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The fecal bacterial microbiota of healthy and sick newborn foals

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Abstract

Background: The fecal bacterial microbiota of normal foals and foals with enterocolitis has been characterized using next-generation sequencing technology; however, there are no reports investigating the gut microbiota in foals hospitalized for other perinatal diseases.

Objective: To describe and compare the fecal bacterial microbiota in healthy and sick foals using next-generation sequencing techniques.

Animals: Hospitalized (17) and healthy foals (21).

Methods: Case-control study. Fecal samples were collected from healthy and sick foals on admission. Sick foals were further divided into sick nonseptic (SNS, n = 9) and septic (n = 8) foals. After extraction of DNA, the V4 region of the 16 S rRNA gene was amplified using a PCR assay, and the final product was sequenced with an Illumina MiSeq.

Results: Diversity was significantly lower in healthy than sick foals ($P < .05$). The bacterial membership (Jaccard index) and structure (Yue & Clayton index) of the fecal microbiota of healthy, septic, and SNS foals were similar (AMOVA, $P > .05$). Bacterial membership (AMOVA, $P = .06$) and structure (AMOVA, $P = .33$) were not different between healthy and sick foals. Enterobacteriaceae, *Enterococcus*, and *Streptococcus* were among the 5 more abundant taxa identified in both groups.

Conclusion and Clinical Importance: Higher fecal microbiota diversity in sick than healthy foals might suggest a high exposure to environmental microorganisms or an unstable colonic microbiota. The presence of microorganisms causing bacteremia in foals in a high relative abundance in the feces of foals suggests the intestine might play an essential role in the causation of bacteremia in foals.

KEYWORDS

Aerococcus, Enterobacteriaceae, *Enterococcus*, equine neonates, sepsis, *Streptococcus*

Abbreviations: AMOVA, analysis of molecular variance; GIT, gastrointestinal tract; LDA, linear discriminatory analysis; LefSe, linear discriminant analysis effect size; PCoA, principal coordinates analysis; SNS, sick nonseptic foals

1 | INTRODUCTION

Sepsis is the most common cause of morbidity and death in newborn foals. The sources and mechanisms of neonatal infection in foals are not well characterized but can be acquired horizontally from the environment or via vertical transmission. Microorganisms causing neonatal sepsis are acquired before, during, and after parturition, with different trends in causative microorganisms reported at various geographic areas.[1-5] Factors associated with the development of sepsis in equine neonates include maternal disorders, abnormal gestation length, failure of transfer of passive immunity (FTPI), poor environmental hygiene, and inadequate umbilical care.[6] Maternal disorders including dystocia, placentitis, gastrointestinal or respiratory diseases have an essential role in the development of bacteremia in foals[7-9]; however, the importance of other routes of infection such as umbilical structures, or the gastrointestinal tract (GIT) and respiratory system are not completely understood.

The bacterial colonization of the GIT of newborn foals appears to follow the classic pattern of colonization of neonatal calves[10] and infants,[11,12] with rapid changes occurring between day 1 and 3 and, day 3 and day 10 after parturition.[13,14,15] Initially, there is rapid colonization by facultative anaerobes followed by strictly anaerobic bacteria.[15-18] Associated with the changes in bacterial communities, there is an increase in richness (number of taxa present in a sample), evenness (proportional abundances of the taxa present in a sample) and diversity (mathematical calculation that accounts for the richness and evenness of a sample).[11,12,14,16] This bacterial shift is likely associated with exhaustion of oxygen supplies in the GIT, creating an anaerobic milieu.[12] During the first days of the neonatal infant and foal's life, the GIT comprises of a myriad of bacterial communities that include potential pathogens. Although the association between the GIT microbiota and neonatal foal sepsis is still not well-understood, in humans, some studies suggest that gastrointestinal dysbiosis can predispose children to neonatal sepsis.[19-22] Little is known about the alterations in the bacterial communities present in the GIT of septic foals and their association with disease. However, most bacterial isolates from blood cultures of septic foals are microorganisms likely present in the GIT (ie, *Escherichia coli*, *Enterococcus* spp., *Enterobacter* spp., *Streptococcus* spp., *Staphylococcus* spp., *Actinobacillus* spp., *Pasteurella* spp., *Pseudomonas* spp., and *Salmonella* spp). This suggests that the GIT microbiota might be an important factor in the pathogenesis of neonatal foal sepsis. The objective of this study was to describe the fecal bacterial microbiota of healthy newborn foals and compare it with sick nonseptic (SNS) and septic foals admitted to a neonatal intensive care unit.

2 | MATERIALS AND METHODS

2.1 | Ethical considerations

This study was approved by the Institutional Animal Care and Use Committee of Iowa State University and adheres to the principles for the humane treatment of animals in veterinary clinical investigations as stated by the American College of Veterinary Internal Medicine and National Institutes of Health guidelines. Owner consent was obtained before inclusion in the study.

2.2 | Animals

Healthy foals from the Iowa State University teaching herd were used as study controls. Healthy foals were <7 days of age with no evidence of disease based on clinical exam, complete blood count (CBC; Advia 212Qi Hematology Analyzer, Siemens Healthineers, Malvern, Pennsylvania), chemistry profile (Vitros 4600 Chemistry System, Ortho Clinical Diagnostics), fibrinogen (Vitros 4600 Chemistry System, Ortho Clinical Diagnostics), and serum IgG (DVM Rapid Test II- Multi-Test Analyzer, MAI Animal Health, Elmwood, Wisconsin) concentrations (>800 mg/dL). Foals of any breeds and sex were included. Hospitalized foals <7 days of age, admitted during the 2018 and 2019 foaling season were included as cases. Foals were classified as septic if they had a positive blood bacterial culture or a sepsis score >11.[23] Foals with negative blood culture presented for other diseases such as neonatal maladjustment syndrome, meconium impaction, and trauma were classified as sick nonseptic (SNS). In addition, hospitalized foals were classified as survivors (foals that survived until discharge from the hospital) and nonsurvivors (foals that died or were euthanized because of grave prognosis). Foals euthanized because of financial limitations were excluded from our study. Foals treated with antimicrobials, blood products or IV fluids, glucocorticoids or other medications before admission were excluded.

2.3 | Sample size calculation

A minimum sample size of 6 foals per group was considered to detect a 25% difference in operational taxonomic unit (OTU) counts with a power of 0.8 and a confidence level of 0.95, assuming a normal distribution with a mean ± SD OTU count of 2000 ± 300 per sample.[24]

2.4 | Sample collection and processing

Fecal samples were obtained from the rectum using a cotton swab on admission before administration of any treatment. The samples were snap frozen in liquid nitrogen and stored at −80°C until processing. Samples were thawed and bacterial DNA was extracted from feces using the Thermo Scientific KingFisher (KingFisher Flex Purification System, ThermoFisher Scientific) instrument with magnetic bead technology following the manufacturer's recommendations.[25] After DNA extraction, samples were transported overnight to the Environmental Sample Preparation and Sequencing Facility at Argonne National Laboratory for sequencing. Amplification of the V4 region of the 16 S rRNA gene was complete following a previously published PCR protocol.[26] The library pool was sequenced with an Illumina MiSeq for 250 cycles from each end.

2.5 | Data analysis

Mothur software (https://mothur.org) was used for bioinformatic analysis following a standard operating protocol.[27,28] Sequences were binned (genus level) into phylotypes after cleaning and filtering and being identified using the Ribosomal Database Project classifier. Normalization of the sequence count was completed using a random subsampling and assessment of the sample coverage was performed using the Good's coverage index.[29] Relative abundances of the main taxa (relative abundance >0.5%) were calculated and compared using a Wilcoxon rank test and *P*-values were adjusted for multiple comparisons.[30] Taxa enriched in fecal samples of each group was identified using the Linear discriminant analysis effect size (LEfSe) based on $P < .05$ and a linear discriminant analysis (LDA) score >2.[31] Differences in fecal microbiota were evaluated using the health condition (eg, healthy, septic and SNS foals) as the main outcome of interest. Diversity (Inverse Simpson's index), evenness (Shannon's evenness index) and richness (Chao-1 index) were used to assess alpha-diversity (differences within the groups) and a Wilcoxon rank test or the Steel-Dwass test for multiple comparisons was used for comparison between groups. Beta-diversity (differences between samples) indices Jaccard[32] and Yue and Clayton[33] were calculated to identify differences in community membership (a measured that only asses for the number of shared genera, but not their abundance) and structure (a measured that asses for the number of shared genera and their relative abundances), respectively. The differences between groups were assessed using an analysis of molecular variance test (AMOVA) and Principal coordinate analysis (PCoA) plots were constructed to investigate for the presence of clustering of the samples (JMP 16.1.0, SAS Institute). The number of different meta-communities or enterotypes into which the data could be clustered was determined using Dirichlet multinomial mixture model (DMM) using the Laplace approximation for selecting the best number of metacommunities.[34]

3 | RESULTS

3.1 | Animals

The study sample was comprised of 21 healthy, 8 septic and 9 SNS foals. In the healthy group 10 foals were Thoroughbred (TB), 9 were Quarter Horse (QH), 1 Connemara pony and 1 Belgian. The septic group included 4 TB and 3 QH, while the SNS group was comprised of 5 TB, 2 QH, 1 Percheron and 1 Pony of the Americas. All sick foals were admitted with their dam and none of the foals was reported to have received either colostrum or milk replacer before admission. The age of the foals at the time of sampling was similar between groups ($P > .2$, for all comparisons). Five of the 8 septic foals had a positive blood culture and 3 had a negative blood culture but the sepsis score was >11. Organisms cultured form the blood samples included Alpha-hemolytic *Streptococcus*, *Enterococcus mundtii*, *Streptococcus dysgalactiae ss equisimilis*, Coagulase-negative *Staphylococcus*, *Escherichia coli*, *Psychrobacter* sp., *Enterococcus faecalis*, *Aerococcus viridans*. The SNS foals were admitted to the hospital for the following complaints: failure to nurse after birth (n = 3), neonatal maladjustment (n = 2), tendon contracture (n = 2), weak foal post-dystocia (n = 1), and omphalitis and diarrhea (1). Septic foals were admitted with complaints of enterocolitis (n = 2), neonatal maladjustment (n = 2), prematurity (n = 2), pneumonia (n = 1) and 1 foal was presented for sepsis. The serum immunoglobulin G concentration for healthy foals (1719 mg/dL ± 690) was higher than for SNS (1034 mg/dL ± 549) and septic (418 mg/dL ± 313) foals. All 9 SNS survived to hospital discharge, but 3 septic foals did not survive hospitalization.

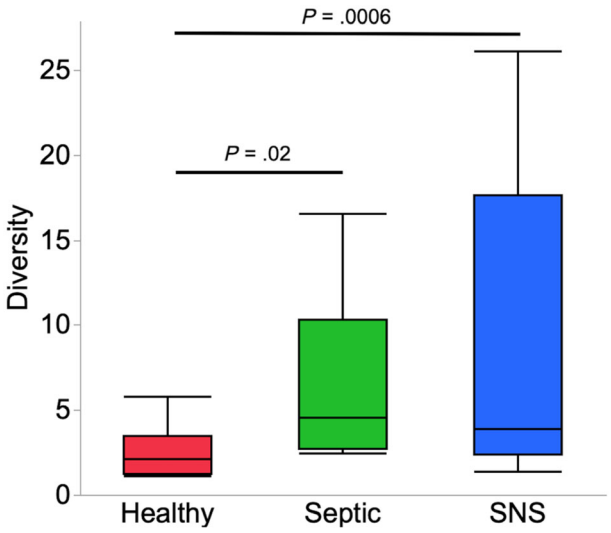

FIGURE 1 Diversity of the fecal microbiota of healthy, sick nonseptic (SNS) and septic foals admitted to a neonatal intensive care unit of a teaching hospital. The whiskers mark the 95th and 5th percentiles. The *P*-values were obtained using the nonparametric Steel-Dwass test for multiple comparisons

3.2 | Sequence analysis

A total of 4 541 730 good quality sequences were used for the final analysis (mean sequences per sample: 119 519 per sample; SD: 172 080; median: 101 347; range: 13 862-1 111 548). The sequences were rarified to a uniform count number of 13 000 sequences per sample. Adequate subsampling was determined by the Good's coverage obtained for all samples (median: 99.9%; range: 99.8%-99.9%).

3.3 | Alpha and beta-diversity measurements

The richness (Chao-1 index) was similar among healthy, septic, and SNS foals ($P > .1$, for all comparisons). The evenness (Shannon evenness index) was significantly higher in septic than in healthy foals ($P < .02$). The diversity (Inverse Simpson's index) was also significantly higher in septic ($P < .005$) and SNS ($P < .02$) than in healthy foals (Figure 1). Alpha-diversity indices were similar between SNS and

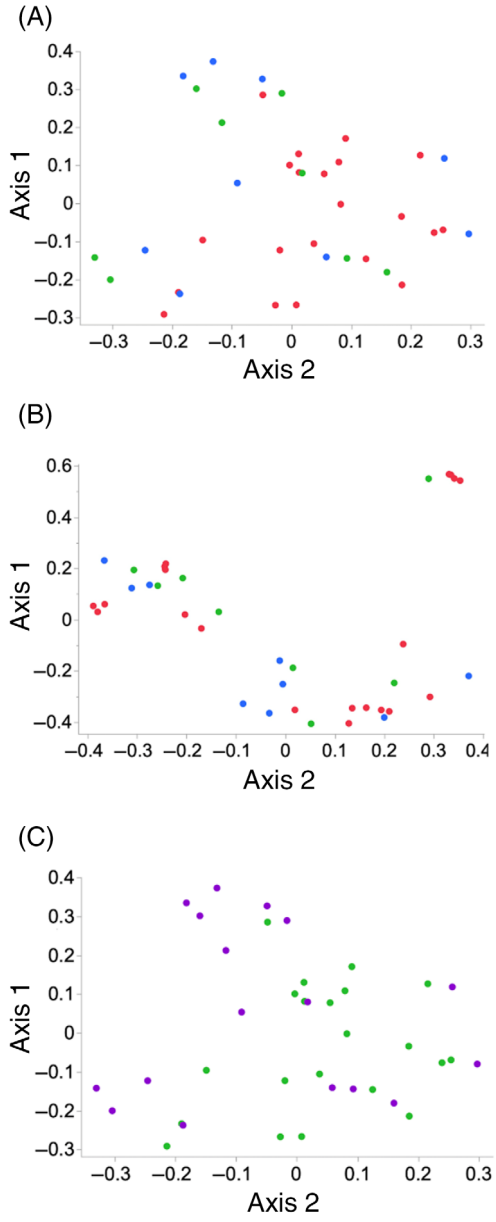

FIGURE 2 Principal coordinate analysis (PCoA) of bacterial community (A) membership (Classic Jaccard analysis) and (B) structure (Yue and Clayton analysis) of healthy (red dots, n = 21) sick nonseptic [SNS] (blue dots, n = 9) and septic foals (green dots, n = 8). The community membership and structure of healthy, SNS and septic foals was similar (AMOVA, $P = .16$ and $P = .35$, respectively). Panel C displayed a Principal coordinate analysis (PCoA) of bacterial community membership (Classic Jaccard analysis) of healthy (green dots, n = 21) and sick foals (purple dots, n = 17). Comparison of healthy and sick foals revealed no differences in community membership (AMOVA $P = .06$) and structure (AMOVA $P = .33$)

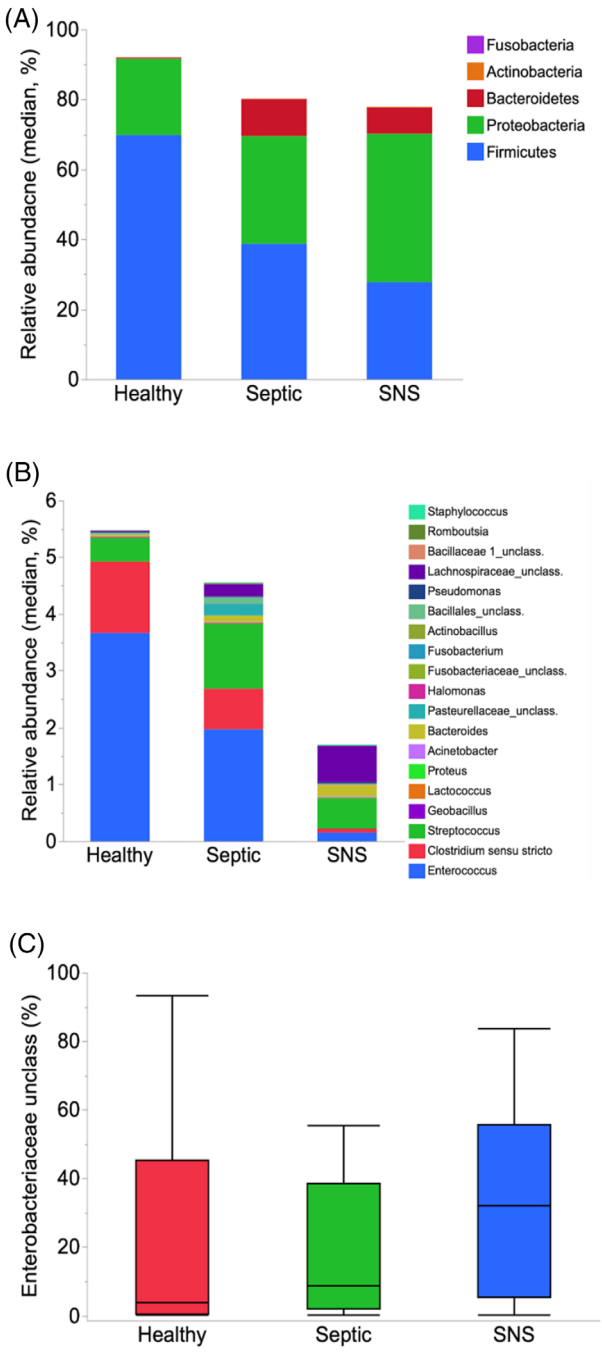

FIGURE 3 Median relative abundance of predominant bacteria at the phylum (A) and genus (B) level identified in feces of healthy (n = 21), sick nonseptic (SNS, n = 9) and septic foals (n = 8). The 6 most abundant phyla and 19 most abundant genera are displayed. (C) Relative abundance of the taxa Enterobacteriaceae identified in feces of healthy, sick nonseptic and septic foals

septic foals ($P > .8$, for all comparisons). When SNS and septic foals were grouped as sick foals, the richness ($P = .09$), evenness ($P < .01$), and diversity ($P < .01$) were higher in sick compared to healthy foals.

The bacterial membership (Jaccard index) and structure (Yue & Clayton index) of the fecal microbiota of healthy, septic, and SNS foal was similar (AMOVA, $P = .16$ and $P = .35$, respectively; Figure 2A,B). However, when SNS and septic foals were grouped as sick foals, the bacterial membership (Jaccard index; AMOVA, $P = .06$) but not in the structure (Yue & Clayton index; AMOVA, $P = .33$), was different between groups. Visualization of Jaccard PCoA plot showed a separate clustering of samples from sick and healthy foals (Figure 2C).

3.4 | Relative abundance and LEfSe analysis

Thirty different phyla were detected, with Firmicutes (51.3%), Proteobacteria (35.7%), Bacteroidetes (5.4%), Fusobacteria (3.5%) and Actinobacteria (2.3%) accounting for more than 98% of total sequences. Seventy-three different classes, 126 orders, and 270 families were identified with 4 classes, 7 orders, and 15 families comprising for >84% of sequences at each taxonomic level. A total of 737 genera were identified with 70 of those present at relative abundance of >0.1%.

The relative abundances of the most abundant phyla, families and genera detected in healthy, septic and SNS foals are presented in Figure 3. At the phylum level, Verrucomicrobia relative abundance was higher in septic and SNS foals compared to the healthy ones ($P < .02$, for both comparisons). At the genus level, an unclassified genus from the Enterobacteriaceae family had the highest relative abundance in all groups but no differences among groups were identified (Figure 3C). The relative abundance of Enterococcus was higher in healthy than SNS foals ($P < .02$), but similar to the septic foal group. The relative abundance of an unclassified genus of the family Pasteurellaceae was higher in septic foals than healthy and SNS foals ($P < .03$, for both comparisons), while Lactobacillus abundance was higher in SNS and septic foals than the healthy counterparts ($P < .03$). Overall, the fecal relative abundance of the genera detected in blood culture of individual septic foals was not different than that identified in healthy foals. However, the relative abundance of *Aerococcus* in the feces of a bacteremic foal positive for this bacterium was higher compared to the healthy foals (Figure 4).

The LEfSe analysis (LDA > 2, $P < .05$) detected an enrichment in *Enterococcus* (Firmicutes) and Pasteurellaceae (Proteobacteria) in healthy foals. *Lactobacillus* (Firmicutes), *Facklamia* (Firmicutes) and unclassified genus of the family Sphingobactarieaceae (Bacteroidetes) were enriched in septic foals. In SNS foals *Akkermansia* (Verrucomicrobia), *Phascolarctobacterium* (Firmicutes), *Porphyromonas* (Bacteroidetes) and unclassified genus of the family Porphyromonadaceae (Bacteroidetes) were enriched (Figure 5). LEfSe analysis after grouping septic and SNS foals as sick foals identified an unclassified genus of the family Lachnospiraceae (Firmicutes) to be enriched in healthy foals while *Akermancia*, *Lactobacillus*, Phascolarcbacterium, *Atopostipes* (Firmicutes), and unclassified genera of the phylum Verrucomicrobia and Bacteroidetes to be enriched in sick foals.

3.5 | Metacommunities

The DMM model grouped all samples into 1 meta-community or enterotype (minimum Laplace value is for a *K* value of 1).

4 | DISCUSSION

In our study, both Gram-positive (ie, *Streptococcus*, *Enterococcus*, *Staphylococcus*) and Gram-negative (*Escherichia coli*, *Psychrobacter*

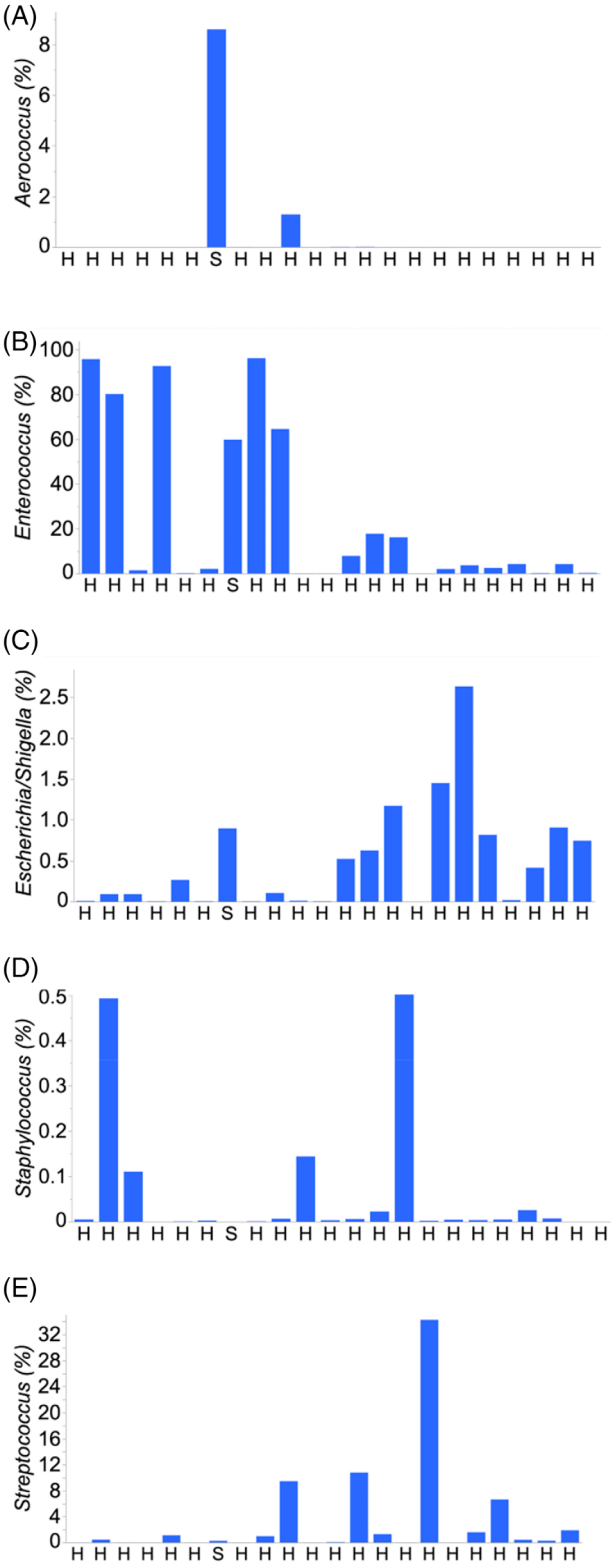

FIGURE 4 Relative abundance of the genera detected in blood culture of 5 septic (S) foals compared with healthy counterparts (H, n = 21)

spp., *Aerococcus* spp.) bacteria were isolated from blood cultures from septic foals. These findings agree with several studies documenting that Enterobacteriaceae (eg, *Escherichia coli*, *Klebsiella*

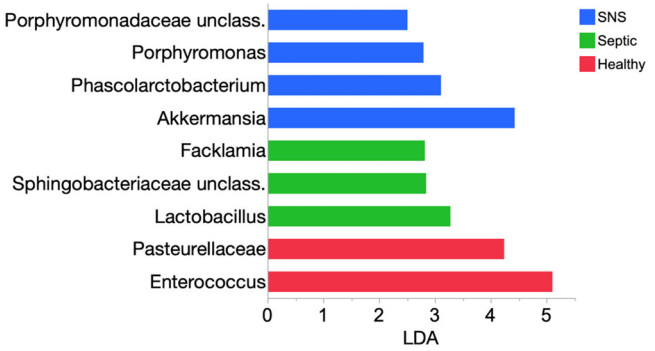

FIGURE 5 Plot from linear discriminant analysis effect size (LEfSe) indicating enriched bacterial genera in fecal samples of healthy (n = 21), sick nonseptic (SNS, n = 9) and septic (n = 8) foals. Cut-off of the linear discriminant analysis (LDA) > 2

spp., *Salmonella* spp., *Enterobacter* spp.), Pasteurellaceae (eg, *Actinobacillus* spp.) and *Pseudomonas* spp., and, are the most frequent Gram-negative bacteria isolated from bacteremic foals, while *Streptococcus* spp., *Enterococcus* spp., and *Staphylococcus* spp. are the most common Gram-positive bacteria.[2,3,5,35,36] Although umbilical infections have historically been considered a common source of sepsis in foals,[6] the hypothesis that the GIT microbiota could represent a source of sepsis in foals has been discussed by clinicians for many years. This hypothesis has been supported by reports indicating that Gram-negative enteric bacteria are the most frequent bacteria isolated from bacteremic foals.[2,3,5,35,36] In our study Enterobacteriaceae, *Enterococcus*, and *Streptococcus* were among the 5 more abundant taxa identified in both healthy and septic foals. In addition, *Pseudomonas* spp., *Enterobacter* spp., and *Actinobacillus* were among the 30 highly abundant taxa identified in feces of our foals. This is in agreement with a previous study showing that in healthy foal of 24 hour of age *Escherichia/Shigella*, *Streptococcus*, *Enterococcus* and *Klebsiella* are the most abundant genera in feces.[13] The presence of microorganisms causing bacteremia in foals in a high relative abundance in the GIT provides indirect evidence to suggest that translocation of bacteria from the intestine might play an essential role in the causation of sepsis in newborn foal. After birth, the foal's gastrointestinal intercellular permeability is high, allowing absorption of nutrients, proteins (eg, immunoglobulins), and leukocytes.[37-40] This permeability reduces substantially during the first 24 hours.[41] This can explain, at least in part, the increased risk of foals with failure of transfer of passive immunity to develop bacteremia and sepsis because of improper colostrum intake. Translocation of bacteria from the GIT into the systemic circulation can also be increased in newborn and premature foals with an immature enteric cell network[42,43] or foals exposed to hypoxic events during parturition[44] as the permeability of the gut in these foals appears to remain high for longer period of time. Foals with gastrointestinal inflammation are also at high risk of bacterial translocation from the GIT.[5,45,46]

In the present study, significant differences in alpha, but not beta-diversity were identified in the fecal bacterial microbiota of septic foals compared with their healthy counterparts. Specifically, a greater richness, evenness, and diversity was present in septic foals compared to healthy ones. These results differed from the current knowledge that a high diversity is associated with healthy GIT in different species[26,47-50] while a lower diversity is associated with late-onset sepsis in human neonates.[19-22] An explanation for high diversity in sick foals could be that healthy foals had a more stable colonic microbiota than septic and SNS foals. This could lead to a higher detection of bacteria from the upper GIT in the feces of septic and SNS foals which are usually obscured by colonic bacteria.[51] This hypothesis can be supported by the differences in bacterial membership (Jaccard index) between healthy and sick foals (ie, SNS and septic foals) because those differences result from the presence/absence of low abundant or rare taxa in each group.[32] The unstable colonic microbiota of sick foals might be related to the lack or reduced consumption of colostrum as demonstrated by a lower serum IgG concentration compared to healthy foals. Colostrum and its immune components play an important role in the development and establishment of the gastrointestinal microbiota in calves.[52-54] Therefore, it is possible that the lack of colostrum consumption in septic and SNS foals could have delayed the establishment and colonization of the colonic microbiota allowing the detection of upper GIT microbiota or transient bacteria in the fecal samples. This ultimately led to increased richness and diversity, and differences in bacterial membership. Another explanation for the higher diversity in SNS and septic foals is that they were exposed to a higher load of environmental organisms, which might increase the richness, and therefore the diversity of the fecal microbiota. The mare's skin and the respiratory, reproductive, and gastrointestinal systems along with those present in the soil, bedding, feed and feces are sources for bacteria transiting and colonizing the gastrointestinal system of the newborn foal.[6] Thus, it is possible that an exposure to a high microbial load by foals with inadequate colostrum intake result in a higher diversity and predispose foals to sepsis. In fact, poor sanitary conditions and highly contaminated environments are considered a risk factor for the development of neonatal sepsis in foals as the gastrointestinal tract is the major port of entrance for bacteria causing sepsis.[6,55]

Limitations to the study exist because of the nature of working with client-owned animals in a clinical setting. First, The small number of foals and inclusion of animals with different diseases in the SNS and sepsis group with known interindividual variability of the gastrointestinal microbiota could increase the type II error and decreased the changes of identifying differences between groups.[56,57] The paired septic, SNS and heathy foals were housed in dissimilar environmental conditions, and they were not matched based upon signalment or age. These factors have the potential to shape the fecal microbiota beyond expected interindividual variation. Additionally, foals were enrolled in the study with fecal samples collected in varying states of disease (eg, sepsis, multiple organ dysfunction) and with different diseases. This may represent a confounding variable, as the fecal microbiota could potentially vary based upon disease state. Finally, microbiota studies and in essence hypothesis generating rather than hypothesis driven studies and therefore our results are descriptive and need to be interpret taking it into consideration.[58] Despite of this limitation, our study

indicates that the fecal microbiota of healthy and sick foals differs significantly likely associated with effects of colostrum intake and sepsis itself on the gastrointestinal microbiota.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Approve by Iowa State University IACUC, 18-373.

HUMAN ETHICS APPROVAL DECLARATION

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

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