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# Topography of the respiratory, oral, and guttural pouch bacterial and fungal microbiotas in horses

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## Abstract

**Background:** The lower respiratory tract microbiota of the horse is different in states of health and disease, but the bacterial and fungal composition of the healthy respiratory tract of the horse has not been studied in detail.

**Hypothesis:** The respiratory tract environment contains distinct niche microbiotas, which decrease in species richness at more distal sampling locations.

**Objective:** Characterize the bacterial and fungal microbiotas along the upper and lower respiratory tract of the horse.

**Animals:** Healthy Argentinian Thoroughbred horses (n = 11) from the same client-owned herd.

**Methods:** Prospective cross-sectional study. Eleven upper and lower respiratory tract anatomical locations (bilateral nasal, bilateral deep nasal, nasopharynx, floor of mouth, oropharynx, arytenoids, proximal and distal trachea, guttural pouch) were sampled using a combination of swabs, protected specimen brushes, and saline washes. Total DNA was extracted from each sample and negative control, and the 16S rRNA gene (V4) and ITS2 region were sequenced. Community composition, alpha-diversity, and beta-diversity were compared among sampling locations.

**Results:** Fungal species richness and diversity were highest in the nostrils. More spatial heterogeneity was found in bacterial composition than in fungal communities. The pharyngeal microbiota was most similar to the distal tracheal bacterial and fungal microbiota in healthy horses and therefore may serve as the primary source of bacteria and fungi to the lower respiratory tract.

**Conclusions and Clinical Importance:** The pharynx is an important location that should be targeted in respiratory microbiota research in horses. Future studies that investigate whether biomarkers of respiratory disease can be reliably detected in nasopharyngeal swab samples are warranted.

**Abbreviation:** BAL, bronchoalveolar lavage.

KEYWORDS
16S, equine respiratory tract, ITS2, microbiome, next generation sequencing

## 1 | INTRODUCTION

The "united allergic airway" concept that connects asthma and allergic rhinitis in humans, where a high proportion of patients have coexisting upper and lower airway disease,[1-5] does not seem to apply to horses with regard to inflammation of the respiratory system.[6] As in humans,[7] evidence exists in horses that the lower airway bacterial communities play a role in the pathogenesis of asthma,[8,9] although bacterial overgrowth is not a common finding.[10] In addition, it appears that disease exacerbation alters the adaptation of the fecal microbiota to changes in both environment and diet,[11] raising questions about microbial crosstalk between the respiratory and gastrointestinal systems. Indeed, the oropharynx has been reported as the primary source for bacterial respiratory pathogens in humans, with the mouth and oropharynx having the most similarity to the composition of the lung microbiota.[12,13] Interestingly, in healthy humans the nasal microbiota seems to contribute little to the composition of the lung microbiota.[12,13] Given the anatomical differences between the respiratory tracts of horses and humans, species-specific differences in respiratory tract microbiota topography should be studied.

Limited information is available regarding the topography of the respiratory tract microbiota of horses.[8,9,14,15] A recent culture-based study investigated the prevalence of fungi in respiratory samples of horses diagnosed with mild asthma and found that the presence of fungi in tracheal wash cytology doubled the likelihood of a horse having mild asthma.[16] Given that the overall bacterial composition of the respiratory tract contributes to various disease states, it is logical to question the role of the respiratory fungal microbiota in the healthy horse.

The bacterial and fungal communities in the respiratory tract of the healthy horse should be comprehensively mapped. Clarification of the relative contributions of the different upper respiratory tract bacterial and fungal microbiotas to community composition in the lower respiratory tract of horses would facilitate better understanding of the possible etiopathological pathways involved in the development of respiratory disease in horses. Given that differences have been detected in association with disease states in the lower respiratory tract of horses, and that it is more practical in field settings for clinicians to take samples from the upper respiratory tract, it is also of interest to determine if a location exists in the upper respiratory tract that is similar in community composition to that of the lower respiratory tract. These findings could enable development of a field test for diagnosis of respiratory disease endotypes in horses based on respiratory biomarkers.

Our hypothesis was that the respiratory tract environment contains distinct niche microbiotas, which decrease in species richness at more distal sampling locations. The horse has an elongated soft palate that results in obligate nasal breathing and we hypothesized that, unlike in humans, the oral cavity would contribute less to the lower respiratory tract community structure of healthy horses and therefore would be more dissimilar to the proximal and distal trachea than to the pharynx. Our objectives were to: (i) characterize the bacterial and fungal microbiotas present along the upper and lower respiratory tract of horses, and (ii) determine if a location exists in the upper respiratory tract that is similar in community composition to that of the lower respiratory tract.

## 2 | METHODS

### 2.1 | Animals

Eleven Argentinean Thoroughbred horses (9 mares, 2 geldings; body condition score [BCS], 4-5/9, median age, 10, interquartile range [IQR], 7-12 years) were studied in a prospective cross-sectional study. Horses were judged to be healthy based on complete physical and respiratory examinations. The horses were used for polo games, at a competition level of fitness, and had no history of decreased performance, health issues, or respiratory infections. They also did not have any history of coughing or nasal mucus discharge after exercise. All horses were from the same herd and turned out together in a 30-acre grass paddock. Their diet consisted of pasture supplemented with senior feed (Purina Equine Senior Horse Feed, Purina Canada, Woodstock, Canada), which was fed on the ground. All horses were vaccinated 3 years before sampling, and were treated with an anthelmintic product biannually (spring and fall). Three of the 11 horses received phenylbutazone (2.0-4.0 mg/kg PO q12h) on an as needed basis, usually for 2 days after a polo game. Horses did not receive any treatment within at least 36 hours of data collection.

### 2.2 | Sampling procedures

Sampling was completed a minimum of 36 hours after exercise. All samples were collected in an indoor facility on a single day in June (ambient temperature range, 9.2-17.1°C; relative humidity, 47%-83%). Horses were premedicated with acepromazine maleate (0.07-0.08 mg/kg IM or IV), and sedated with xylazine hydrochloride (0.4-0.5 mg/kg IV) and butorphanol tartrate (0.05-0.1 mg/kg IV). The order of sample collection was consistent for all 11 horses. All procedures were performed using sterile gloves and standard aseptic techniques. After sedation, bilateral nasal swab samples were collected first, followed in succession by samples of the deep nasal passages and dorsal pharynx (nasopharynx, using a long, guarded uterine swab). The ventral aspect of the tongue (floor of the mouth), caudal area of the hard palate (oropharynx), arytenoids and proximal trachea then

were sampled as described below. Finally, the distal trachea and guttural pouch were sampled in that order to prevent potential contamination, using aspiration techniques (described below). After sample collection, a bronchoalveolar lavage (BAL) was performed.

Nasal swab and oral swab samples of the tongue and hard palate were collected using 15.2 cm sterile flocked Nylon swabs (ESwabTM, Copan Diagnostics, Murrieta, California) and stored in 1 mL Amies transport media (ESwabTM, Copan Diagnostics, Murrieta, California). Two nasal swabs were obtained per horse (1 per nasal cavity). Oral samples were taken after fitting a mouth gag opener; the tongue sample (floor of the mouth) was taken from the frenulum area on the ventral aspect of the tongue after lifting it by hand. The hard palate oral sample was taken after pushing the tongue down and swabbing the caudal aspect of the hard palate without touching the tongue. Deep nasal swabs, as well as dorsal pharynx (nasopharynx) swabs, were collected as previously described[17] using long guarded swabs (27 cm) with a rayon bud (Dryswab Veterinary Laryngeal, Medical Wire and Equipment, Corsham, England) and immediately placed into Amies transport media (1.0 mL). Two deep nasal swabs (1 per nasal cavity) and 1 pharyngeal swab were obtained per horse. A 1.3 m video endoscope (GIF-130, Olympus Canada, Inc, Richmond Hill, Canada) then was introduced into the nasal passage while avoiding contact with mucus, and the arytenoids and proximal trachea were visualized sequentially and sampled using a guarded brush inserted through the sterilized biopsy channel of an endoscope. Precautions were taken to extend the tip of the guarded brush a sufficient distance ahead of the endoscope tip before pushing the brush out, ensuring initial contact with the area of interest. A transendoscopic tracheal wash then was performed as previously described,[18] with the following modifications. A self-manufactured, sterilized, triple-sheathed sterilized plastic catheter was passed through the sterilized biopsy channel of a 1.3 m video endoscope (GIF-130, Olympus Canada, Inc, Richmond Hill, Canada) until 5 to 6 cm emerged from the distal end. Sterile saline (approximately 3 mL) was pushed through the catheter into a sterile 10 mL plain tube and stored at −20°C, to be used as a negative control. The tubing then was retracted until shielded in the endoscope. The endoscope then was introduced into the ventral meatus of the nose, and passed down into the trachea, advancing to approximately 90 cm from the nares. The triple-sheathed catheter then was advanced through the biopsy channel until the end was observed to be protruding approximately 5 to 6 cm from the distal end of the endoscope, and the walls of the tracheal lumen were lavaged with approximately 10 mL sterile saline. The fluid then was aspirated and immediately transferred into a sterile 10 mL plain tube and stored at 4°C. The BAL was performed as previously described.[8,9] Briefly, a balloon-tipped sterile BAL tube (BAL300, Mila International, Florence, Kentucky) was inserted until wedged against the wall of a bronchus, and 2 boluses (250 mL/bolus) of sterile isotonic saline (0.9% NaCl) solution were sequentially instilled. Lavage fluid was recovered, and 2 10-mL aliquots were immediately stored at 4°C. Another small aliquot of BAL fluid was centrifuged using a Cytospin (90*g* for 5 min) and the obtained slides were stained with modified Wright-Giemsa stain. A differential count of BAL fluid cells was performed on a minimum of 400 cells as previously described.[19]

For each sampling technique, matching negative control swabs, brushes, or fluid aspirate also were obtained on each collection day (n = 2). The tip of the swabs or brushes were exposed to the stable air, whereas the fluids were run through the endoscopic or BAL tubes and collected for storage and analysis of negative controls.

All swabs and fluid samples were kept at 4°C immediately after collection, processed within 10 hours and transferred to −80°C storage pending DNA extraction. At processing of the swabs, each rayon tip was removed from the Amies transport media, which then was transferred into a sterile 1.5 mL microfuge tube, and centrifuged (Eppendorf Centrifuge 5424, Eppendorf AG, Hamburg, Germany) at 13 000 rpm for 5 minutes. The resulting pellet and tip were stored in the microfuge tube at −80°C pending DNA extraction.

## 2.3 | DNA extraction

Total DNA was extracted from all samples using a DNeasy Blood and Tissue kit (Qiagen, Inc, Mississauga, ON, Canada) as previously described.[8] Extracted DNA was stored at −80°C until amplification and sequencing. Blank negative controls (kit only) were included in triplicate during DNA extraction.

## 2.4 | Amplification and sequencing

The 16S amplicon PCR 515F forward primer (5′GTGCCAGCMGCCGCGGTAA) and 806R reverse primer (5′GGACTACHVGGGTWTCTAAT) were used to amplify the V4 hypervariable region. The ITS amplicon PCR ITS1F forward primer (5′CTTGGTCATTTAGAGGAAGTAA) and ITS2 reverse primer (5′GCTGCGTTCTTCATCGATGC) were used. All amplification and sequencing steps were carried out at Genome Quebec (McGill University and Genome Quebec Innovation Centre, Quebec, Canada).

## 2.5 | Bacterial sequence processing

Sequencing data were processed as previously described[20] using cutadapt v2.3[21] and DADA2 v1.10[22] as implemented in R v3.5.1.[23] All sequencing reads were trimmed from the 3′ end down to 220 bases per read. The 220 base length was selected based on the sequencing data quality profiles and to ensure adequate overlap of paired-end reads after removal of sequencing primers. The forward and reverse 5′ 16S primers, as well as low-quality ends, were trimmed from the sequencing data using cutadapt in paired-end mode with a maximum allowed error rate of 0.1 and a quality cutoff of 20. Reverse complement primers were not trimmed because the trimmed read length was 220 base pairs. Because the approximate length of the V4 region is 254 base pairs, reverse complements of the forward and reverse 5′ primers were trimmed off and therefore not present in the data. Sequencing data quality was assessed using FastQC v0.11.8[24] and the individual sample quality reports then were compiled into a

comprehensive report using MultiQC v1.7.[25] Once the data quality was deemed acceptable, DADA2 was utilized to filter and trim reads, infer exact sequence variants, and assign taxonomy to variants. Default parameters were used for all DADA2 functions unless expressly mentioned. Sequencing reads were filtered using a maximum expected error of 2. A parametric error model then was estimated using a form of unsupervised machine-learning with 100 million sequences each for the forward and reverse reads separately. Sequencing reads then were dereplicated. Exact amplicon sequence variants (SVs) were inferred for each sample using the DADA2 sample inference algorithm and the estimated error model. Samples were pooled together for sample inference to increase sensitivity to SVs present at extremely low frequencies. Full, denoised sequences were obtained by merging the inferred forward and reverse reads. A sequence variant (SV) table, which is functionally similar to an operational taxonomic unit table, was assembled from the denoised sequences. Chimeric sequences then were removed from the table. A taxonomy table was assembled by assigning taxonomy to each SV in the SV table using the Ribosomal Database Project (RDP)[26] taxonomic database for DADA2.[27] All species level assignment was accomplished using the DADA2::addSpecies function, with exact matching used to assign species when possible.

### 2.6 | Fungal sequence processing

Sequencing data were processed using cutadapt v2.3 and DADA2 v1.10 as implemented in R v3.5.1. The forward and reverse 5′ ITS2 primers, as well their 3′ reverse complements, were trimmed from the sequencing data using cutadapt in paired-end mode with a maximum allowed error rate of 0.1. From this point forward, sequencing data were processed the same as described above for the 16S rRNA sequences unless otherwise noted. Briefly, sequencing data quality was assessed and compiled into a single comprehensive report. Sequencing reads were filtered using a maximum expected error of 2 and minimum length cutoff of 50 bases. A parametric error model then was estimated using a form of unsupervised machine-learning. Sequencing reads were dereplicated. Exact amplicon SVs were inferred for each sample using the DADA2 sample inference algorithm and the estimated error model. Samples were pooled together for sample inference. Full, denoised sequences were obtained by merging the inferred forward and reverse reads. An SV table was assembled from the denoised sequences. Chimeric sequences then were removed from the table. A taxonomy table was assembled by assigning taxonomy to each SV in the SV table using the General Fasta release files from the UNITE ITS database.[28] All species level assignment was accomplished using the DADA2::addSpecies function, with exact matching used to assign species when possible.

### 2.7 | Statistical analyses

Downstream analyses were performed in R using multiple functions from phyloseq v1.26.1,[29] ggpubr v0.2.4,[30] RVAideMemoire v0.9-74,[31] and vegan v2.5-6.[32] Two separate objects (1 for bacteria and 1 for fungi) were constructed from the SV and taxonomy tables in R using phyloseq for subsequent analysis. A prevalence filter was applied to the bacterial phyloseq object such that only SVs present in ≥1% of the samples remained. The fungal phyloseq object was not prevalence filtered. Mean relative abundance and beta-diversity measures were calculated using the prevalence-filtered bacterial data and the unfiltered fungal data; alpha-diversity measures were calculated using unfiltered data for both bacteria and fungi.

To facilitate downstream analyses, the left and right nostrils and the left and right deep nostrils were combined into location metagroups (nostrils and deep nostrils) based on preliminary analyses and anatomical/functional similarity among sampling locations. Preliminary analyses incorporated pairwise comparisons of the different sampling locations and included a permutational multivariate analysis of variance (PERMANOVA) and an analysis of similarities (ANOSIM), as detailed below (Additional files 1-3).

Species richness (Chao1) and diversity (Shannon) were calculated separately for bacterial and fungal samples as implemented in phyloseq. Pairwise comparisons of alpha-diversity measures were made using Wilcoxon rank-sum tests to compare alpha-diversity group means among sampling location metagroups as implemented in ggpubr. Adjustments for multiple comparisons were made using the Holm method.

A pairwise analysis of similarities (ANOSIM) using a Bray-Curtis dissimilarity index as implemented in vegan was used to evaluate compositional similarities among different sampling locations and location metagroups for bacteria and fungi separately. An individual ANOSIM was performed for each pairwise sampling location comparison and each sampling location metagroup comparison. All *P*-values were adjusted for multiple comparisons using the Holm method as implemented in vegan. A pairwise permutational analysis of variance (PERMANOVA) using a Bray-Curtis dissimilarity index as implemented in the RVAideMemoire package was used to evaluate compositional differences among different sampling locations for bacteria and fungi separately. The RVAideMemoire package was used to correct for multiple comparisons using the Benjamini & Hochberg method.

## 3 | RESULTS

### 3.1 | Respiratory tract status

The BAL fluid differential cell count was within the reference range recommended by the American College of Veterinary Medicine (ACVIM) consensus statement[33] for all horses enrolled in the study (Supplemental Table 1). This result rules out lung inflammation and confirms the healthy status of the horses.

### 3.2 | Baseline sequencing data

A total of 242 (16S rRNA = 121; ITS2 = 121) samples were collected from 11 healthy horses. Sequencing of the 16S rRNA region yielded

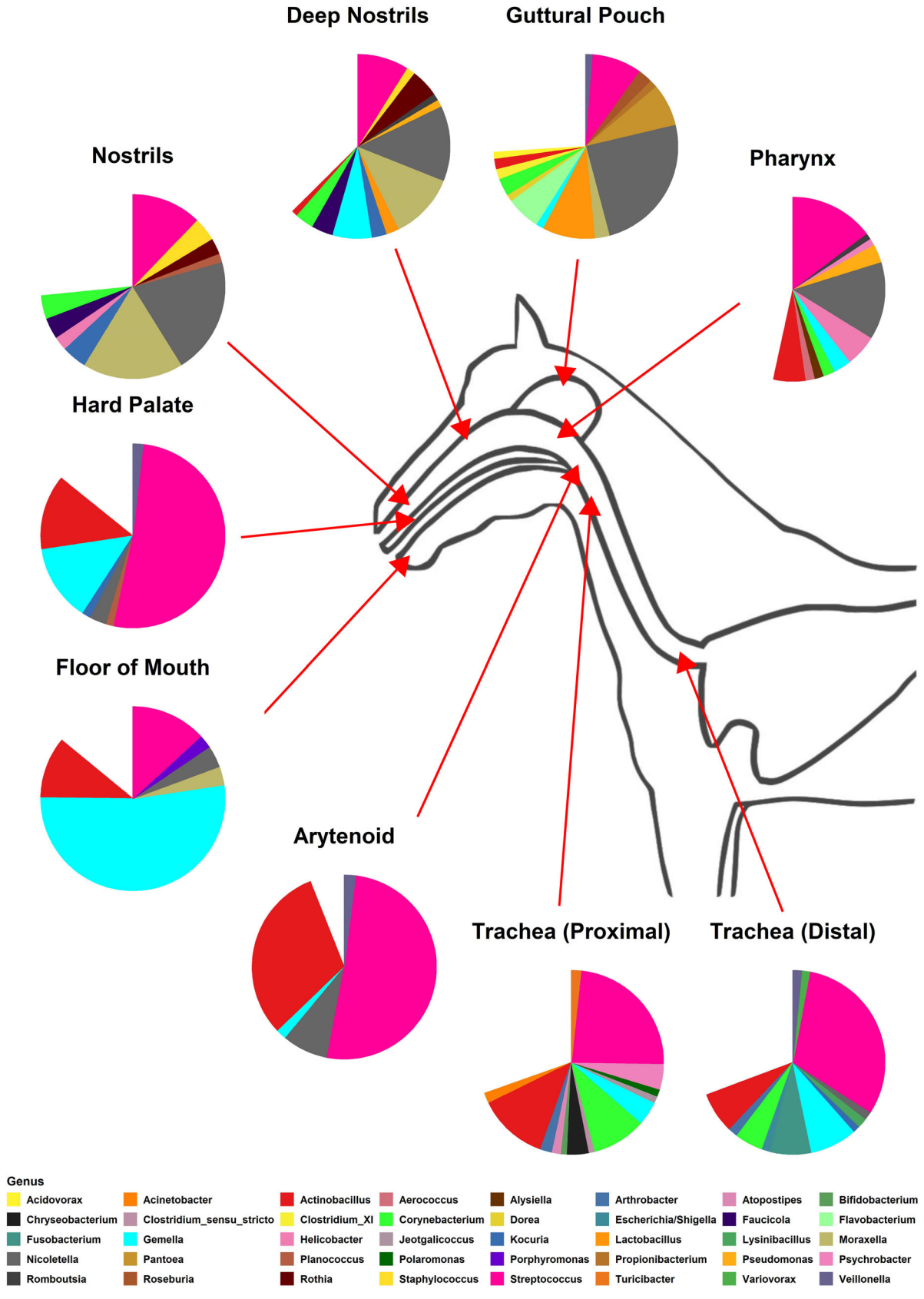


**FIGURE 1** Mean relative abundance of bacteria present at ≥1% abundance at the genus level of different upper and lower respiratory bacterial communities for 11 healthy horses

11 822 646 reads from 1 run with an average Phred quality score of 32.7 before upstream processing. Sequencing of the ITS2 region yielded 7 278 077 reads from 1 run with an average Phred quality score of 31.4 before upstream processing. Six individual ITS2 samples (arytenoid = 1; guttural pouch = 2; pharynx = 2; trachea [proximal] = 1) from 6 different horses were removed from the study for having insufficient sequencing depth (<500 sequencing reads/sample). After processing with DADA2 and removing the 6 aforementioned samples, a total of 6 703 120 16S rRNA reads (average, 55 398 reads per sample; range, 12 884-111 756) and 3 828 542 ITS2 reads (average, 31 641 reads per sample; range, 1201-68 927) remained across all samples. From the 16S rRNA sequences, 11 531 unique SVs were identified across all samples. After removal of all SVs that did not belong to the kingdom Bacteria, a total of 10 429 16S rRNA SVs remained. Furthermore, after 1% prevalence filtering 2516 16S rRNA SVs remained across all samples. From the ITS2 sequences, 1970 unique SVs were identified across all samples.

Along with the various study samples, 18 negative control samples (16S rRNA = 9; ITS2 = 9) were sent for targeted amplicon sequencing. It was determined that, based on the composition and low numbers of sequences in each individual sample, no significant contamination of the study samples resulting from improper sample collection or storage techniques or from the DNA extraction and targeted amplicon sequencing processes had occurred. Study sample sequencing results therefore were not adjusted for possible contaminants.

### 3.3 | Characterization of the respiratory tract bacterial microbiota

The most abundant bacterial phyla across all sampling locations were *Firmicutes* (39.72%), *Proteobacteria* (35.85%), *Actinobacteria* (16.83%), *Bacteroidetes* (2.75%), and *Chlorflexi* (1.17%; Supplemental Table 2), but the order of these phyla by mean relative abundance differed by sampling location (Supplemental Figure 1). *Proteobacteria* was most abundant in the nostrils, deep nostrils, pharynx, and guttural pouch, whereas *Firmicutes* was most abundant on the floor of the mouth, hard palate, arytenoid, and trachea (proximal and distal).

The 5 most abundant bacterial genera across all sampling locations were *Streptococcus* (21.45%), *Nicoletella* (11.15%), *Gemella* (9.05%), *Actinobacillus* (7.75%), and *Moraxella* (6.00%; Supplemental Table 2); but the order of genera by mean relative abundance differed by sampling location (Supplemental Figure 2). *Streptococcus* was most abundant in the pharynx, arytenoid, hard palate, and trachea (both proximal and distal; Figure 1). *Nicoletella* was most abundant in the guttural pouch, nostrils, and deep nostrils, whereas *Gemella* was most abundant on the floor of the mouth.

The 5 most prominent bacterial species across all sampling locations were *Nicoletella semolina* (11.15%), *Gemella haemolysans/morbillorum/sanguinis* (8.76%), *Actinobacillus capsulatus* (5.86%), *Streptococcus minor/pluranimalium* (2.21%), and an *Actinobacillus* SV ambiguously identified to >5 species (1.50%; Supplemental Table 2); but the order of species by mean relative abundance differed by sampling location (Supplemental Figure 3).

### 3.4 | Characterization of the respiratory tract fungal microbiota

The most abundant fungal phyla across all sampling locations were *Ascomycota* (49.72%) and *Basidiomycota* (44.69%; Supplemental Table 3). *Ascomycota* was most abundant in the nostrils, deep nostrils, pharynx, guttural pouch, arytenoid, and proximal trachea, whereas *Basidiomycota* was most abundant on the floor of the mouth, hard palate, and distal trachea (Supplemental Figure 4).

The 5 most abundant fungal genera across all sampling locations were *Vishniacozyma* (26.6%), *Mycosphaerella* (9.75%), *Capnocheirides* (5.58%), *Preussia* (3.08%), and *Phaeococcomyces* (2.4%; Supplemental Table 3); but, the order of genera by mean relative abundance differed by sampling location (Supplemental Figure 5). *Vishniacozyma* was most abundant in all locations except for the pharynx and guttural pouch, where *Mycosphaerella* and *Rhodotorula* were most abundant, respectively (Figure 2).

The 5 most prominent fungal species across all sampling locations were *Vishniacozyma victoriae* (17.93%), *Mycosphaerella tassiana* (9.75%), *Epicoccum dendrobii* (1.99%), *Preussia africana* (1.33%), and *Vishniacozyma carnescens* (1.33%; Supplemental Table 3), but the order of species by mean relative abundance differed by sampling location (Supplemental Figure 6).

### 3.5 | Comparison of bacterial and fungal microbiota structure within and among sampling locations

Bacterial species richness (Chao1) was highest in the nostrils compared to all other location metagroups, but it was only significant ($P < .05$) for the arytenoid and guttural pouch after correcting for multiple comparisons (Figure 3; Supplemental Table 4). The highest bacterial diversity (Shannon index) was observed in the deep nostrils and trachea (both proximal and distal), with significantly higher diversity in the trachea compared to the arytenoid and both oral locations (Figure 3; Supplementary Table 5). Fungal species richness and diversity was highest in the nostrils compared to all other location metagroups, but the results were not significant ($P \geq .05$) for the hard palate and floor of the mouth after correcting for multiple comparisons (Figure 4; Supplemental Tables 6 and 7).

Bacterial community composition was dissimilar between all location metagroups ($P < .05$) except for between the pharynx and proximal trachea ($P = .2$), but after correcting for multiple comparisons the pharynx also was not dissimilar to the distal trachea ($P = .06$; Supplemental Table 8). The distal trachea was most similar to the pharynx (R statistic = .14), but the pharynx was more similar to the proximal trachea (R statistic = .04) than any other sampling location (Figure 5). Significant ($P < .05$) overlap was noted among multiple sampling location fungal communities (Supplemental Table 9). After correcting for multiple comparisons, the pharynx was only dissimilar to the nostrils and hard palate ($P = .04$). Notably, the pharynx, arytenoid, proximal trachea, and distal trachea were all very similar to each other

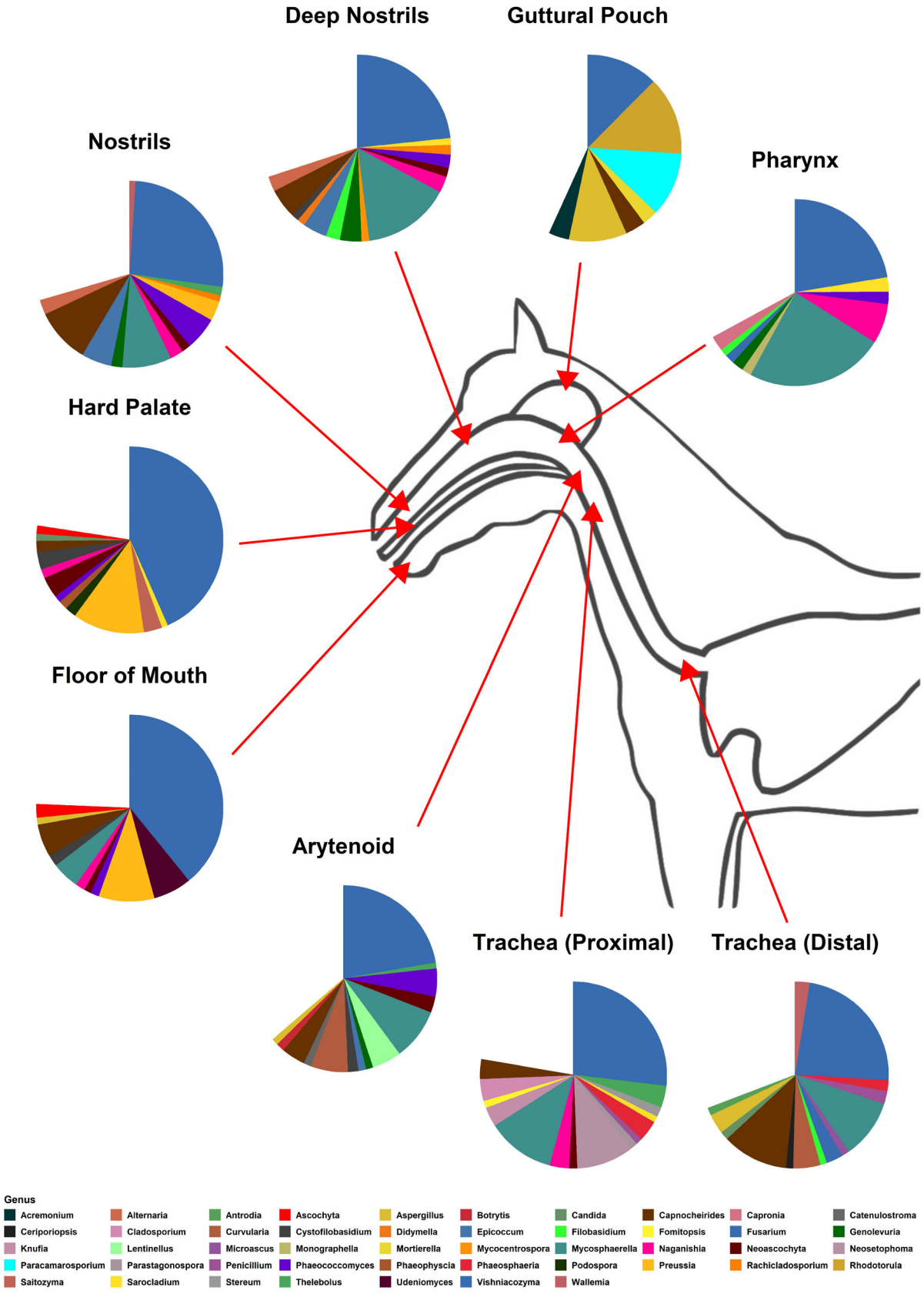


**FIGURE 2** Mean relative abundance of fungus present at ≥1% abundance at the genus level of different upper and lower respiratory fungal communities for 11 healthy horses

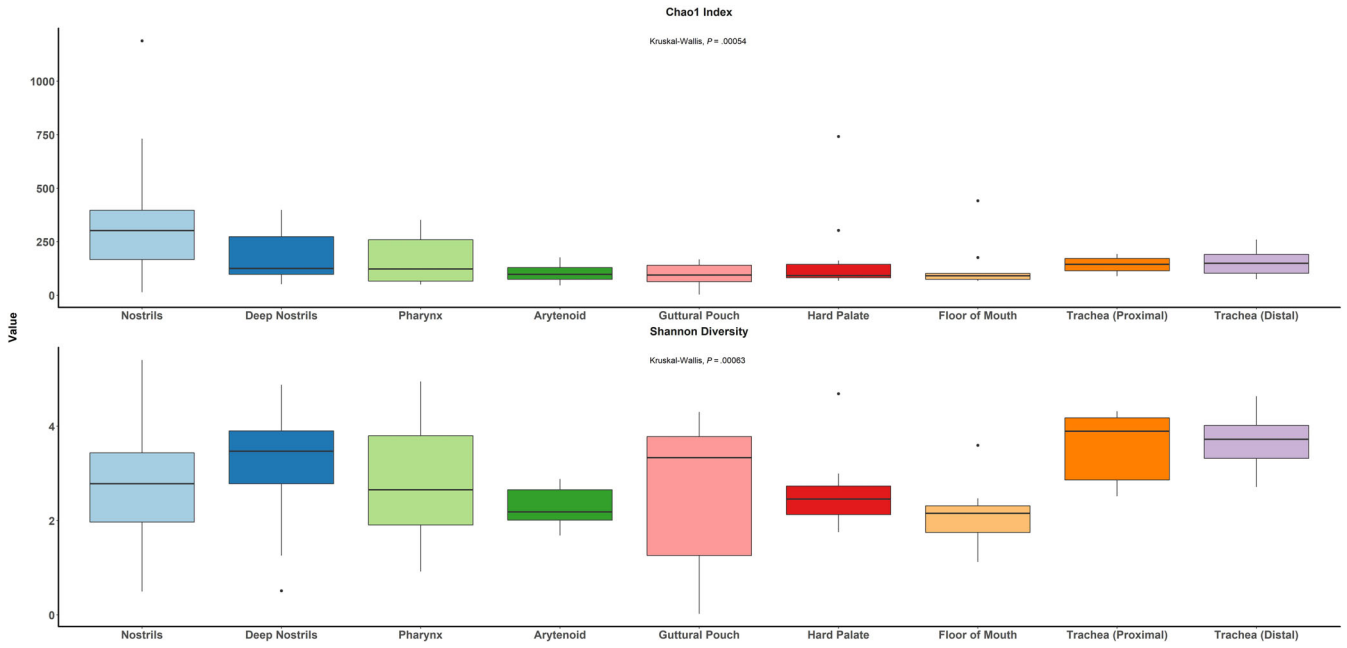


**FIGURE 3** Species richness (Chao1) and diversity (Shannon index) of different upper and lower respiratory bacterial communities for 11 healthy horses

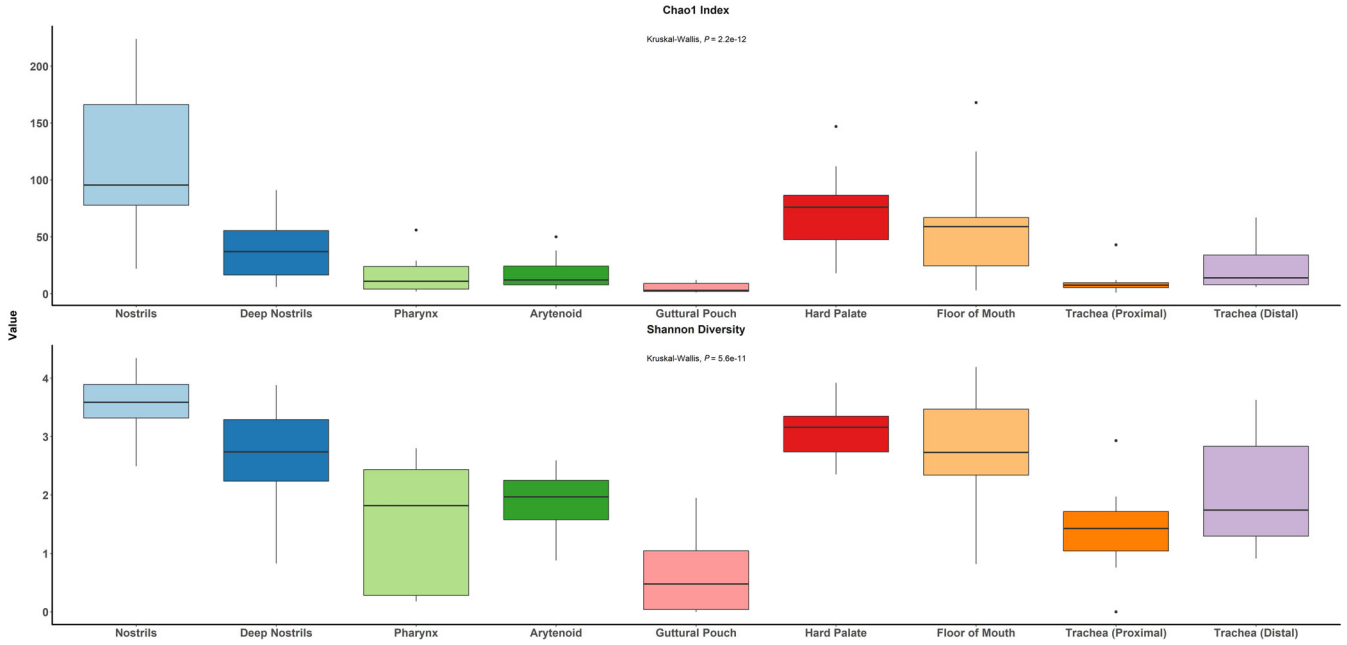


**FIGURE 4** Species richness (Chao1) and diversity (Shannon index) of different upper and lower respiratory fungal communities for 11 healthy horses

(Figure 6). All comparisons among these locations yielded R statistics <0.

## 4 | DISCUSSION

We described and compared the bacterial and fungal microbiota of different niches present along the upper and lower respiratory tract of healthy horses. Community composition and diversity differed by sampling location, although substantially more spatial heterogeneity was observed in bacterial composition as compared to fungal communities. A variety of bacterial genera contributed to these differences, including *Streptococcus*, *Nicoletella*, *Gemella*, *Actinobacillus*, and *Moraxella*. We showed that the bacterial and fungal microbiotas of the pharynx were compositionally more similar to the proximal and distal trachea than any other sampling site in the upper respiratory tract.

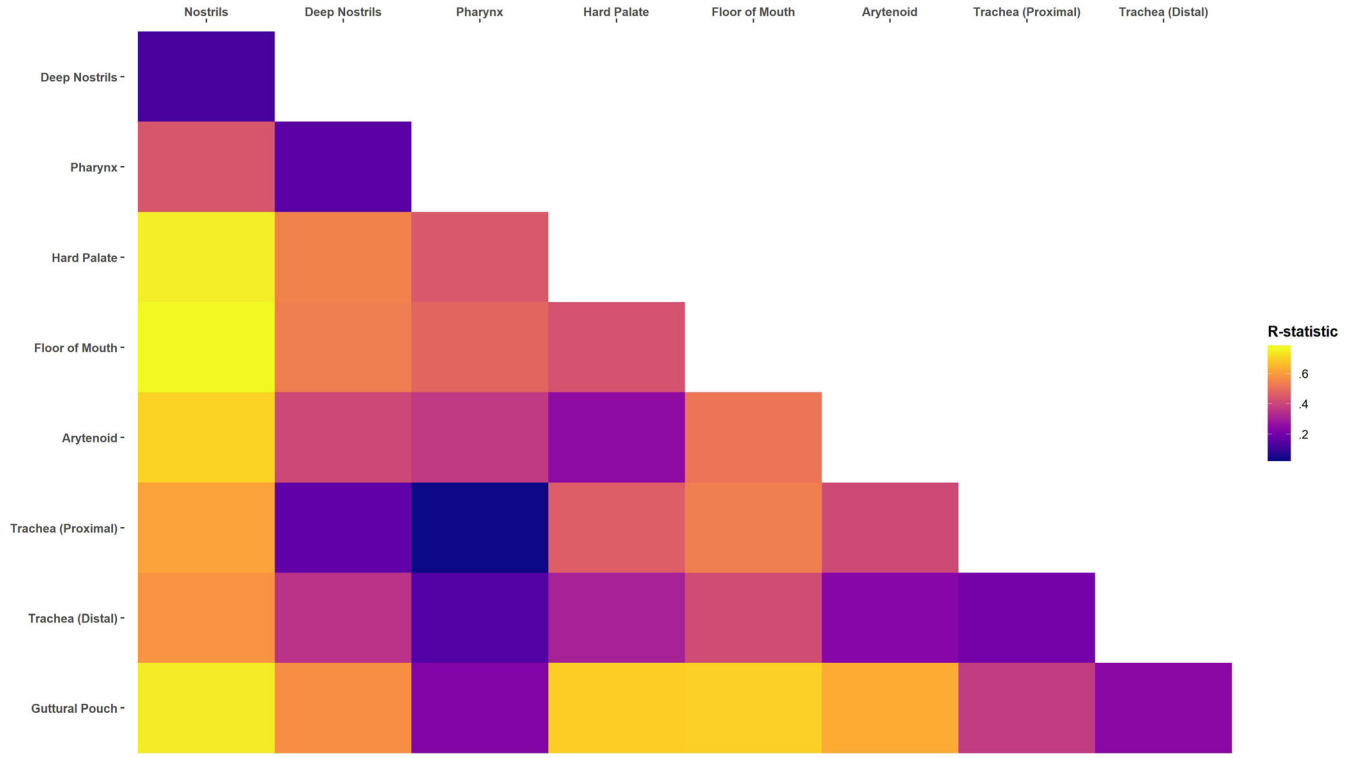


**FIGURE 5** Analysis of similarities results for pairwise comparisons of different upper and lower respiratory bacterial communities for 11 healthy horses

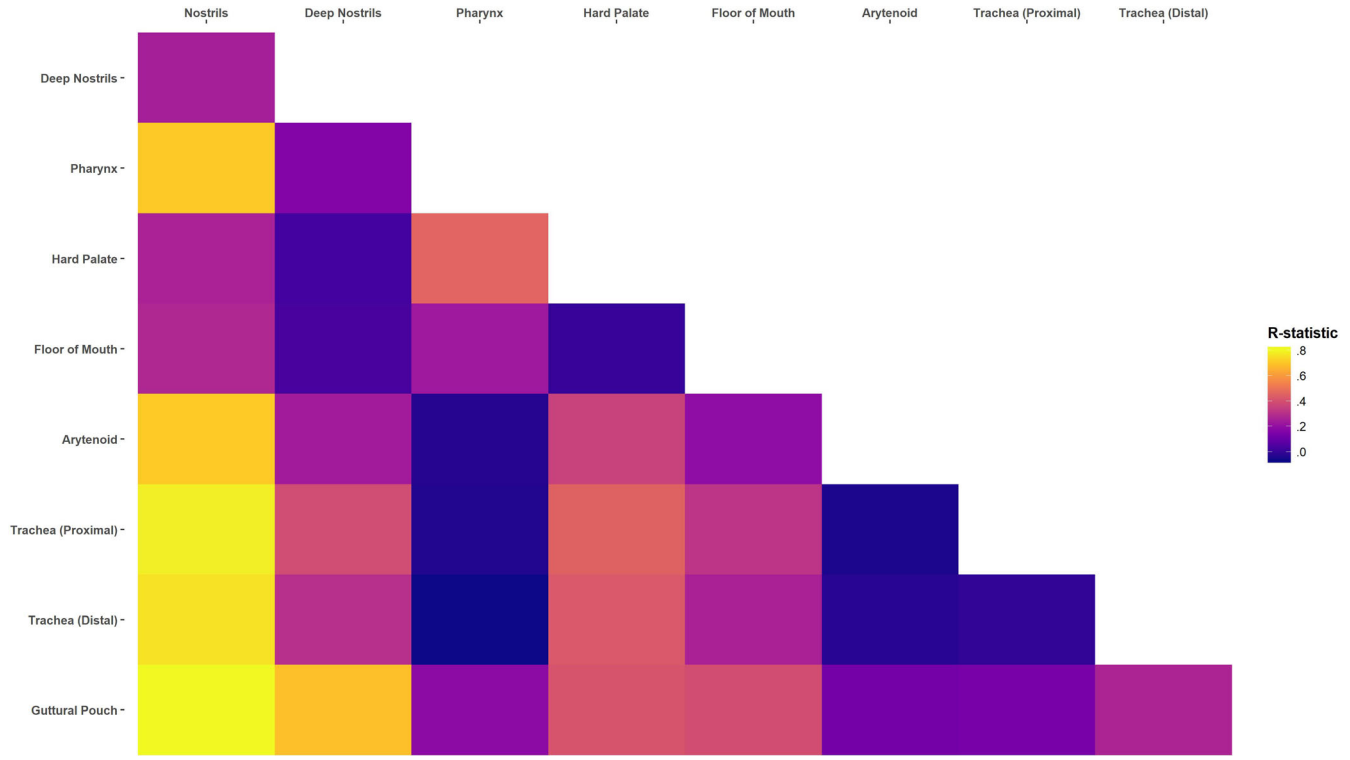


**FIGURE 6** Analysis of similarities results for pairwise comparisons of different upper and lower respiratory fungal communities for 11 healthy horses

This finding indicates that the pharynx potentially is a major source of microbes that translocate to the lower respiratory tract and is an important location that should be investigated when performing respiratory microbiota research in horses.

The characterization of distinct niche bacterial and fungal microbiotas present throughout the upper and lower respiratory tract of healthy horses is a novel finding. Differences in community structure between the upper and lower respiratory tract microbiotas have been reported in both healthy horses and those with lower airway inflammation.[8,9,15] However, these studies have only investigated a limited number of locations, comparing the pulmonary (BAL), oral, and nasal microbiomes (flush samples),[9] nasal swabs with transendoscopic tracheal aspirates,[15] or nasopharyngeal swabs with percutaneous transtracheal washes.[15] Our findings concur with these reports, as well as investigations in humans[12,13] and ruminants,[20,34] which reported distinct bacterial niches and spatial variation.

Recently, the topography of the respiratory tract microbiota of cattle was characterized. Consistent with our findings, discrete sampling locations had different microbiota composition,[20] which can be attributed to physiological and biochemical differences, including pH, $CO_2$ concentration, temperature, epithelial cell types, mucosal thickness, and luminal immune cells, throughout the respiratory tract.[35-37] In healthy cattle, the nasopharyngeal microbiota is most similar to the lung.[20] Similarly, we showed that the microbiota of the pharynx was more compositionally similar to the proximal and distal trachea than any other sampling site in the upper respiratory tract. We also found significant compositional overlap in the fungal microbiota among different niches of the respiratory tract, with the pharynx, arytenoid, proximal trachea, and distal trachea having no significant difference among location metagroups. Indeed, all comparisons among these locations yielded R statistics <0, which suggests more difference in community composition among samples within locations rather than among locations (i.e., differences among horses were larger than differences among various niches in the respiratory tract within the same horse).

Despite finding significant variation in the microbiota among sampling locations, we found little variation between anatomically similar locations (ie, left and right nostrils, left and right deep nostrils). This aspect was a strength of our study design because rather than assuming both nostrils to have identical community structure, we obtained bilateral nasal and deep nasal swab samples. Upon initial analysis, we confirmed that no significant difference existed between the 2 sides and then combined them as a single site for subsequent analysis. Our results were consistent with findings bilaterally in both humans[36] and cattle.[20]

In healthy humans, it appears that the nasal microbiota contributes little to the composition of the lung microbiota. Rather, the oropharynx shares most similarity with the lung community structure.[12,13] Given that horses are obligate nasal breathers, it was interesting to find that the largest bacterial diversity occurred in the deep nostrils and trachea (both proximal and distal), with significantly higher diversity in the trachea compared to the arytenoid and both oral locations. Similar to cattle, the oral microbiotas had less compositional overlap with the lung than with the nasopharynx.[20] However, despite the horse being an obligate nasal breather, microbial communication does seem to occur between the respiratory and gastrointestinal systems. Exacerbation of asthma in horses has been reported to alter adaptation of the fecal microbiota to changes in environment and diet,[11] but the clinical relevance of this finding remains to be determined.

An essential management component for the prevention and treatment of chronic airway inflammation in horses is the environment, in particular minimization of respirable particulate matter.[38,39] It is unknown whether airway dysbiosis perpetuates respiratory inflammation or occurs secondary to a chronic inflammatory process disrupting the healthy airway.[40] Our study therefore sought to characterize the topography of the respiratory, oral, and guttural pouch microbiotas in the healthy horse to better understand the communities present at various discrete locations in the respiratory system. However, all horses enrolled in our study resided on a single property and were fed the same feed. Although this study design enables comparison of interindividual variation at sampling locations while minimizing cofounding factors, considerable care should be taken when extrapolating findings to the horse population in general. Given the microbial communication between the respiratory and gastrointestinal systems, it is likely that changes in environment and diet impact composition of the respiratory microbiome.

Although characterizing the community structure of the healthy airway of horses was our primary aim, investigation of pathogenic species commonly associated with respiratory diseases of horses was also of interest. Alterations in the airway microbiota of horses have been reported in association with asthma,[8,9] and significant increases in *Streptococcus* spp., *Actinobacillus* spp., *Acinetobacter* spp., and *Mycoplasma* spp. previously have been associated with mild asthma.[8,41,42] Bacterial overgrowth of potential pathogens however is not a common finding associated with chronic airway inflammatory disease.[10] Dysbiosis of the lower airways has been suggested to result either from chronic inflammation or drug administration or to contribute to the perpetuation of lower airway inflammation and clinical signs.[10] We did not detect *Streptococcus zooepidemicus* in any sample. *Streptococcus equi* subsp *equi* was present in the nostrils, pharynx, and arytenoid of 1 horse, the distal trachea of another horse, and the arytenoid of a third horse, but none of these were in high abundance and therefore are not indicated in the abundance figures. We did not find *S. equi* subsp *equi* in any guttural pouch samples. *Rhodococcus* was identified in several different sampling locations in multiple horses, but because the average abundance was <1%, it was not reported in Figure 1. One horse had 11.65% *Rhodococcus* in its deep left nostril and 4.24% in its distal trachea. We also identified *Aspergillus fumigatus* in the pharynx of 1 horse at 0.45% relative abundance.

*Nicoletella semolina*, a *Pasteurellaceae*, is commonly found in the airways of horses,[43] and has been isolated from horses both with and without respiratory disease.[44,45] It has been associated with tracheal inflammation using culture based techniques,[46] and has been postulated to be an opportunistic pathogen like other species of the *Pasteurellaceae*.[47] However, it has not been found to be significantly

increased in asthmatic horses during exacerbation,[43] has been isolated at similar frequencies in healthy horses and those with respiratory disorders,[45] and the high prevalence of *Nicoletella* observed in our study of healthy horses using high-throughput sequencing suggests that it is primarily a commensal of the respiratory tract. However, its potential contribution to the perpetuation of airway dysbiosis requires further investigation.

A higher prevalence of potential pathogens may have been present than was identified. A limitation of 16 S sequencing is the ability to accurately identify microbes constituting the microbiota to a clinically relevant taxonomic level.[48] Future studies might consider using whole genome shotgun sequencing for enhanced detection of bacterial species, increased detection of diversity, and improved accuracy of species detection.[48] The unclassified sections shown in Figures 1 and 2 as uncolored sections of the pie graphs represent bacteria and fungi that were classified at the genus level but had an average relative abundance of <1%. Another limitation of our study design is that it was not adequately powered to analyze bacterial and fungal cooccurrence. We obtained a maximum of 11 samples for any given anatomical sampling location, which was not enough for patterns to emerge in the rudimentary analysis performed (not reported). Future studies should compare the fungal and bacterial communities of the pharynx, nostrils, and lungs to determine any patterns that could arise. A strength of our study was the rigorous negative controls that were included. Potential for contamination between upper and lower respiratory sampling locations was minimized by using a sterilized endoscope and custom protected specimen brushes with a cellulose plug and triple-sheathed catheters. Sample collection, DNA extraction, and DNA amplification and sequencing techniques did not introduce significant contamination, as evidenced by the negative control samples that were included.

## 5 | CONCLUSIONS

The bacterial and fungal microbiotas of the pharynx were more similar to the proximal and distal trachea than any other sampling site in the upper respiratory tract. This finding indicates that the pharynx is potentially a major source of microbes that translocate to the lower respiratory tract and an important location that should be investigated when performing respiratory microbiota research in horses. Future studies that investigate whether biomarkers of respiratory disease can be reliably detected in nasopharyngeal swab samples are warranted.

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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

This study was conducted in strict accordance with the recommendations of the Canadian Council of Animal Care. The University of Calgary Veterinary Animal Care Committee approved the study (#AC17-0036) and written consent was obtained from the horses' agent.

### HUMAN ETHICS APPROVAL DECLARATION

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

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## SUPPORTING INFORMATION

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

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