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Comparison of the nasopharyngeal bacterial microbiota of beef calves raised without the use of antimicrobials between healthy calves and those diagnosed with bovine respiratory disease

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ABSTRACT

The role of the respiratory bacterial microbiota in the pathogenesis of bovine respiratory disease (BRD) is still not well defined, limiting our understanding of the disease. Specifically, there is no information on the nasopharyngeal bacterial microbiota of cattle raised without antimicrobials. The objective was to characterize and compare the nasopharyngeal bacterial microbiota in feedlot cattle raised without antimicrobials that were healthy or diagnosed with BRD. Newly-received feedlot cattle (arrival bodyweight ± SD = 218 ± 37 kg) with BRD (n = 82) and pen-matched controls (n = 82) were clinically examined and sampled by deep nasopharyngeal swab (DNS). DNA was extracted from each DNS and the 16S rRNA gene (V4) was sequenced. Alpha and beta diversity were compared between health groups and among 3 days-on-feed (DOF) groups (group A = 3–12 DOF; group B = 13–20 DOF; group C = 21–44 DOF). Observed species richness was lower ($P = 0.031$) in cattle with BRD compared to healthy ones. Both health status ($P = 0.007$) and DOF groups ($P < 0.001$) were sources of variation in microbiota composition. Differences between health groups were driven by multiple sequence variants, including *Mycoplasma bovis*, *Histophilus somni*, and several *Moraxella* spp. Notably, *M. bovis* was more frequently identified in cattle with BRD. *M. bovis* identification was also higher in cattle sampled at later DOF. The increased identification of *M. bovis* in cattle with BRD reaffirms a potentially significant role for this bacterium in respiratory health.

1. Introduction

The nasopharyngeal bacterial microbiota of beef cattle and its relation to bovine respiratory disease (BRD) has garnered increased attention in recent years (Timsit et al., 2016). Although host, viral, and environmental factors play an important role in respiratory health, it is now generally accepted that the composition and stability of the nasopharyngeal bacterial microbiota can also predispose or protect cattle against BRD (Holman et al., 2015a,b; Timsit et al., 2018).

Currently, the primary method in North America for controlling BRD in feedlot cattle is the mass administration of injectable antimicrobials upon or soon after arrival at the feedlot (also known as metaphylaxis) (Checkley et al., 2010; Ives and Richeson, 2015). This control measure aims at reducing the abundance of pathogenic bacteria in the nasopharynx of cattle, including *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni*, and *Mycoplasma bovis* (Holman et al., 2018; Frank and Duff, 2000). For example, in a recent study (Holman et al., 2018) parenteral injection of oxytetracycline on arrival at the feedlot significantly reduced the relative abundance of *Mannheimia* spp. from feedlot entry to 60 d. Furthermore, both oxytetracycline and tulathromycin treated cattle had a significantly lower relative abundance of *Mycoplasma* spp. at feedlot exit compared with the in-feed antimicrobial-only group. Unfortunately, injectable antimicrobials do not just target pathogenic bacteria, and it is known that antimicrobial use can have an impact on the entire respiratory microbiota, including commensals (Holman et al., 2018).

There are currently no data in the scientific literature on the composition of the nasopharyngeal bacterial microbiota of healthy feedlot

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cattle and those diagnosed with BRD that are raised without antimicrobials (*i.e.* natural cattle). This knowledge is important as the beef industry is beginning to move away from the use of antimicrobials, especially for metaphylaxis. Therefore, the objective of this study was to characterize and compare the nasopharyngeal bacterial microbiota in beef feedlot cattle, raised without the use of antimicrobials, that were either healthy or diagnosed with BRD.

2. Materials and methods

This study was conducted in strict accordance with the recommendations of the Canadian Council of Animal Care (Olfert et al., 1993). The University of Calgary Veterinary Sciences Animal Care Committee reviewed and approved the research protocol for this study (AC14-0192).

2.1. Study animals

Crossbred beef-breed steer (n = 128) and heifer (n = 36) calves (arrival bodyweight ± SD = 218 ± 37 kg) that were raised from birth without the use of antimicrobials (*i.e.* natural program) were enrolled at a feedlot located in southern Alberta between November and December 2014. Calves arrived at the feedlot directly from calf-ranches during the fall of 2014. On arrival at the feedlot, calves were processed according to standard feedlot protocols and received a topical avermectin (Bimectin™, Bimeda-MTC Animal Health Inc., Cambridge, Ontario) and a clostridial vaccine (Ultrabac® 7/Somubac®, Zoetis Canada Inc., Kirkland, Québec).

After arrival processing, calves were commingled with other calves of the same sex and housed in large, outdoor dirt-floor pens with capacities between ~250-300 cattle/pen. Twice daily, calves were fed a barley-based diet formulated to meet or exceed nutrient requirements. This diet did not contain any in-feed antimicrobials. Feed bunks were visually inspected and evaluated every day prior to feeding and feed deliveries were adjusted accordingly to ensure that calves had access to sufficient feed to allow for *ad libitum* consumption.

2.2. Study design and case definition

Experienced feedlot personnel visually evaluated calves daily for signs of clinical disease. Any calves that presented signs associated with clinical BRD (*e.g.* depression, difficulty breathing, cough, nasal discharge, ocular discharge) were pulled from their pen and walked to a feedlot hospital facility for further evaluation. For each suspected BRD case, one apparently healthy pen-mate was selected from the pen based on ease of pulling the animal from the pen and was walked to the same feedlot hospital facility as the suspected sick calf for further evaluation.

At the hospital, once it was determined that there had been no prior treatment for clinical BRD or other disease during the feeding period, a study technician specifically trained by an experienced veterinarian examined each calf for inclusion to the study. This included a visual assessment and grading of the calf for clinical signs associated with BRD, specifically depression, cough, nasal discharge, and ocular discharge, as well as measurement of rectal temperature. Calves that had a measured rectal temperature ≥ 40.0 °C and exhibited two or more of the aforementioned specific signs associated with BRD were enrolled in the study to the case group (referred to from here on as the BRD group). Calves that had a temperature < 40.0 °C and did not exhibit any signs associated with BRD were enrolled in the study to the control group (referred to from here on as the CTRL group). If the apparently healthy pen-mate did not meet the criteria for enrolment in the CTRL group, it was returned to its pen and replaced until a calf meeting the criteria was found. Once a calf was enrolled to the study, it was sampled by deep nasopharyngeal swab (DNS).

2.3. Sampling procedures

Deep nasal swab samples were collected as described (McMullen et al., 2018) using long, guarded swabs (27 cm) with a rayon bud (MW 124, Medical Wire & Equipment, Corsham, United Kingdom). Briefly, paper towel was used to thoroughly wipe out one nostril from each calf in order to remove potential debris. A DNS with the swab retracted behind the guard was inserted into the previously cleaned nostril, into the nasopharynx. The nasopharynx was sampled by extending the swab beyond the guard and vigorously moving it back and forth against the mucosal surface. After retracting the swab behind the guard, the entire DNS was removed from the calf's nasal passageway. The swab was then extended beyond the guard and the rayon tip inserted into a transport tube containing liquid Amies transport media, where it was removed from the rest of the swab using scissors. All samples were immediately stored in a polystyrene cooler on ice packs for transport.

Samples were initially collected and stored in a refrigerator at the University of Calgary. Groups of samples were then shipped on ice packs in a polystyrene cooler to the Agriculture and Agri-food Canada (AAFC) Lethbridge Research and Development Center in Lethbridge, Alberta within 48 h. After arrival at the AAFC center, swabs were removed from the Amies transport media and placed into microcentrifuge tubes individually with 1 mL aliquots of 20% glycerol/80% brain heart infusion (BHI) broth. The transport tubes were centrifuged at 2000 x g for 5 min. The supernatant was removed, and the pellets were suspended in the 200 µL of glycerol/BHI broth and added to the tube with the swab. Each microcentrifuge tube was vortexed for 30 s. Samples were frozen at -80 °C until use.

2.4. DNA extraction

A commercially available extraction kit (DNEasy® Blood & Tissue Kit, QIAGEN Inc., Mississauga, Ontario) was used to extract total DNA from all samples, as described (McMullen et al., 2018). Briefly, the swabs were removed from each sample tube and placed in individual sterile tubes on ice. The sample tubes containing glycerol/BHI broth, from which the tips were removed, were centrifuged at 5000 x g for 5 min. Supernatant was pulled out of the sample tube using a pipette and disposed. Each swab was returned to its original respective tube. The tips and pellets were suspended in 180 µl of an enzymatic lysis buffer containing lysozyme (100 mg/ml) and mutanolysin (25,000 U/ml). Each sample mixture was vortexed and incubated at 37 °C for 1 h. Next, an ethanol-free lysis buffer (200 µl) and proteinase K (25 µl) were combined with each mixture. Each sample mixture was individually vortexed and incubated for 30 min at 56 °C. Approximately 300 mg of sterile 0.1 mm zircon/silica beads were added into each sample mixture and beaten using a TissueLyser LT (QIAGEN) for 5 min at 30 Hz. Each mixture was then centrifuged at 13,000 x g for 5 min. In the same tube, the resulting supernatant was mixed with ethanol (200 µl) and each tube was vortexed. The DNEasy Blood & Tissue Kit protocol, in accordance with the instructions provided by the manufacturers, was used from this point forward to finish the extraction process. DNA extractions performed within the same day used the same reagents. A negative control sample was included for each day of extractions, which involved DNA extraction steps as outlined above minus the presence of sample material (n = 13 negative controls).

2.5. Amplification and sequencing

Metagenomic sequencing (16S rRNA) for all DNA samples was performed at Génome Québec, located in Montréal, Québec, as previously described (McMullen et al., 2018). The V4 hypervariable region of the 16S rRNA gene was amplified using primers 515 F (ACACTGAC GACATGGTTCTACA) and 806R (TACGGTAGCAGAGACTTGGTCT). Each primer was modified to include adapters designed to bind DNA to a flow cell for sequencing, as well as index barcodes to allow for library

multiplexing. DNA was amplified using a 25 µL reaction mixture that contained each primer at a concentration of 0.6 µM, deoxynucleoside triphosphate at a concentration of 0.2 mM, dimethyl sulfoxide at a concentration of 5%, TAQ 5U-µl polymerase at a concentration of 0.02 U/µL, 17.35 µL of distilled water, and 3 µL of DNA. Polymerase chain reaction (PCR) entailed an initial denaturation step at 94 °C for 2 min. Initial denaturation was followed by 33 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s, finishing with an extension step at 72 °C for 7 min. Verification of DNA barcoding and amplification were performed separately on 2% agarose gels. A Quant-iT™ PicoGreen® dsDNA Assay Kit (Thermo Fisher Scientific, Waltham, Massachusetts) was used to quantify total DNA amplified.

DNA libraries were set up by pooling 25 ng of individual samples together. All libraries were cleaned with AMPure XP beads (Beckman Coulter, Brea, California) at a ratio of 0.85. A Quant-iT PicoGreen dsDNA Assay Kit and a universal KAPA Library Quantification Kit for Illumina® Platforms with Revised Primers and Kapa SYBR® Fast (Kapa Biosystems, Wilmington, Massachusetts) were then used to quantify the libraries. Average fragment size was established using a LabChip GX (PerkinElmer, Waltham, Massachusetts) instrument. In order to ameliorate unbalanced base composition, 10% of the PhiX control library was added to the amplicon pool (final loading concentration of 5.5 pM) prior to DNA sequencing. A MiSeq Reagent Kit v2 (500 cycles) (Illumina, Inc., San Diego, California) was used according to the instructions provided by the manufacturer to perform 16S rRNA gene amplicon sequencing. Additionally, LNA™ modified custom primers (Exiqon, Copenhagen, Kingdom of Denmark) were included in the amplicon sequencing process (Primer read 1 – ACACTGACGACATGGT TCTACA; primer read 2 – TACGGTAGCAGAGACTTGGTCT; Primer index read – AGACCAAGTCTCTGCTACCGTA). Following sequencing, Génome Québec demultiplexed the libraries and removed all adapters and index barcodes from the sequence data.

2.6. Sequence processing

Sequencing data were processed using cutadapt v1.18 (Martin, 2011) and DADA2 v1.6 (Callahan et al., 2016) as implemented in R v3.4.3 (R Development Core Team, 2010). Forward and reverse 5′ 16S primers, as well as low-quality ends, were trimmed from the raw sequencing data using cutadapt in paired-end mode with a maximum allowed error rate of 0 and a quality-cutoff of 20. Reverse compliment primers were not trimmed as the targeted read length was 250 × 250 base pairs; since the approximate length of the V4 region is 254 base pairs, reverse compliments of the forward and reverse 5′ primers were never sequenced and therefore were not present in the data. Sequencing data quality was then assessed using FastQC v0.11.7 (Andrews, 2014). A random subset of samples was selected for analysis, resulting in the generation of individual sample quality reports which were compiled into one comprehensive report using MultiQC v1.4 (Ewels et al., 2016). Once the quality of the data was deemed acceptable, DADA2 was utilized to filter and trim reads, infer exact sequence variants, and assign taxonomy to variants, as previously described (McMullen et al., 2018). Default parameters were used for all DADA2 functions unless noted otherwise. Reads were filtered using a maximum expected error of one. A parametric error model was then estimated through a form of unsupervised machine-learning. This estimation was performed using 2 million sequences each for the forward and reverse reads separately. Sequencing reads were then dereplicated. Exact amplicon sequence variants (SVs) were inferred for each sample independently using the DADA2 sample inference algorithm and the estimated error models. This algorithm does not call for singletons during the SV inference process. Full, denoised sequences were obtained by merging the inferred forward and reverse reads. An SV table, which is functionally similar to an operational taxonomic unit table, was assembled from the denoised sequences. Chimeric sequences were then removed from the table. A taxonomy table was assembled by assigning taxonomy to each SV in the SV table using the RDP (Cole et al., 2014) taxonomic database for DADA2 (Callahan, 2017). All species level assignment was accomplished using the DADA2::addSpecies function, with exact matching used to assign species when possible. When exact matching of an SV to a species was not possible, all potential matching species were listed for the SV.

2.7. Statistical analyses

Downstream analyses were performed in R v3.5.1 (R Development Core Team, 2010) using multiple functions from phyloseq (McMurdie and Holmes, 2013), ggpubr (Kassambara, 2017), RVAideMemoire (Hervé, 2018), and vegan (Dixon, 2003). An object was constructed from the SV and taxonomy tables in R using phyloseq for subsequent analysis. A prevalence filter was applied to the phyloseq object such that only SVs present in ≥ 1% of the samples remained.

In order to take into account the influence of DOF (Timsit et al., 2017), calves were categorized into one of three artificially constructed groups based on the DOF of each calf at the time of study enrollment using percentiles of 33.33% and 66.67%.

Relative abundance, proportion, and beta-diversity measures of the microbiota were calculated using prevalence filtered data; alpha-diversity measures were calculated using unfiltered data. A Wilcoxon rank-sum test was used to compare relative abundance group means between BRD-CTRL groups. Pairwise comparisons of relative abundance group means were also made between BRD-CTRL and DOF groups using Wilcoxon rank-sum tests, correcting for multiple comparisons using the Holm method as implemented in the R package. Fisher's exact test was used to compare, between BRD-CTRL and DOF groups, the proportion of calves whose microbiota contained BRD pathogens, including *Mannheimia haemolytica, Pasteurella multocida, Histophilus somni*, and *Mycoplasma bovis*. A calf had a count of 1 if the specific bacterium being tested for was present at a relative abundance of ≥ 1%. When a Fisher's exact test was significant for a specific bacterium, a pairwise comparison of BRD-CTRL and DOF groups was performed, correcting for multiple comparisons using the Holm method as implemented in the RVAideMemoire package in R.

Observed species richness and Shannon diversity were calculated for both BRD and CTRL groups as implemented in phyloseq. A Wilcoxon rank-sum test was used to compare alpha-diversity group means between groups. Pairwise comparisons of alpha-diversity measures were also made for both the BRD and CTRL groups based on DOF group, and were adjusted for multiple comparisons using the Holm method as implemented in the ggpubr package in R.

A permutational multivariate analysis of variance (PERMANOVA) using a Bray-Curtis dissimilarity index was used as implemented in vegan to evaluate the effects of BRD-CTRL group, DOF group, and sex, as well as any potential interactions between these terms, on microbiota composition. Group dispersion homogeneity (beta dispersion) was evaluated using a permutational analysis as implemented in vegan. A pairwise PERMANOVA utilizing a Bray-Curtis dissimilarity index as implemented in the RVAideMemoire package was used to analyze the effects of DOF group and BRD-CTRL group on microbiota composition by comparing different DOF groups within the BRD and CTRL groups, as well as by comparing the BRD and CTRL groups within each DOF group. The RVAideMemoire package corrected for multiple comparisons using the Holm method. Pairwise comparisons of beta dispersion were tested using a permutational analysis as implemented in vegan and were adjusted for multiple comparisons using the Holm method as implemented in the R package. To determine which SVs drove differences in beta-diversity, data were ordinated using non-metric dimensional scaling (NMDS) and a Bray-Curtis dissimilarity index as implemented in phyloseq. The ordinations were plotted and the NMDS coordinates of each sample relative to one other were correlated utilizing Spearman's rank-order correlation with the within sample relative abundance of the prevalence filtered SVs. This resulted in two

correlation coefficients for each SV, one for each NMDS axis. The top 10 SVs were then selected based on the highest combination of the two correlation coefficients for each SV having a relative abundance of ≥ 1%.

3. Results

3.1. Health data

A total of 164 calves (steers = 128; heifers = 36) were enrolled in the study. The average DOF at the time of study enrollment was 17.6 days. Average rectal temperature was 40.7 °C for the BRD group and 39.4 °C for the CTRL group. For DOF group, there were 51 calves in group A (3–12 DOF), 58 calves in group B (13–20 DOF), and 55 calves in group C (21–44 DOF).

3.2. Baseline sequencing data

A total of 7,240,915 reads were obtained across all samples from one sequencing run (BRD = 3,675,409; CTRL = 3,565,506) with an average Phred quality score of 35.9 (same for both BRD and CTRL groups) prior to upstream processing. After processing with DADA2, a total of 4,988,778 reads remained across all samples (BRD = 2,507,511; CTRL = 2,481,267), with an average coverage of 30,419 reads (range = 16,121-41,849) per sample (BRD = 30,579; CTRL = 30,259). From these sequences, 2,700 unique SVs were identified across all samples. After removal of all SVs that did not belong to the kingdom Bacteria, a total of 2,669 SVs remained. Furthermore, after 1% prevalence filtering 786 SVs remained across all samples.

Along with DNS samples, 13 negative control samples were sent for metagenomic sequencing. The sequencing results for each negative control were assessed individually, and it was determined that, based on the composition of the reads and the extremely low numbers of sequences returned for each sample, there was no obvious contamination of the calf samples due to the DNA extraction and metagenomic sequencing processes (Table S1). Therefore, there was no need to adjust the study calf DNA sequencing results for possible contaminants.

3.3. Characterization of the nasopharyngeal bacterial microbiota

Among both BRD-CTRL groups, the most prominent phyla were Proteobacteria (69.35%), Tenericutes (22.51%), Firmicutes (3.33%), Actinobacteria (2.33%), and Bacteroidetes (2.31%). The order of the top three phyla by abundance for both the BRD and CTRL groups was Proteobacteria, Tenericutes, and Firmicutes. However, Actinobacteria was the next most abundant followed by Bacteroidetes in the BRD group, whereas Bacteroidetes was the next most abundant followed by Actinobacteria in the CTRL group (Fig. 1).

The most prominent identified genera across both BRD-CTRL groups were *Mycoplasma* (22.16%), *Moraxella* (19.53%), *Histophilus* (19.02%), *Psychrobacter* (9.82%), *Mannheimia* (6.27%), *Pasteurella* (4.38%), *Pseudomonas* (1.83%), and *Alysiella* (1.04%). However, the order of these genera by abundance was different between BRD-CTRL groups (Fig. 1). The top three most abundant genera for both groups were *Histophilus*, *Moraxella*, and *Mycoplasma*, though the order of these genera differed by group. In the BRD group, the order was *Mycoplasma* (27.00%), *Histophilus* (21.38%), and *Moraxella* (17.59%). These genera accounted for 99.35%, 32.34%, and 26.61% of their corresponding phyla within the BRD group, respectively (Table S2). Comparatively, the order in the CTRL group was *Moraxella* (21.50%), *Mycoplasma* (17.25%), and *Histophilus* (16.62%). These genera accounted for 29.91%, 97.04%, and 23.12% of their corresponding phyla within the CTRL group, respectively (Table S3).

H. somni (BRD = 21.38%; CTRL = 16.62%) was the most abundant species across both groups (Fig. 1), accounting for 100.00% of all identified *Histophilus* spp. in both groups. The second and third most abundant species in the BRD group were *Mycoplasma agalacitae/bovis* (12.94%), accounting for 47.94% of all identified *Mycoplasma* spp., and *Psychrobacter halophilus/marincola/maritimus/psychrophilus/submarinus* (9.24%), accounting for 96.51% of all identified *Psychrobacter* spp. In contrast, the second most abundant species in the CTRL group was *P. halophilus/marincola/maritimus/psychrophilus/submarinus* (9.61%), accounting for 95.48% of all identified *Psychrobacter* spp., while *Moraxella bovoculi* (8.76%) was the third most abundant, accounting for 40.75% of all *Moraxella* spp.

3.4. Comparison of nasopharyngeal bacterial microbiota structure between BRD and CTRL groups

Relative abundance did not differ between BRD-CTRL groups ($P \geq 0.05$) for *H. somni*, *M. haemolytica*, *Mannheimia caviae/glucosida/haemolytica*, *M. agalactiae/bovis*, *Mycoplasma bovirhinis*, or *P. multocida*, nor did it differ between BRD-CTRL groups based on DOF group ($P \geq 0.05$). However, the proportion of samples that contained *M. agalacitae/bovis* at a relative abundancy of > 1% differed between BRD-CTRL groups ($P < 0.001$), with a higher proportion in the BRD group (43.90%) compared to the CTRL group (18.29%) (Table 1). Additionally, the proportion of samples that contained *M. agalacitae/bovis* at a relative abundancy of > 1% was different by DOF group ($P < 0.001$) (Fig. 2; Table S4). Pairwise comparisons showed significantly ($P < 0.05$) higher proportions of *M. agalacitae/bovis* for DOF group B and C compared to group A in the BRD group and for group C compared to group A in the CTRL group (Table S5).

Observed species richness was higher in the CTRL group compared

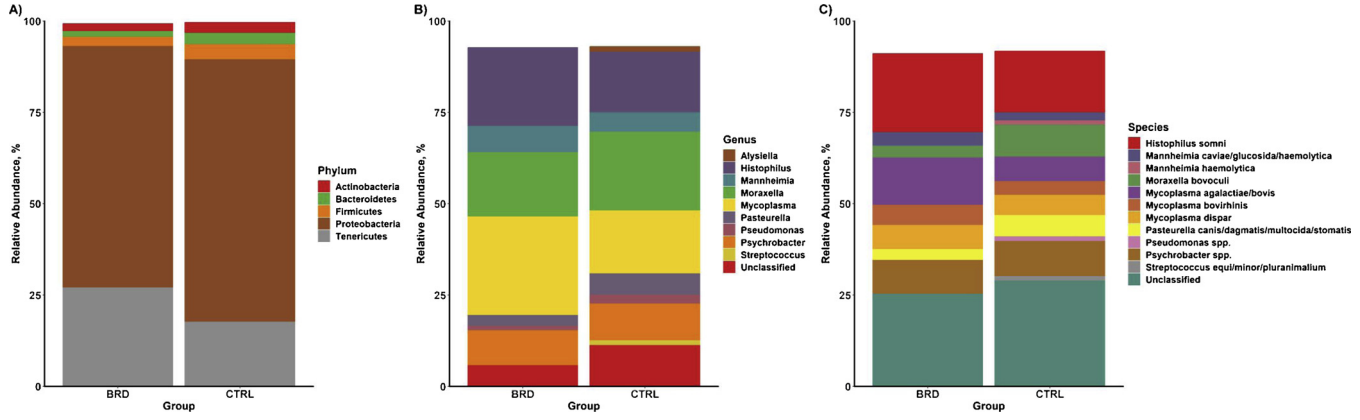

Fig. 1. Relative abundance of nasopharyngeal bacteria (present at ≥ 1% abundance) at the phylum (A), genus (B), and species (C) level for beef calves raised without the use of antimicrobials that were either healthy or diagnosed with BRD.

Table 1
Proportion of nasopharyngeal microbiota that contained bacterial respiratory pathogens (present at ≥ 1% abundance) for beef calves raised without the use of antimicrobials that were either healthy (CRTL) or diagnosed with BRD.

Bacterial organism	BRD (%)	CTRL (%)	P value*
Histophilus somni	63.41	48.78	0.083
Mannheimia haemolytica	12.20	9.76	0.804
Mannheimia caviae/glucosida/haemolytica	26.83	14.63	0.082
Mycoplasma agalactiae/bovis	43.90	18.29	< 0.001
Mycoplasma bovirhinis	51.22	34.15	0.040
Pasteurella multocida	28.05	31.71	0.733

* P value reflects comparison between BRD and CTRL groups.

to the BRD group (P = 0.031); however, Shannon diversity was not significantly different between groups (P ≥ 0.05) (Fig. 3). Based on PERMANOVA analyses, BRD-CTRL group (P = 0.007) and DOF group (P < 0.001) were significant sources of variation in microbiota profile (Table S6; Fig. 4). The top 10 SVs that drove differences in beta-diversity between the BRD and CTRL groups included SVs classified as *M. agalacitae/bovis*, *H. somni*, and *M. bovoculi*, among others (Fig. 4; Table S7). Sex was not a significant source of variation, nor were there any significant interactions between BRD-CTRL groups, DOF groups, or sex (P ≥ 0.05). After a pairwise comparison, DOF remained a significant (P < 0.05) source of variation between groups A and C for BRD-CTRL groups (Table S8; Fig. S1).

4. Discussion

This study assessed and compared the composition of the nasopharyngeal bacterial microbiota of beef calves raised without the use of antimicrobials between healthy calves and those diagnosed with BRD. We showed that bacterial microbiota composition and diversity differed between groups. These differences were driven by multiple different SVs, including *M. bovis*, *H. somni*, and several *Moraxella* spp. Notably, although relative abundance of *M. bovis* did not differ between groups, the proportion of animals with this bacterium was higher in cattle with BRD. The proportion of calves with *M. bovis* also differed based on DOF for both groups, with higher proportions observed at later days in the feeding period.

There were multiple strengths in this study. Negative control samples were included in the DNA extraction process, reducing concerns over potential confounding laboratory contamination. As well, the DADA2 approach inferred exact SVs with single-nucleotide resolution, allowing for a more accurate and in-depth view of the microbiota when compared to traditional methods of binning sequences into operational taxonomic units (OTUs), including taxonomic identification at the species level. However, a mock bacterial microbiota was not included in the study as a positive control for estimating any potential biases introduced during PCR and sequencing.

An indirect comparison of this study and previous case-control studies that used cattle raised by conventional industry practices, *i.e.* using on-arrival metaphylaxis (Holman et al., 2015a; Timsit et al., 2018), showed many similarities in bacterial microbiota composition. Though exact compositions differ, similar phyla (Tenericutes, Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria) and genera (*Histophilus*, *Mannheimia*, *Pasteurella*, *Psychrobacter*, *Mycoplasma*, *Pseudomonas*, *Streptococcus*, *Moraxella*) were found for all cattle in relatively high abundance. These potential similarities between "conventional" calves and calves raised without the use of antimicrobials highlights a gap in knowledge regarding the impact of antimicrobials on the composition of the nasopharyngeal bacterial microbiota. A recent study on the fecal bacterial microbiota of feedlot cattle showed that other factors (changes in diet, geography, exposure to foreign pathogens, *etc.*) may affect the microbiota more than metaphylactic drugs (Doster et al., 2018). Perhaps this is similar for the upper respiratory bacterial microbiota; however, further research is needed to fully understand the impact of our current management practices on the composition and stability of the upper respiratory tract microbiota.

The difference in microbiota composition and diversity observed between BRD and CTRL groups is in agreement with previous findings reported in conventionally raised feedlot cattle (Timsit et al., 2018). Indeed, in a recent study by Timsit et. al., 2018, bacterial diversity in the upper and lower airways was reduced in calves that had BRD compared to healthy cattle. Furthermore, Timsit et. al., 2018 also found that *M. bovis* explained some of the differences between BRD and healthy cattle, a finding similar to the present findings which confirms the importance of this bacterium in the pathogenesis of BRD in feedlot cattle. It is noteworthy that numerous other studies have reported *Mycoplasma* as one of the most abundant genera in feedlot cattle (Nicola et al., 2017; Stroebel et al., 2018; Timsit et al., 2016; Zeineldin et al., 2017). However, it seems that, among *Mycoplasma, M. bovis* is the only species to be more frequently identified and/or abundant in BRD *versus* healthy cattle, reinforcing the importance of reporting (when possible) results at the species level for *Mycoplasma.*

The relative abundance and proportion of calves with *M. haemolytica*, *H. somni*, and *P. multocida* did not differ between BRD-CTRL groups, which disagrees with previous findings based on bacterial culture (Allen et al., 1992; Timsit et al., 2017). This discrepancy may be explained by the fact that 16S rRNA sequencing can be less sensitive than culturing to detect specific changes in bacterial communities. For example, in a previous study on cattle nasopharyngeal microbiota, *Pasteurella* was at very low levels (< 0.05% of the sequences) and only in a few individuals based on 16S rRNA sequencing, whereas it represented 17.1% of bacteria cultured on selective media (Holman et al.,

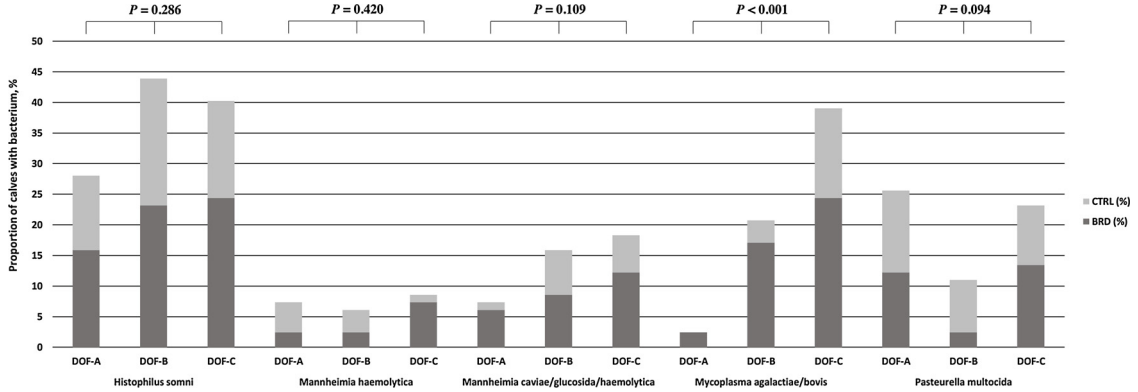

Fig. 2. Proportion of calves whose nasopharyngeal microbiota contained bacterial respiratory pathogens (present at ≥ 1% abundance) by days-on-feed (DOF-A = 3–12 days; DOF-B = 13-20 days; DOF-C = 21–44 days) for beef calves raised without the use of antimicrobials that were either healthy (CTRL) or diagnosed with BRD.

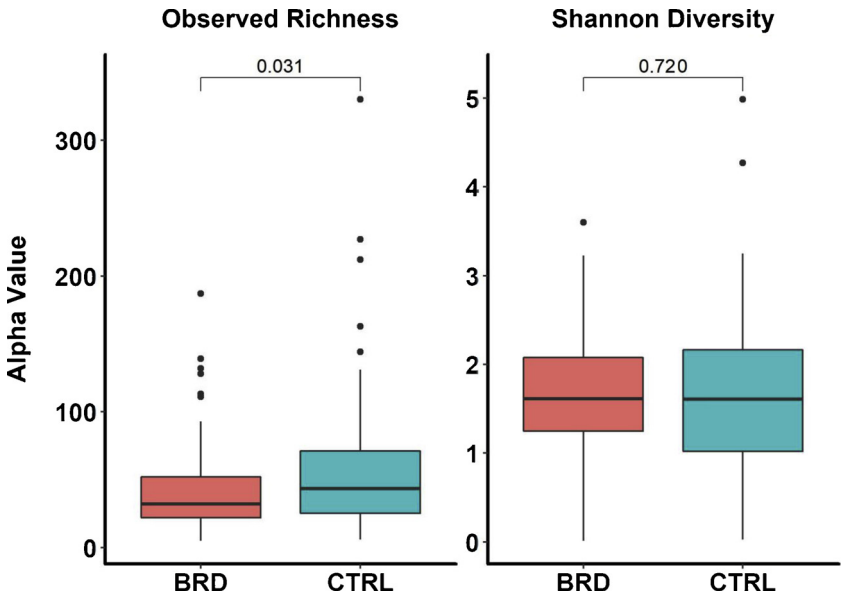

Fig. 3. Observed species richness and Shannon diversity of nasopharyngeal bacteria for beef calves raised without the use of antimicrobials that were either healthy or diagnosed with BRD.

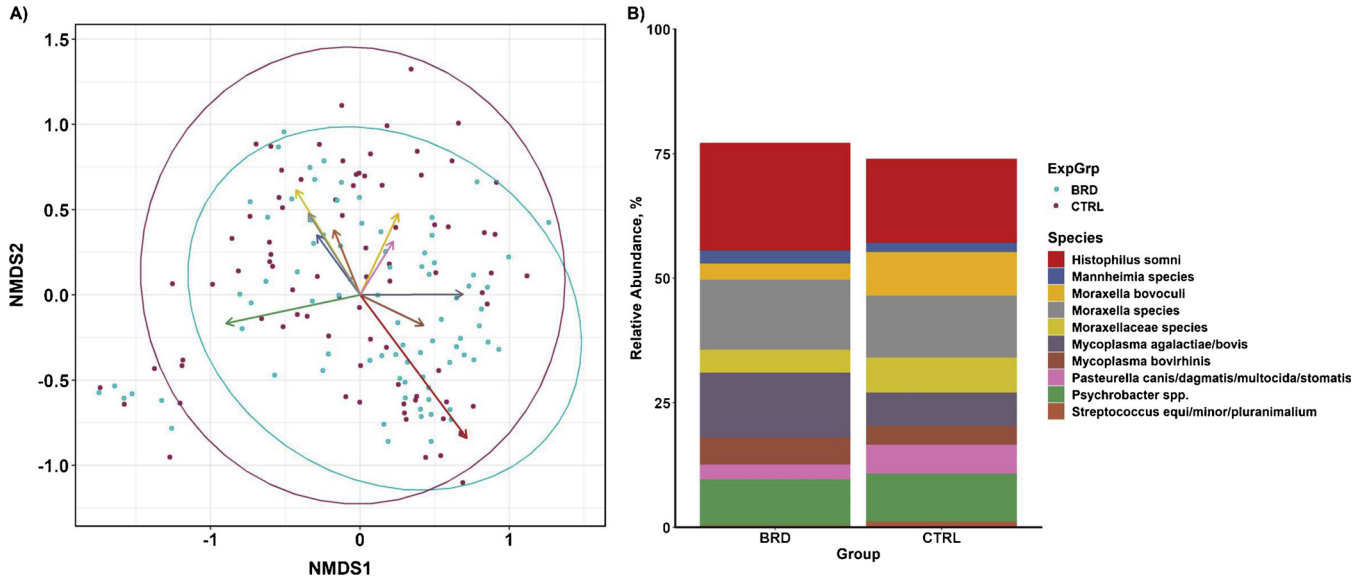

Fig. 4. The top 10 nasopharyngeal bacteria sequence variants (SVs) correlated with differences in beta-diversity observed for beef calves raised without the use of antimicrobials that were either diagnosed with or did not develop BRD. Fig. 4A represents an ordination of relative abundance data using non-metric multi-dimensional scaling and a Bray-Curtis dissimilarity index with vectors pertaining to the top 10 SVs and the correlation between their relative abundance and the differences in observed beta-diversity. Fig. 4B represents the relative abundance of the top 10 SVs.

2015b). Regardless, these findings might also suggest that the simple presence or relative abundance of these pathogens in the nasopharynx does not necessitate that cattle will develop BRD. While the nasopharynx is thought to act as a reservoir for BRD pathogens, other factors, such as stress or exposure to foreign pathogens, also play a significant role in the development of BRD. In addition, it is possible that other bacterial niches of the respiratory tract, such as the tonsils (Frank et al., 1986), may play an important role in influencing the microbiota of the lower respiratory tract and the development of BRD. In order to modulate the bacterial microbiota of the respiratory tract to prevent and control BRD, it is important that we understand the dynamics of bacterial species in the respiratory tract in its entirety.

Microbiota composition also differed based on DOF group for both BRD-CTRL groups. Interestingly, pairwise comparisons showed that this variation arose between DOF groups A and C. While these differences were driven by both commensal and pathogenic bacteria, there was a notable increase in the proportion of calves with *M. bovis* in DOF groups B and C. A study by Stroebel et. al., 2018 showed an increase in *M. bovis* after feedlot arrival and hypothesized that this increase was associated with mass medication with tildipirosin, an antimicrobial with a low activity against *M. bovis*. That there appears to be an increase in *M. bovis* in the present study for cattle not treated with antimicrobials on arrival seems to contradict this hypothesis and indicates that *M. bovis* spreads among cattle even without mass medication with an antimicrobial upon arrival at a feedlot.

5. Conclusions

The nasopharyngeal bacterial microbiota of beef calves raised without the use of antimicrobials differed between healthy calves and

those diagnosed with BRD, as well as among DOF groups. The increased proportion of calves with BRD that harbored *M. bovis* in their nasopharynx reaffirms a potentially significant role for this bacterium in beef cattle respiratory health.

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Authors' contributions

CM did laboratory work, analyzed the data, and wrote the first draft of the manuscript. ET and KO trained field technicians to collect DNS samples, designed the study, and provided support for data analysis and writing the manuscript. TA assisted in the study design and provided technical support for laboratory work. FVDM and GP assisted in study design. All authors reviewed and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.02.030.

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