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Disparity in the nasopharyngeal microbiota between healthy cattle on feed, at entry processing and with respiratory disease

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ABSTRACT

Bovine respiratory disease (BRD) is one of the most serious causes of health and economic problems in the beef production industry, especially in recently weaned, intensely raised and newly transported feedlot cattle. While the importance of upper airway structure and function in the susceptibility of the lower respiratory tract to colonization with potential pathogens is well established, the role of the mucosal microbiota in respirtatory health is less well defined. The objective of this study was to characterize the nasopharyngeal microbiota of feedlot cattle at entry into a commercial feedlot, during initial management processing, and to compare the dynamics of change in these microbial communities between clinically healthy calves and those that develop BRD within the first month after entry. Deep nasopharyngeal swabs were collected from randomly selected healthy calves (n = 66) during initial handling and processing at the feedlot, and again at the initial diagnosis of BRD (n = 22). Clinically healthy pen matched controls calves (n = 10) were sampled at the same time as the BRD affected animals. Genomic DNA was extracted from each sample, and the 16S rRNA gene V1-V3 hypervariable region was amplified and sequenced using the Illumina MiSeq platform. Across all the samples, the predominant bacterial phyla were *Proteobacteria*, *Firmicutes* and *Actinobacteria.* While the predominant genera were *Moraxella*, *Mycoplasma* and *Acinetobacter*. Linear discriminant analysis (LDA) effect size (LEfSe) revealed significant differences in bacterial taxa between healthy and BRD affected calves. Discriminant analysis revealed that the nasopharyngeal microbiota in feedlot calves at entry and in BRD affected calves were distinct from pen matched healthy calves. While the temporal dynamics of this shift were not examined in this study, it is possible that the observed changes in mucosal microbiota are linked to the increased susceptibility of calves to BRD during the first month after entry in to the feedlot. Additional studies are needed to examine the trajectory of change in nasopharyngeal microbial communities from entry to disease onset, and to explore the impact of other factors such as diet transition, commingling, vaccination and housing on the nasopharyngeal microbiota of growing cattle.

1. Introduction

Bovine respiratory disease (BRD) is a serious, ongoing health and economic problem, especially in intensely raised and newly transported feedlot cattle (Buckham Sporer et al., 2008). The annual prevention and therapeutic costs of BRD to the cattle industry have been estimated at over 1 billion dollars (McVey, 2009). Despite many advancements in management and therapeutics, BRD and its sequelae, continues to be a leading cause of animal morbidity, mortality, welfare concern and production loss to the industry (Apley, 2014). The etiopathogenesis of BRD is complex, and various predisposing factors such as stress, nutritional changes and environmental conditions (weaning, transportation, stocking density and commingling) combine to impact host susceptibility to BRD (Hodgson et al., 2012). Multiple pathogens have been implicated in the pathogenesis of BRD. For instance, viral agents combine to impair respiratory mucosal immune defenses, thereby allowing colonization by bacterial pathogens (Hay et al., 2016). Clinical research shows that *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, *Arcanobacterium pyogenes*, *Trueperella pyogenes* (Griffin et al., 2010) as well as *Mycoplasma bovis*, *Mycoplasma bovirhinis*,

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Mycoplasma dispar and Ureaplasma diversum (Klima et al., 2014) are the most commonly isolated pathogens from calves that develop BRD. Interestingly, most of these bacteria are considered transient residents of the upper respiratory tract of healthy cattle (Magwood et al., 1969), but become opportunistic pathogens when viral infections and the neurohumoral effects of stress, impair the systemic and local host immune defenses (Griffin et al., 2010). The use of metaphylactic antimicrobial treatment upon entry to the feedlot has been a significant advance in the management of BRD caused by these bacterial pathogens (Taylor et al., 2010). However, with increasing concerns regarding the overall efficacy of current antibiotics, and the development of antimicrobial resistance, new management strategies for optimizing mucosal health and immune defenses, are required.

In view of the important role of the nasopharyngeal microbial phenotype (microbiota) and genotype (microbiome) in respiratory health, an understanding of normal microbial community structure, and its perturbation in disease states, could provide critical insights into the pathogenesis of respiratory tract infectious disease, as well as inform the development of new management approaches to optimize bovine respiratory health (Allen et al., 2014). Our current knowledge of bovine respiratory tract microbial communities has relied heavily on conventional culture-based methods, with a focus on identifying the presence of major pathogens rather than on the overall structure of the microbial community (Francoz et al., 2015). The purpose of this study was to characterize the dynamics of change of the nasopharyngeal microbial community structure of feedlot calves in the commercial feedlot, by amplifying and analyzing the hypervariable region (V1-V3) of the 16S-rRNA gene. The study compared the nasopharyngeal microbiota of four populations of cattle; calves at entry processing that later developed BRD (BRD-E), calves that developed BRD within one month after entry at the time of initial diagnosis (BRD-D), calves at entry processing that remained healthy (Healthy-E) and healthy calves at the time matched to disease identification in the BRD calves (Healthy-D).

2. Materials and methods

2.1. Animals and sample collection

The study population consisted of Charolais feedlot calves (n = 135, mean body weight at entry 247 ± 33.8 kg) that had been recently transported from a university-owned cow-calf operation (Dixon Springs, southern Illinois, USA), to the commercial and research university feedlot at South Farms Beef cattle and Sheep Field Laboratory (Urbana, IL, USA). All calves in this group were processed within 24 h after arrival. The calves did not receive any antimicrobial drugs prior to arrival, or at initial processing. During processing, each calf was restrained in a hydraulic chute, weighed, ear tagged, vaccinated with a modified live virus vaccine against IBR, BVDV (Types I and II), BPIV-3 and BRSV (5 ml IM; Bovi-Shield Gold FP5 L5 HB Cattle Vaccine, Zoetis Animal Health) and dewormed with a topical anthelminthic (Noromectin Pour-On Solution, Norbrook® Inc. USA). The use of animals for this study was approved by University of Illinois Institutional Animal Care and Use Committee (IACUC Protocol: #15064).

Physical examinations were performed on each calf during processing and included demeanor, rectal temperature, presence of ocular or nasal discharge and respiratory rate. A more detailed respiratory system examination was performed using a non-invasive, computer-aided evaluation of lung sounds, and ultrasonographic assessment of the pleural surface and superficial lung tissue. For each animal the lung sounds were recorded at a standardized location in the 5th intercostal space of the right hemithorax using a commercially available, automated Whisper stethoscope (Whisper®, Geissler Corp, Plymouth, MN). After collection, the recorded sounds were interpreted by an accompanying software package that converts the measurements in to a 5-point lung score scale (Mang et al., 2015). A thoracic ultrasound evaluation was performed with a 4.5 MHz linear probe (EasiScan™, BCF Innovative Image Technology, LLC, USA), using a standardized scanning process along the 5th-11th intercostal spaces of the right hemithorax (Buczinski et al., 2014).

The head of each calf was manually restrained, and a deep nasopharyngeal swab were collected from the left nostril using a 33-inch-long, double-guarded PVC culture swab (Kalayjian Industries, Inc. U.S.A.) (Godinho et al., 2007). Briefly, the calf's nostrils were cleaned with a disposable wipe before collection. The nasopharyngeal swab was carefully inserted into the ventral meatus of the nose, and advanced approximately 2/3rds of the dorsal head length. Once in place, 1–2 inches of the swab was exposed by withdrawing the outer sleeve, and then firmly rotated 360° against the pharyngeal wall for 20–30 s. Once completed, the cotton tipped swab was retracted back in to the outer sheath, and the whole swab was removed gently from the animal's nose. The cotton swabs were then broken off into sterile 2-mL cryo-tubes and transported on dry ice to the laboratory pending further procedures.

Following initial processing, the calves were maintained in the university beef unit according to established health care protocols. During the study period the calves were grouped in 16 ft × 64 ft pens, at a stocking density of approximately 30 animals per pen. Calves were fed a total mixed ration (20% silage, 20% modified wet distillers grains with soluble, 10% dry supplement, and 50% high moisture corn) and given free access to water.

Following entry, all of the calves were monitored daily for signs of respiratory disease according to industry-standard protocols (anorexia, nasal discharge, change in respiratory pattern, rectal temperature ≥ 40 °C and Whisper lung score ≥ 3). Deep nasopharyngeal swabs were collected from any calves showing clinical signs of BRD, just prior to treatment (n = 22). In addition, clinically healthy, pen-matched controls calves (n = 10) were sampled at the same time. After sampling, the calves with BRD were treated with a single dose of tilmicosin (10 mg/kg SC; Micotil, Elanco Animal Health) according to label instructions. Immediately after being sampled and treated the calves were returned to their original group pen and monitored for clinical improvement.

2.2. Genomic DNA extraction

A sub-group of healthy calves (n = 66) was selected from the original entry cohort (n = 135), based on rectal temperature (< 102.9°F), normal lung scores (1), and the absence of abnormal nasal discharge, ultrasonographic evidence of lung pathology, or evidence of traumatic injury or other disease at the time of initial processing. The nasopharyngeal samples from these calves, along with samples from the BRD calves and their healthy, age and pen-matched controls, were processed for DNA extraction using the power® Fecal DNA isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, each cotton swab was removed from the cryo-tube and added to a dry bead tube with 750 µl of Bead Solution, heated at 65 °C for 10 min, and settled in Bullet Blender 24 Gold tube holder (Next Advance, Inc., Averill Park, NY, USA). The tubes were vortexed at maximum speed for 10 min to achieve microbial cell disruption. The power® Fecal DNA isolation Kit protocol was used to complete the extraction according to manufacturer instructions. The DNA integrity of each sample was evaluated by agarose gel electrophoresis and the concentration was quantified using a Nanodrop™ spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) at wavelengths of 260 and 280 nm.

2.3. Fluidigm access array amplification of the V1-V3 hypervariable region of 16S rRNA genes and illumina sequencing

DNA extracted was subject to Fluidigm Access Array Amplification (Fluidigm Corp.). Prior to amplification all DNA samples were measured on a Qubit fluorometer (Life technologies, Grand Island, NY,

USA). 16S rRNA gene amplicons of were generated using a Fluidigm Access Array (Fluidigm Corporation, South San Francisco, CA, USA) in combination with Roche High Fidelity Fast Start Kit (Roche, Indianapolis, IN, USA) as mentioned in (Welly et al., 2016). The primer sequences F28-2-for (ACACTGACGACATGGTTCTACA) and R519-2-rev (TACGGTAGCAGAGACTTGGTCT) were used to amplify the V1-V3 hypervariable region of 16s rRNA gene. CS1 forward tag and CS2 reverse tag were added according to the Fluidigm protocol. The final harvested product was then quantified on a Qubit fluorometer the quality of the amplicons regions was assessed using a Fragment Analyzer (Advanced Analytics, Ames, IA, USA) to confirm amplicon regions and sizes. DNA samples were then pooled in equal amounts according to product concentration. The pooled samples were then size selected on a 2% agarose E-gel (Life technologies, Grand Island, NY, USA) and extracted using Qiagen gel purification kit (Qiagen, Valencia, CA). Cleaned size-selected pooled products were run on an Agilent Bioanalyzer to confirm appropriate profile and average size. The final pooled Fluidigm libraries were transferred to the DNA Services lab at the W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign for Illumina sequencing. The Illumina MiSeq platform (Illumina, San Diego, CA, USA) was used to sequence the V1- V3 region of the 16S rRNA gene according to the Illumina instructions.

2.4. Sequence data processing and statistical analysis

The sequence run generated Illumina base call (bcl) files were converted into demultiplexed, compressed fastq files using bcl2fastq 1.8.4 (Illumina, CA). A secondary pipeline was used to decompress the fastq files, generate quality score plots (FastX Tool Kit), and to report the number of reads per sample per library. The bcl files were also processed in bcl2fastq 1.8.4 without demultiplexing, and then sorted by initial PCR-specific primer using a custom in-house pipeline. After preprocessing, sequences were processed and analyzed using an open-source software package (Quantitative Insights into Microbial Ecology (QIIME) software, version 1.9) (http://qiime.org/) (Caporaso et al., 2010). Chimeric sequences were detected and removed using UCHIME (Edgar et al., 2011). The remaining sequences were clustered into operational taxonomic units (OTUs) using open reference, OTU selection protocol (97% identity cutoff) with the UCLUST algorithm (Edgar, 2010), and were taxonomically assigned against the Greengenes database, to the genus level (McDonald et al., 2012). Additionally, we generated a species-level OTU table using the MiSeq Reporter Metagenomics Workflow. The MiSeq Reporter classification is based on the Greengenes database and the output of this workflow is a classification of reads at multiple taxonomic levels: kingdom, phylum, class, order, family, genus, and species.

The core microbiota of nasopharyngeal samples from all calves was also determined at the genus level. Since the inter-individual variability resulted in a limited core microbiota, we identified the core microbiota as the OTUs present in more than 50% of animals across all samples. Bacterial diversity (an estimate of bacterial community richness in a sample) was calculated within QIIME using the Chao1, observed species and Phylogenetic Diversity (PD) whole tree. Due to unequal sequencing depth between the different calves groups, and to standardize sequence counts across samples, all samples were randomly subsampled and rarefied to 1000 sequences per sample using QIIME.

To determine the changes in NP bacterial communities over time and with disease, the relative abundance of nasopharyngeal bacterial phyla, bacterial genera and bacterial diversity metrics (Chao1, observed species and PD whole tree) between the different groups (BRD-E, BRD-D, Healthy-E and Healthy-D) were compared using non-parametric Wilcoxon/Kruskal-Wallis Tests fitted in JMP 12.12 (SAS Institute Inc.). Differences with a value of P-value ≤ 0.05 were considered significant.

To further explore the dynamics of change in these microbial communities between clinically healthy calves (Healthy-D) and those that develop BRD (BRD-D) within the first month after entry, the OTU relative abundance values were analyzed using the linear discriminant analysis (LDA) effect size (LEfSe) algorithm to identify OTUs that display significant differences between clinically healthy calves and those that develop BRD (Segata et al., 2011). Additionally, a cladograms were produced using the online LEfSe tool. The algorithm first used the non-parametric factorial Kruskal-Wallis test to detect taxa with significantly different abundance, followed by pairwise Wilcoxon test to detect biological consistency between the two groups, and then used LDA to estimate the effect size of each differentially abundant feature.

We then compared all of calf groups (BRD-E, BRD-D, Healthy-E and Healthy-D) with each other by stepwise discriminant analysis using JMP 12.2 (SAS Institute Inc.). For this analysis, the relative abundances of different bacterial genera in each sample was used as a covariate, and the calf groups (BRD-E, BRD-D, Healthy-E and Healthy- D) was used as the categorical variable. In our study, variables were removed from the models in a stepwise manner until only variables with a p value of 0.05 were kept in the final model. The discriminant analysis was used to determine how equivalent samples, from animals in different groups, were differentiated from one another, and was illustrated using canonical loading plots.

Fastq data obtained in the current study were uploaded to the sequence read archive (SRA) on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/sra) to make the files available for public databases with a bioproject accession number PRJNA318938.

3. Result

3.1. Overall 16S rRNA, V1-V3 hypervariable region, sequence analysis

The combined sequence analysis from the nasopharyngeal samples all cattle resulted in a total of 1,297,074 sequences. The mean sequence per nasopharyngeal sample was 91,567, the SD was 12716.412 and comprised 562 OTUs (97% identity cutoff) across all samples. In terms of relative abundance, taxonomic analysis revealed a total of 15 different bacteria phyla, and 165 bacterial genera, across all samples.

3.2. Characterization of the nasopharyngeal microbiota within the different calf groups (BRD-E, BRD-D, healthy-E and healthy-D)

Across all the samples the 6 most predominant phyla as *Proteobacteria* (34.82%), *Firmicutes* (18.56%), *Actinobacteria* (17.21%), *Bacteroidetes* (12.05%), *Tenericutes* (11.18%), and *Fusobacteria* (1.18%) (Fig. 1). All other classified OTUs belonged to bacterial phyla comprising less than 1% of the total abundance represented as others/unassigned taxa (Fig. 1). The relative abundance of each phylum between individuals was highly variable across all the nasopharngeal samples (Fig. S1).

At the genus level, the most abundant bacterial genera across all samples, were *Moraxella* (10.86%), *Mycoplasma* (10.71%), *Acinetobacter* (9.69%), *Rathayibacter* (5.04%) Promicromonospora (4.40%) and *Mannheimia* (4.07%). Other fairly common bacterial genera were *Solibacillus* (3.51%), *Clostridium* (3.30%), and *Corynebacterium* (3.79%), *Pasteurella* (1.88%) (Fig. 2). All other classified OTUs that comprised less than 1% of the total abundance and belonged to bacterial genera were classified as 'other' or 'unassigned' (Fig. 2). There was a high inter-individual variability in the composition of the nasopharngeal microbiota across all the individuals (Fig. S2).The relative abundance of the most common fifty bacterial genera across different groups are shown in (Table S1).

In an effort to explore the trajectory of NP microbial community change within the different calves groups, non-parametric Wilcoxon/Kruskal-Wallis Tests were used to compare the mean relative abundance of the most abundant bacterial phyla and genera comprising > 1% for all four calf groups (BRD-E, BRD-D, Healthy-E and Healthy- D). At the phylum level, there were statistically significant differences

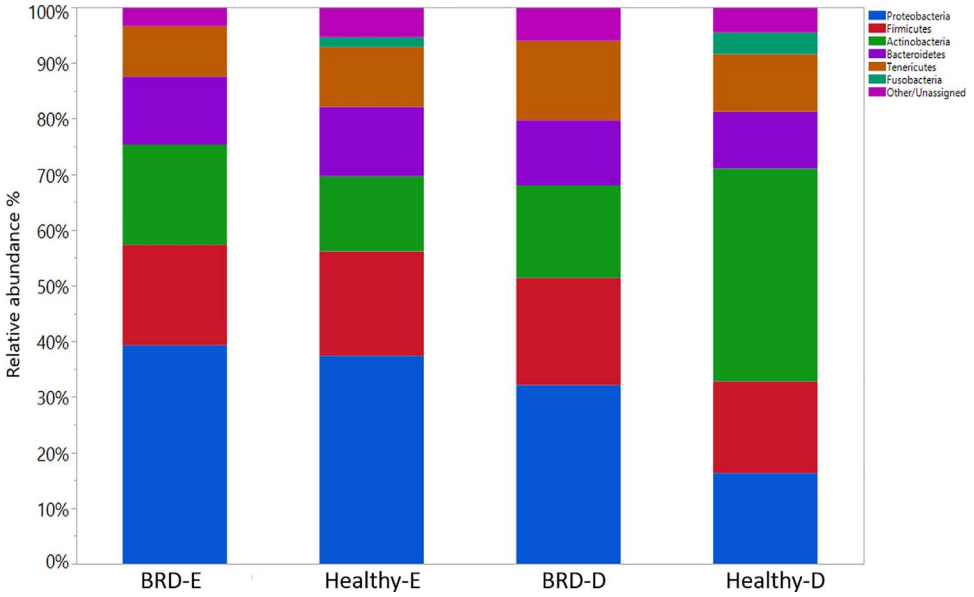

Fig. 1. Relative abundance of bacterial 16S rRNA gene sequences at the phylum level observed in the nasopharyngeal swabs from calves at entry processing that later developed BRD (BRD-E), calves that developed BRD within one month after entry at the time of initial diagnosis (BRD- D), calves at entry processing that remained healthy (Healthy- E) and healthy calves at the time matched to disease identification in the BRD calves (Healthy- D). Only the bacterial phyla representing populations that averaged more than 1% of the relative abundance across all samples are displayed. All other classified OTUs comprising less than 1% of the total abundance are represented as others/unassigned taxa.

between the four groups in the abundance of *Proteobacteria* ($p = 0.0406$), *Actinobacteria* ($p = 0.041$) and *Fusobacteria* ($p = 0.005$). While, at the genus level, there were statistically significant differences between the four groups in the abundance of *Acinetobacter* ($p = 0.041$), *Rathayibacter* ($p = 0.001$), *Corynebacterium* ($p = 0.006$), *Solibacillus* ($p = 0.004$) and *Turicibacter* ($p = 0.008$).

3.3. Dynamics of change in the nasopharyngeal microbiota between clinically healthy calves and those that develop BRD during the first month after entry

To evaluate the potential change in nasopharyngeal microbiota associated with BRD, we compared the nasopharyngeal microbial communities between calves that develop respiratory disease during the first month after arrival (BRD-D), and pen matched clinically healthy calves (Healthy-D). We found marked differences in relative abundance of certain taxa between these populations based on non-parametric Wilcoxon tests. At the phylum level, we observed a predominance of *Proteobacteria* in BRD-D calves [32.12% versus 16.32% in clinically healthy calves ($p = 0.054$)] (Fig. S3). While, in Healthy-D calves we observed a predominance of *Actinobacteria* [38.29% versus 16.58% in BRD-D calves ($P = 0.034$)] (Fig. S3) and *Fusobacteria* [3.86% versus 0.03% in BRD-D calves ($P = 0.063$)] (Fig. S3).

At the genus level, in BRD-D calves we observed a statistically significant predominance of *Acinetobacter* [12.54% versus 2.16% in Healthy-D calves ($p = 0.0059$)], *Solibacillus* [3.71% versus 0.02% in Healthy-D calves ($p = 0.0018$)], and *Pasteurella* [2.38% versus 0.03% in Healthy-D calves ($p = 0.047$)]. While, in Healthy-D calves we only observed a statistically significant predominance of *Rathayibacter* [20.09% versus 3.96% in BRD-D calves ($p = 0.0051$)], as depicted in (Fig. 3).

Additionally, the difference in the microbial composition relative abundance between the Healthy-D and those BRD-D were compared using the Linear Discriminant Analysis (LDA) Effect Size (LEfSe) algorithm (Fig. 4). The nasopharyngeal samples indicator OTUs from the Healthy-D calves were clustered into the bacterial taxa *Rathayibacter*, *Cellulomonadaceae* and *Bacteroidales*. The nasopharyngeal sample indicator OTUs from BRD-D calves were clustered into the taxa *Arcanobacterium*, *Actinomycetaceae*, *Microbacterium*, *Lysinibacillus*, *Solibacillus*, *Bacillus*, *Comamonas*, *Burkholderiales*, *Neisseriales*, *Acinetobacter*, *Moraxellaceae*, *Pseudomonadales*, *Stenotrophomonadaceae*, *Xanthomonadaceae* and *Xanthomonadales* (Fig. 4).

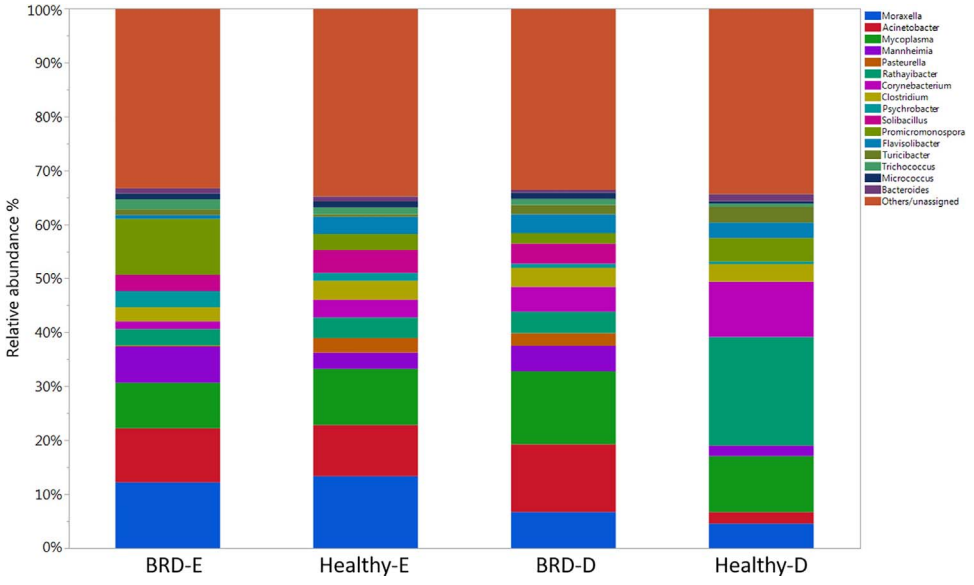

Fig. 2. Relative abundance of bacterial 16S rRNA gene sequences at the genus level observed in the nasopharyngeal swabs from calves at entry processing that later developed BRD (BRD-E), calves that developed BRD within one month after entry at the time of initial diagnosis (BRD- D), calves at entry processing that remained healthy (Healthy- E) and healthy calves at the time matched to disease identification in the BRD calves (Healthy- D). At genus level, only those bacterial species representing populations that averaged more than 1% of the relative abundance across all samples are displayed. All other classified OTUs comprising less than 1% of the total abundance are represented as others/unassigned taxa.

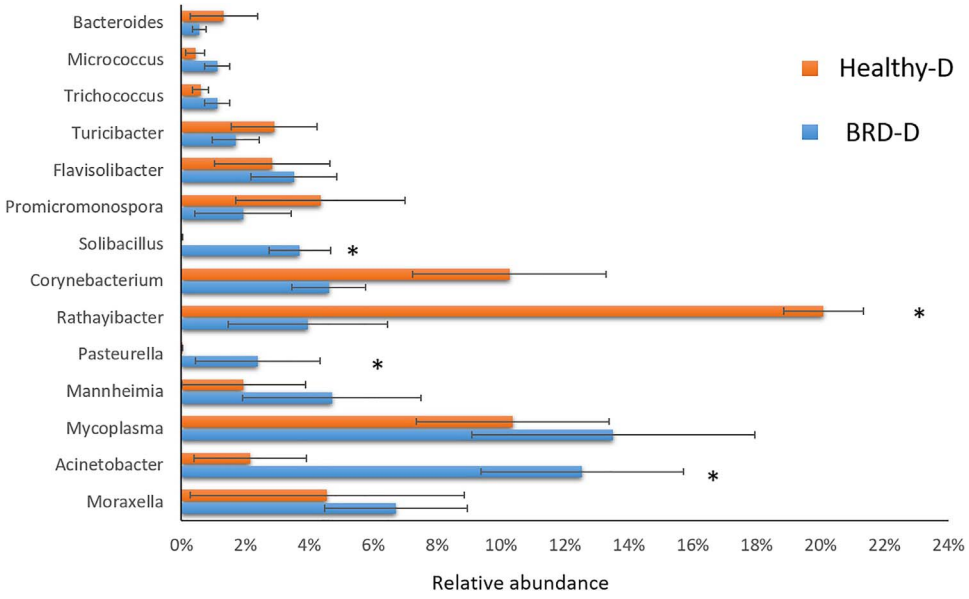

Fig. 3. Bar graphs illustrating the relative abundance of top bacterial genera observed in the nasopharyngeal swabs from calves that developed BRD within one month after entry at the time of initial diagnosis (BRD- D) and pen matched clinically healthy calves (Healthy- D). Error bars represent standard errors. The asterisks indicate significant differences between BRD affected calves and pen matched clinically healthy calves.* $p \leq 0.05$.

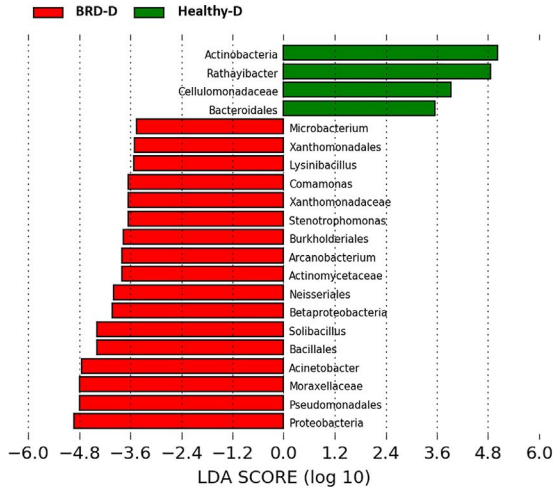

Fig. 4. LEfSe comparison of the nasopharyngeal microbiota in calves that developed BRD within one month after entry at the time of initial diagnosis (BRD- D) and pen matched clinically healthy calves (Healthy- D) depicting the top operational taxonomic units (OTUs) with the highest linear discriminant analysis (LDA score log10 ≥ 2.0). These graphical outputs were generated by the publicly available LEfSe visualization modules. LEfSe scores can be interpreted as the degree of consistent difference in relative abundance between the two classes of analyzed microbial communities.

3.4. Discriminant analysis

Stepwise discriminant analysis were used to compare all four calf groups (BRD-E, BRD-D, Healthy-E and Healthy- D) with one another. This analysis showed that the nasopharyngeal microbial profile of the BRD-E, BRD-D and Healthy-E calves were each distinct from the Healthy-D calves (Fig. 5).

3.5. Bacterial diversity indices and core microbiota of nasopharyngeal microbiota

Bacterial diversity and richness of the nasopharyngeal microbiota were measured using several metrics namely Chao1, observed species (number of taxa) and PD whole tree (Fig. 6; Table 1). There were no statistically significant differences ($p < 0.05$) in microbial diversity or richness between the groups (BRD-E, BRD-D, Healthy-E and Healthy-D).

The core microbiota of the nasopharyngeal samples from all calves was also determined. In view of the high inter-individual variability, we limited our delineation of the core microbiota to include only those OTUs that were present in more than 50% of all samples. With this definition we observed a core nasopharyngeal microbiota of *Mycoplasma*, *Moraxella*, *Acinetobacter*, *Corynebacterium*, *Clostridium*, *Solibacillus*, *Turicibacter*, *Bacteroides* and *Blautia*.

4. Discussion

The cooperative and mutually beneficial interactions between microbial communities and their hosts have been recognized for many years. It is widely accepted that mucosal bacterial populations act, not only as a reservoir of potential pathogens, but also in regulating local and systemic immune homeostasis through a complex cross talk with the epithelium, local immune system and mucosal neuronal pathway (Corthésy et al., 2007; Gensollen et al., 2016). Many of the principles of host-microbiota relationships have arisen from studies in the gastrointestinal tract (Holmes et al., 2011). More recently, there is a growing appreciation of the respiratory tract as a unique ecosystem hosting many distinct microbial populations that interact together and affect host immune responses (Timsit et al., 2016). In feedlot cattle, the composition of the nasopharyngeal microbiota has been shown to play an important role in determining respiratory tract health (Holman et al., 2015b).

An important aim of this study was to compare the nasopharyngeal microbiota of four populations of cattle (BRD-E, BRD-D, Healthy-E and Healthy- D) during first month of the feedlot production cycle. Since the window of BRD susceptibility is high during this initial 4-week post-entry period (Booker et al., 2008), it is anticipated that a clear understanding of the dynamics of change in the nasopharyngeal microbiota during this transitional stage of the feed cycle may provide critical insights into the pathogenesis of BRD, and inform new approaches for optimizing health management.

The cattle in this study all originated from the same cow-calf enterprise, and had been exposed to similar management and environmental challenges, and so the pattern of nasopharyngeal microbial communities was anticipated to be relatively consistent across individuals during initial processing. In line with other studies, the most predominant nasopharyngeal phyla were *Proteobacteria* and *Firmicutes* (Holman et al., 2015a,b). Interestingly this is also comparable with other species (Huse et al., 2012). At the genus level, the most prevalent bacterial genera were *Moraxella*, *Mycoplasma*, *Acinetobacter*. The high abundance of these particular bacterial taxa at entry processing was particularly striking since this same combination of genera was found at

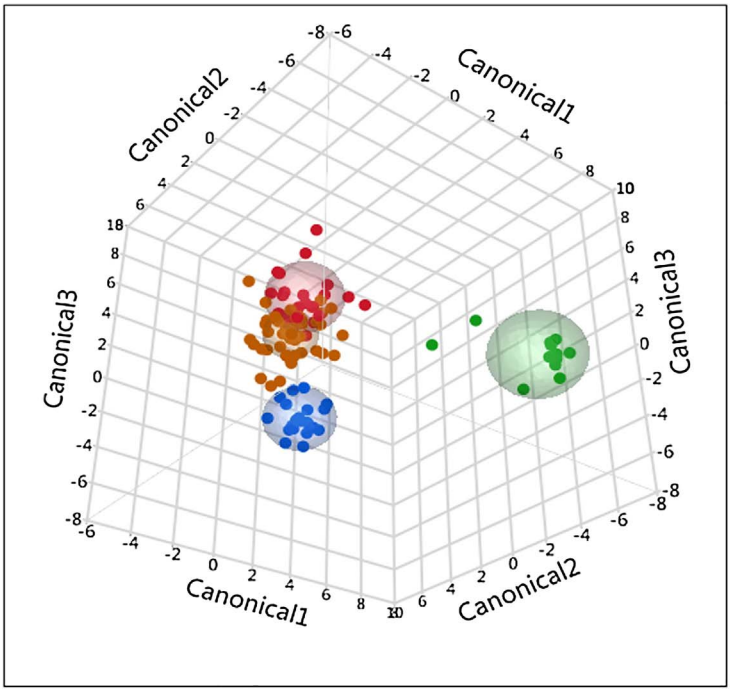

Fig. 5. Discriminant analysis of nasopharyngeal microbiota in calves at entry processing that later developed BRD (BRD-E), calves that developed BRD within one month after entry at the time of initial diagnosis (BRD- D), calves at entry processing that remained healthy (Healthy- E) and healthy calves at the time matched to disease identification in the BRD calves (Healthy- D). Each dot represents an individual calf, and the circles indicate the discriminate variation between groups. Different mean relative abundances of bacterial genera in nasopharyngeal microbiota were used as covariates, and sampling groups were used as categorical variables. Differences in the nasopharyngeal microbial profiles of the different groups are illustrated by canonical 1, 2 and 3.

high predominance in those calves that developed BRD within one month after arrival.

The genus *Mycoplasma* was also present at a high relative abundance in nasopharyngeal samples from all animals, across different groups. This substantiates the notion that, while *Mycoplasma* species may on occasion contribute to increased morbidity and mortality in feedlot cattle (Caswell et al., 2010), they are common inhabitants of the bovine respiratory tract (Magwood et al., 1969; Maunsell and Donovan, 2009; Timsit et al., 2016), and can be considered as part of the core nasopharyngeal microbiota of both healthy and diseased cattle. It is likely that the *Mycoplasma* populations contained mainly non-pathogenic organisms such as *Mycoplasma dispar* and *Mycoplasma bovirhinis* (Magwood et al., 1969). The high relative abundance of *Mycoplasma* populations (Fig. S4) in this study also highlights the advantage of using culture-independent techniques, since these organisms are notoriously fastidious, and difficult to growth ex vivo (Holman et al., 2015b).

In addition to characterizing the nasopharyngeal microbiota at entry, we evaluated the dynamics of change in nasopharyngeal microbial communities between clinically healthy calves and those that develop BRD within the first month of entry in to the feedlot. Our results revealed a predominance of *Proteobacteria*, *Firmicutes* and *Tenericutes* phyla in samples obtained from BRD affected calves compared to samples from clinically healthy calves. Futhermore, we found more *Acinetobacter*, *Solibacillus* and *Pasteurella* in samples from BRD affected calves compared to clinically healthy calves. Although *Mycoplasma*, *Moraxella*, *Mannheimia* and *Pasteurella* are the most well-known pathogens responsible for BRD in feedlot cattle (Booker et al., 2008; Griffin et al., 2010), our results did not show an increase in relative abundance of *Mannheimia* between the diseased and healthy calves. The higher relative abundance of *Acinetobacter* species (Fig. S5) suggest that this taxa may also be, in some way, associated with the development of BRD, and warrants additional study. It is important to note that there was no attempt to sub-classify any of the microbes by other techniques (e.g. serotyping), and so additional conclusions regarding the potential virulence of any specific taxa could not be made.

Acinetobacter genus is a realtively new, heterogeneous group of pathogens that play a significant role in the colonization and infection of patients admitted to hospitals (Dijkshoorn et al., 2007). While *Acinetobacter* species have been implicated in a diverse array of diseases, such as urinary tract infection and secondary meningitis, their predominant role is as a causal agent of nosocomial respiratory disease, particularly ventilator-associated pneumonia in patients confined to hospital intensive care units (Bergogne-Bérézin and Towner, 1996).

The different bacterial diversity indices suggest that the bovine nasopharyngeal microbial communities in the animals in this study were rich and diverse, though not as diverse as other body sites

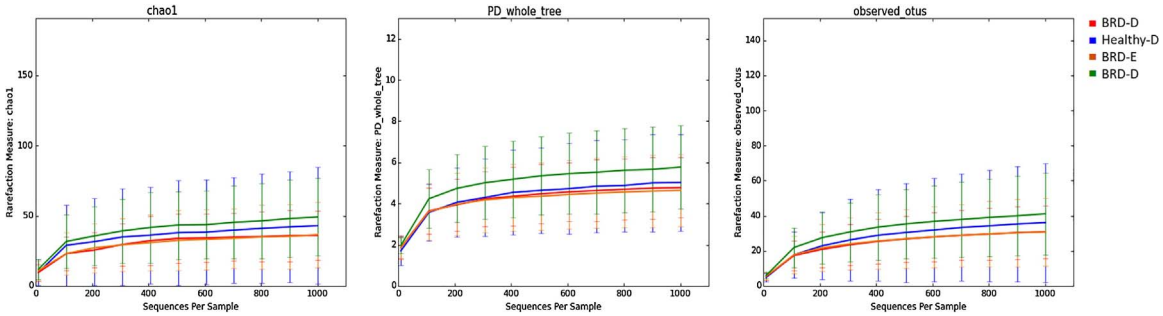

Fig. 6. Rarefaction curves of 16S rRNA gene sequences obtained from nasopharyngeal samples from calves at entry processing that later developed BRD (BRD-E), calves that developed BRD within one month after entry at the time of initial diagnosis (BRD- D), calves at entry processing that remained healthy (Healthy- E) and healthy calves at the time matched to disease identification in the BRD calves (Healthy- D). The graphical lines represent the mean and error bars represent standard deviations. The analysis was performed on a randomly selected subset of 1000 sequences per sample.

Table 1
Bacterial diversity indices (Chao1, PD whole tree and observed species) measures for the nasopharyngeal microbiota of calves that later developed BRD at entry processing (BRD-E), calves that developed BRD within one month after entry at the time of initial diagnosis (BRD- D), calves at entry processing that remained healthy (Healthy- E) and healthy calves at the time matched to disease identification in the BRD calves (Healthy- D). The data are presented as the mean ± standard deviation. There were no statistically significant differences in different bacterial diversity indices between the different groups ($p > 0.05$).

Bacterial Diversity indices	BRD-E	Healthy-E	BRD-D	Healthy-D	P value
Chao1 index	49.12 ± 27.57	36.58 ± 23.19	36.01 ± 17.72	42.99 ± 41.65	0.474
PD whole tree	5.77 ± 2.03	4.64 ± 1.76	4.77 ± 1.47	5.02 ± 233	0.182
Observed species	41.16 ± 23.17	30.83 ± 13.39	30.88 ± 15.08	36.11 ± 33.89	0.516

(Oikonomou et al., 2013). There were no statistically significant differences in microbial diversity or richness between clinically healthy calves, calves sampled at entry, or those that developed BRD within one month after entry (Table 1). These results are different from previous studies that have shown that the nasopharyngeal microbiota in healthy feedlot cattle has a more rich and diverse population than that of BRD affected calves (Holman et al., 2015a).

Despite the uniform management conditions, there was a high inter-individual variability in the composition of the nasopharyngeal microbiota at both the phylum and genus level (Figs. S1, S2). This is perhaps expected, especially in feedlots husbandry systems, as the upper respiratory tract is constantly exposed to many and various bacteria from the surrounding environment (Holman et al., 2015b). This high inter-individual variability resulted in a limited core microbiota, which is similar to that described in studies in people in which only two OTUs accounted for 17% of sequences in the anterior nares (Huse et al., 2012).

In the present study, a total of 1,297,074 sequences were resulted from the sequencing analysis with an average of 91,567 sequences per sample. At present, the analysis and interpretation of microbiome data sets of this magnitude are a major challenge for researchers (Edrington et al., 2012). In our study, we used stepwise discriminant analysis to help interpret this data, and to identify bacterial genera that significantly discriminated between calves in different experimental groups. This approach has been used successfully to visualize metagenomic data and identify bacterial taxa of interest for further analysis (Lima et al., 2015), to disclose microbial community profiles across different disease phenotypes (Willing et al., 2010), and to relate disease status with future growth performance in calves (Oikonomou et al., 2013). In our study, discriminant analysis showed that the nasopharyngeal microbial profiles of the BRD-E, BRD-D and Healthy-E calves were distinct from those observed in the Healthy-D calves (Fig. 5). This clear shift in the microbiota composition supports the notion that the trajectory of change of nasopharyngeal microbial community structure in the weeks following entry and processing is associated with the development of BRD during the early phase of growth in the feedlot environment. Similar changes in composition of the nasopharyngeal microbiota of feedlot cattle during the first weeks after arrival at a feedlot have been previously described (Timsit et al., 2016; Holman et al., 2017). At this stage of our understanding, these patterns do not establish a causal relationship between microbial community structure and the development of BRD, but may simply be a bioindicator of overall mucosal health in these animals. However, it does seem likely that the mutiple stressors (e.g. commingling, transportation and diet transition), associated with the adjustment of recently arrived cattle to the feedlot environment, would impact the mucosal immune status, upper respiratory epithelial health, and stability of the nasopharyngeal microbiota in these animals (Loerch and Fluharty, 1999), thereby altering disease susceptibility. In addition to neuroendocrinological stress, viral agents have also been shown to be able to induce nasopharyngeal dysbiosis through alteration of host defense mechanisms (Caswell, 2014). Recent research in people has demonstrated that dietary changes and disturbances in the gastrointestinal microbiota can also alter the respiratory microbiota (Madan et al., 2012). Further studies are needed to determine the potential role and mechanisms by which the changes in nasopharyngeal microbiota observed in this study are related to the long term respiratory health and susceptibility to disease in growing cattle.

5. Conclusions

Overall, the results of this study support the conclusions of other investigators, that the nasopharyngeal microbiota at entry in to a feedlot could serve as a key player in the pathophysiology of BRD. While the large sample size used in this study (n = 66 at entry during initial processing) substantiates the identity of the overall nasopharyngeal microbial populations in this group of animals, the prolonged time lapse (30 days) between specific sample time points prevents us from drawing conclusions regarding the process and mechanism by which the trajectory of change in microbial communities differs between healthy and diseased animals. An understanding of the intrinsic and extrinsic factors that impact the post-entry maturation of the nasopharyngeal microbiota will be important in determining whether management strategies can be used to encourage a sustain the microbial ecology of the respiratory tract during this important and determinative phase of production.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2017.07.006.

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