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Equine Hematology

Equine Blood Microbiome in a Cohort of Clinically Healthy Trail Riding Horses

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ABSTRACT

Background: Emerging research suggests the presence of a blood microbiome in clinically healthy individuals. Characterizing bacterial composition and abundance in blood from a group of healthy horses is of clinical interest.

Hypothesis/Objectives: Horses in a closed herd environment will have blood microbiomes with similarities among individuals.

Animals: Twenty trail-riding horses of different breeds and ages living in relative isolation on a dry lot pasture in Colorado at 7680 ft elevation.

Methods: Cross-sectional study. Blood was collected from the jugular vein into serum, blood collection, and EDTA tubes. Samples were submitted to external laboratories for microbiome analysis and routine blood tests (CBC, serum biochemistry).

Results: Venous blood is not sterile in healthy horses. A total of 293 bacterial genera were identified in these samples, whereas most horses had 55 to 70 genera. The most dominant taxa were *Gardnerella*, *Sporomusaceae*, *Kapabacteriales*, *Beijerinckiaceae*, *and Phascolarctobacterium*. Principal coordinate analysis, investigating microbial structure diversity, identified large variability with no obvious clustering, indicating dissimilarity among bacterial populations in different horses. All blood samples contained genera with pathogenic potential for horses, such as *Bacteroides* spp., *Clostridium* spp., *Peptostreptococcus* spp., *Streptococcus* spp., and *Staphylococcus* spp.

Conclusions and Clinical Importance: Clinically healthy horses had a diverse blood microbiome. Despite the relative isolation of the horses, their blood microbiota varied markedly among individuals. Investigating the bacteria in clinically healthy horse blood provides new insight into possible microbiome shifts that may result in clinical disease.

1 | Introduction

Blood conventionally has been considered free from microbes in the absence of systemic infection. Recent research however has challenged this view. In recent studies investigating the microbiome of blood in humans, resident microbiota were found in clinically healthy individuals [1, 2]. Within the studied blood samples from humans, diverse communities of bacteria were present, which suggests a possible core blood microbiome [1]. This data also prompted microbiota research in the veterinary field with a focus on blood from dogs, cats, and cows. The examined blood of healthy individuals from these species also suggests the presence of resident bacterial populations [3–5].

Abbreviations: CBC, complete blood count; DNA/RNA, deoxyribonucleic acid/ribonucleic acid; EDTA, ethylenediaminetetraacetic acid; IACUC, Institutional Animal Care and Use Committee; MRSA, methicillin-resistant *Staphylococcus aureus*; NGS, next-generation sequencing.

The gut microbiome of horses has been extensively investigated because of the complex nature of the gastrointestinal fermentation system in horses, with the presence of fungi, parasites, protozoa, archaea, viruses, and bacteria combined with inherent susceptibility to dysbiosis [6]. Previous studies also have described the effects of dental diseases and procedures in horses on bacteremia changes [7]. However, relatively little is known about the microbial populations that may be found in the blood of healthy horses that are apparently free of infectious disease. This lack of knowledge highlights the need for assessment and characterization of bacteria in the horse blood microbiome. Establishing a baseline of the abundance and diversity of bacteria could provide insight into possible microbiome shifts that result in disease or promote gastrointestinal as well as systemic health. We aimed to characterize bacterial composition and abundance in blood from clinically healthy, and relatively isolated, trail-riding horses using 16S next-generation sequencing (NGS). We hypothesized that horses living in a relatively isolated herd would have similar blood microbiomes.

2 | Materials and Methods

2.1 | Sample Collection and Standard Blood Analysis

A cohort of clinically healthy hack horses utilized for light trail rides ($n = 20$) was investigated in a cross-sectional study. The horses were geldings ($n = 7$) and mares ($n = 13$), estimated to be between 3 and 25 years of age, and mixed breeds (quarter horse crosses). They were kept on a dry lot pasture with an indoor stable in Colorado at 7680 ft elevation where every horse had resided for at least 2 years before the study. No new horses were introduced to the herd in over 2 years with little to no exposure to outside horses and herds. At the time of sample collection, their diet consisted of grass hay with alfalfa and grains, fed in shared round feeders with shared water troughs. The horses were deemed clinically healthy based on examination from afar, physical examination, and if the following variables were within the normal reference ranges for adult horses: temperature (99.5°–101.5° F), heart rate (28–40 beats per minute), and respiration rate (8–16 breaths per minutes) [8].

Blood samples were collected from members of the cohort in January 2023 in a stable where the ambient temperature was −4°C. Blood (10–12 mL) was collected from the jugular vein using a vacutainer system after the site was aseptically prepared (70% isopropyl alcohol). Serum, Zymo Research DNA/RNA Shield Blood Collection tubes (3 mL) and EDTA tubes were filled in this order, allowing the serum tube sample to serve as an initial withdrawal of blood to minimize potential contamination with skin microorganisms. Samples were packaged and shipped immediately to third-party laboratories. The serum and EDTA samples were sent to Zoetis for serum biochemistry profile and CBC. The blood collection tubes (DNA/RNA Shield Blood collection Tubes, Zymo Research, Irvine CA) were shipped to CosmosID (CosmosID Inc., Germantown, MD) for 16S amplicon

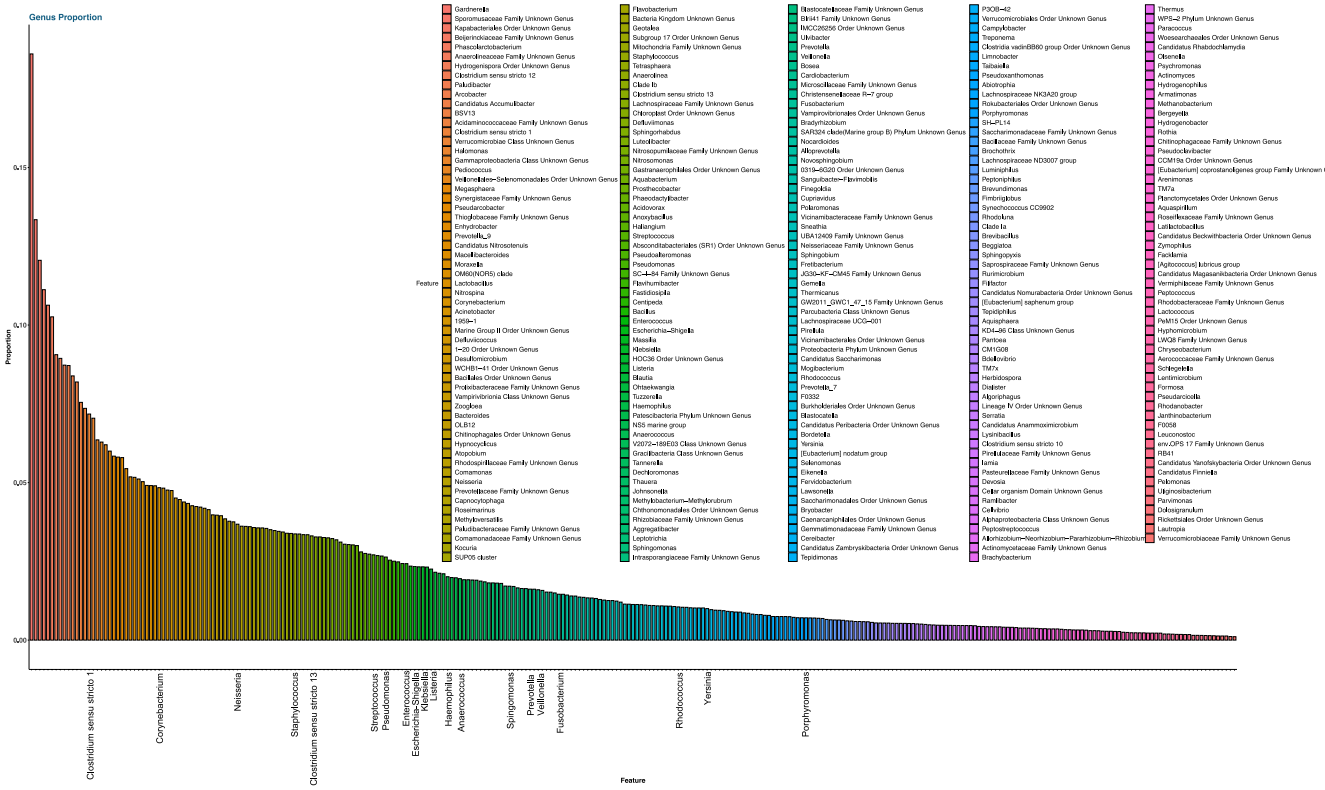


FIGURE 1 | Summary of bacterial richness and community composition. Rank abundance of bacterial genera across all horses expressed as the mean relative abundance of each genus. All bacterial genera are arranged in order of relative abundance from left to right across each column. Bacterial genera shown on the *x*-axis represent genera in the CosmosID database encountered in previous studies in horses considered as known or potential pathogens.

sequencing [9]. The blood collection tubes contained a buffer that allowed for storage at ambient temperature for at least 1 year.

2.2 | DNA Extraction and Analysis of Blood Microbiome via NGS

The following methods, as provided by CosmosID, were used for the study to sequence and analyze the DNA and graph the resulting microbiome data.

2.2.1 | DNA Extraction Methods

The DNA was extracted from 20 blood samples using the Qiamp DNA Blood Mini Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions.

2.2.2 | Library Preparation and Sequencing Methods

Isolated genomic DNA was quantified using an Equal bit 1× dsDNA HS Assay Kit (Vazyme Biotech Co., Nanjing, China). Fifty nanograms of isolated genomic DNA was amplified by PCR using proprietary primers (Admera Health LLC, South Plainfield, NJ) covering hypervariable regions V3 and V4. Primer selection and design were chosen to achieve comprehensive taxonomic coverage and elimination of spike-in to gain maximal data. Final library quantity was assessed using Qubit 2.0 (Thermo Fisher Scientific, Waltham, MA) and quality was assessed by TapeStation D1000 ScreenTape (Agilent Technologies Inc., Santa Clara, CA). Illumina 8-nt dual-indices were used. Equimolar pooling of libraries was performed based on quality control (QC) values and sequenced on an Illumina MiSeq (Illumina, San Diego, CA) with a read length configuration of 250 base pairs (bp) for 0.1 M paired-end reads per sample (500 K in each direction).

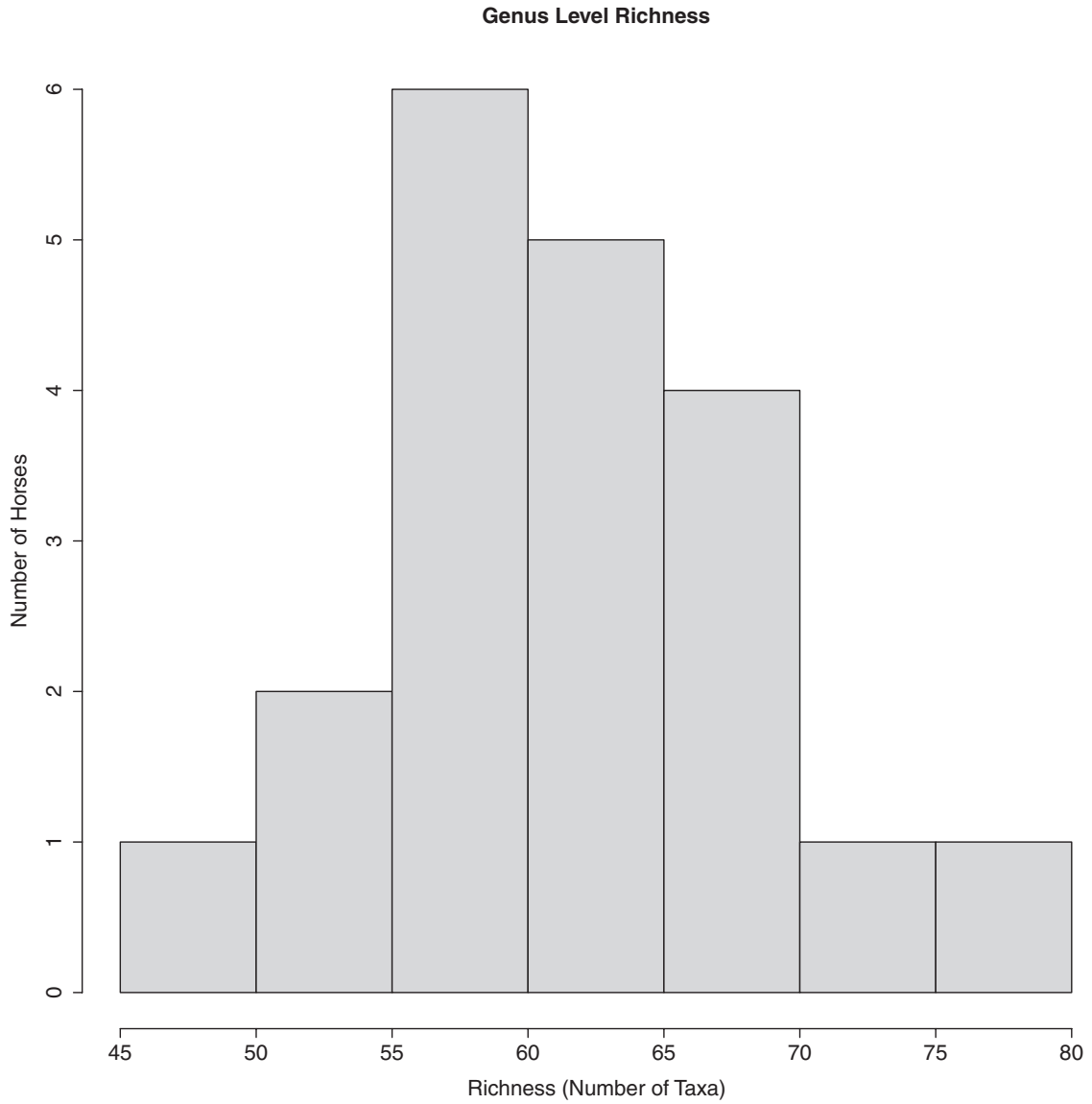
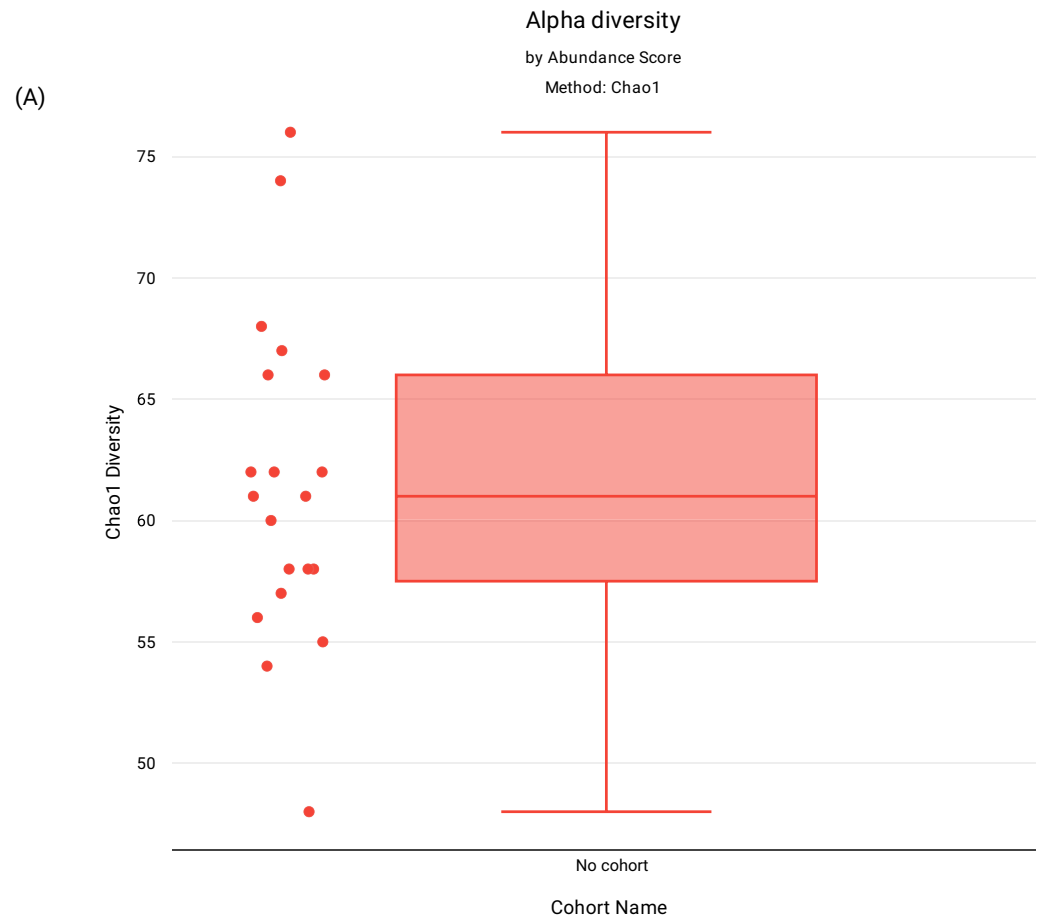


FIGURE 2 | Histogram of the number of bacterial genera identified per horse ($n = 20$).



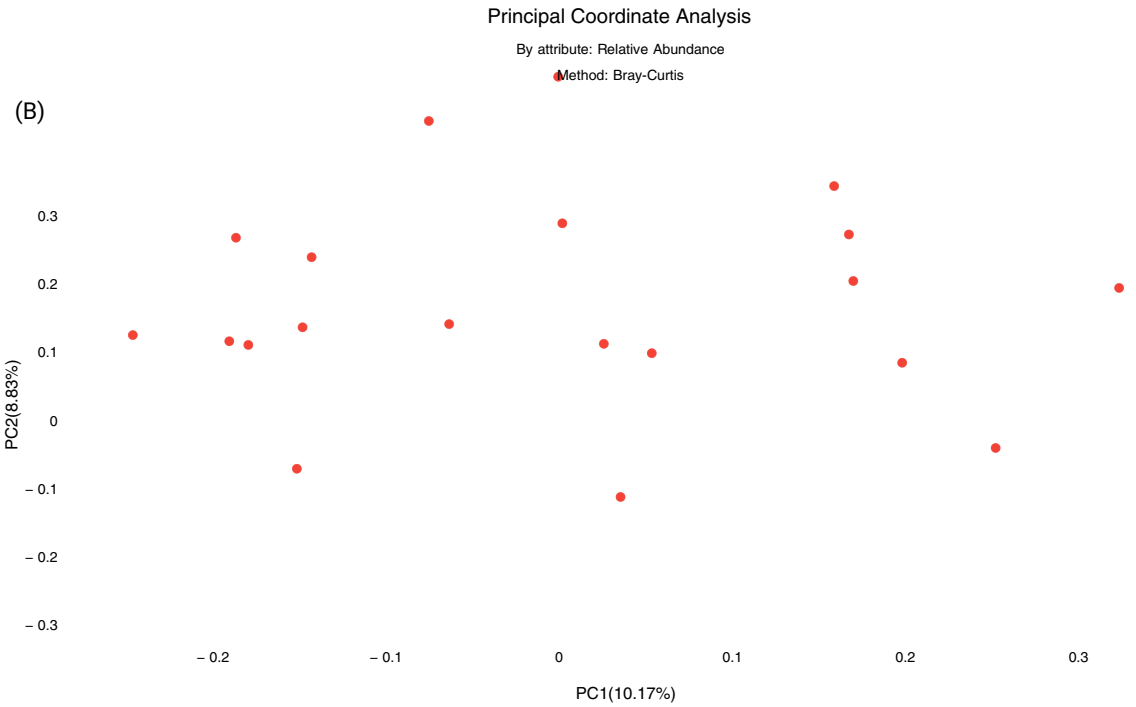


FIGURE 3 | Microbial abundance and diversity analyses of equine blood ($n = 20$). (A) Alpha diversity (by abundance score) shows microbial taxonomic diversity in individual blood samples. (B) Beta diversity plot (Principal Coordinate Analysis; Bray–Curtis dissimilarity) investigates microbial structure diversity, reflecting large variability of the data with no obvious clustering.

2.3 | Bioinformatics Analysis Using CosmosID-HUB

The CosmosID-HUB Microbiome's 16S workflow utilized the DADA2 algorithm [10] as its core engine and Nextflow ampliseq pipeline [11] definitions to run it on the company's cloud infrastructure. Briefly, primer removal was done using Cutadapt, and quality trimming parameters were passed to DADA2 to ensure that the median quality score over the length of the read exceeded a certain Phred score threshold [12]. Within DADA2, forward and reverse reads were each trimmed to a uniform length based on the quality of reads in the sample. Higher quality data generally resulted in longer reads. DADA2 used machine learning with a parametric error model to learn the error rates for the forward and reverse reads, based on the premise that correct sequences should be more common than any one error-variant. DADA2 then applied its core sample inference algorithm to the filtered and trimmed data, applying these learned error models. Paired-end reads then were merged if they had at least 12 bases of overlap and were identical across the entire overlap. The resulting table of sequences and observed frequencies were filtered to remove chimeric sequences (those that exactly match a combination of more prevalent parent sequences). Taxonomy and species-level identification (where possible) were conducted with DADA2's naive Bayesian classifier, using the Silva version 138 database.

2.4 | Custom Figure Creation and Pathogen Identification

All custom figures were created by CosmosID using R (R Core Team, 2013). Histograms were made using base R functions.

FIGURE 4 | Relative abundance bubble map of bacterial species in a single horse. The individual (blood sample from horse 3) had the highest blood microbiome diversity with 76 species detected. Circular diameter represents the abundance (%) of individual bacterial species.

Barplots were made using ggplot2 [13]. Species richness was calculated using the package vegan [14]. Horse-specific pathogens were identified using the Equine Disease Communication Database along with literature sources [6, 15–23].

3 | Results

3.1 | Evaluation of Health Status Using Conventional Blood Testing

The clinically healthy status of enrolled horses was confirmed based on physical examination, CBC, and serum biochemistry results. No clinically relevant abnormalities were identified (Table S1).

3.2 | Microbial Analysis of Equine Blood

Blood samples from this cohort of clinically healthy horses ($n = 20$) were not sterile. A total of 293 bacterial genera were identified across the sample set (Figure 1). Most horses had between 55 and 70 bacterial genera (no horse had 0), as shown in Figure 2. The top five most dominant bacterial taxa were *Gardnerella* (genus), *Sporomusaceae* (family), *Kapabacteriales* (order), *Beijerinckiaceae* (family), and *Phascolarctobacterium* (genus) as shown in Figure 1.

Alpha diversity by abundance score (Figure 3A) showed microbial taxonomic diversity in individual blood samples with a median of 61 bacterial species. A beta diversity plot (Figure 3B), using principal coordinate analysis based on Bray–Curtis dissimilarity to investigate microbial structure diversity, identified substantial variability in the data with no obvious clustering, indicating dissimilarity between the bacterial populations in blood from different horses.

Figure 4 shows a relative abundance bubble map from the horse with the highest number of bacterial species in its blood. This individual level map shows *Bacteroides acidifaciens* (species), *Gammaproteobacteria* (class), *and Luteolibacter* (genus) as the three bacterial taxa with the third highest relative abundance. Potential pathogens were detected in 1 to 20 horses per genera, with *Corynebacterium*, *Moraxella*, *Neisseria, Staphylococcus*, and *Streptococcus* present in all samples (Table 1), which align with the heat map of microbial taxonomy and abundance across individual samples, as shown in Figure 5. All blood samples contained bacterial genera with pathogenic potential for horses.

4 | Discussion

We determined that the blood of clinically healthy horses from a herd living in relative isolation contained diverse microbiomes. Considering the relative isolation of the herd, the bacterial diversity was surprisingly heterogeneous. The dissimilarity among bacterial populations in the blood microbiome of different horses was contrary to the study hypothesis. Given the proximity and shared resources, the diversity shown within individuals and across the herd overall is of interest, while also considering the low-stress environment, relatively low-impact job, and ample access to resources. The fact that all blood samples contained potential pathogens prompts the question of what the disease thresholds are for the presence and prevalence of different bacteria, especially those known to be disease-causing.

Blood samples included several commonly identified and clinically relevant bacterial species, such as *Bacteroides* spp. [20], *Clostridium* spp. [24], *Peptostreptococcus* spp. [16], *Streptococcus* spp. [25], and *Staphylococcus* spp. [26]. *Bacteroides* spp., for example, is an important pathogen in horses and comprises an estimated 8% of anaerobic bacterial infections. Horses infected with *Bacteroides* spp. typically have paraoral and lower respiratory tract infections, causing pneumonia and pleuropneumonia [20]. *Peptostreptococcus* spp. is a pathogen that has been isolated from both normal pharyngeal tonsilar surfaces and from horses with tooth abscesses. It has been identified in chronic infections of the upper respiratory tract, ears, sinuses, and teeth, as well as the female reproductive system, intraabdominal infections, and

TABLE 1 | Bacterial genera identified in the blood that might contain opportunistic pathogenic species.

Genus	Number of horses
Corynebacterium	20
Moraxella	20
Neisseria	20
Staphylococcus	20
Streptococcus	20
Fusobacterium	19
Acinetobacter	18
Actinomyces	17
Prevotella	16
Lawsonella	15
Pseudomonas	14
Campylobacter	10
Bacteroides	7
Peptostreptococcus	3
Stenotrophomonas	3
Clostridium_sensu_stricto_12	2
Escherichia-Shigella	2
Rhodococcus	2
Bordetella	1
Clostridium_sensu_stricto_1	1
Clostridium_sensu_stricto_10	1
Clostridium_sensu_stricto_13	1
Enterococcus	1
Mycobacterium	1

soft tissue abscesses [16]. Although some species of *Clostridium* are nonpathogenic, many are known to cause severe disease in horses and other mammals, including *C. tetani*, *C. botulinum*, *C. perfringens*, *C. piliforme*, and *C. novyi* type B [24]. *Streptococcus* spp. is another clinically relevant bacterial species in horses because of the species *S. equi*. The clinical implications of strangles and the necessity of tracking horses that are carriers of *S. equi* are of utmost importance [25]. We also identified *Staphylococcus* spp. Although it is a bacterial species commonly found on the skin of horses, there also is existing evidence of methicillin-resistant *Staphylococcus aureus* (MRSA) infections and outbreaks in horses and in veterinary clinic settings [26].

Our data also highlights an interesting species-spanning aspect because there are public health implications given the presence of bacterial species with zoonotic disease potential (e.g., MRSA) in seemingly healthy horses. Previous studies on blood microbiomes have focused on dogs, cats, cows, and humans, and this literature also supports the presence of bacteria in the blood of clinically healthy individuals [3–5]. Horses are unique as compared to other investigated species because they are hind-gut fermenters, rather than non-fermenters or fore-gut fermenters. Further investigation is indicated to help understand if being a hind-gut fermenter influences the blood microbiome and the clinical relevance this difference may have. There is ongoing discussion on the impact of bacterial diversity and abundance on the competition and commensalism of microorganisms in the body [27]. Here, we found that the most prevalent taxa (*Bacteroides acidifaciens*, *Gammaproteobacteria*, *and Luteolibacter*) in the horse with the highest number of bacterial species in the blood belonged to the commensal equine gut microbiome.

The horses examined in our study were clinically free from disease, despite having diverse and abundant bacterial species present in their blood. Establishing a reference on the microbial abundance and diversity in blood from clinically healthy horses contributes new insight into possible microbiome shifts that could serve as new biomarkers to predict clinical disease. Data on the blood microbiome using 16S NGS may help clinicians in their decision-making process by offering information beyond conventional culture and sensitivity analyses [28]. This is especially important when considering that many bacterial species

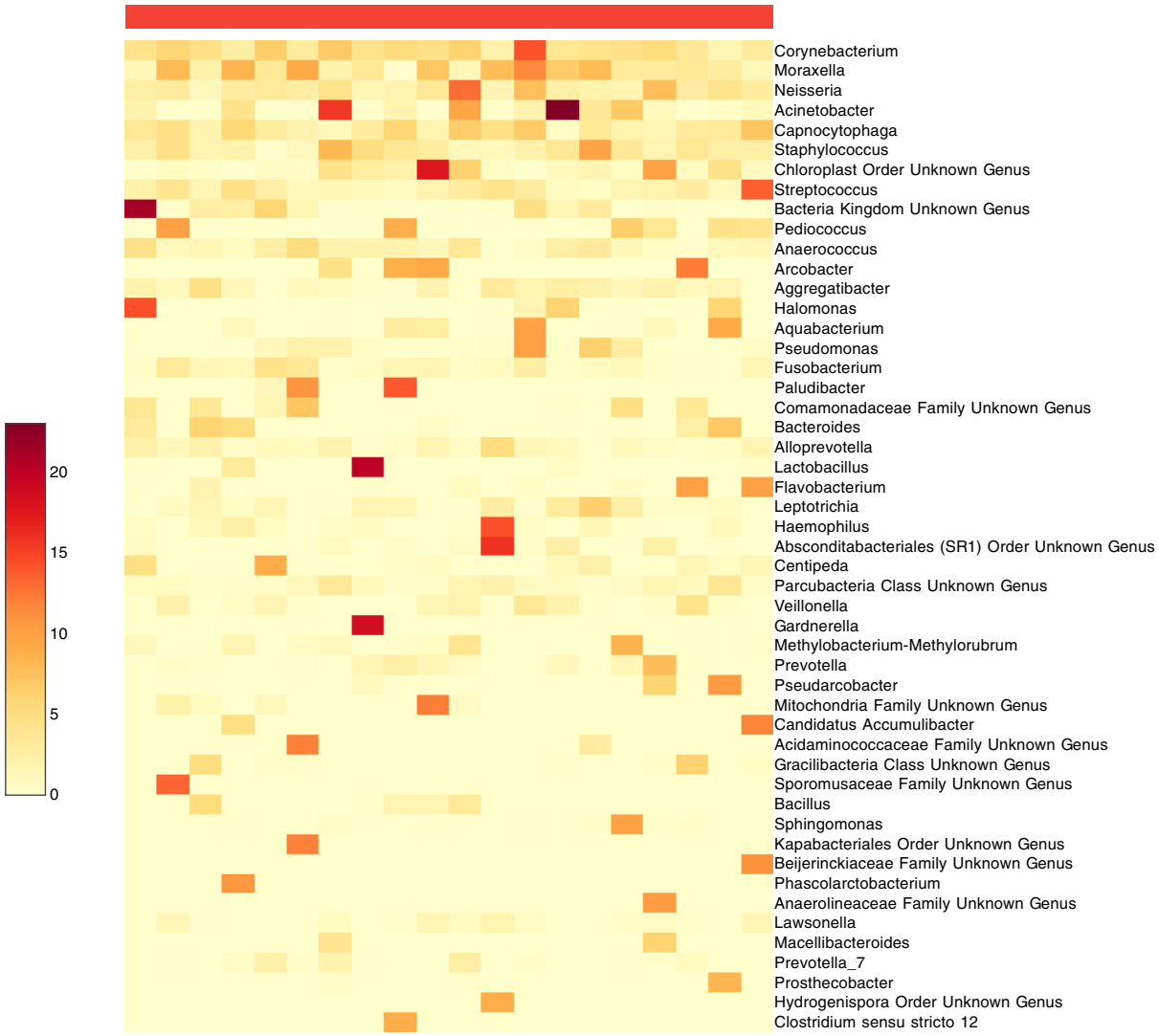


FIGURE 5 | Heat map of microbial taxonomic abundance and composition in horse blood. The columns represent individual horses (H) from a single cohort consisting of 20 horses. Some bacterial genera were detected in most samples; however, the map reflects large variability between blood samples.

cannot be cultured. Using NGS in conjunction with bacterial cultures in horses that display signs of illness may help identify the true causative bacteria and thus the appropriate antimicrobials for targeted treatment.

Our study had some limitations. The cohort of subjects is one group in a secluded high-altitude location in Colorado. It would be beneficial and informative to investigate horses from other locations and with other stress levels, such as racing horses. Other variables to consider are the extent of the variety of breeds (mixed versus purebred) and the ages represented in this cohort. These factors may have as much, if not more, influence on bacterial populations than the surrounding environment. Other potential limitations include possible contamination during sample collection or processing. Notably, there were no clinically relevant abnormalities in neutrophil numbers on the CBCs performed when establishing the health status of the horses. When interpreting NGS data, it is important to note that the identification of bacterial DNA does not necessarily correspond with live bacteria at the time of examination and sampling. However, this information gap may be partially complemented by also performing a culture, while acknowledging the inherent limitation of this methodology.

Our study had two important conclusions. First, unlike other species studied, horses in a closed environment with a shared diet maintain microbiome diversity. Second, many pathogenic organisms seemingly lie dormant within the host. The organisms may await conditions that favor their proliferation, such as stress, transport, and high performance. Previously, it was believed these organisms were present in the environment alone, but our data indicate that some reside in the systemic circulation of horses. Two horses (H12 and H16) exhibited mildly increased neutrophil counts, but their microbiomes did not differ substantially from those of other horses in terms of pathogens. Almost 2 years after the original sample collection, the herd remains clinically healthy.

Overall, our results support the presence of a consistent core microbiome endogenous to equine blood. However, the number of individuals included in our study does not exclude the possibility that the equine blood microbiome also might be the result of transient and sporadic translocation of commensal microbes from other body sites into the bloodstream.

Disclosure

Authors declare no off-label use of antimicrobials.

Ethics Statement

Approved by the Western University of Health Sciences Institutional Animal Care and Use Committee (ref. # R21IACUC036). Authors declare human ethics approval was not needed.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

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