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Nasopharyngeal bacterial and fungal microbiota in normal horses and horses with nasopharyngeal cicatrix syndrome

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Abstract

Background: The nasopharyngeal bacterial and fungal microbiota of normal horses and those with nasopharyngeal cicatrix syndrome (NCS) are unknown.

Hypotheses/Objectives: To describe the microbiota from nasopharyngeal washes of healthy horses and of horses acutely affected with NCS.

Animals: Twenty-six horses acutely affected with NCS horses and 14 unaffected horses.

Methods: Prospective, observational cohort study. Horses were recruited by investigators through personal communications in central Texas. Bacterial (16s RNA) and fungal (internal transcribed spacer) microbiota from nasopharyngeal washes were evaluated. Polymerase chain reaction for detection of *Pythium insidiosum* was performed.

Results: Results indicated that 6 fungal genera (*Alternaria*, *Bipolaris*, *Microascus*, *Spegazzinia*, *Paraconiothyrium*, *Claviceps*) and 1 bacterial genera (*Staphylococcus*) were significantly different between affected and unaffected horses. The fungal genus *Bipolaris* had increased abundance in NCS affected horses and on NCS affected farms. *Pythium insidiosum* was absent in the nasopharyngeal wash of all horses, irrespective of health status.

Conclusion and Clinical Importance: Significant differences were identified in the fungal microbiota in horses affected with NCS and farms affected with NCS compared to those unaffected. Therefore, *Bipolaris* warrants further investigation.

KEYWORDS
equine, pharyngeal scar, pythium, Texas

1 | INTRODUCTION

Equine nasopharyngeal cicatrix syndrome (NCS) is a condition of horses, characterized by catarrhal inflammation and airway obstruction affecting the proximal trachea, arytenoids, larynx, nasopharynx, or nasal passages.[1] Horses present with acute inflammation and variable clinical signs, which often progresses to a circular web-like

Abbreviations: ASVs, amplicon sequence variants; ITS, internal transcribed spacer; NCS, nasopharyngeal cicatrix syndrome; PCoA, principal coordinate analysis; PCR, polymerase chain reaction.

scarring in chronic stages of disease causing life-threatening respiratory distress.[1] This condition is prevalent in central and southeast Texas although the cause remains unknown.[2] Housing horses exclusively in a stall is protective against development of NCS and the duration of pasture turnout has a dose-related effect.[2] This association with pasture environment combined with the narrow geographical distribution of disease suggests an environmental cause.[2] Consequently, investigation of the upper respiratory tract of horses acutely affected with NCS compared to unaffected horses might help identify a potential cause for this condition. Importantly, given the geographic overlap of disease, 1 proposed etiologic agent of this disease is *Pythium insidiosum* and vaccination against it to prevent NSC is practiced without evidence of efficacy. Permanent tracheostomy and euthanasia are the only options for management of severe cases.[3]

The pharyngeal bacterial flora of healthy horses and horses affected by pharyngitis vary greatly in composition between horses[4-6] with differences in the nasal bacterial microbiota between healthy horses and those affected by equine herpes virus-1 (EHV-1).[7] This highlights the need for understanding how the upper respiratory microbiota changes in health and disease in horses. In people and in cattle, there is comprehensive understanding of the nasopharyngeal microbiota.[8-11] The bacterial microbiota in equine nasal passages and sinuses is largely uncultivable, aerobic bacteria.[12] Therefore, next generation sequencing including 16srDNA and internal transcribed spacer (ITS) region offers better characterization of the nasopharyngeal bacterial and fungal microbiota as compared with traditional culture approaches.[13,14] Variability in the microbiome of humans is affected by geographic location, population density, hormones, stress, environment, season, and diet affecting the richness of bacteria.[15-17] In the absence of knowledge of the causative agent for NCS, development of effective strategies to treat or prevent the disease will remain elusive. Thus, the first objective of this study is to compare the upper respiratory tract bacterial and fungal microbiota, including *Pythium insidiosum*, between horses affected with NCS and healthy horses. The second objective of this study is to describe the bacterial and fungal microbiota from nasopharyngeal washes of healthy horses.

2 | MATERIALS AND METHODS

2.1 | Study sample

Local horse operations in central Texas were recruited through personal communications with the investigators to participate in the study. Farms with horses demonstrating signs of acute NCS and farms within close proximity (10 miles) with healthy horses that provided written informed consent for participation were included. All horses with NCS from a given farm were included and a convenience sample of healthy controls were selected for inclusion. Data collected for each horse included signalment (age, breed, sex), clinical signs, stable/pasture management,

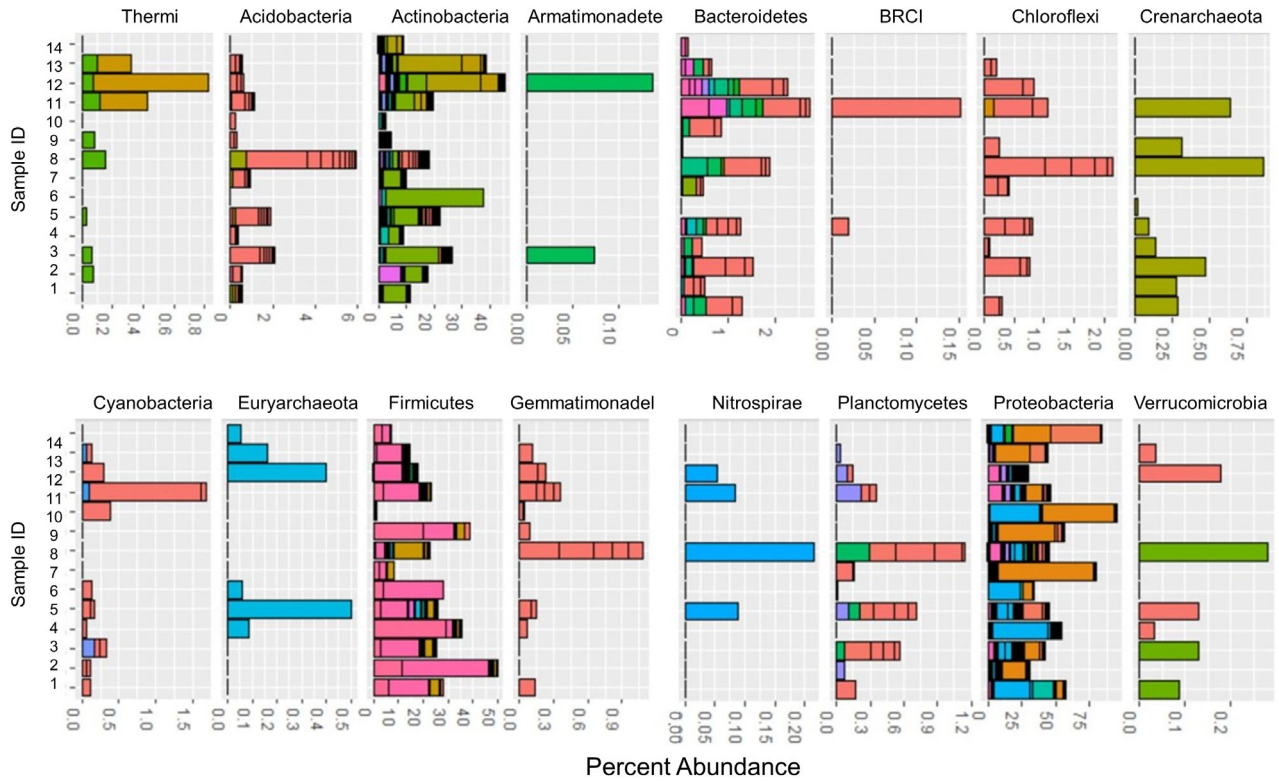

FIGURE 1 Bacterial phyla identified in 14 clinically healthy horses. Facet bar plots depicting the phylum of bacteria identified by 16s rRNA sequencing. Individual plots for each phylum are represented, with % abundance on the x-axis and each healthy horse (1-14) depicted on the y-axis. Color variety shows the diversity within the phylum. The phyla with the highest abundance include Proteobacteria, Firmicutes, Actinobacteria, Acidobacteria, and Bacteroidetes

dietary roughage, and endoscopic findings as determined by 2 veterinary observers (C.M. Whitfield-Cargile and M.C. Coleman), with clinical experience in evaluating horses with NCS. Cases of acute NCS were defined as horses having both clinical signs and endoscopic changes consistent with acute disease. Included horses with NCS had at least 1 of the following clinical signs at the time of examination: nasal discharge, cough, respiratory stridor, or respiratory distress. Additionally, endoscopic examination revealed nasal, pharyngeal, laryngeal, or proximal tracheal inflammation with diphtheritic membrane formation as described with NCS.[2] When available, apparently healthy horses on the same farm with a normal upper respiratory tract endoscopic examination were identified for inclusion as unaffected horses. All data were collected during a 2-week period of time in the month of April. Horses were excluded from the study if they had a reported history of NCS or endoscopic evidence of chronic NCS, including scar formation of the nasal, pharyngeal, or tracheal mucosa, deformation of the epiglottis, inflammation of the arytenoid cartilage, thickening of the vocal cords, scarring of the salpingopharyngeal openings, or rostral deviation of the palatopharyngeal arch. Healthy horses treated with antimicrobials within 30 days of evaluation, with a current illness, or transport within 2 weeks of evaluation were also excluded from the study. This study was approved by the Institutional Animal Care and Use Committee (IACUC-2016-0328) and the Clinical Research and Review Committee of Texas A&M University.

2.2 | Sample processing

A nasopharyngeal wash was performed on all horses by introducing an 8-french polypropylene nasal cannula (Argyle Suction Catheter, Covidien, Mansfield, Massachusetts) into 1 nostril and passing this catheter to the nasopharynx. Sixty milliliters of sterile saline was infused through the catheter and the wash collected out of the nostril into a sterile plastic container. No effort was made to clean the nose before collection to best characterize the microbial population of the nasopharynx in situ and not introduce changes by cleaning the nostril. The samples were placed immediately in dry ice and transported to −80°C on the day of collection.

2.3 | DNA isolation

DNA was extracted from nasopharyngeal wash samples using the QIAamp Fast DNA Stool Mini Kit (Qiagen) according to manufacturer's protocol, with slight modifications. Briefly, 2 to 4.5 mL of nasal wash sample was centrifuged at maximum speed for 5 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended in 0.5 mL Inhibitex buffer and 50 mg each of sterile/DNase free 0.1- and 0.5-mm silica zirconium beads. The sample was homogenized for 90 seconds

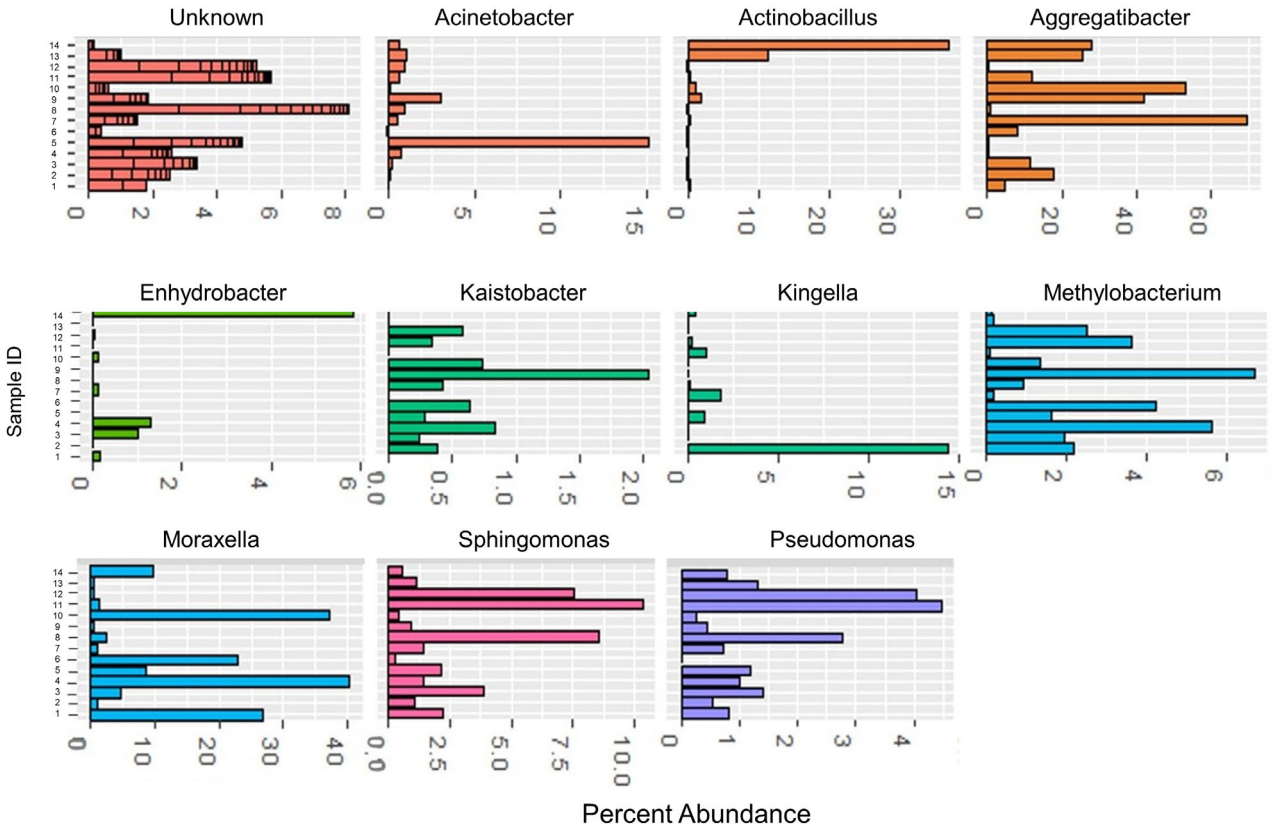

FIGURE 2 Bacterial genera with >2% abundance within phylum Proteobacteria in clinically healthy horses. Facet bar plots depicting the phylum Proteobacteria with each plot representing genera with greater than 2% abundance, with % abundance on the x-axis and each horse (1-14) represented on the y-axis

(2 cycles of 45 seconds) at 6.5 m/s using a FastPrep FP120 cell disrupter (Qbiogene, Carlsbad, California). The sample was then heated at 95°C for 5 minutes before DNA extraction as per manufacturer's protocol (isolation of DNA from feces for pathogen detection). Isolated DNA was quantified using an ND-1000 Nanodrop spectrophotometer and stored at −80°C until use. Contemporaneous negative controls were not collected for samples nor were negative controls introduced in the DNA isolation or sequencing steps. Potential contaminants were identified computationally postsequencing, described below.

2.4 | Sequencing

Amplification and sequencing of the V4 variable region 16S rRNA gene was commercially performed (www.mrdnalab.com, Shallowater, Texas).[18] Briefly, samples were barcoded and polymerase chain reaction (PCR) primers 515F/806R were used in a 28 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen) under the following conditions: 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds, and 72°C for 1 minute, with a final elongation step at 72°C for 5 minutes. A DNA library was prepared according to Illumina TruSeq DNA library preparation protocol. Sequencing was performed on a MiSeq (Illumina) following the manufacturer's guidelines. Similarly, ITS-1-2 region was amplified and sequenced.[19] Samples were barcoded and PCR primers were used under the conditions described above.

2.5 | Analysis of sequencing data

The software Quantitative Insights Into Microbial Ecology-2 (QIIME2; https://qiime2.org) (ver 2019.1) was used for sequence processing and analysis. The raw sequence data were de-multiplexed, and low-quality reads were filtered using database's default parameters. Sequences were then assigned to amplicon sequence variants (ASVs) using dada2 against the Greengenes database (ver. gg_13_5) filtered at 97% identity for 16S rRNA sequences and UNITE database (ver. 7.2) for ITS-1 sequences. Count tables with assigned taxonomy and phylogenetic trees constructed in QIIME2 were exported to R (ver. 3.6.2) where phyloseq (ver 1.24.2) was used for further analyses.[20] Contaminating ASVs, based on frequency compared with DNA concentration, were identified using R package decontam.[21] Rarefaction was not performed, consistent with recommendations of others and our previous work.[22] Principal coordinate analysis (PCoA) plots and bar charts of taxonomic assignments were made in phyloseq. The ASV count tables were collapsed to the genera level and the R package corncob (ver 0.1.0) was used to test for differential abundance of both bacterial and fungal genera between normal horses and horses

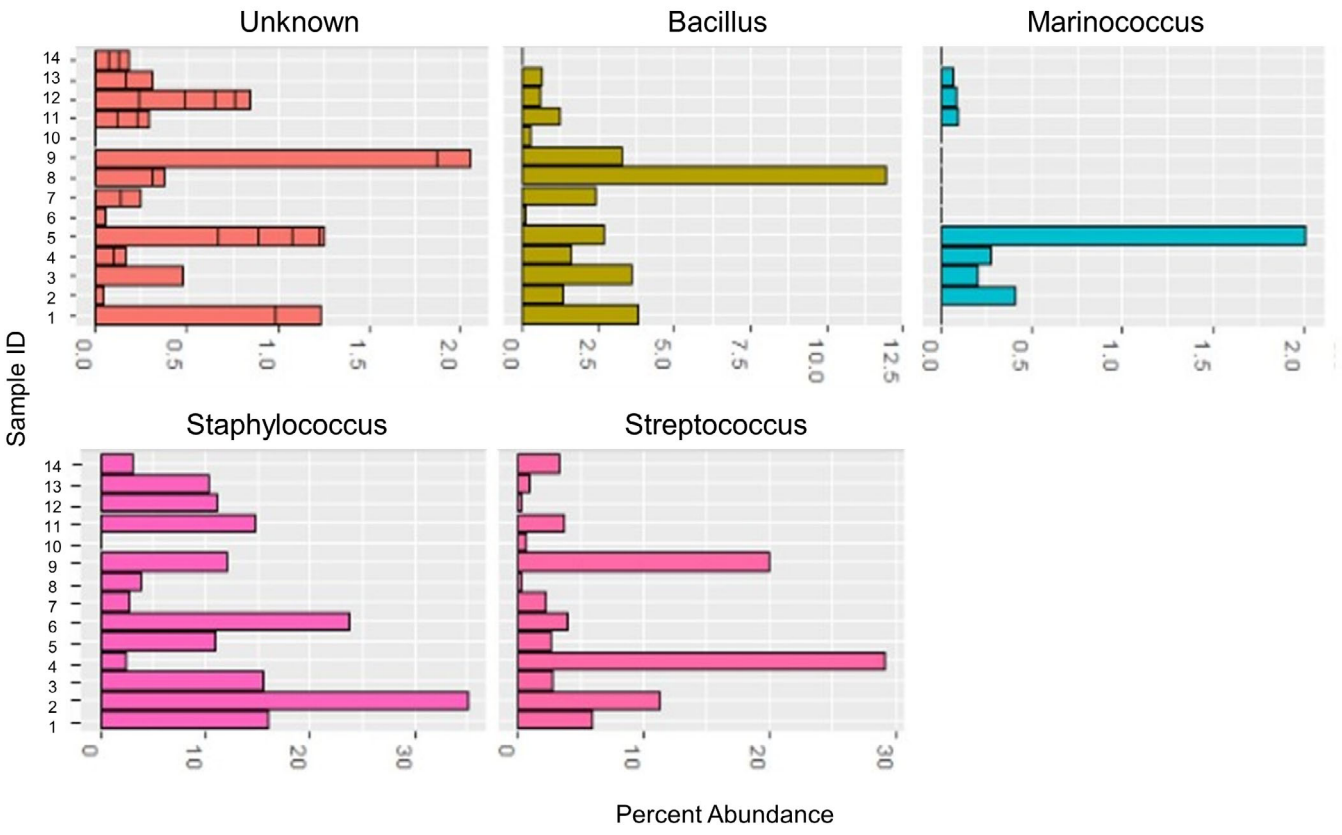

FIGURE 3 Bacterial genera with >2% abundance within phylum Firmicutes in clinically healthy horses. Facet bar plots depicting the phylum Firmicutes with each plot representing genera with greater than 2% abundance, with % abundance on the x-axis and each horse (1-14) represented on the y-axis

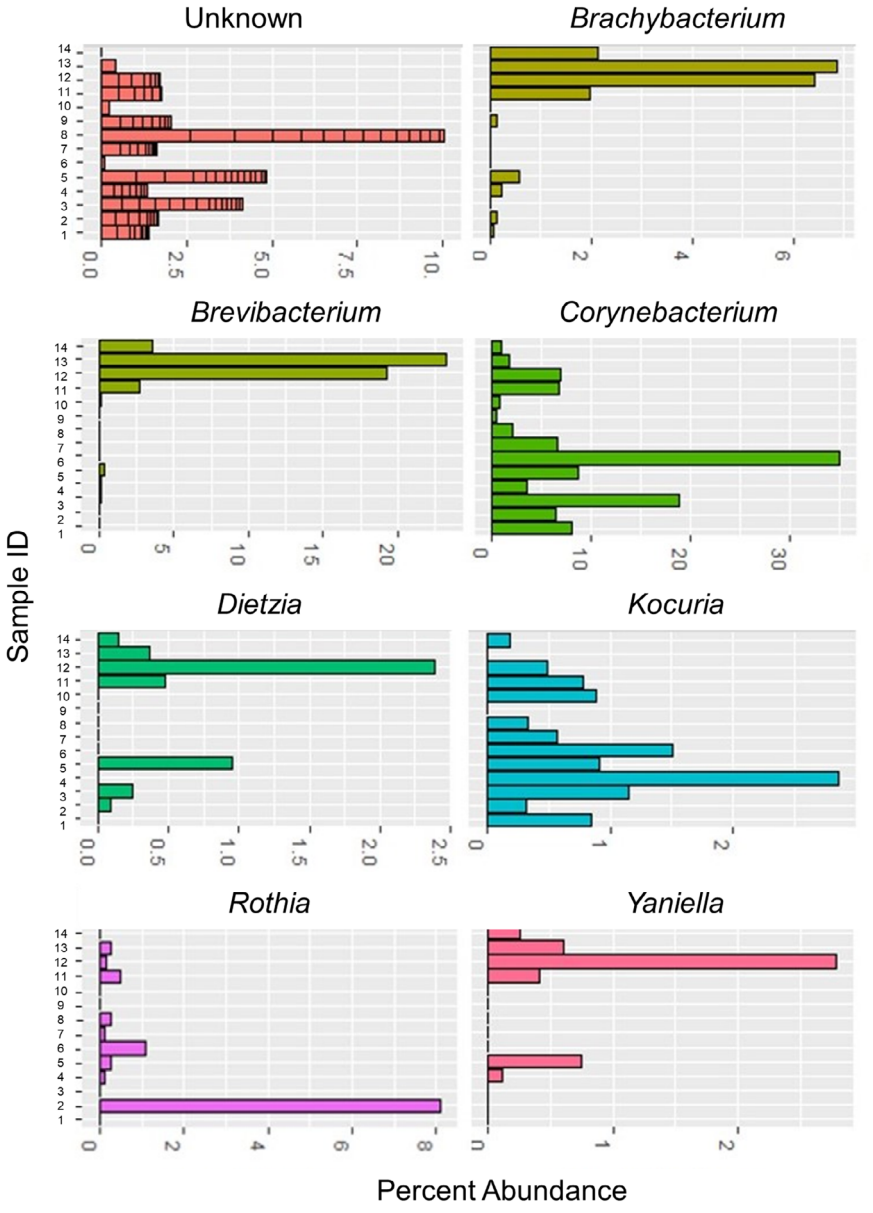

FIGURE 4 Bacterial genera with >2% abundance within phylum Actinobacteria in clinically healthy horses. Facet bar plots depicting the phylum Actinobacteria with each plot representing genera with greater than 2% abundance, with % abundance on the x-axis and each horse (1-14) represented on the y-axis

affected with NCS.[23] This package uses a beta-binomial regression model to determine differential abundance and Benjamini-Hochberg procedure for false discovery rate corrected *P* value.

2.6 | PCR assay for the detection of *Pythium insidiosum*

DNA from a positive control tissue sample was extracted using the DNeasy Blood & Tissue kit (Qiagen). Briefly, 25 mg of tissue from a cutaneous lesion, confirmed on culture and histopathology as positive for *Pythium*, was homogenized using a mortar and pestle. Negative controls (RNAse-free water) were used throughout this entire PCR process. Small particles were resuspended in ATL buffer and Proteinase K, and DNA was extracted as per manufacturer's protocol (purification of total DNA from animal tissues). Isolated DNA was quantified using an ND-1000 Nanodrop spectrophotometer and stored at −80°C until use.

2.7 | PCR from nasopharyngeal wash samples

PCR detection of *Pythium insidiosum* was carried out as previously described, with slight modifications.[24] Briefly, 30 ng of isolated nasal wash DNA or positive control were used as template in 20 μL amplification reactions containing 10 pmol of each primer, 200 μM of each dNTP, 2 μL of 10X PCR buffer, and 5 units of Taq DNA polymerase. Amplifications were carried out in a T-100 thermal cycler (BioRad) using the following conditions: initial denaturation at 94°C for 3 minutes, followed by 36 cycles of denaturation at 94°C for 45 seconds, annealing at 64°C for 30 seconds, extension at 72°C for 60 seconds, and a final extension at 72°C for 10 minutes. Amplification products resolved in a 2% agarose gel

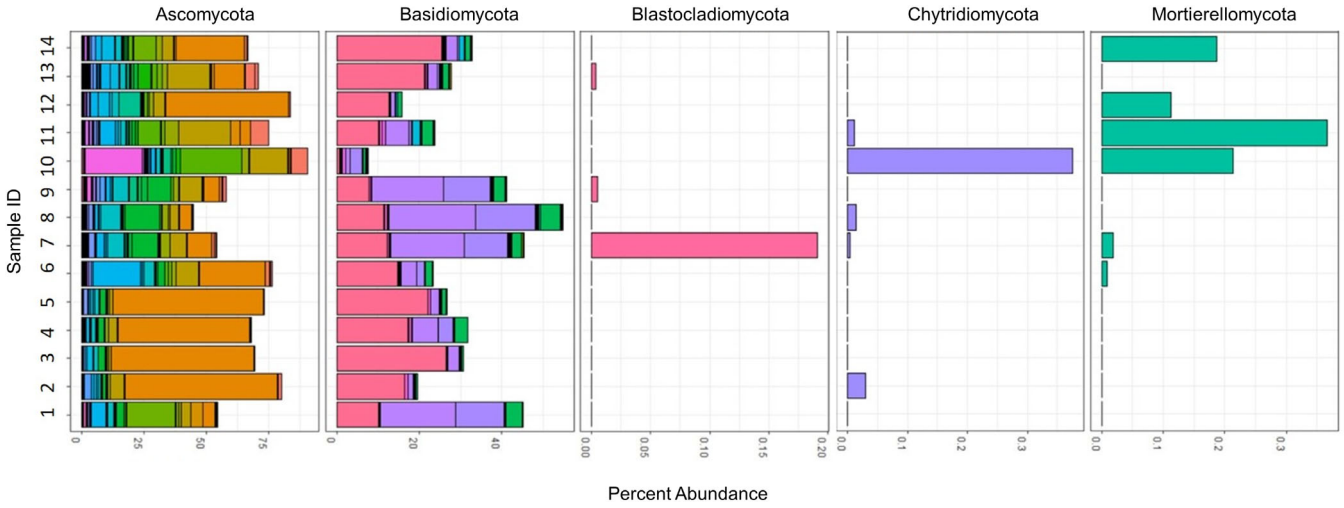

FIGURE 5 Phylum of fungus identified by ITS1 sequencing in clinically healthy horses. Facet bar plots depicting the phylum of fungus identified by ITS1 sequencing with greater than 1% average abundance, with % abundance on the x-axis and each horse (1-14) represented on the y-axis

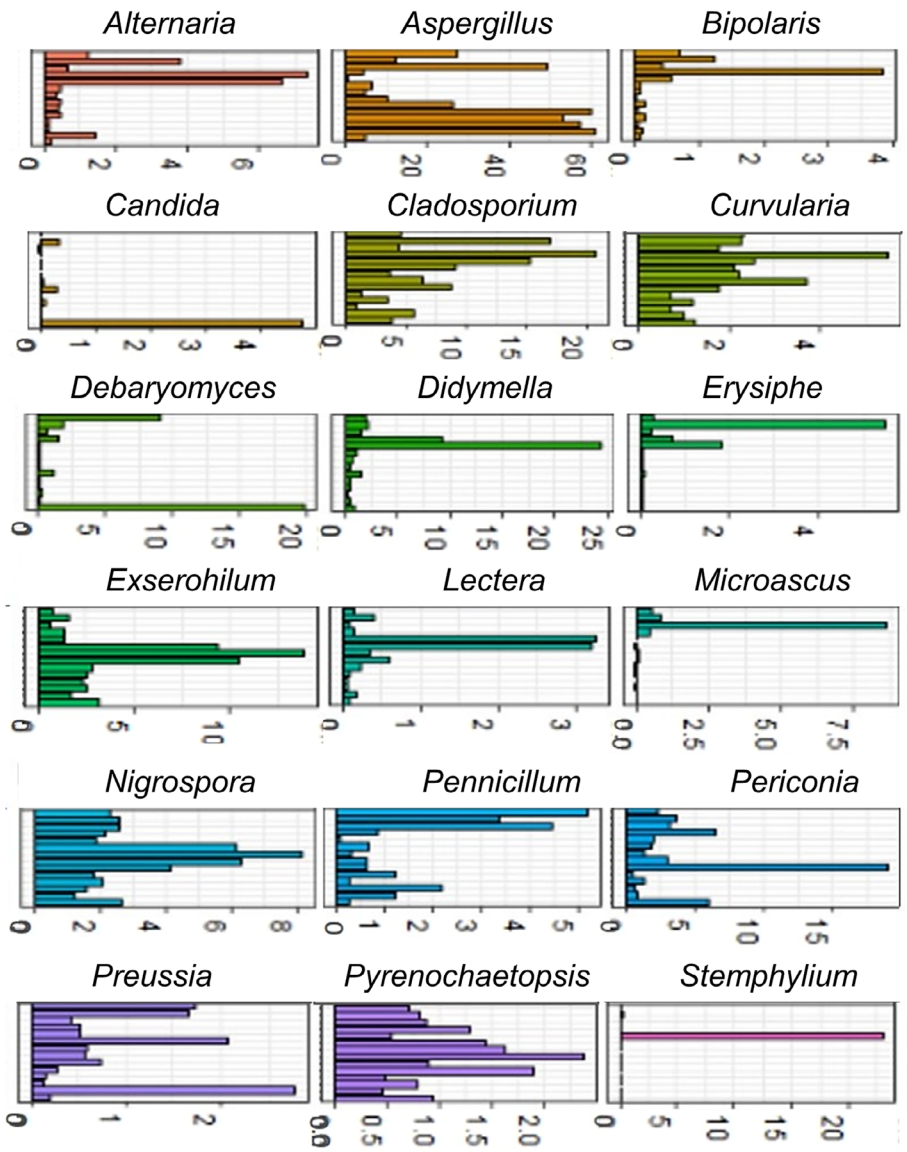

FIGURE 6 Fungal genera with >2% abundance within phylum Ascomycota in clinically healthy horses. Facet bar plots depicting the phylum Ascomycota with each plot representing genera with greater than 2% abundance, with % abundance on the x-axis and each horse (1-14) represented on the y-axis

and visualized using a ChemiDoc Touch Imaging System (BioRad) under ultraviolet light.

3 | RESULTS

3.1 | Animals

A total of 40 horses from 7 farms were included in the study: 26 horses with NCS from 6 farms and 14 healthy horses from 3 farms. Affected and unaffected horses from the same farm were identified in only 2 of the included farms, with 4 horses in each group at 1 farm and 1 horse in each group at the other farm. One farm with no affected horses was recruited for inclusion of controls. The mean age ± SD of horses with NCS was 15.6 ± 6 years (range, 4-29 years) and the mean age ± SD of healthy control horses was 13 ± 7 years (range, 1-26 years) ($P = .23$). Of the NCS horses, there were 17 geldings and 9 mares. Of the healthy horses, there were 11 geldings, 2 mares, and 1 stallion. Horses with NCS included Quarter Horses (n = 22) and Arabians (n = 4). Healthy horses included Warmbloods (n = 5), Quarter Horses (n = 3), Arabian (n = 1), Fjords (n = 2), Morgan (n = 1), Andalusian (n = 1), and Thoroughbred (n = 1).

The duration of time stalled or turned out to pasture was reported by owners. There was no significant difference ($P = 1.0$) in the number of hours spent at pasture between groups, with a mean of 15 ± 8 hours for affected horses and 15 ± 5 hours for healthy horses. Except for 1 horse in the healthy group, all horses were offered coastal Bermuda hay; information regarding concentrates in the diet was not recorded. Horses with NCS presented with the following clinical signs: nasal discharge (15), coughing (11), resting tachypnea defined as >24 breaths per minute (3), and exercise intolerance (1). Location of endoscopic lesions included the pharynx (24), nasal passages (16), epiglottis (13), larynx (8), arytenoids (10), and trachea (1).

3.2 | Bacterial microbiota of the nasopharynx of healthy horses

Count tables were filtered to contain only ASVs present >0.01% across all samples and further collapsed to the genera level. These data were then transformed to relative abundance to describe bacterial microbiota of the nasopharynx of the healthy horses. The bacterial phyla present in the nasopharynx are presented in Figure 1. The bacterial phyla that represented >2% of the bacterial population of the microbiota were then individually characterized at the genera level. The phyla meeting this criterion included Proteobacteria, Firmicutes, Actinobacteria, Acidobacteria, and Bacteroidetes. The remaining phyla were present in low abundance (<2%) and characterized primarily by 1 or 2 genera. Proteobacteria was the most prevalent phylum and contained 60 different genera with *Aggregatibacter*, *Moraxella*, *Methylobacterium*, *Pseudomonas*, and *Sphingomonas* being the most prevalent genera within this phylum (Figure 2). There were 29 genera represented in Firmicutes with *Staphylococcus*, *Streptococcus*, and *Bacillus* representing 18%, 10%, and 3.5% of the total bacterial microbiota, respectively (Figure 3). Finally, the Actinobacteria phylum contained 31 different genera with *Corynebacterium* being the most prevalent across all samples (Figure 4). *Brachybacterium* and *Brevibacterium* were variably represented, with high relative abundance in some samples. The genera making up the

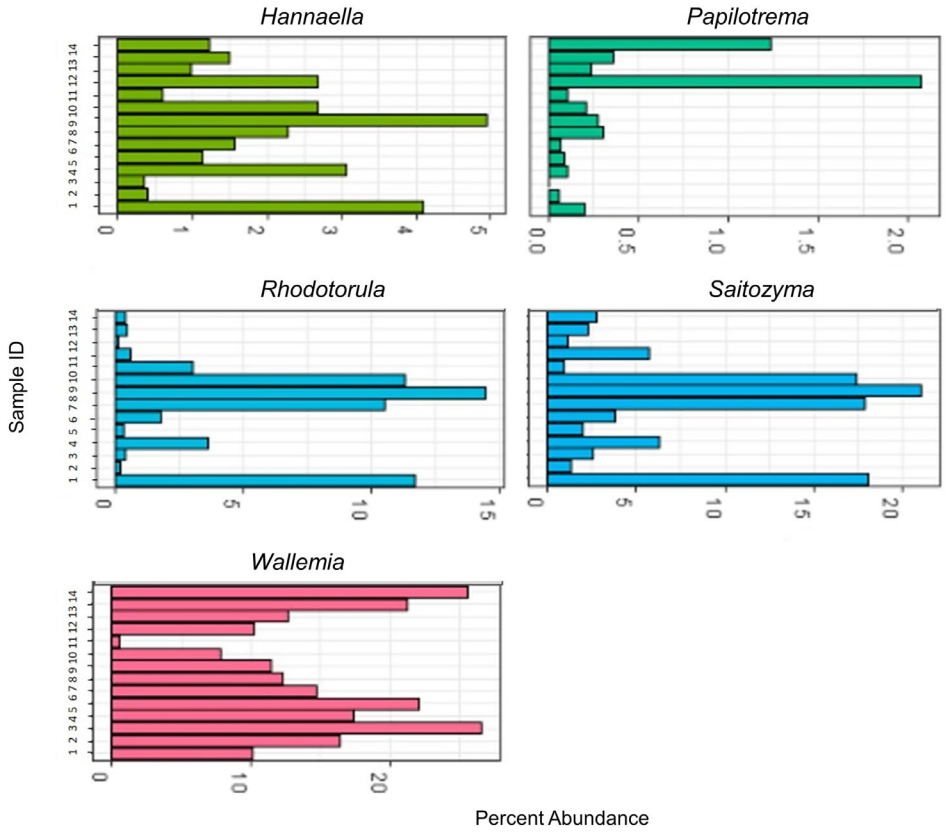

FIGURE 7 Fungal genera with >2% abundance within phylum Basidiomycota in clinically healthy horses. Facet bar plots depicting the phylum Basidiomycota with each plot representing genera with greater than 2% abundance, with % abundance is on the x-axis and each horse (1-14) represented on the y-axis

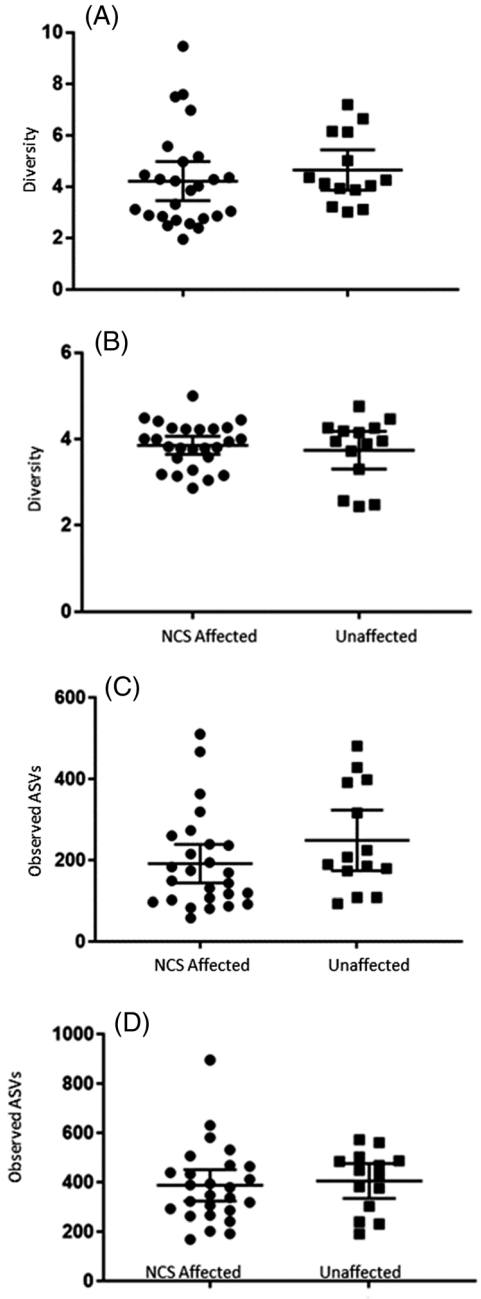

FIGURE 8 Alpha diversity and richness measures for 16S rRNA and ITS sequencing. Scatterplots of (A) Shannon diversity metric of 16s rRNA, (B) numbers of observed ASVs of 16s rRNA, (C) Shannon diversity metric of ITS, and (D) numbers of observed ASVs of ITS sequencing for 26 horses with acute nasopharyngeal cicatrix (NCS) represented as black circles and 14 unaffected horses represented as black squares. The long horizontal line represents the mean and short horizontal lines represent the 25% interquartile range. There was no difference between groups for either measure, with $P = .61$ and $P = .74$, respectively. ASVs, amplicon sequence variants; ITS, internal transcribed spacer

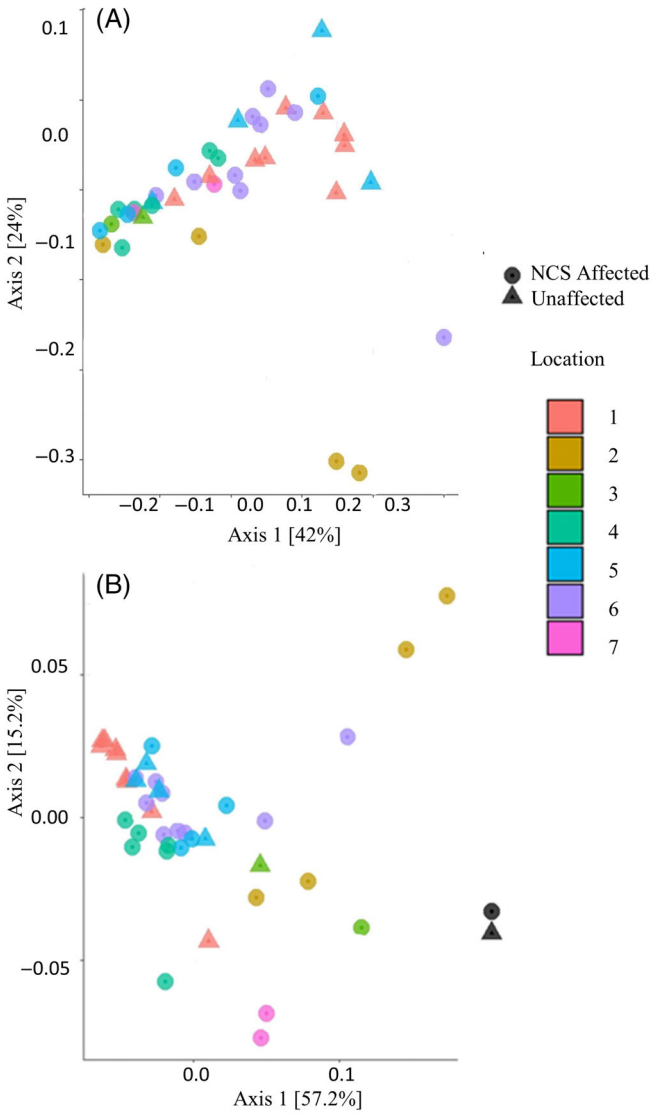

FIGURE 9 Principal coordinate analysis (PCoA) plots constructed based on unifrac distance metric for 16s rRNA and ITS sequencing. Principal coordinate analysis plots depicting the between sample diversity for (A) 16s rRNA and (B) ITS fungal sequencing for 26 horses with acute nasopharyngeal cicatrix (NCS) represented as circles and 14 unaffected horses represented as triangles. Colors represent individual farms (1-7). There was no visually obvious clustering. ITS, internal transcribed spacer

least dominant phyla (Bacteroidetes and Acidobacteria) are shown as Figures S2 and S3. Taken together, these data indicate that the primary bacterial genera comprising the nasopharyngeal microbiota of healthy horses consists of *Moraxella*, *Staphylococcus*, *Streptococcus*, and *Corynebacteria*. To a lesser extent, *Methylbacteria*, *Pseudomonas*, and *Bacillus* also contribute.

3.3 | Fungal microbiota of the nasopharynx of healthy horses

Count tables generated from ITS1-2 sequencing were filtered to contain only ASVs present >0.01% across all samples and further collapsed to the genera level. These data were transformed to relative

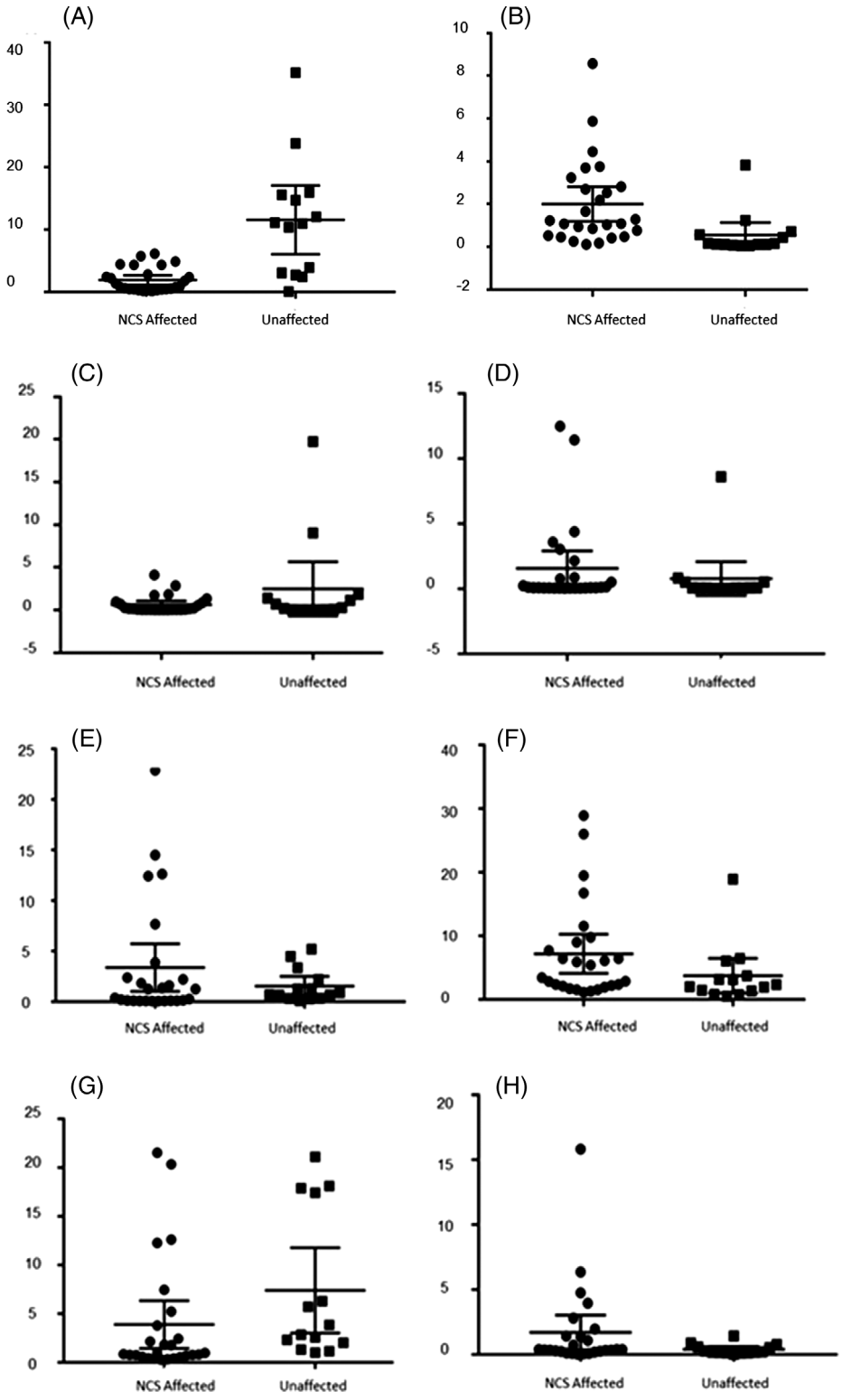

FIGURE 10 Differential abundance of bacterial and fungal microbiota comparing horses with nasopharyngeal cicatrix (NCS) and horses unaffected with NCS. Scatter plots representing the significant differential abundance at the level of genera (represented in >1% abundance) for bacterial (A) and fungal organisms (B-H). Group is represented on the x-axis and percent relative abundance is represented on the y-axis

abundance to describe the fungal microbiota of the nasopharynx of healthy horses. The phyla (colored by genera) that make up the fungal portion of the nasopharyngeal microbiota is shown in Figure 5. The fungal microbiota is overwhelmingly represented by 2 phyla: Ascomycota and Basidiomycota. The genera making up these phyla are shown in Figures 6 and 7, respectively. The Ascomycota phylum is predominately characterized by *Aspergillus*, although *Cladosporium*, *Nigrospora*, *Curvularia*, *Exserohilum*, and *Penicillium* also consistently contribute to this phylum. Similarly, the Basidiomycota phylum is primarily characterized by the genus *Wallemia*, although the genera *Rhodotorula* and *Saitozyma* also contribute to this phylum. Taken together, the fungal component of the nasopharyngeal microbiota of healthy horses is primarily characterized by *Aspergillus*, *Wallemia*, *Rhodotorula*, and *Saitozyma*.

TABLE 1 Table representing bacterial and fungal taxon comparing horses with and without nasopharyngeal cicatrix

Phylum	Class	Order	Family	Genus	FDR *P* value	Relative abundance	Log 2 difference
Firmicutes	Bacillio	Bacillales	Staphylococcaceae	Staphylococcus	8.77^{-02}	0.41	−1.2963
Ascomycota	Dothideomycetes	Pleosporales	Periconiaceae	Periconia	1.34^{-02}	5.96	0.2642
Ascomycota	Eurotiomycetes	Euritiales	Aspergillaceae	Penicillium	4.66^{-02}	2.72	0.3219
Ascomycota	Leomycetes	Erysiphales	Erysiphaceae	Erysiphe	1.52^{-02}	0.09	0.4005
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Bipolaris	4.04^{-02}	1.49	0.2016
Ascomycota	Sordariomycetes	Microascales	Microascaceae	Microascus	1.52^{-02}	1.28	0.2141
Ascomycota	Saccharomycetes	Saccharomycetale	Debaryomycetaceae	Debaryomyces	5.41^{-05}	1.28	−1.0291
Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	Spegazzinia	1.22^{-07}	1.22	0.4854
Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	Paraconiothyrium	1.06^{-03}	0.87	0.2868
Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	Stemphylium	2.63^{-18}	0.78	0.4436
Ascomycota	Dothideomycetes	Pleosporales	Curcurbitariaceae	Pyrenochaetopsis	4.66^{-02}	0.78	−0.2863
Ascomycota	Dothideomycetes	Pleosporales	Sporomiaceae	Preussia	1.60^{-02}	0.49	−0.7612
Ascomycota	Sordarimycetes	Hypocreales	Clavicipitaceae	Claviceps	1.20^{-11}	0.44	0.5849
Ascomycota	Leotiomycetes	Erysiphales	Erysiphaceae	Podosphaera	5.80^{-04}	0.37	−0.8889
Ascomycota	Leotiomycetes	Helotiales	Haetomellaceae	Pilidium	1.17^{-02}	0.19	0.0285
Ascomycota	Leotiomycetes	Helotiales	Elotiaceae	Articulospora	7.23^{-03}	0.03	0.4436
Ascomycota	Dothideomycetes	Pleosporales	Tetraplosphaeriaceae	Unidentified	1.06^{-03}	0.01	0.1890
Ascomycota	Eurotiomycetes	Chaetothyriale	Herpotrichiellaceae	Phialophora	1.43^{-03}	0.01	0.1890
Basidiomycota	Tremellomycetes	Tremellales	Trimorphomycetaceae	Saitozyma	1.77^{-02}	5.1	−0.3584
Basidiomycota	Tremellomycetes	Tremellale	Rhynchogastremataceae	Papiliotrema	8.70^{-03}	0.3	−0.2009
Basidiomycota	Tremellomycetes	Filobasidial	Filobasidiaceae	Naganishia	1.52^{-02}	0.09	0.4329
Basidiomycota	Agaricomycetes	Agaricales	Strophariaceae	Psilocybe	4.66^{-02}	0.13	−0.1202

Note: Within the 16S data (highlighted in orange), only *Staphylococcus* was significantly differentially abundant between horses affected with NCS and those unaffected with NCS. Within the ITS data (highlighted in blue), there were 21 differentially abundant genera between horses affected and unaffected with NCS. A negative Log 2 difference indicates a loss of organism in the NCS affected horses relative to unaffected horses.
Abbreviations: FDR, false discovery rate; ITS, internal transcribed spacer; NCS, nasopharyngeal cicatrix syndrome.

TABLE 2 Representation of fungal taxon comparing premises affected with and without nasopharyngeal cicatrix

Class	Order	Family	Genus	FDR *P* value	Relative abundance	Log 2 difference
Dothideomycetes	Pleosporales	Pleosporaceae	Alternaria	1.49^{-05}	3.99	3.2352
Dothideomycetes	Pleosporales	Pleosporaceae	Bipolaris	2.38^{12}	1.49	3.5137
Sordariomycetes	Microascales	Microascaceae	Microascus	1.02^{-15}	1.28	4.1937
Dothideomycetes	Pleosporales	Didymosphaeriaceae	Spegazzinia	1.18^{-04}	1.22	2.8773
Dothideomycetes	Pleosporales	Didymosphaeriaceae	Paraconiothyrium	9.58^{-04}	0.87	0.8559
Sordariomycetes	Hypocreales	Clavicipitaceae	Claviceps	3.37^{-04}	0.44	6.0932

Note: Within the ITS data, there were 6 fungal genera that were significantly abundant across premises, controlling for effects of farm. These fungal genera were present more abundantly in NCS affected compared to unaffected horses.
Abbreviations: FDR, false discovery rate; ITS, internal transcribed spacer; NCS, nasopharyngeal cicatrix syndrome.

3.4 | Difference between healthy horses and horses affected with NCS

phyloseq was used to examine both alpha and beta diversity between healthy horses and horses affected with NCS. There was no significant difference in the Shannon alpha diversity measures within either the bacterial or fungal microbiota (Figure 8). Similarly, there were also no differences in richness (observed ASVs) between the groups. Beta diversity was analyzed both visually and quantitatively. Specifically, PCoA plots were constructed based on the unweighted UniFrac distance metric (Figure 9). Visually, there was no obvious evidence of clustering, but permutational multivariate analysis of variance (PERMANOVA) using the weighted UniFrac distance matrix and 999 permutations, revealed significant clustering of samples by both

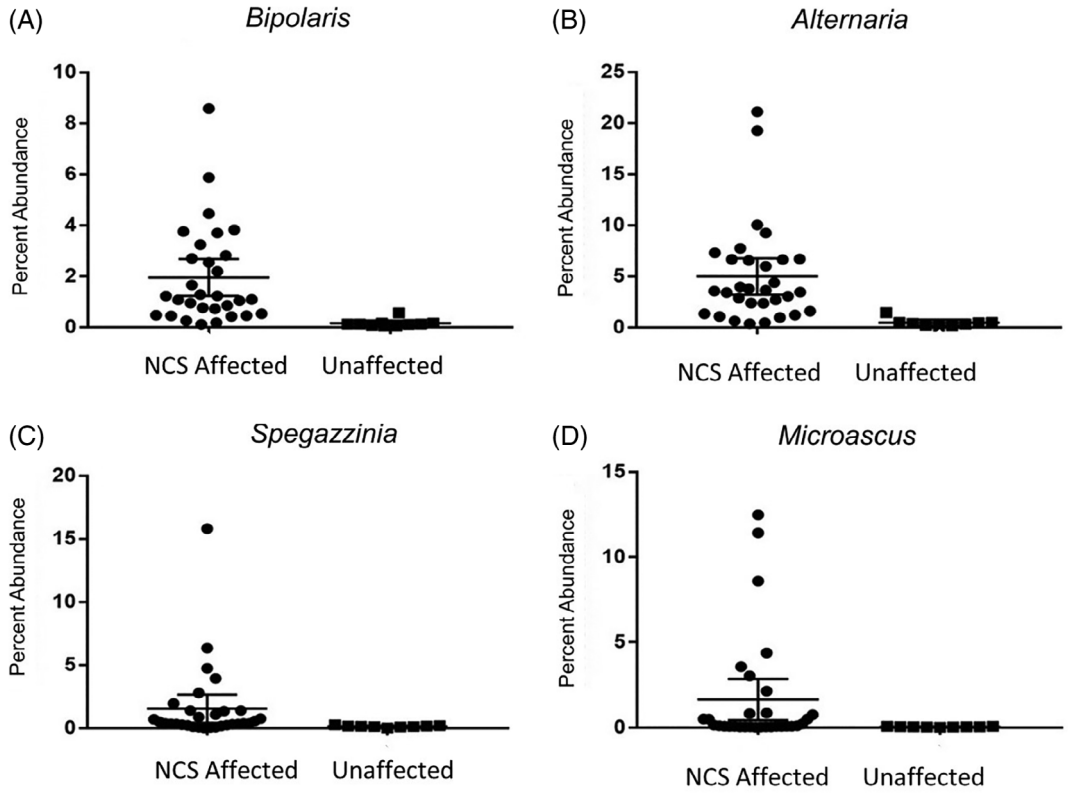

FIGURE 11 Differential abundance of fungal microbiota comparing affected premises. Scatter plots representing the significant differential abundance at the level of genera comparing farms affected (affected NCS) with those farms unaffected with disease, when controlling for individual farm. Group is represented on the x-axis and percent abundance is represented on the y-axis. NCS, nasopharyngeal cicatrix syndrome

location (ie, farm) (16s $R^2 = 0.303$, $P = .001$; ITS $R^2 = 0.28$, $P = .001$) and disease (16s $R^2 = 0.059$, $P = .003$; ITS $R^2 = 0.46$, $P = .002$), although the magnitude of these effects was small.

In order to detect differential abundance between healthy horses and horses affected with NCS, the R package Corncob was used on ASV count tables collapsed to the genus level, controlling for the effect of location on abundance.[23] Within the 16S data, only the genus *Staphylococcus* was differentially abundant (Figure 10), characterized by a loss of *Staphylococcus* in horses affected with NCS (Table 1). There were 21 fungal genera that had significant differential abundance between healthy horses and horses affected with NCS (Table 2). Among these, 7 had an overall abundance greater than 1% including *Bipolaris*, *Debaryomyces*, *Microascus*, *Penicillium*, *Periconia*, *Saitozyma*, and *Spegazzinia* (Figure 10). Due to the environmental association of NCS,[2] we then investigated taxa that were differentially abundant between premises housing affected horses and those premises without affected horses. This was performed with the R package corncob, as described above. Analysis revealed 6 fungal genera that were significantly different including *Alternaria*, *Bipolaris*, *Microascus*, *Spegazzinia*, *Paraconiothyrium*, and *Claviceps* (Table 2). The distribution of these taxa across premises is depicted in Figure 11. Collectively, these data suggest that there are differences in the nasopharyngeal microbiota between horses affected and unaffected with NCS as well as differences with regards to premises housing NCS-affected horses and those premises without affected horses. This difference is almost exclusively related to the fungal component of the microbiota and not the bacterial component. *Bipolaris*, *Microascus*, and *Alternaria* each had a similar magnitude of difference between affected and unaffected premises. At the horse level, *Bipolaris* also had the greatest magnitude of difference between affected and unaffected horses (Figure 12).

3.5 | *Pythium insidiosum*

There are anecdotal suggestions that the oomycete *Pythium insidiosum* is the causative agent of NCS. This organism is not included in the UNITE database, thus qPCR on DNA isolated from the nasopharyngeal washes was performed. *Pythium* was identified in the positive control but was not identified in nasopharyngeal samples from any of the horses included in our study (Figure S4). These data suggest that *Pythium* is not part of the normal nasopharyngeal microbiota of horses, nor can it be found in horses acutely affected with NCS.

4 | DISCUSSION

This study describes the nasopharyngeal bacterial and fungal microbiota of healthy horses and compares these findings to horses affected with NCS. The results of this study indicate that the bacterial population of

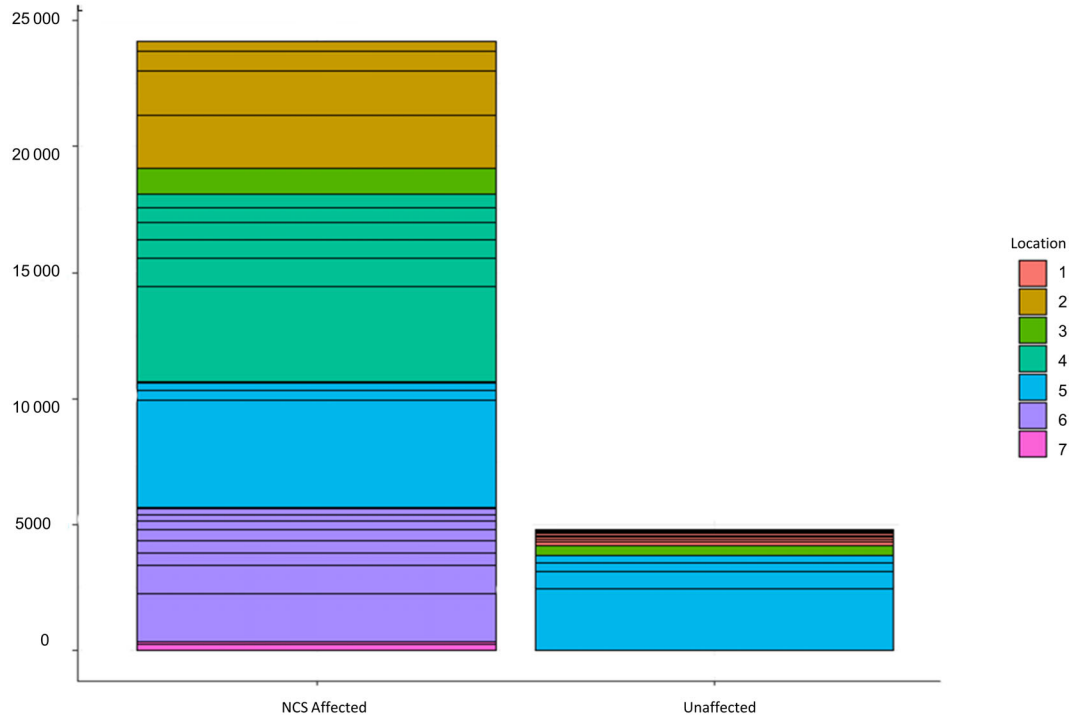

FIGURE 12 Abundance of *Bipolaris* by location, horse, and disease status. Stacked bar plot with each farm represented as a different color. Each black line, within each location, represents an individual horse at that location. The numbers of ASVs, cumulative, are shown on the y-axis with group (NCS affected or unaffected) on the x-axis. ASVs, amplicon sequence variants; NCS, nasopharyngeal cicatrix syndrome

the nasopharynx in healthy horses is diverse, though primarily occupied by the genera of *Staphylococcus*, *Streptococcus*, *Moraxella*, and *Corynebacteria*. Several studies have investigated the microbiota of the respiratory tract of healthy and diseased horses including tracheal wash samples, sinus lavage, and nasal swab.[6,12] Despite a difference in geographic location, our findings are similar to those in the upper and lower respiratory tract of healthy horses in Canada in which Proteobacteria, Firmicutes, Actinobacteria, and Bacteroides represented over 95% of the total bacterial abundance, with Proteobacteria representing the greatest abundance.[5] Similarly, sinus lavage samples from healthy horses from the same geographic region as the current study also demonstrated a predominance of Proteobacteria, with Firmicutes and Actinobacteria also well represented.[12]

The current study revealed that the fungal microbiota of the nasopharynx of healthy horses is largely characterized by *Aspergillus*, *Wallemia*, *Rhodotorula*, *Saitozyma*, and *Cladosporium*. This is again similar to the aforementioned study in which *Aspergillus* was considered part of the normal equine sinus.[12] This finding is not surprising given that *Aspergillus* is associated with opportunistic upper and lower respiratory disease in the horse[25,26] and it is the most common fungal organism of hay.[27-29] The significance of the other identified fungal organisms is unknown. *Wallemia* is generally considered a food-borne pathogenic fungi, with rare reports of its association with allergological conditions in people such, as farmer's lung disease and cutaneous infections,[30] and no known association to equine respiratory disease. In a previous study, its abundance in nasal swabs and tracheal washes of horses was decreased with nebulized saline and dexamethasone separately.[6] Fungal infection caused by *Rhodotorula* in people generally occurs in association with immunosuppression[31] and *Saitozyma* is poorly identified in the literature in both human and veterinary medicine. *Cladosporium* in humans is among the most important allergenic fungi associated with allergic rhinitis and respiratory arrest in asthmatic patients, and has also been described to cause subcutaneous and deep infections in humans and animals.[32] This fungal organism is commonly found in the environment, well recognized as an allergen in people, and has been identified in equine nasal washes and paranasal sinuses.[6,12,28,29] There are differences in microbiota between health and disease. One study found firmicutes and proteobacteria in nasal passages with lower abundance of proteobacteria in virally affected horses with EHV-1.[7] Healthy horses and horses affected by low grade pharyngitis have a pharyngeal microbiota that includes *Corynebacterium* spp., *Staphylococci* spp., *Nocardia* spp., *Moraxella* spp., and *Enterobacter* spp. These findings were variable between horses and could have suggested transitory microflora.[4]

When comparing horses affected with NCS and unaffected horses, distinct differences in the fungal microbiota were identified. Specifically, differential abundance of *Bipolaris* was increased in affected horses compared to unaffected horses. The only difference identified in the bacterial microbiota was a decrease in *Staphylococcus* in horses affected with NCS compared to unaffected horses. The importance of this finding is unknown; however, as a commensal organism, it is likely that this decrease is an effect of an underlining disease process. In the absence of overgrowth of other bacterial organisms, the role of the bacterial microbiota in causing disease is

likely insignificant. There is a decrease in the abundance of bacteria in the acute stages of some respiratory diseases.[17]

Considering the suspicion of an environmental component to NCS, comparison was also made between farms affected and unaffected with NCS when controlling for farm and irrespective of disease status. Again, the differential abundance of *Bipolaris* was increased in horses residing on affected farms. Additionally, the differential abundance of *Alternaria* was increased in affected farms. This fungus that grows mostly in warm and humid regions is opportunistically pathogenic to humans causing bronchial asthma, allergic sinusitis, and rhinitis.[33,34] In horses, there are several reports of *Alternaria* associated nodular dermatitis and cutaneous granulomas.[35,36] Additionally, a previous study reported an increase in differential abundance of *Alternaria* in nasal swabs and tracheal washes of horses that were nebulized with steroids.[6]

Based on the apparent importance of this organism when considering both disease status and location, *Bipolaris* is of particular interest. *Bipolaris* is a ubiquitous, dematiaceous fungus present in air, soil, and plants, specifically in hot climates including the southern states of the United States, Australia, and India.[37] Though typically considered a plant pathogen, it is increasingly recognized as a cause of disease in people, including meningitis,[38] keratitis,[39] endarteritis,[40] and disseminated infection in neonates.[41] However, the most common location of infection with *Bipolaris* is the nasal sinuses, specifically, *Bipolaris spicifera* identified as the most common cause of allergic fungal sinusitis in the southwestern United States.[42,43] While it is not possible to assess causality in the present study, this fungal organism warrants further consideration in association with NCS in horses. There is only 1 report of *Bipolaris hawaiiensis* in a horse causing a fungal granuloma resulting in chronic invasive fungal rhinitis.[44] Additionally, *Bipolaris spicifera*, a closely related agent, causes superficial ophthalmic and dermatologic infections.

Due to similarities in geographic distribution and suspicion of an environmental acquired etiologic agent in NCS, it has been proposed that *Pythium insidiosum* is the causative agent of NCS and vaccination against *Pythium* has been proposed as a treatment or prophylaxis of NCS in horses; however, no evidence exists to support this claim.[45-48] Pythium *insidiosum* was evaluated via validated PCR method and the agent was not identified in any sample, suggesting that this etiologic agent is unlikely to be the cause of NCS in horses.

Several important limitations in this study should be discussed. Description of the normal fungal and bacterial nasopharyngeal microbiota was geographically limited to horses in central Texas. Extrapolation to other geographic regions should be done with caution. The importance of inclusion of negative controls in microbiota sequencing is gaining increased recognition. Unfortunately, negative controls during the collection, extraction, and amplification process were not obtained. We attempted to address this computationally using the frequency method within the R package decontam. While this approach has some value, likely combining this approach with a prevalence approach, based on negative controls, would have improved our ability to identify contaminants. The contaminants we did identify were present in low numbers and had no impact on our analyses. In addition, while unidentified contaminants could alter our description of normal horses, they would likely not have impacted our data regarding differences between affected and unaffected horses as the contaminants would be present in all samples. A further limitation is that diagnosis of NCS is based on clinical signs and endoscopic findings. The endoscopic appearance of chronic NCS is more distinct, with a particular web-like scarring appearance. In acute cases, it is possible that misclassification of disease occurs, as the clinical signs and endoscopic findings are less characteristic and could resemble other causes of pharyngeal inflammation. Repeated evaluation or a longitudinal study to confirm development of chronic changes and more definitively diagnose NCS would have been ideal; however, our findings were supported by geographic distribution of disease, clinical findings, and presence of herd involvement in the cases included in this study. Importantly, inclusion of only acutely affected horses with NCS in this study was important in that once chronic scar tissue forms, the lesions remain present and the duration of time for initial infection is difficult to ascertain. If NCS is caused by an etiologic agent, it is likely that it would not be detectable in the chronic stage of disease. There is no described gold standard technique to investigate microbiota from the nasopharynx in horses, although we strongly believe the nasopharyngeal wash may be an optimal method as it samples a large nasal and nasopharyngeal surface area compared to a nasopharyngeal swab. Nasopharyngeal cicatrix affects the entire upper respiratory tract, including everything from the proximal trachea to the nasal passages, suggesting that the microbiota information of this entire region is important. In this study, all horses except for 1 healthy horse were fed the same type of hay but their grain was not recorded. This is a potential limitation as in humans, there is evidence that the gastrointestinal microbiome can be associated with lower airway immunology and in horses it could represent a role between health and disease.[15,49] Importantly, in central Texas farms horses with no history of being affected by NCS could be challenging to identify. Upon recruiting horses into the study, unaffected horses were difficult to identify, resulting in few farms in which unaffected horses could be included, and differences in breed distribution between groups. Finally, our sample size was small, which might have impaired our ability to detect differences between groups, resulting in a type 2 error; however, our samples size is similar to other studies demonstrating important differences in the respiratory microbiota of horses in health and disease.[5,6]

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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

Approved by the IACUC (IACUC-2016-0328) and the Clinical Research and Review Committee of the College of Veterinary Medicine & Biomedical Sciences at Texas A&M University.

HUMAN ETHICS APPROVAL DECLARATION

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

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