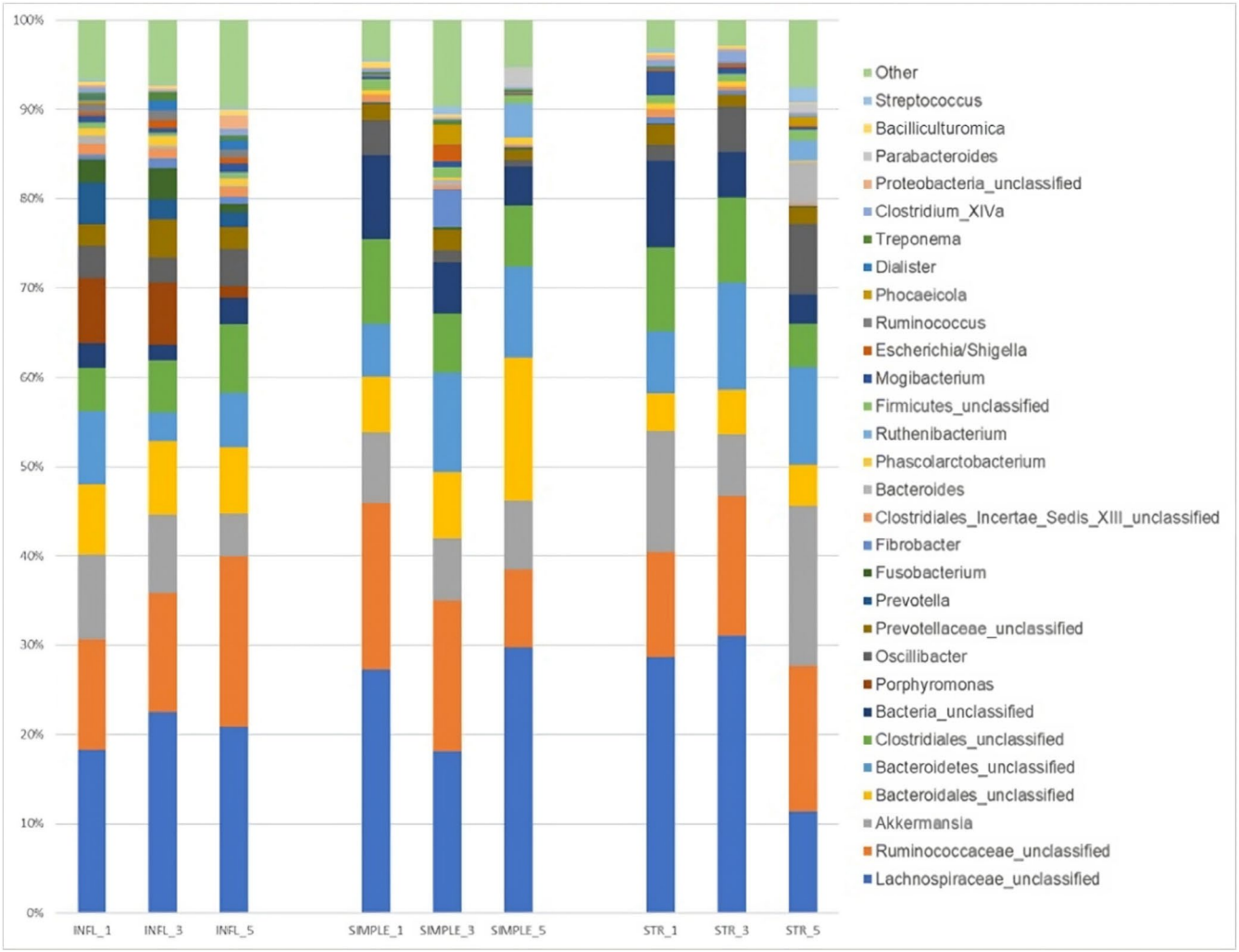

FIGURE 3 | Bar chart depicting the mean relative abundance (RA) of the main bacterial genera found in the fecal content of horses with different diagnostic groups of intestinal disease during hospitalization (Days 1, 3, 5). Inflammatory (INFL), simple obstruction (SIMPLE), and strangulated obstruction (STR) groups.

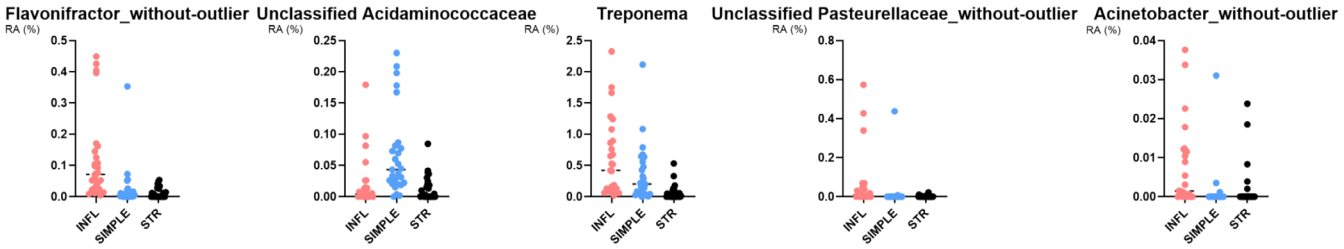

FIGURE 4 | Dot plots demonstrating individual relative abundances of the bacterial taxa statistically different between types of colic revealed by the Linear discriminant analysis Effect Size (LEfSe). Inflammatory (INFL), simple obstruction (SIMPLE), and strangulated obstruction (STR) groups. RA: Relative abundance. For the ease of visualization one outlier has been removed in: *Flavonifractor*, *Acinetobacter*, and *unclassified Pasteurellaceae group*.

with admission in horses with LI pathologies (strangulated and non-strangulated colic obstructions with medical and surgical treatments), suggesting that this genus could be an indicator of disruption in fermentative digestion [11].

Although no differences in alpha diversity indices were observed among colic types on D1 and D3, at D5, horses in the INFL group had significantly higher richness and diversity indices (Shannon and Simpson) in feces compared with horses with colon displacement. These findings suggest that horses with intestinal inflammation had a larger number of different bacterial populations that were also more evenly distributed compared with horses with other types of colic. Most previously published studies reported decreased fecal microbial diversity in horses with intestinal inflammation [5, 15, 17, 24, 25]. However, these studies described fecal microbiota findings at a single

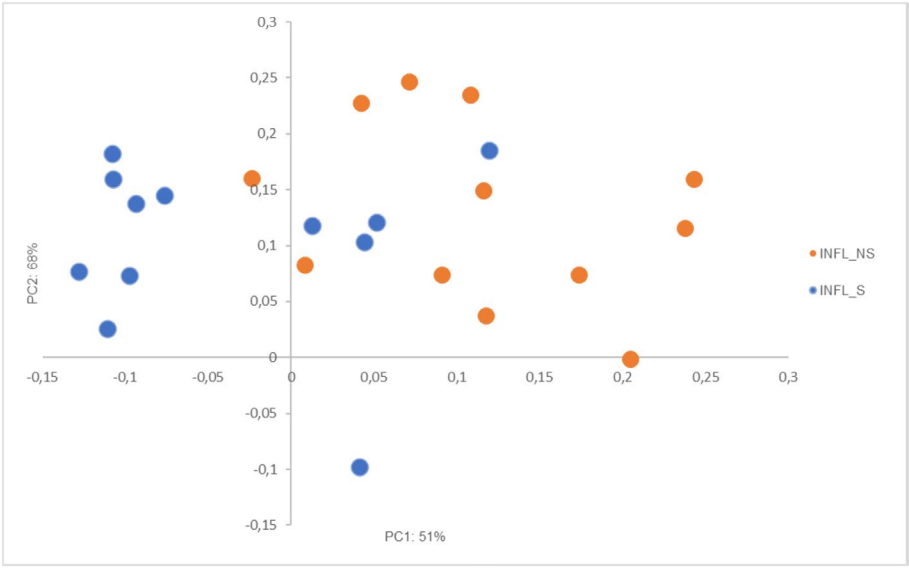

FIGURE 5 | Principal coordinate analysis (PCoA) representing bacterial membership (Jaccard Index) in the feces of horses with intestinal inflammatory disease which survived (INFL_S) and did not survive (INFL_NS) hospitalization. A statistical difference ($p = 0.001$) assessed by the AMOVA test was found between groups after removal of one outlier horse.

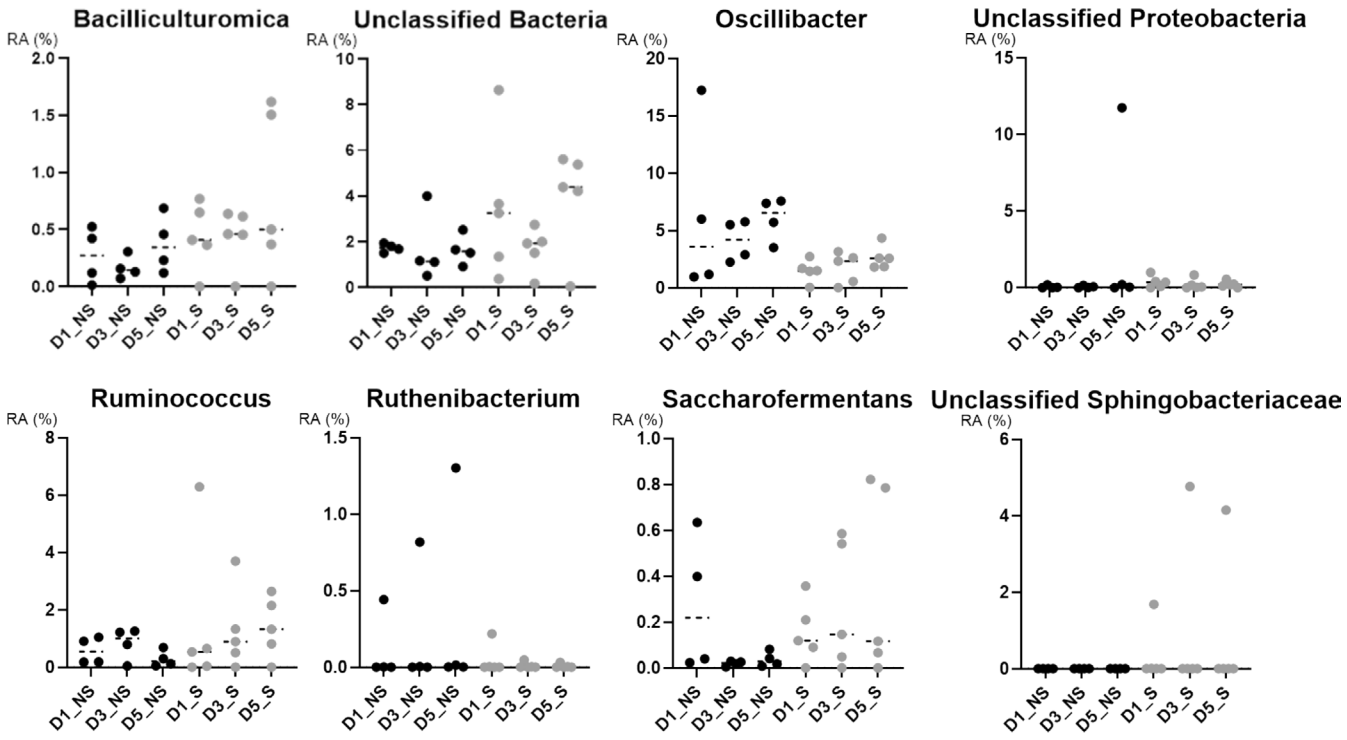

FIGURE 6 | Dot plots demonstrating individual relative abundances of the bacterial taxa statistically different between outcome groups: Survivors (S) and non-survivors (NS) for horses with inflammatory intestinal disease (INFL) during hospitalization (Days 1, 3 and 5) assessed by the Linear discriminant analysis (LDA) Effect Size analysis. All LDA scores > 3.

time point mostly on admission. We assessed fecal microbiota for 5 days, and variations in patient management should be considered when interpreting results. All horses in the INFL group were offered roughage from D1, whereas the other colic groups were subjected to some degree of underfeeding for therapeutic or pathological (i.e., postoperative ileus) reasons. Withholding feed has been linked to a decrease in species number and diversity [26], potentially contributing to the decreased richness and diversity observed in the SIMPLE and STR groups at D5 compared with the INFL group. Other factors, such as antimicrobial use, may affect microbiota diversity [15, 26]. None of the horses in the INFL group received antimicrobials or underwent surgery, whereas 2 out of the 9 horses in the SIMPLE group and all horses in the STR group did. Differences in antimicrobial use and anesthesia might have influenced the diversity results [13, 27]. However, within the SIMPLE group, all horses showed

decreased diversity, not only those that received antimicrobials and underwent surgery. Moreover, HOMOVA identified no significant difference ($p = 0.59$), suggesting a homogeneous distribution of samples within the SIMPLE group. Therefore, antimicrobials and surgery in the 2 samples within the SIMPLE group did not seem to have substantially impacted the overall group.

We did not include a direct comparison with a group of healthy control horses, which limits our ability to definitively determine whether the observed microbial changes reflect a general trend in local colitis cases or a specific alteration associated with colic. However, because of substantial inter-individual variability in the microbiota of horses, including a traditional control group would have presented additional challenges. A more suitable control would have been a stable companion under identical conditions, including diet, hospitalization duration, and treatments, which was not feasible within the constraints of our study. As a result, we used a within-subject comparison by monitoring the evolution of the fecal microbiota from the same horse, with the baseline sample at admission serving as a reference point. Although this approach offers valuable insight into the temporal microbiota dynamics, the lack of a separate healthy control group may limit broader interpretation of our findings.

A recent study found decreased richness between admission samples and samples from the first 3 days of hospitalization in horses with large and small intestinal obstruction (strangulated and simple obstructions) [11]. Comparing results with other studies is complicated in microbiota research, mostly because of methodological differences, including data processing and variable degrees of inflammation and severity of gastrointestinal disease among cases [28]. Therefore, larger multicenter studies using standardized analyses are necessary to better understand microbial shifts in horses with intestinal diseases.

One important feature of our study is the longitudinal evaluation of microbiota during the first 5 days of hospitalization. Although PERMANOVA found no differences in community composition between D1 and D5, LEfSe analysis indicated decreased relative abundances of unclassified *Clostridiales incertae sedis XIII*, unclassified Ruminococcaceae, *Bacilliculturomica*, and unclassified Proteobacteria between D1 and D5. In the SIMPLE group, 4 of 9 horses were still fasting at D3 and receiving laxatives, which could have affected the fecal microbiota until D5. The family *Ruminococcaceae* consists of fiber-degrading butyrate producers that are important in horses [29]. Therefore, the decrease in *Ruminococcaceae* might be associated with fasting. Increased abundance of Proteobacteria has been associated with dysbiosis in horses [5, 8]. Proteobacteria also have been identified out as a marker of intestinal disease in humans [30–32]. A previous study reported that this phylum increased in pregnant mares that developed colic caused by large colon volvulus [8]. In our study, Proteobacteria were significantly more abundant on admission in the SIMPLE group and decreased over time, suggesting a normalization of dysbiosis during hospitalization. However, another study reported a different evolution of Proteobacteria during hospitalization, with an increase from admission to day 3 in large intestinal lesions [11]. The specific reason for this finding is unclear, but we found that the LI colic comprised both strangulated and non-strangulated groups of colic in this study. Comparing results among studies is challenging when group definitions differ. Moreover, in our study we described an unclassified Proteobacteria that could be a different species compared with the previous study [11].

One limitation of DNA sequencing is the lack of absolute quantification, and the increase in Proteobacteria relative abundance might be caused by a decrease in normal microbiota members such as Firmicutes and Bacteroidetes [33–36]. Therefore, more studies using absolute quantification methods such as quantitative PCR are required.

Our study also sought to find potential fecal microbiota features indicative of prognosis. Unfortunately, mortality was uneven across the different colic groups and did not reflect clinical impressions. One inclusion criterion was to include only horses hospitalized for 5 days, but in the STR group, mortality often occurred before 5 days, making survival comparisons between groups impossible. These observations led us to perform this analysis only in the INFL group. However, the number of horses for comparison was relatively small (5 survivors vs. 4 non-survivors). Beta diversity membership and structure identified significantly different microbiota profiles between survival and non-survival groups ($p = 0.001$) after excluding an outlier. This outlier had clinical signs of colic symptoms for 5 days, the longest among all patients, whereas other horses had clinical signs for < 48 h. A previous study found lower abundances of Proteobacteria and lower richness and diversity in horses with colic onset > 60 h [11], which also was observed in the outlier horse (relative abundance of 0%). Both findings suggest that colic duration might impact the fecal microbiota of horses. The LEfSe analysis identified increased Bacilliculturomica and Saccharofermentans in surviving horses. The genus Saccharofermentans has been associated with health in microbiota studies of horses [37, 38]. Another study found several bacterial taxa capable of discriminating horses with colitis that survived ($n = 27$) from those that did not ($n = 28$) [17]. None of the taxa identified in that study coincided with those highlighted in the INFL group of our study. This difference possibly is explained by differences in methodologies used among studies [28]. An important point to emphasize is that the use of AMOVA, based on the analysis between survivors and non-survivors within the INFL colic group, did not account for the duration of hospitalization. Therefore, future studies should further explore the relationship between microbiota and survival to establish any potential associations. The small number of horses and large interindividual variability in the relative abundances of some discriminant taxa should be considered when interpreting our results. Although the sample size in our study was comparable to those of other studies in horses, it remained relatively modest, potentially limiting its representativeness for a broader population of horses with colic. Achieving uniformity in the horse population is challenging because many factors can influence the microbiota of horses [14, 39]. Treatments including antibiotics, mineral oil, enteral fluids, abdominal surgery, and withholding of feed potentially can introduce confounding effects that complicate the interpretation of changes observed during hospitalization.

Using fecal samples to evaluate microbiota changes in the cecum and colon remains a topic of debate. Fecal samples are readily available and obtained using minimally invasive methods and

have been used as proxies for the large colon microbiota of healthy horses [12, 40–42]. However, a previous study found decreased species richness in colonic compared with fecal samples from horses admitted with large colon lesions, concluding that fecal samples collected at admission may not accurately represent colonic microbiota changes [9]. Moreover, retention times of ingesta in the cecum and large colon are estimated to be 35 h, thus serial fecal sampling every 48 h could be necessary to detect colonic microbiota changes in fecal samples [43]. For this reason, we collected feces every 48 h. Despite these limitations, our study brings more information to the field of gastroenterology in horses, specifically the search for microbiota alterations among different presentations of colic.

In conclusion, horses with diarrhea exhibited significant differences in both alpha and beta diversity compared with other colic types. Short-term hospitalization did not have a substantial impact on the fecal microbiota of horses with colic. Although certain bacterial genera were closely associated with specific types of colic, the relationship between microbiota composition and survival outcomes remains uncertain and requires further investigation.

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Disclosure

Authors declare no off-label use of antimicrobials.

Ethics Statement

Authors declare no Institutional Animal Care and Use Committee or other approval was needed. Authors declare human ethics approval was not needed.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

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