Comparison of sampling and diagnostic techniques for recovery of *Mannheimia haemolytica* and respiratory microbiome characterization from feedlot cattle

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Abstract

1.1 Background:

Bovine respiratory disease (BRD) is caused by interactions among host, environment, and pathogens. The current standard for antemortem pathogen identification in cattle with BRD is deep-guarded nasopharyngeal swabbing, which is challenging, costly, and waste generating. The objective was to compare the ability to recover *Mannheimia haemolytica* and compare microbial community structure using 29.5 inch (74.9 cm) deep-guarded nasopharyngeal swabs, 16 inch (40.6 cm) unguarded proctology swabs, or 6 inch (15.2 cm) unguarded nasal swabs when characterized using culture, real time-qPCR, and 16S rRNA gene sequencing. Samples for aerobic culture, qPCR, and 16S rRNA gene sequencing were collected from the upper respiratory tract of high-risk cattle 2 weeks after feedlot arrival.

1.2 Results

There was high concordance of culture and qPCR results for all swab types (results for 77% and 81% of samples animals completely across all 3 swab types for culture and qPCR respectively). Microbial communities were highly similar among samples collected with different swabs types, and differences identified relative to treatment for BRD were also similar. Positive qPCR results for *M. haemolytica* were highly concordant (81% agreed completely), but samples collected by deep-guarded swabbing had higher C_t values (Kruskal-Wallis analysis of variance on ranks, $P < 0.05$; Dunn-test for pairwise comparison with Benjamini-Hochberg correction, $P < 0.05$) and lower frequency of positive compared to nasal and proctology swabs (McNemar's Chi-square test, $P < 0.05$).

1.3 Conclusions

Though slight differences existed among different types of swabs collected from individual cattle, nasal swabs and proctology swabs offer comparable results to deep-guarded nasopharyngeal swabs when identifying and characterizing *M. haemolytica* by culture, 16S rRNA microbiome, and qPCR.

2 Background

Bovine respiratory disease (BRD) is one of the leading causes of morbidity and mortality in cattle, leading to significant economic loss in feedlot operations (Griffin, 1997). BRD is a complex disease involving the interaction between environmental factors, host immunity, and microbial pathogens (Taylor et al., 2010; Griffin et al., 2010). Though BRD is a multifactorial disease, the involvement of bacterial pathogens leads to antimicrobials being the primary treatment for suspected BRD, as well as being used for disease control and prevention (USDA, 2012; Ives & Richeson, 2015). Widespread antimicrobial use to treat and control BRD has led to concerns over increased prevalence of antimicrobial resistance in isolates of BRD pathogens (Lubbers & Hanzliek, 2013; Stanford et al., 2020).

The most frequently isolated bacterial pathogen in feedlot cattle with BRD is *Mannheimia haemolytica* (*Mh*) (Klima et al., 2014). Though *Mh* can be found in healthy animals (Confer, 2009), isolation of *Mh* from the upper respiratory tract in groups of animals affected by BRD is associated with isolation of *Mh* in the lungs (Godhino et al., 2007). While aerobic culture is a mainstay of diagnostic procedures used to detect *Mh* which allows characterization through *in vitro* susceptibility testing and whole genome sequencing, use of culture-independent molecular techniques is beneficial to help decrease time for diagnosis, and to enhance detection of pathogens that are difficult to culture (Loy et al., 2018). Real-time qPCR has been reported for *Mh* (Loy et al., 2018), which allows for identification and quantification of *Mh* within a sample without the need for culture. Further, it is becoming increasingly important to study pathogens within the context of entire microbial communities, as is possible through metagnomic sequencing (Holman et al., 2015; Holman et al., 2018; McMullen et al. 2020).

Perhaps the most common technique for antemortem detection of BRD pathogens is the use of long (29.5-inch; 74.9 cm) double-guarded swabs originally designed for uterine culture in mares. A perceived advantage of using these swabs is the ability to sample deep in the nasopharynx with less likelihood of contamination from the nares and rostral airways, allowing easier identification of important respiratory bacteria with double-guarded swabs compared to short (6-inch) nasal swabs, which are commonly used to sample cattle for respiratory viral pathogens. In some studies, nasopharyngeal culture showed higher agreement with lower airway sampling in calves affected by BRD than culture of the nasal passages (Godhino et al., 2007). However, other studies have shown high agreement between both nasal and nasopharyngeal culture of *Mh* when compared to lower airway culture in acutely ill dairy and beef calves (Doyle et al., 2017; DeRosa et al., 2000). When evaluating the upper respiratory microbiome of healthy cattle, greater agreement has been shown between the nasopharyngeal bacterial community and the lung community than between the nasal passages and lungs (McMullen et al., 2020). However, deep nasopharyngeal sampling with the double-guarded swabs requires more technical skill and knowledge of anatomy than sampling with nasal swabs, and frequently requires firm restraint of the head to be accomplished. One possible alternative to these sampling techniques is a long (16-inch; 40.6 cm) large-tipped swab, designed for human proctological sampling. These swabs are long enough to reach the nasopharynx but more flexible than double-guarded swabs, potentially easing passage through the upper airways. The much larger swab head on the proctology swab (14 mm diameter x 35 mm length) also has the potential to collect a larger volume of respiratory secretions when compared to the smaller swab heads (5 mm diameter x 15 mm length) of the both long double-guarded swabs and short (6-inch; 15.2 cm) swabs commonly used for collection of microbial samples (Figures S1-S2). While either 16-inch proctology swabs or 6-inch nasal swabs are easier to use and less expensive, relative to the 29.5-inch double guarded swabs, the degree to which results for these 3 swab types agree has not been reported.

The objective of this study was to compare the use of long double guarded swabs of the nasopharynx, short swabs of the nasal passage, and long proctology swabs of the nasal passage and nasopharynx for the recovery and characterization of *Mh* in feedlot cattle when evaluated using culture for isolation and susceptibility testing, and culture-independent methods (qPCR, 16S rRNA gene sequencing). Importantly,

this study addresses the use of these sample and testing strategies within the context of BRD and *Mh* culture status.

3 Methods

Study population and sampling

Two groups each consisting of 60 beef-type steers and bulls were purchased from a livestock auction market located in central Texas, shipped to the West Texas A&M University Research Feedlot on May 14 and May 21, 2020, where they were enrolled in this study (n=120). Upon arrival at the feedlot, cattle received an ear tag with an individual identification number and were processed following standard practices of many feedlots. Briefly, tildipirosin (Zuprevo, Intervet Inc., Summit, NJ), a long-acting macrolide, was administered to every animal at 4 mg/kg subcutaneously for BRD metaphylaxis. Animals were vaccinated against clostridial (Calvary 9, Merck Animal Health, Omaha, NE) and respiratory bacterial pathogens (Once PMH, Merck Animal Health, Omaha, NE), given a zeranol growth implant (Ralgro, Merck Animal Health, Summit, NJ) and given anthelminthic therapy with albendazole (Valbazen, Zoetis, Kalamazoo, MI) and ivermectin with corsulon (Ivermectin Plus, Durvet, Inc., Blue Springs, MO). Animals were also tested to identify any animals persistently infected with bovine viral diarrhea virus (BVD-PI), and any BVD-PI animals were removed from the study. Bulls were castrated and given meloxicam at 1.1 mg/kg orally the day following metaphylaxis, vaccine, and anthelminthic administration (Table S1).

Pens were monitored daily by trained feedlot personnel to identify animals with BRD, and animals were assigned a BRD clinical score of 0-4 based on visual signs of disease (Perino & Apley, 1998; Table S2). Cattle were removed from pens if they had a clinical score of $\geq$ 2. Animals were classified as BRD positive if they had a rectal of temperature $\geq$ 40°C and/or a clinical score of $\geq$ 3. Animals were treated for BRD with antimicrobials based on the feedlot protocol (Table S2). The animals were on feed for 213 and 255 days for group 1 and group 2, respectively.

On day 14 after arrival, when a high prevalence of *M. haemolytica* shedding was expected (Woolums, 2018), cattle were processed through a chute, where they were weighed and restrained for sampling. Six different nasal and nasopharyngeal samples (three from the left and three from the right) were obtained as previously described (Godinho et al., 2007). Briefly, the external nares were cleaned with a paper towel to remove superficial secretions and dirt, and both internal nasal passages were then swabbed with the 6-inch (15.2 cm) rayon fiber nasal swabs (NS) (SP130D, Starplex Scientific Corporation, St. Louis, MO). After collecting nasal swabs, the 16-inch (40.6 cm) rayon fiber proctology swab (PS) (816-100, Puritan, Guilford, ME) or the 29.5 in (74.9 cm) cotton fiber deep-guarded swab (DG) (E9-5200, Continental Plastic, Delavan, WI) were used to sample the left and right nasal and nasopharyngeal passages; the order of collection of the proctology and deep-guarded swabs was randomized. All swabs collected via the left nostril were placed in modified Amies transport media (Starplex Scientific Corporation, St. Louis, MO) and used for aerobic bacterial culture, identification, and antimicrobial susceptibility testing. All swabs collected via the right nostril were placed in 100% ethanol and were used for DNA extraction and

subsequent analyses with 16S rRNA gene sequencing and qPCR. All samples were kept on ice and transported to the laboratory for processing immediately after collection.

The unique animal ID was incorrectly recorded for two enrolled animals, which prevented extraction of corresponding data regarding animal weight and health records. Additionally, three swab samples intended for DNA analyses (two deep-guarded swab sample and one proctology swab sample), and one deep-guarded swab intended for culture were damaged during transport to the laboratory and could not be analyzed. DNA extraction from one deep-guarded swab sample failed, as well. These data are therefore missing from the results.

3.1 Culture, microbial identification, and susceptibility testing

Swabs collected in modified Amies media were directly streaked onto one quadrant of a plate of tryptic soy agar (TSA) with 5% sheep blood (Remel, Lenexa, KS), and sterile disposable loops (Remel, Lenexa, KS) were used to streak the rest of the plate for bacterial isolation. Plates were incubated at 37°C with 5% CO_2. At 24 and 48 hours of incubation, plates were monitored for growth consistent with *Mh* (2-3 mm, round, raised, light-grey, smooth, shiny colonies with faint β hemolysis). If colonies consistent with such growth were present, catalase, oxidase, and indole tests were performed. If preliminary biochemical tests were consistent with *Mh* (catalase-positive, oxidase-positive, and indole-negative), a single colony was randomly selected by choosing the *Mh*-like colony closest to a mark made at a random position on the bottom of the media plate and subcultured onto a new blood agar plate and returned to the incubator at the above conditions. After 24 hours, subcultures were monitored for colony phenotype and biochemical tests consistent with *Mh*. If present, 5-7 colonies were randomly selected with a sterile disposable loop and suspended into 1.5 mL of Brain Heart Infusion broth (B-D, Franklin Lakes, NJ) and 30 % glycerol (Thermofisher, Waltham, MA). The same loop was then used to streak one half of another blood agar plate which was then incubated as described above for 24 hours then shipped on ice to University of Nebraska-Lincoln Veterinary Diagnostic Center (UNL-VDC) to confirm identity and for antimicrobial susceptibility testing. Primary plates with no suspected *Mh* growth at 48 hours were considered negative for *M. haemolytica.*

At UNL-VDC, a single colony from the shipped plate was subcultured overnight on blood agar to ensure pure growth which was then used to confirm *Mh* identification and antimicrobial susceptibility testing. Matrix assisted laser desorption-ionization time-of-flight mass spectroscopy (MALDI-TOF) was used to confirm *Mh* identity as well as MALDI-TOF biomarker based genotyping of *Mh* isolates (Loy & Clawson, 2017).

Antimicrobial susceptibility testing was performed at UNL-VDC using semi-automated broth microdilution via the Sensititre system (ThermoFisher, Waltham, MA) and the bovine/porcine panel containing gamithromycin and tildipirosin (BOPO7F Vet AST Plate, ThermoFisher, Waltham, MA). Results were interpreted according to breakpoints for *Mh* in BRD from the Clinical and Laboratory Standards Institute

(CLSI, 2018). Isolates were characterized as multidrug resistant (MDR) if they were not susceptible to antimicrobial(s) from ≥ 3 antimicrobial classes (Sweeney et al., 2018). Because the concentration range for ampicillin on the BOPO7 plate does not include CLSI breakpoints, only minimum inhibitory concentration (MIC) was recorded, and ampicillin resistance classification was not included in determination of isolates as MDR.

DNA extraction

Metagenomic DNA was isolated from swab samples using a QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany). Following isolation, DNA was quantified (ng/uL) using a Qubit Flex fluorometer (ThermoFisher, Waltham, MA).

3.2 qPCR Sample Preparation and Reaction Conditions

From the DNA extracted DNA, two 400 ng DNA aliquots were sent to Mississippi State for qPCR. Samples from one aliquot were diluted in Low-Tris TE buffer to an estimated final concentration of 8 ng/µL. Final concentrations were measured on Qubit 4 fluorometer, and the mean DNA concentration was 6.73 ± 2.00 ng/µL.

All reactions, including samples and controls, were run in triplicate using a QuantStudio 3 Real-Time PCR instrument (Thermofisher, Waltham, MA) and the following reaction mixture: 40 ng (mean=41.5 ng, SD=4.7 ng) of metagenomic sample DNA, 10 µL of PerfeCTa SYBR Green FastMix Low ROX (Quantabio, Beverly, MA), 1 µL each of F and R primer for *Mh* leukotoxin D gene *(lktD)* (F-CTGCAACAAAGCCGATATCTTT, R- TACGACTGCTGAAACCTTGAT) (Loy et al., 2018), and molecular grade H_2O to reach a final volume of 20 µL. Five positive controls of triplicate 10-fold dilutions DNA extracted from pure growth of *Mh* confirmed by Sensititre GNID (Thermofisher, Waltham, MA) were included on each 96-well MicroAmp plate (4316813, Thermofisher, Waltham, MA). Also included on each plate, negative controls consisting of reaction mixture of molecular grade H2O in place of template DNA. Additionally, controls with no primer added, and no master mix controls added were included. Amplification occurred under the following conditions: 95°C for 5 minutes, then 45 cycles of 95°C for 15 seconds, and 60°C for 45 seconds.

Cycle threshold (C_t) was determined using QuantStudio Experiment Design and Analysis Software, then reviewed manually. Melt curves were used to check reaction specificity. Samples containing triplicates with C_t values differing by more than two were removed, and the remaining technical duplicates were used to determine mean C_t. In order to maximize agreement between qPCR and bacterial culture, culture was used as a gold standard, and qPCR sensitivities, specificities, and accuracies were calculated at a range of C_t (25-40; Additional File 1). Samples with $C_t \leq 30$ were considered positive for *Mh*. For quantitative statistical analyses, samples with no amplification were considered to have C_t=50, as done previously (Loy et al., 2018).

3.3 16S rRNA library preparation, and sequencing

Preparation of libraries for sequencing of the V3-V4 region of 16S rRNA was conducted as previously described (Illumina, 2013). The V3-V4 region of the 16S rRNA gene was amplified using the 341F (5' – CCTACGGGNGGCWGCAG – 3') and 805R (5' – GACTACHVGGGTATCTAATCC – 3') primer pair (Integrated DNA Technologies, Inc, Coralville, IA) and sequencing libraries were prepared using the Nextera IDT kit (Illumina, San Diego, CA). The resulting pooled amplicon library was sequenced on an Illumina NovaSeq instrument using paired-end chemistry (2 x 250bp) at the University of Colorado Anschutz Medical Campus' Genomics and Microarray Core.

Bioinformatics and statistics

Demultiplexed paired-end reads generated from 16S rRNA gene sequencing were imported in QIIME2 version 2020.11 (Bolyen et al., 2019). Amplicon sequence variants (ASVs) were generated using DADA2 (Callahan et al., 2016), which was also used to filter reads for quality, remove chimeric sequences, and merge overlapping paired-end reads. Forward and reverse reads were truncated at 248 bp and 250 bp, respectively. Taxonomy was assigned using a Naïve Bayes classifier trained on the Greengenes version 13_8 99% OTUs database (DeSantis et al., 2006), where sequences had been trimmed to include only the base pairs from the V3-V4 region bound by the 341F/805R primer pair. Reads mapping to chloroplast and mitochondrial sequences were filtered from the representative sequences and ASV table using the 'filter-seqs' and 'filter-table' functions, and a midpoint-rooted phylogenetic tree was generated using the 'q2-phylogeny' pipeline with default settings, which was used to calculate phylogeny-based diversity metrics. Data and metadata were then imported into phyloseq (McMurdie & Holmes, 2013) using the 'import_biom' and 'import_qiime_sample_data' functions and merged into a phyloseq object. The proportion of reads mapped to each taxonomic rank can be found in Table S2. ASV counts for each sample were then normalized using cumulative sum scaling (Paulson et al., 2013) and beta-diversity was analyzed using generalized UniFrac distances (Lozupone et al., 2011, Chen et al., 2012). From these distances, principal coordinates analysis (PCoA) was performed and plotted, and a permutational multivariate analysis of variance (PERMANOVA) was used to test for significant differences in community structure using the vegan (Oksanen et al., 2019) and pairwiseAdonis (Arbizu, 2017) packages. To ensure significant differences were not the result of unequal dispersion of variability between groups, permutational analyses of dispersion (PERMDISP) were conducted for all significant PERMANOVA outcomes using the vegan package. Further, the relative abundances of ASVs within each sample were calculated and plotted using phyloseq. Differences in relative abundance were tested using a pairwise Wilcoxon rank-sum test with a Benjamini-Hochberg correction for multiple comparisons in R version 3.6.0.

Summary statistics of arrival weight, number of animals treated for BRD overall and number treated at time of sampling, and days on feed (DOF) until their first BRD treatment were calculated using R version 4.0.3 (R Core Team, 2020). Comparisons between the two sampling groups were made using Wilcoxon rank-sum test for continuous outcome variables (arrival weight and DOF until first treatment) and Chi-square test for binary response variables (treatment for BRD during feeding period and treatment for BRD at the time of sampling) in the rstatix package in R (Kassambra, 2021). Cochran's Q test was used to

compare isolation of *Mh* by swab type using SAS software v 9.4 (SAS Institute, Cary, NC). If differences were found using Cochran's Q test, pairwise comparisons using McNemar's Chi-square test were performed with the rstatix package.

Comparisons of C_t between swab types and *Mh* culture status were assessed using Kruskal-Wallis analysis of variance by ranks using rstatix (Kassambra, 2021). If differences in C_t were found, pairwise comparisons were tested with a Dunn test with Benjamini-Hochberg correction for multiple comparisons in the rstatix package (Kassambra, 2021). Differences in qPCR positive ($C_t \leq 30$) and negative ($C_t > 30$) rates between swab types were tested using Cochran's Q test in SAS software v 9.4 (SAS Institute, Cary, NC), with *post hoc* comparisons tested with pairwise McNemar's Chi-square in rstatix.

4 Results

4.1 Cattle population

At the time of sampling (14 DOF), the mean body weight of all animals was 261.2 kg (SD = 12.2 kg). A total of 36% (43/118) of calves were treated for BRD at least once during the feeding period (Additional File 2). There was a greater number of calves treated for BRD in the first group than the second group of calves, with 50.0% (30/60) and 22.4% (13/58) treated respectively (Table 1; Chi-square test, P=0.003). The median day until first treatment for all sampled calves was 10 days, and there was not a statistically detectable difference between groups (Wilcoxon rank-sum test, p=0.188). Only one calf received his first antimicrobial treatment for BRD after day 40 (Figures S3-S5).

Table 1
Descriptive statistics of weight, number of animals treated, and days to first treatment. Values in the same column width different superscripts indicate significant difference (Chi-square; ab, p=0.003)

Group	Animals (n)	Mean weight (kg)	SD weight (kg)	Animals Treated Total (n)	Median days to first treatment	Range of days to first treatment
Group 1	60	260.1	11.7	30^{a}	9.5	6-116
Group 2	58*	262.3	12.8	13^{b}	13	6-22
All	118*	261.2	12.2	43	10	6-116

* Unique animal IDs were mis-recorded for two cattle preventing the ability to link feedlot records regarding weight and BRD occurrence.

4.2 Culture results and isolate characterization

Overall, *Mh* was isolated by culture from 67.5% (81/120) of cattle: 55.0% (67/119) of DG, 56.3% (66/120) of NS, and 56.7% (68/120) of PS, with significantly higher frequency of *Mh* isolation in group 1 than group 2 for each swab type (Table S3; Chi-square test, $P<0.05$). All 201 *Mh* isolates were identified as genotype 2, and nearly all isolates were MDR (98.5%, 198/201; Additional Files 2-2 & Figure 1). Three isolates were pansusceptible, and these isolates were from the different swabs from the same animal (Animal 2490, Figure 1). Frequency of *Mh* isolation was not statistically different among swab types (Table S3; Cochran's Q test, $p=0.86$). There was complete concordance in culture results for the 3 sampling methods for 77% of cattle (92/119); two concordant positive and 1 discordant negative result was found in 11% of cattle (13/119), and 1 discordant positive result was identified in the remaining 12% of cattle (14/119) (Table 2).

Table 2
Concordance of swab types for culture and qPCR with culture and qPCR pattern of swabs. Percentage is out of total swabs with results from all 3 swab types.

Concordance	Isolation Pattern (DG, NS, PS)	Culture Frequency	Culture Percentage	qPCR Frequency	qPCR Percentage
Full	YYY	53	77.3 %	53	81.0 %
	NNN	39		41	
Two Yes	YYN	3	10.9 %	1	12.9 %
	YNY	5		0	
	NYY	5		14	
Two No	NNY	4	11.8 %	1	6.0 %
	NYN	4		4	
	YNN	6		2	
	Total	119*	100 %	116*	99.9 %

*Samples from 1 swab for culture (DG) and 4 swabs for qPCR (3 DG and 1 PS) were damaged in transport or failed DNA extraction so samples from these animals were not used in concordance analysis. Abbreviations: DG, double-guarded swab; NS, nasal swab; PS, proctology swab; Y, culture/qPCR positive; N, culture/qPCR negative.

4.3 qPCR

Mh was detected by qPCR in 65.0 % (78/120) of all cattle: 48.7 % (57/117) of DG, 62.5 % (75/120) of NS, and 58.8 % (70/119) of PS (Table S4). *Mh* was identified in significantly fewer samples collected with DG swabs as compared to either NS and PS in both group 1 and group 2, with group 2 having significantly fewer animals *Mh* positive than group 1 for each swab (Table S4; McNemar's Chi-square, $P<0.05$). Similarly, C_t was significantly different among swab types (Figure 2; Kruskal-Wallis test, $P < 0.05$), and

median C_t for DG was significantly higher when compared to both NS and PS (Additional File 1 & Figure 2; pairwise Dunn test with Benjamini-Hochberg correction, P =0.011 & P=0.019, respectively). There was complete concordance among qPCR between swabs for 81.0 % of animals (94/116); two concordant positive and 1 discordant negative result was found in 12.9 % of animals (15/116), and 1 discordant positive result with 2 concordant negative results was identified in the remaining 6.0 % of animals (7/116) (Table 2). When evaluating the swabs' ability to identify *Mh* by qPCR in animals treated or not treated for BRD, there was significant difference in median C_t value in between swab types in animals not treated for BRD (Figure 3; Kruskal-Wallis analysis of variance on ranks, P=0.03); however, pairwise comparisons revealed no significant difference within BRD negative animals (Figure 3; pairwise Dunn test with Benjamini-Hochberg correction, P>0.05). When evaluating the effect of *Mh* culture on the ability to identify *Mh* by qPCR, swabs that came from animals who were culture positive had significantly lower median C_t compared to animals that were culture negative, and this was true for all swab types (Figures 4 & 5; Wilcoxon rank-sum test, P<0.05).

4.4 16S rRNA gene sequencing

4.1.1 Overall differences in microbial community composition

The effect of sample collection method (i.e., DG, NS, or PS) on microbial community composition was analyzed with PERMANOVA and Principal Co-ordinates Analysis. Based on generalized UniFrac values, microbial community structured differed significantly between samples collected with each swab type (Table S5; PERMANOVA; P-adj. < 0.05). However, PCoA illustrated that the community structure of samples collected with DG swabs was the most unique, while the amount of variation explained by collecting samples with NS versus PS was exceedingly small (>2%) and that those communities were very similar (Figure 6).

To further compare differences in microbial communities resulting from the three sampling methods, the relative abundance of phyla representing more than 1% of the overall community across all samples were compared. Except for the relative abundance of Proteobacteria in samples collected using NS and PS, there were significant differences in the relative abundances of all six phyla among the different sample types (Figure 6; pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction, P < 0.05). However, the prevalence of these six phyla followed the same order across all three swab types, with Tenericutes being the most abundant phyla followed by Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria.

4.1.2 Characterizing microbial shifts related to clinical BRD

Differences in microbial composition, as they related to the occurrence of BRD were visualized at the taxonomic level of order, based on the normalized proportion of ASVs within individual samples (Figure 7). Each swab type demonstrated a similar shift between BRD-negative and BRD-positive animals: an increased abundance of the order Mycoplasmatales coupled with decreases in abundance of Pseudomonadales, Clostridiales, and Bacteroidales. Of the six phyla representing greater than 1% of the overall microbial community, four differed significantly in abundance between BRD-negative and -positive animals when sampled using DG swabs. Only two phyla differed if samples were collected using NS, while four differed significantly when sampled with PS (Figure 8; Kruskal-Wallis analysis of variance on ranks, $P < 0.05$). All three collection methods demonstrated a significant difference in Tenericutes, which was the most abundant phylum across all samples and was almost exclusively composed of the order Mycoplasmatales. However, sample collection using NS was less effective in characterizing changes within less abundant phyla than samples collected using DG swabs or PS.

To illustrate potentially important differences among the different sample types, the relative abundances were further examined for six families (Mycoplasmataceae, Moraxelleceae, Ruminococcaceae, Lachnospiraceae, Chitinophagaceae, and Bacteroidaceae) and three genera (*Mannheimia, Pasteurella*, and *Histophilus*) that were differentially abundant between BRD-negative and -positive animals, or were of specific clinical interest. Generally, the same trend within these taxa was observed across samples collected with all three swab types. The family Mycoplasmataceae was in significantly higher abundance in BRD-positive animals when sampled with all three swab types (Figure 9A; Kruskal-Wallis analysis of variance on ranks; $P < 0.05$). Mycoplasmataceae was also overwhelmingly the most abundant ASV at the family level, representing over 50% of the total microbial population across all sample types and virtually 100% of all Tenericutes. Additionally, the relative abundance of the two genera of Mycoplasmataceae detected in this study were compared between BRD-positive and BRD-animals. *Mycoplasma* comprised the vast majority (> 97%) of Mycoplasmataceae, and was significantly higher in BRD positive animals in samples collected with any swab type (Figure S6; Kruskal-Wallis analysis of variances on ranks; $P > 0.05$). The relative abundance of *Ureaplasma*, which was greatest in samples collected with DG swabs, did decrease in BRD positive animals (Figure S6). However, because of its low abundance (~1%) and large variation among individual animals the difference was not significant within any swab type (Kruskal-Wallis analysis of variances on ranks; $P > 0.05$).

The families of Moraxellaceae (almost exclusively composed of the genus *Moraxella* and unclassified Moraxellaceae) and Chitinophagaceae demonstrated the largest decrease in abundance in BRD-positive animals (Figure 9B). The decrease in Moraxellaceae was only significant when sampled using DG swabs and PS, while Chitinophagaceae decreased significantly using all three swab types (Figure 9B; Kruskal-Wallis analysis of variance on ranks; $P < 0.05$). The families Ruminoccaceae, Bacteroidaceae, and Lachnospiraceae also decreased in BRD-positive animals, though the differences were smaller and largely only significant when samples were collected using PS (Figure 9B; Kruskal-Wallis analysis of variance on ranks; $P < 0.05$). Due to their clinical relevance, the relative abundance of the genera *Mannheimia, Pasteurella, Histophilus* and their family Pasteurellaceae were also compared between BRD-

negative and -positive animals, but there were no differences in abundances among any of the sampling methods (Figure S7; Kruskal-Wallis analysis of variance on ranks; $P > 0.05$).

4.1.3 Characterizing microbial shifts in *M. haemolytica* culture-positive animals

As *Mh* is widely considered one of the most important respiratory pathogens of cattle, the different sampling methods were compared regarding the ability to capture differences in microbial abundances between *Mh* culture-positive and culture-negative animals. ASVs associated with the genus *Mannheimia* represented an average of only 0.56% ± 0.19% (SEM) of the total microbial community in samples collected from *Mh* culture-negative animals, but significantly increased to an average abundance of 13.7% ± 1.22% (SEM) in culture-positive animals (Figure 10; Kruskal-Wallis analysis of variance on ranks; $P < 0.05$). While *Mannheimia* increased, both *Pasteurella* and *Histophilus* decreased significantly in abundance within *Mh* culture-positive animals for all sample types (Figure 10; Kruskal-Wallis analysis of variances on ranks; $P < 0.05$). However, the sample collection method (DG swab, NS, or PS) did not impact the abundance of Pasteurellaceae or *Mannheimia*, as there were no differences within animals of the same *Mh* culture status (Figure 10; pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction, $P > 0.05$).

5 Discussion

This unique study compared three sample collection methods (deep-guarded nasopharyngeal swabs, unguarded 16-inch proctology swabs, and unguarded 6-inch nasal swabs) to identify *Mh* and characterize changes in the microbial community structure within the context of bovine respiratory disease, using a combination of culture-dependent and culture-independent (16S rRNA gene sequencing and qPCR). The results were largely equivalent when comparing samples collected by DG, NS, or PS relative to the detection of *Mh* or characterization of the microbial community composition. This has important ramifications for researchers studying microbial communities of the upper respiratory tract of live cattle because of the significant logistical issues of sample collection under conditions of commercial cattle production. While there were slight differences among the sample types in statistical significance of the results, the trends in *Mh* detection and characterization of *Mh*/BRD-associated shifts in microbial communities were consistent regardless of sample collection method. As BRD is one of the leading causes of morbidity and mortality in cattle and one of the most common reasons for treatment with antimicrobial drugs (Griffin, 1997). Improving methods for investigating BRD pathogens within the context of entire microbial communities is critical to furthering our understanding of this disease, as well as efficiently conducting relevant surveillance. The results presented here provide researchers with justification for choosing a simpler sampling method to characterize bovine respiratory tract microbial communities and the pathogens playing important roles in BRD; however, it is important to note that the concordance was not perfect among sampling methods. Therefore, depending on the clinical or research questions of interest, production or management system of the animals, and disease severity or case definition, different sampling strategies may be indicated. Similar studies in different production classes

or management systems are encouraged to determine if these simpler sampling methods are applicable in broader context (stocker, dairy, or cow-calf operations; no metaphylaxis; etc.)

Variation in the structure of microbial communities inhabiting different segments of the respiratory tract of cattle has been described previously (e.g., nasopharynx or bronchoalveolar) (Zeineldin et. al 2017a). DG swabs have been used by investigators to specifically sample the nasopharynx without contamination from the more rostral nasal passage, but they are more logistically challenging to use and are more expensive than other swabs used in this study. The short NS employed in this study were easier to use but only sampled the most rostral few inches of the nasal passage. The PS swabs sampled both the nasal passage and the nasopharynx, sampling a region of the upper respiratory tract that was effectively a combination of the regions sampled with the DG and NS. The DG samples exhibited the most unique microbial community structure (Figure 6), but these differences in community structures were largely attributable to differences in abundance of shared taxa and not the presence of different taxa (Figure 7). Interestingly, the relative differences in the abundance of common microbial taxa when comparing NS to PS to DG samples suggests that an ecological gradient may exist within the bovine respiratory tract. Ecological gradients (i.e., pH, salinity, temperature) are well-establish drivers of microbial community structure in environmental microbiology (i.e., Rousk et al. 2010, Crump et al. 2004, Deng et al. 2018), but this concept is largely unexplored within the context of respiratory tracts. Results from this study regarding culture and molecular-based detection of *Mh* are consistent with previous research demonstrating that recovery of the upper respiratory tract is consistent with the culture of the lower respiratory tract in acute cases of BRD and at the group level (Godhino et al., 2007; Allen et al., 1991; Doyle et al., 2017; DeRosa et al., 2000). However, given that this study only explored differences within upper respiratory tract samples, we cannot remark about the consistency in results among DG, PS, and NS and lower respiratory tract sampling methods.

Importantly, the trends for microbial taxa of interest between BRD-positive and -negative animals were essentially the same for each swab type. *Mycoplasma bovis* and *Mannheimia haemolytica* are the pathogens most commonly associated with BRD (Arcangioli et al., 1991; Rosendal & Martin, 1986; Autio et al., 2007), and in this study *Mycoplasma* was significantly more abundant in BRD-positive animals, regardless of sample type. Interestingly, there was no difference in the relative abundance of *Mannheimia* or any other Pasteurellaceae genera believed to be important in BRD at this level of the respiratory tract, in contrast with patterns of pathogen detection in the lower respiratory tissues in cattle with BRD that die (Klima et al., 2014; Booker et al, 2009). One potential reason for these differences is that, in the present study, some cattle were sampled before treatment for disease and even before showing signs of disease in some cases; however, previous work has shown that microbial community is different at arrival in animals that go on to have BRD compared to those animals that remain healthy and that *Mannheimia* did not have increased relative abundance in diseased animals (Zeineldin et al., 2017b). It is also important to note that tildipirosin metaphylaxis could have affected the nasopharyngeal microbiome. The nasopharyngeal microbiome of calves treated with tulathromycin, another macrolide, has been shown to recover by day 12 after administration (Holman et al., 2019). There is little information on the duration that tildipirosin administration would affect the nasopharyngeal microbiota; however, it is reasonable to

speculate that tildipirosin may have a longer duration of effect on the microbiome than tulathromycin, due to the longer half-life of tildipirosin in lung tissue of 10 days (Menge et al., 2011) compared to 8.75 days for tulathromycin (Draxxin product label, Zoetis). Holman et al. (2018) also demonstrated that there was a large effect on the nasopharyngeal microbiome within 2-5 days after administration, meaning BRD treatment shortly before 14 DOF could have some effect on the nasopharyngeal microbiome observed in this study compared to others, as well.

Differences relative to BRD status for other taxonomic orders were less expected as these taxa are not typically considered to be important members of respiratory flora (Figure 9B). However, the decreased abundance of gut-associated taxa such as Ruminococcaceae, Chitinophagaceae, Bacteroidaceae, and unclassified Clostridiales may be the result of decreased rumination leading to decreased transfer to the upper respiratory tract in animals with BRD, which typically have decreased appetite (Griffin et al., 2010).

The high prevalence of antimicrobial resistance in *Mh* isolates was consistent with previous studies involving upper respiratory culture of beef cattle at about 14 days after metaphylactic treatment with long-acting macrolide antibiotics (Snyder et al., 2017; Woolums et al. 2018). This high frequency of isolation of MDR resistant is consistent with MALDI genotyping, as genotype 2b is more commonly associated the presence of antimicrobial resistance genes (Clawson et al., 2016). Clawson et al. also note that these resistance genes in genotype 2b are commonly associated with an integrative conjugative element (ICE). ICEs are mobile genetic elements (MGE) that can transfer to naïve cells via conjugative transfer, but also integrate into the genome of the bacterial host (Johnson & Grossman, 2015). The presence of antimicrobial resistance genes on a MGE capable of inter-species and inter-genera transfer (Michael et al., 2012) may explain the similarity among sample types regarding isolation of MDR *Mh*, and highlights the importance of studying antimicrobial resistance in *Mh* and other BRD pathogens within the context of entire microbial communities and other BRD pathogens.

Culture and qPCR only targeted *Mh*, but the use of 16S rRNA sequencing was a very useful and synergistic investigation approach as it allowed both focused and broad-based investigation of thecomposition of the respirator microbiome. However, it was still limited in the investigation of microbes affecting BRD occurrence as it did not allow investigation of viral agents that are believed to be highly important in the occurrence of this multifactorial disease. Incorporation of additional molecular diagnostics would allow an even broader metagenomic investigation of all microbes (bacterial, archeal, viral) of the respiratory tract, in addition to host factors affecting BRD occurrence (Zhang et al, 2021; Scott et al., 2020 and 2021).

6 Conclusions

Results of this study showed that the three sampling methods evaluated provided highly comparable results regarding evaluation of *M. haemolytica* recovery by culture, detection by qPCR, and for characterization of microbial community structure using 16S rRNA gene sequencing. Further, important differences in microbial community structure that were found identified in relation to BRD status were

reflected similarly in all three sample types. Variations in abundance related to some specific taxa (e.g., for the genus Mycoplasma) may suggest that some sample types may be more appropriate for studies when characterizing specific organisms. Future work should focus on the comparability of these sampling techniques across cattle from different production sectors, as well as the comparability of PS upper respiratory tract sampling with lower respiratory tract sampling methods such as tracheal wash and/or bronchoalveolar lavage.

Abbreviations

ASV, amplicon sequence variants; BRD, bovine respiratory disease; DG, deep-guarded swab; *Mh*, *Mannheimia haemolytica*; NS, nasal swab; PS, proctology swab; qPCR, quantitative polymerase chain reaction.

Declarations

8.1 Ethics approval

Protocols used in this research were reviewed and approved by the West Texas A&M University Institutional Animal Care and Use Committee (Protocol# 2020.04.003).

8.2 Consent for publications

Not applicable

8.3 Availability of data and materials

All data generated or analysed during this study are included in this published article and its additional information files.

8.4 Competing interests

The authors declare that they have no competing interests.

8.5 Funding

This research was funded by Texas A&M University.

8.6 Authors' Contributions

WBC performed sample collection, bacterial isolation, and qPCR, as well as primary authorship of the manuscript. LJP performed bioinformatic analysis and contributed largely to text and figures in metagenomic sequencing sections of the manuscript. JTR is the research manager of the West Texas A&M Research Feedlot and was crucial in management of health of animals as well as final review of the manuscript. CW and JK performed DNA extraction and library prep as well quality control of metagenomic samples. JDL oversaw confirmation of *M. haemolytica* culture identification and susceptibility testing and contributed to final review of the manuscript. SPG was contributed to experimental design and review of the manuscript. KSS consulted on qPCR methods and analysis and contributed to final review of the manuscript. SFC contributed to experimental design and final review of the manuscript. ARW and PSM share senior authorship and were responsible for all aspects of the study, including conceptualization, experimental design, sample collection, analysis, drafting, and review of final manuscript. All authors read and approved the final manuscript.

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Figures

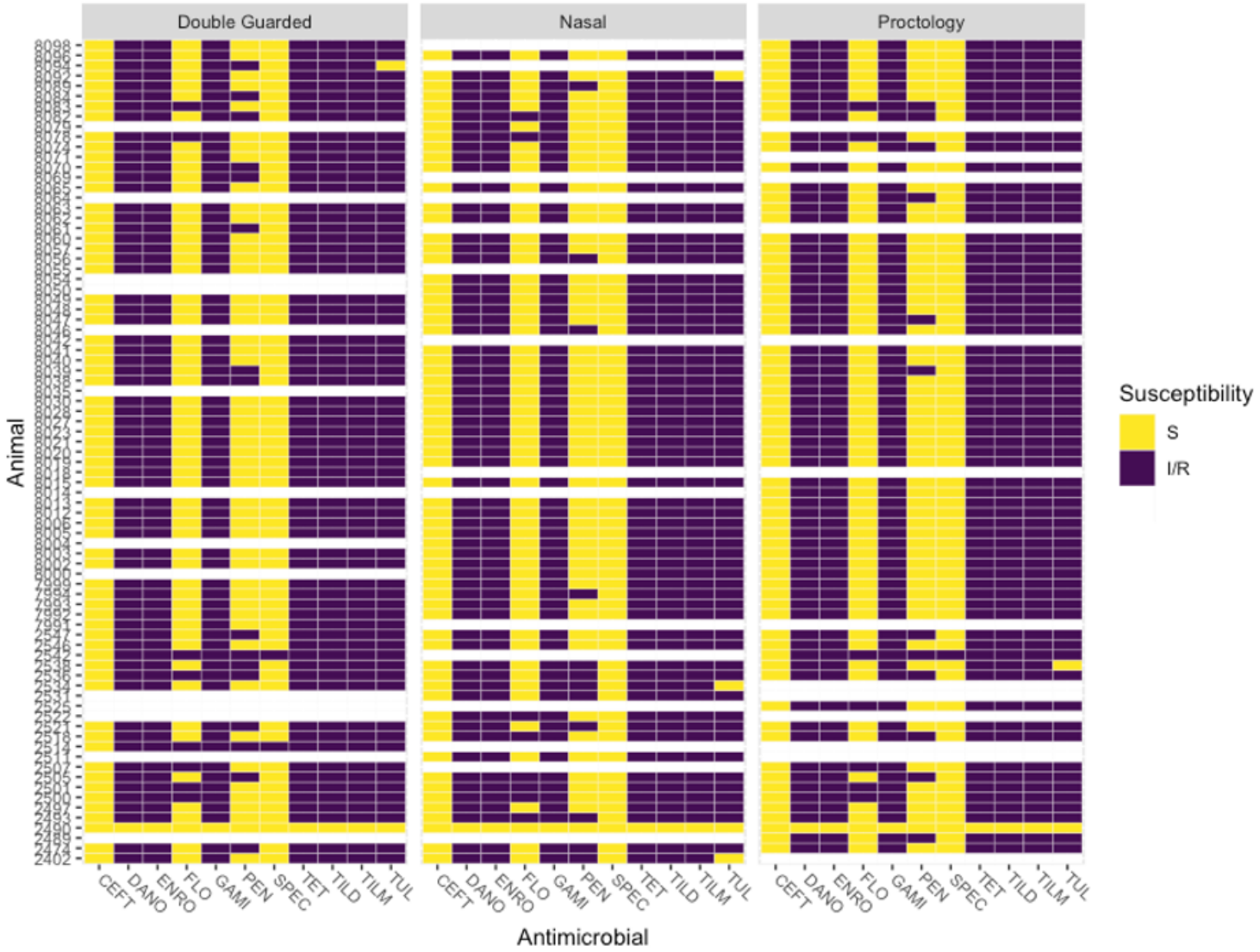

Figure 1

Antimicrobial resistance patterns of *M. haemolytica* isolated from each calf separated by swab type. Each isolate is identified by the calf from which it was isolated. Blank lines indicate calves that did not have *M. haemolytica* isolated from that swab, though it was *M. haemolytica* positive via (an)other swab type(s). Abbreviations: S, susceptible; I/R, intermediate or resistant; CEFT, ceftiofur; DANO, danofloxacin; ENRO, enrofloxacin; GAMI, gamithromycin; PEN, penicillin; SPEC, spectinomycin; TET, tetracycline; TILD, tildipirosin; TILM, tilmicosin; TUL, tulathromycin.

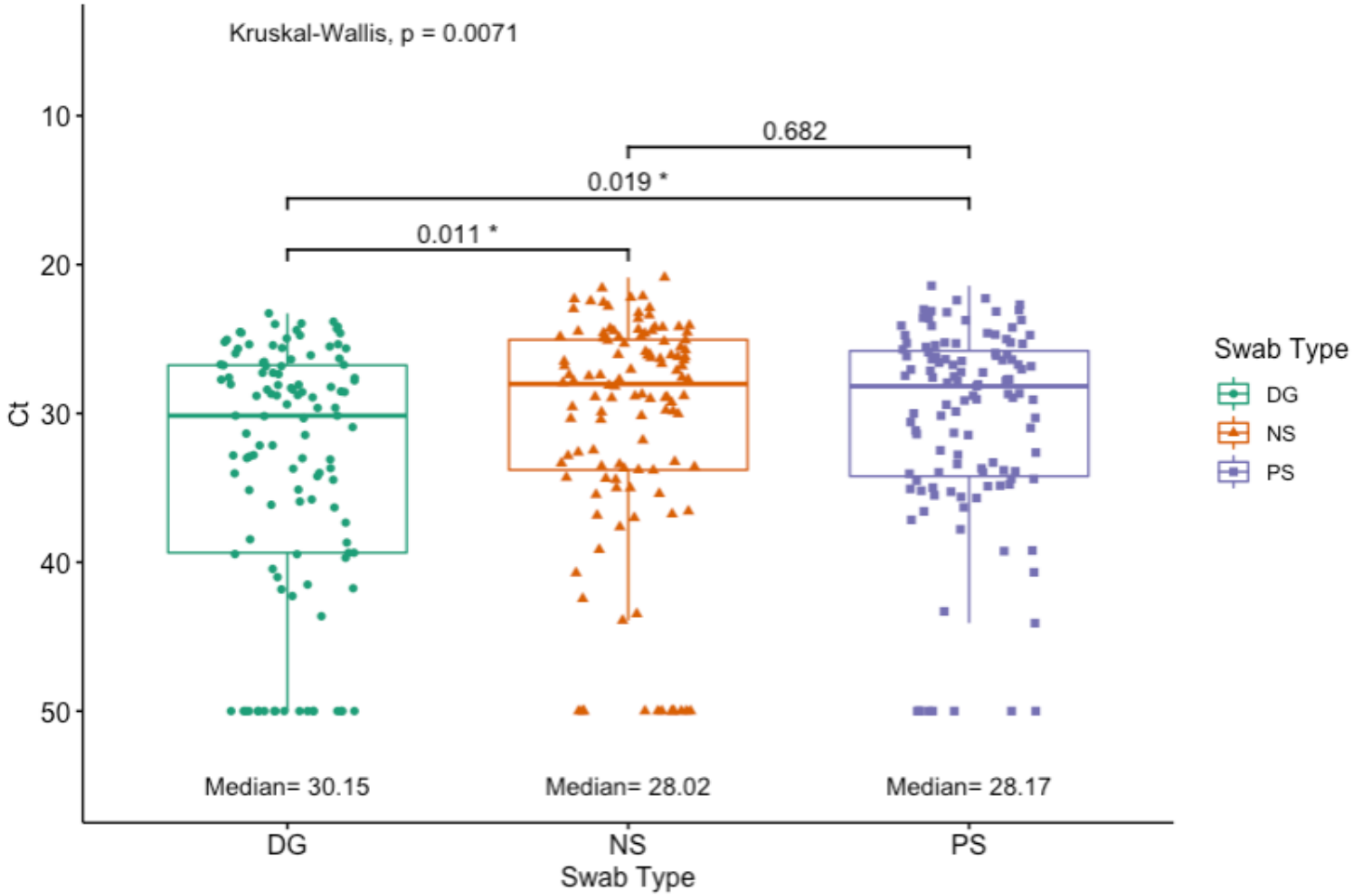

Figure 2

Box and whisker of C_t vs. swab type. (Kruskal-Wallis test, $p < 0.05$; * pairwise Dunn test with Benjamini-Hochberg correction, $p < 0.05$). Key: DG=double guarded swab, NS= nasal swab, and PS=proctology swab. Note reverse y-axis.

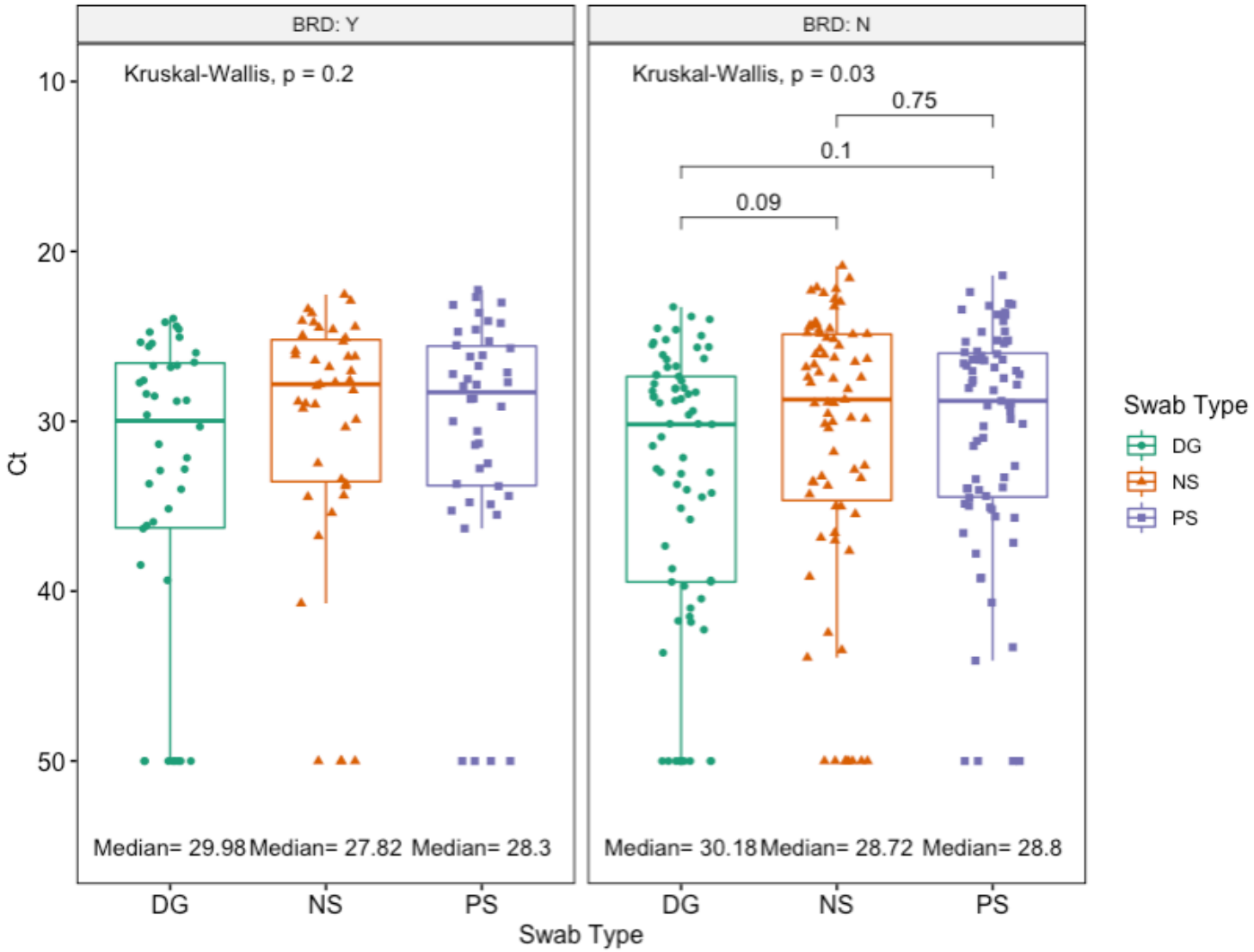

Figure 3

Box and whisker of C_t vs. swab type, separated by BRD treatment (Kruskal-Wallis test, P=0.03 for BRD negative; pairwise Dunn test with Benjamini-Hochberg correction, $P > 0.05$). Key: DG=double guarded swab, NS= nasal swab, and PS=proctology swab, BRD:Y=BRD positive, BRD:N=BRD negative. Note reverse y-axis.

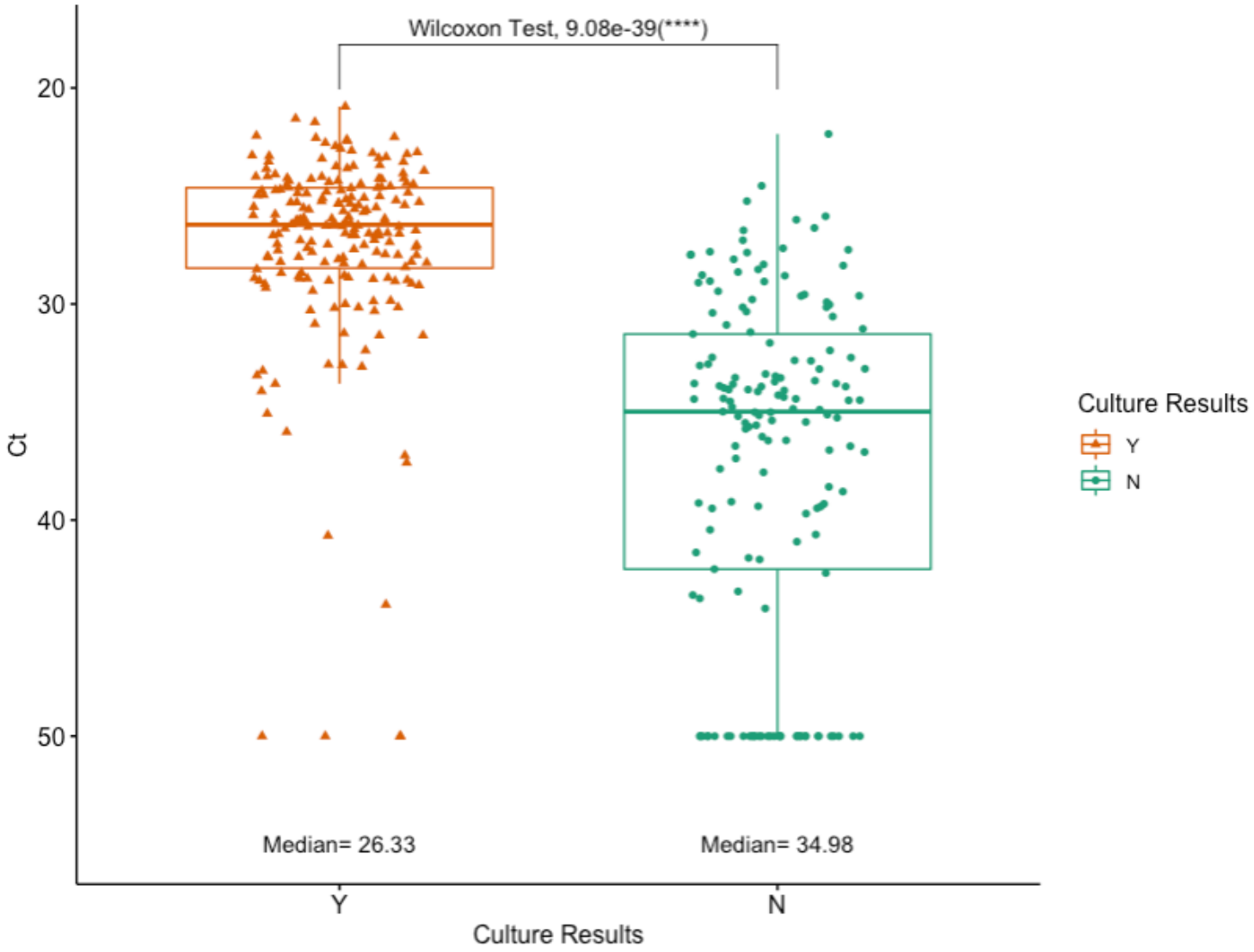

Figure 4

Box and whisker of C_t vs. culture results (Wilcoxon rank-sum test, $p<0.05$). Note reverse y-axis.

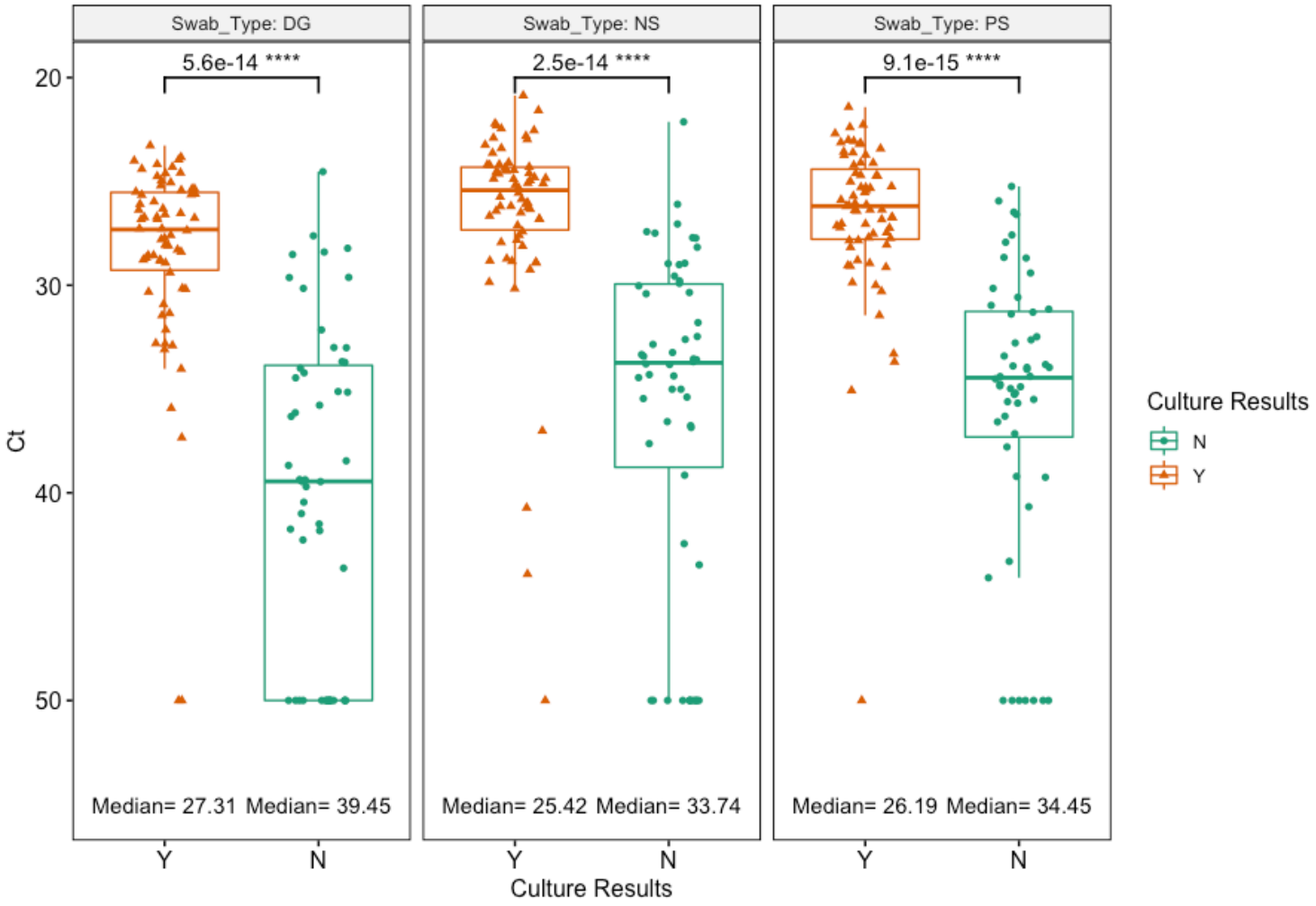

Figure 5

Box and whisker plots of C_t vs sampling group, separated by Swab Type (Wilcoxon rank-sum test, P <0.05).

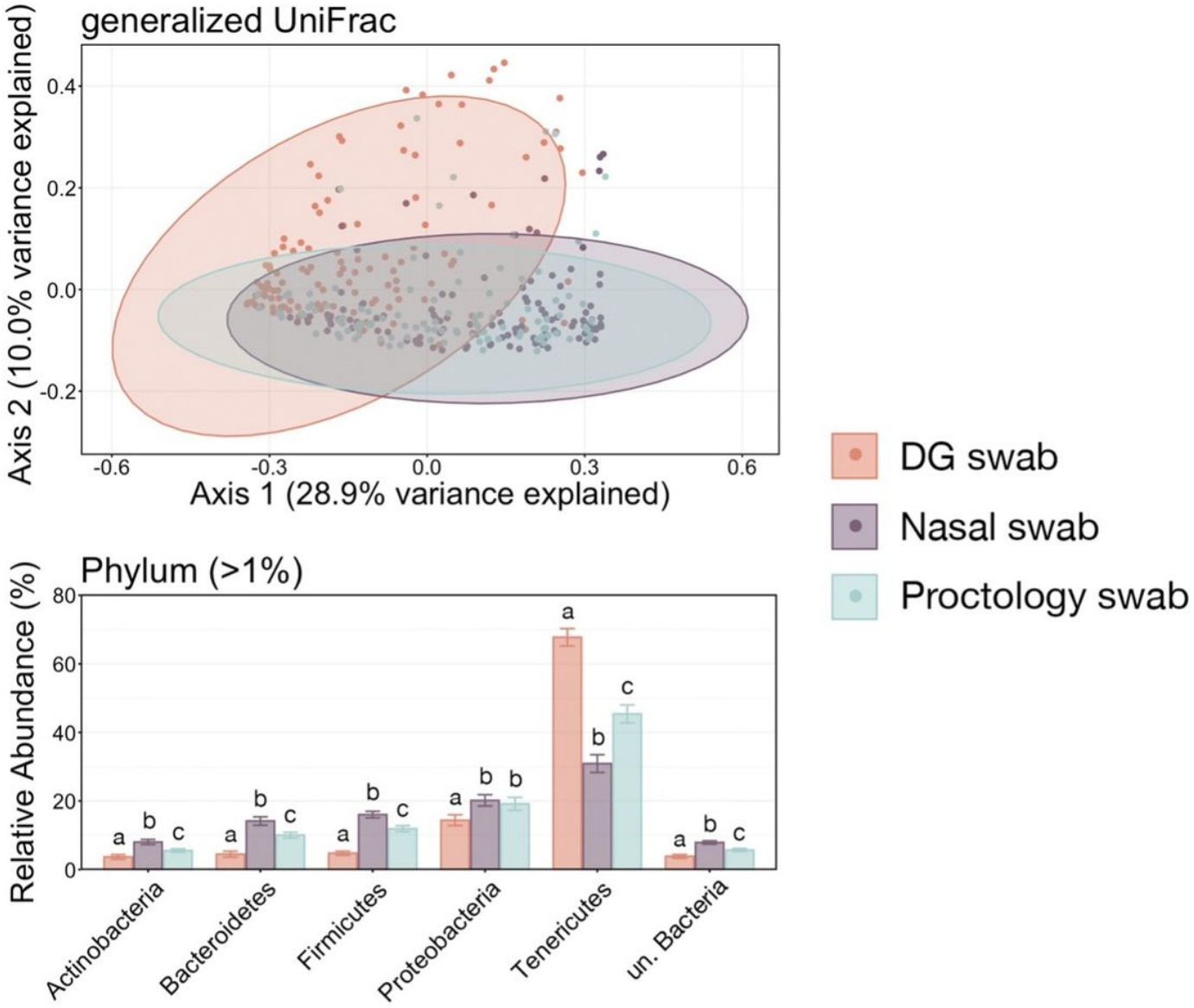

Figure 6

Principal coordinates analysis (PcoA) of generalized UniFrac values and barplot showing the relative abundances of the six phyla representing greater than 1% of the whole community illustrating the variation in microbial community structure across all samples. The PcoA demonstrates clustering of ASVs from microbial communities collected with DG swabs, NS, or PS and shaded areas represent 95% confidence ellipsis for each swab type. Error bars on the barplot demonstrate the standard error of the mean relative abundance for each of the six phyla when sampled using DG swabs, NS, or PS. Significant differences between relative abundances as collected with each swab type are illustrated by different letters (Pairwise Wilcoxon rank-sum test with Benjamini-Hochberg correction, $P < 0.05$).

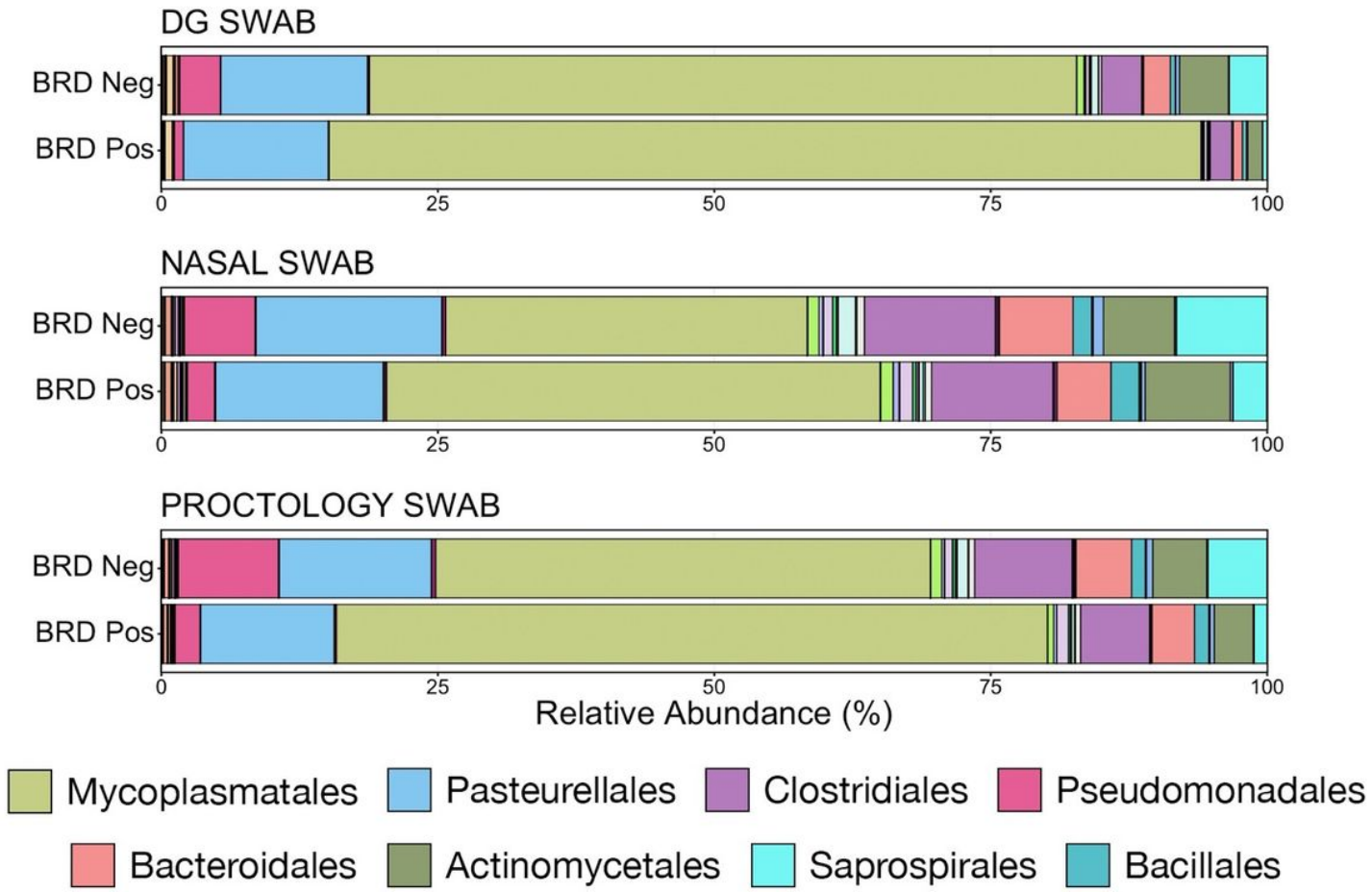

Figure 7

Bar plot illustrating the mean relative abundance of microbial orders within BRD negative or positive animals as sampled with DG swab, NS, or PS. Abundances were normalized to the total number of CSS-normalized ASVs within each sample. The 8 most abundant orders are displayed in the legend.

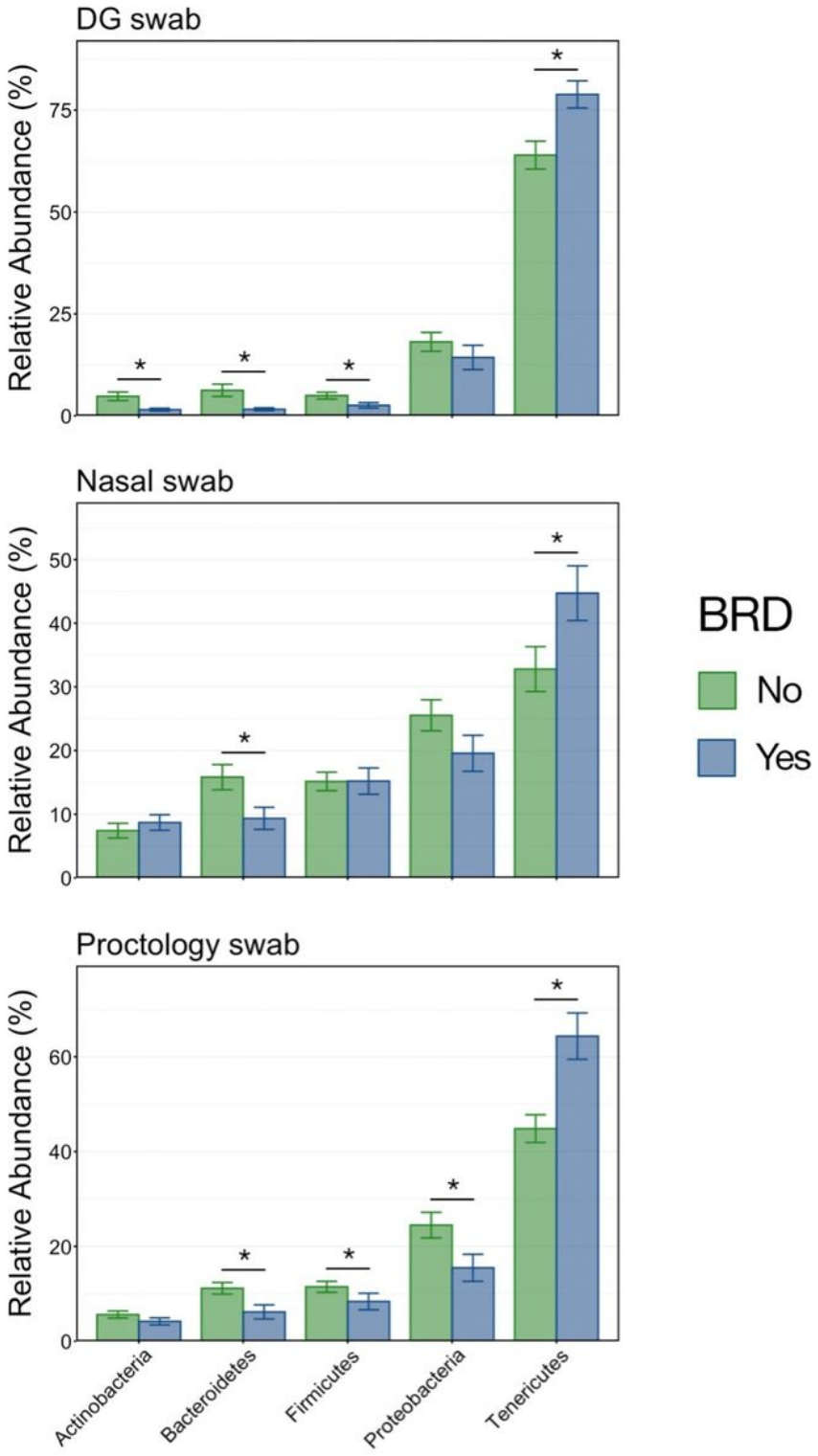

Figure 8

Bar plot demonstrating differences in the relative abundance of each of the 6 most abundant phyla in BRD negative and BRD positive animals as collected with DG swabs, nasal swabs, or proctology swabs. Error bars display the standard error of the mean. Significant differences among relative abundances within each phylum are noted with an asterisk (Kruskal-Wallis analysis of variance by ranks, $p < 0.05$).

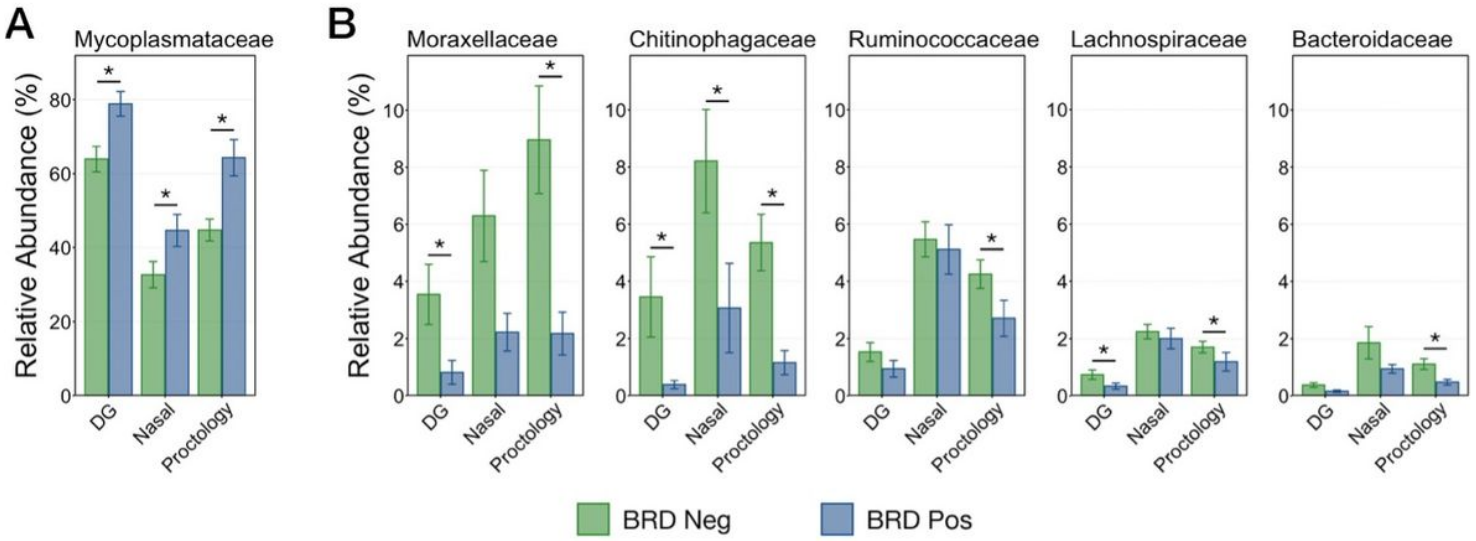

Figure 9

Bar plot demonstrating differences in the relative abundance of microbial taxa of interest within BRD negative and BRD positive animals as collected with DG swabs, nasal swabs, or proctology swabs. Error bars display the standard error of the mean. Significant differences among relative abundances within each phylum and swab type are noted with an asterisk (Kruskal-Wallis analysis of variance by ranks, $p < 0.05$). Note the difference in the relative abundance scale for 4A and 4B.

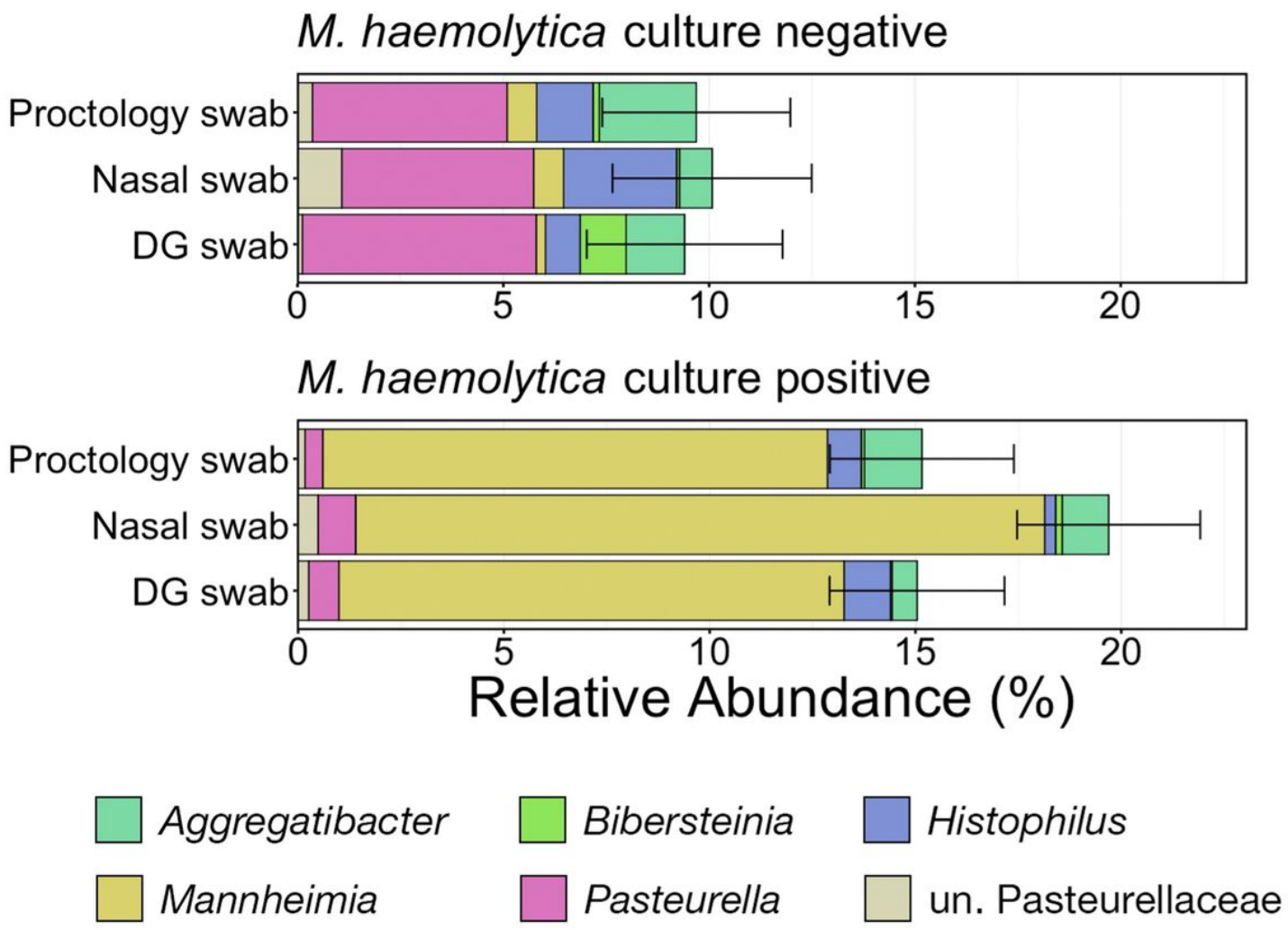

Figure 10

Bar plot showing the relative abundances among all classified taxa for five Pasteurellaceae genera and unassigned Pasteurellaceae ASVs within *M. haemolytica* culture-negative and culture-positive animals, as collected with DG swabs, nasal swabs, or proctology swabs. Error bars demonstrate the standard error of the mean relative abundance of Pasteurellaceae. The six most abundant genera across all samples are displayed in the legend. Abbreviations: un., unclassified

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile15NOV2021.xlsx
- AdditionalFile25NOV2021.xlsx
- AdditionalFile35NOV2021.xlsx
- SupplementaryFigures4NOV2021.docx

- SupplementaryTables4NOV2021.docx