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Biogeography of the Bovine Respiratory Microbiome and Its Relationship with Bovine Respiratory Disease

Jianmin Chai
University of Arkansas, Fayetteville

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by

Jianmin Chai
Hebei North University
Bachelor of Science in Animal Science, 2012
Chinese Academy of Agricultural Sciences
Master of Science in Animal Nutrition and Feed Science, 2015

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University of Arkansas

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Jiangchao Zhao, Ph.D
Dissertation Director

Beth Kegley, Ph.D.
Committee Member

Jeremy G. Powell, DVM, Ph.D.
Committee Member

Jung Ae Lee-Bartlett, Ph.D.
Committee Member

Abstract

Bovine respiratory disease (BRD) is the most common and costly disease in the beef cattle industry, leading to high morbidity, mortality and huge economic loss. Despite the recent advances in vaccination and antimicrobial techniques, no significant health-improved outcomes have developed. Due to a deep investigation of the microbiome, respiratory microbiotas are known to have important roles for host health and disease. However, BRD specific pathogens have not yet been identified since they are found in both healthy and diseased animals. A systemic and comprehensive study of the biogeography of the bovine respiratory microbiome and its relationship with BRD is lacking and urgently needed. In this dissertation, we characterized the biogeography of the bovine respiratory microbiome from a total of 222 samples and identified the microbial composition of the nostrils, nasopharynx and lungs. Signature microbiota for each niche were identified (Chapter III). Shared bacteria among the three niches were observed, and a strong correlation between adjacent sampling niches was found. Next, using a random forest model (Chapter IV), high accuracies of the nasal, nasopharyngeal and lung microbiomes to predict and diagnose BRD were found. A set of bacterial features were identified. A significantly temporal dynamic of the respiratory microbiome was found from feedlot arrival to the onset of BRD, with consistent increases in the abundance of BRD pathogens and consistent decreases of the commensal microbiota. Finally, the spatial microbial movement within the bovine respiratory tract associated with BRD status was clarified (Chapter V). A larger proportion of the lung microbiota was found to be derived from the upper airway community in BRD calves compared to healthy calves, and pathogens in BRD lungs could be predicted by using their abundances in the BRD upper airway. Complex interactions among commensal microbiota were found in healthy calves, while dysbiosis of the microbial community as well as increased pathogen interactions in the airway were found in BRD calves.

All our discoveries from the first (test) trial were validated in the second (validation) animal trial. In conclusion, this comprehensive study further advanced our understanding of the relationship between the respiratory microbiome and BRD. Additionally, nasal swabbing was found as an innovative approach to be used for BRD research. It provides a new research direction into airway disease research and is capable of providing more advanced microbial therapies for bovine respiratory disease.

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Dedication

I would like to dedicate this dissertation to my accomplished professor, Dr. Jiangchao Zhao, who taught me to pursue the truth in research.

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Chapter I. Introduction

Introduction to the Bovine Respiratory Microbiome

Bovine respiratory disease (BRD), a leading cause of morbidity, mortality and economic loss, is one of the largest health challenges facing the modern-day beef cattle industry, especially in newly weaned and transported finished cattle (Nicola et al., 2017). BRD does not only result in increased therapeutic costs, but morbid cattle also grow slower, develop an inefficient feed conversion ratio, and have carcasses of a lower quality grade after slaughter. Despite the recent advances in vaccine and antimicrobial technologies, the morbidity and mortality rates caused by BRD have not decreased over the last 20 years. Antibiotics are commonly administered to cattle determined to be morbid with BRD, and often cattle are treated with different antimicrobials upon subsequent diagnoses (Holman et al., 2019). Although a common practice in the industry, there is little scientific evidence supporting that the administration of antibiotics is effective in reducing the longevity of BRD symptoms or improving health outcomes (Lima et al., 2016). The continued prevalence of BRD in tandem with shifting consumer preferences toward a reduction in livestock antibiotic usage will influence the necessity to improve management practices and develop alternative therapies, such as probiotics, driving the significance of investigative bovine respiratory microbiome studies.

In past decades, next-generation sequencing (NGS) has contributed to the progressive understanding of the roles of the resident microbiota (Man et al., 2017). The respiratory microbiome plays an important role in maintaining health and is the main indicator of disease (Lloyd-Price et al., 2016). The microbial ecosystem aids in maintaining normal host physiology by developing and cooperating with the immune system, metabolizing complex products and providing critical defense against infections of opportunistic pathogens (Gao et al., 2014; Shukla

et al., 2017). Thus, understanding of the respiratory microbiota is critical for better understanding BRD pathophysiology and provides insights into probiotics isolation for alternative therapies.

The respiratory ecosystem contains the upper respiratory tract (URT) and the lower respiratory tract (LRT) at the anatomical and physiological perspectives. The mucosal surface of the respiratory tract is inhabited by niche-specific microbiome communities (Man et al., 2017). Current research relevant to the bovine respiratory microbiome is only at an initial stage. Most culture-dependent studies of BRD have focused on the opportunistic bacterial BRD pathogens in the nasopharynx, such *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis* (Nicola et al., 2017). However, these bacteria have been isolated from both healthy and sick animals. Although several studies have attempted to investigate the nasal and lung microbiomes using NGS, the signature microbiota for BRD have not yet been identified and the microbial movements within the respiratory tract are still unknown. Despite various experimental designs and results in previous studies, the notion that the respiratory microbiome is significantly important to cattle health has been confirmed (Klima et al., 2019; Timsit et al., 2016; Zeineldin et al., 2019; Zeineldin et al., 2017). Furthermore, BRD research implementing the advantages in understanding the pathogenesis of opportunistic infection in the lung can improve the general understanding of the development of respiratory disease for other mammals. This is due to the high prevalence of BRD, feasible intervention in cattle, and shared certain immunologic (e.g., interleukin 8) and anatomic features (e.g., bronchial glands, cartilaginous airways) between cattle and other mammals such as humans (Caswell, 2014). Therefore, it is necessary to deeply investigate the biogeography of bovine respiratory microbiome and its association with BRD.

Objectives of the study

1) To determine the biogeography of bovine respiratory microbiome

2) To identify microbial biomarkers to predict and diagnose BRD

3) To investigate the relationship between spatial dynamics of the respiratory microbiome and BRD status

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Chapter II. Literature Review

The pathogenesis of bovine respiratory disease

Bovine respiratory disease (BRD), also called 'shipping fever or pneumonia', is the most common and costly disease of the modern beef cattle industry (Wolfger et al., 2015). Weaned and transported cattle are at even greater risk for BRD, which contributes to 70 to 80% of dry-lot total morbidity and 10 to 50% of feedlot mortalities, also contributing to a subsequent loss of performance and health (Sanderson et al., 2008). In the feedlot, BRD affects 21% of calves weighing less than 318 kg and 9% of calves weighing at least 318 kg (USDA, 2013). Moreover, BRD diagnosis is usually dependent on trained feedlot personnel and is based on clinical signs, which may overlook some animals need for treatment. Treatment for BRD is known to contribute to the development of antibiotic-resistance of pathogens (Holman et al., 2019). Therefore, the understanding of BRD pathogenesis can potentially provide a foundation for the prevention and treatment of the respiratory disease.

BRD usually infects cattle within four weeks of transportation to a feedlot (Currin et al., 2005). There are multiple clinical symptoms of BRD, which vary greatly, depending on the phase and extent of the disease process. The general signs include depression, inappetence, lethargy and fever. Also, some respiratory signs, including ocular and nasal discharge, coughing, excessive salivation, and abnormal respiratory rhythm, have been observed in BRD cattle (White et al., 2009). Those signals are helpful for BRD diagnosis and reflect the alteration of cattle respiratory physiology. For example, in early BRD cases, mild, tentative, and soft coughing assist in clearing the respiratory tract. The loud and prominent coughing indicating far more chronic and advanced cases may clean phlegm that includes significant loads of the microbiome, resulting in tremendous changes to the respiratory physiological environment. On-site, BRD

diagnosis is based on subjective observations and is dependent on the combination of general symptoms and respiratory symptoms, currently the most practical, economically feasible, and common approach for feedlot cattle. If the above general signs are found in calves, they are separated into holding facilities, and their body temperature is measured. Calves with a rectal temperature greater than 40 ^{o}C (104°F) and noticeable clinical signs usually need therapeutic treatment (Urban-Chmiel et al., 2012). However, this assessment has limited sensitivity and specificity resulting in unnecessary treatment and the delayed or negative detection of BRD in sick animals (Abutarbush et al., 2012). A study found the sensitivity of BRD detection based on subjective observation of clinical signs was only 62%, which indicates that BRD cases often go undetected, or are not detected until later in the disease process when successful intervention is less likely (Kayser et al., 2019; White et al., 2009). Moreover, lung lesions (e.g. pleural adhesions, collapse/consolidation, parenchymal fibrosis, abscesses, or emphysema) resulting from BRD are frequently found at slaughter, often in calves in which clinical BRD has never been detected (Thompson et al., 2006). Logically, lung lesion and BRD status should have some relationship, but this has not yet been discovered and it is possible little to no relationship exists (Taylor et al., 2010). Overall, current methods for the early detection, prognosis, and diagnosis of BRD still have low accuracy.

The pathogenesis of BRD might be the complex synergistic interaction of bacteria and viruses under the influence of various management procedures (i.e., weaning, comingling, transportation and dietary changes), host and environment (Snowder et al., 2006; Taylor et al., 2010) (Figure 1). A harmonious interaction between the host, microbiome and local environment within the airways is needed in healthy cattle. In contrast, a disequilibrium related to microbial dysbiosis and mucosal dysfunction as well as acute or chronic inflammation consequently

generates opportunity for the development of BRD (Hakansson et al., 2018). So far, we know the mechanism of pathogen invasion causes the acute syndrome of BRD after the bovine respiratory tract has been compromised by viral infections, environmental and/or other stress factors (Fulton et al., 2000). The multiple viral agents that cause the development of BRD are *bovine viral diarrhea virus* (BVDV), *bovine respiratory syncytial virus* (BRSV), *infectious bovine rhinotracheitis* (IBR), and *parainfluenza 3 virus* (PI3V) (Klima et al., 2014). These viruses can lead to the increase of colonization and replication of bacterial pathogens. However, the incidence and abundance of these bacteria do not correlate well with the occurrences of BRD. A high abundance of *Mycoplasma* has been observed in healthy steers and those diagnosed with BRD (McDaneld et al., 2018). Likely, the understanding of pathophysiological processes of viral or bacterial infection is complex and necessary. Therefore, a review of current research on community structure and composition of the microbiome of the bovine respiratory tract helps to improve our understanding of the pathobiology of BRD and emphasizes an important research direction.

The microbiome, including the community of all the microscopic cells, viruses, and phages in a specific environment (e.g. gut, lung), plays an important role in animal health and disease (Dickson et al., 2016). Despite the remarkable progress made in recent studies using the Next-Generation Sequencing (NGS) technique, much still remains unknown about the bovine respiratory microbiome. For example, the microbial composition of the airway in healthy cattle is still unclear. The longitudinal changes of the airway microbiome in phases of pre-BRD, the onset of BRD and post-BRD are limited, and most of the BRD microbiology studies have been conducted using a nasopharyngeal swab (NPS) sampling approach (Zeineldin et al., 2017b), invasive and impractical for large epidemiology studies.

Techniques used in the study of the respiratory microbiome

A comprehensive review of techniques is essential for accurate respiratory microbiome investigation and the identification of BRD pathogens. We will briefly describe the methods of sample collection in relation to the specific anatomical structures of the airway. Then, the key points focus on subsequent measurements and data analysis of sequencing, since most of the studies analyzed used the 16S rRNA sequencing technique.

For sample collection, literature reported various methods when comparing the upper (URT) and lower (LRT) respiratory tract. Typically, the sterile swabbing approach is popular for the collection of the microbiome in the URT (Figure 2). There are many types of swabs, for example, cotton swabs with transport medium. Based on previous cattle studies, researchers used short swabs for nasal (17 cm length) and long swabs (84 cm length) for nasopharyngeal sample collection (Nicola et al., 2017; Zeineldin et al., 2017a; Zeineldin et al., 2017b). There were limited studies that investigated the oral microbiome in ruminants. In a lamb study, oropharyngeal samples were collected using cotton-tipped swabs (Glendinning et al., 2017). For oral samples in humans, swabs, mouthwash fluids, and saliva or sputum were collected (Einarsson et al., 2019). The common modalities for collection of LRT samples include bronchoalveolar lavage (BAL), and trans-tracheal aspiration (TTA) (Doyle et al., 2017). TTA utilizes the insertion of a catheter into the trachea and upper airway of the subjects to collect fluid samples lavaged from the lower airway. TTA samples provide molecular information about the trachea and major bronchi for cytologic and culture analysis. BAL is a medical procedure in which a bronchoscope is passed through the mouth or nose and into the lungs for sampling of the lower generation bronchi and alveolar spaces, and is considered a minimally invasive technique (Figure 2) (Woods, 2013). BAL collection has become an invaluable diagnostic tool with

important clinical implications in both opportunistic infections and the pulmonary pathology of immunologic disease, since it samples a large portion of lung parenchyma (approximately 1 million alveoli) (Sanchez Nieto et al., 1995). Pneumonia is an infection that inflames the alveoli. Therefore, using BAL would be a more accurate way for BRD diagnosis or research (Derksen et al., 1989). To date, only a few studies have compared the differences between TTA and BAL. In humans, a report confirmed that both TTA and BAL had significantly greater similarity in pneumonia-positive subjects as compared to the pneumonia-negative subjects (Kalantar et al., 2019). In horses, a study concluded that either the TTA or BAL method was sufficient to detect airway neutrophilia that had a positive correlation with lower respiratory diseases, which meant their neutrophil percentages correlated well in samples with naturally occurring respiratory disease of varying status and healthy subjects (Rossi et al., 2018). In dairy cattle, a previous report suggested that nasal swabs, nasopharyngeal swabs and BAL had similar abilities to determine the bacterial pathogens including *M. haemolytica*, *P. multocida*, or *M. bovis* in the lower airways of dairy calves with acute BRD. However, BAL was validated as the superior method to detect the viral agents (BRSV) compared to NS or NPS and TTA (Doyle et al., 2017). Regarding LRT sampling through the URT, it is inferred that BAL introduces a risk of contamination, but multiple sources suggest that this effect is minimal and can be ignored (Dickson et al., 2016). Moreover, some researchers collected lung tissue after slaughter for microbiome analysis, but this is still limited because of the cost of euthanasia and lack of processing facilities as well as the disturbed factor from lung tissue to the microbiome (Johnston et al., 2017). Additionally, an improved method of airway brushing to obtain bronchial epithelial cells for the estimation of host immune response to bacteria has been reported (Segal et al., 2016). Overall, the most useful sampling method should be dependent on research goals, and the

type and the status of the suspected disease process (Rossi et al., 2018). Regarding factors including cost, labor, and injury to animals, sampling on the URT, especially the nasal or oral cavities, would be the optimal choice if the URT microbiome has a higher accuracy for BRD prediction and diagnosis.

Previous studies analyzed the respiratory microbiome or the pathogens using culture dependent techniques (Hildebrandt, 1888; Hoeprich, 1970; Sellart-Altisent et al., 2007). However, these techniques only enable the detection of a small fraction of the microbiome. Also, various molecular techniques have been used to quantify the specific microbes within the respiratory microbial community, including immunohistochemistry (Sachse et al., 1993), and real-time PCR (Sachse et al., 2010). The advent of the NGS technique has allowed us to more adequately study the microbiome. Due to the low biomass of specimens in the LRT, the lungs have been considered as a minimal source of bacteria in the past (Dickson et al., 2016). However, the first application of culture-independent techniques with high depth confirmed that healthy lungs harbor microbiota and the community structure of patients with respiratory disease was different from healthy subjects using the BAL sampling approach (Hilty et al., 2010). The rate of subsequent publications in this area has grown exponentially, and most have focused on the human airway, but limited studies have also investigated bovine. Since massive economic loss is incurred due to BRD, and cattle are one of the key food sources of human society, the bovine respiratory system and its accompanying microbiome should be analyzed, and the research approaches or advantages of effective sample collection could subsequently be used as a model for other species and human analysis. Several recent studies using the NGS techniques have revealed that the bovine respiratory microbiome is much more complex than previously appreciated. For example, using 16S rRNA gene sequencing, a study characterized the

dissimilarities of the microbiota of the upper and lower respiratory tracts of calves, in both healthy individuals and those with clinical signs of respiratory disease (Nicola et al., 2017). Also, research that combines culture-dependent and independent approaches has worked to find a more complete picture of the bacterial colonization in the lung and the impacts of disease (Segal et al., 2016; Stearns et al., 2015). Therefore, NGS and its combination with other techniques has become the most popular method to measure the respiratory microbiome. With the improvement of sequencing techniques, multiple omics (i.e., metagenomics, metaproteomics, metatranscriptomics, and metabolomics) are growing to become useful analysis techniques. These multi-omics approaches allow researchers to develop a deeper understanding of the respiratory microbiome and its association with health and disease. In humans, Dai et al. started to use metagenomics analysis for respiratory samples from children with pneumonia and children considered healthy, and found that more unknown microbial components and host-microorganism interactions are also important for health (Dai et al., 2019). Zhang et al. performed metatranscriptomics on nasal and throat swab samples and indicated that the host changed the respiratory microbial community composition and overall gene expression resulting in the alteration of antibiotic resistance gene expression when infected with influenza (Zhang et al., 2020). However, due to many factors such as cost, technique error, and time of analysis etc., NGS is still the most popular approach. Therefore, introducing the data processing of NGS provides insights to other researchers.

Big data analysis post NGS is another key point in respiratory microbiome analysis. After obtaining sequencing data from Illumina MiSeq /HiSeq or 454 pyrosequencing platforms (Caporaso et al., 2011; Huws et al., 2016), these sequences are processed through a series of quality control steps and classified to genus- or species- level based on available databases. The

software, including quantitative insights into microbial ecology (QIIME) and mothur, have been widely used (Bolyen et al., 2018; Caporaso et al., 2010; Kozich et al., 2013). The microbiome sequences are classified to the operational taxonomic unit (OTU) using RDP (Cole et al., 2013), Greengenes (McDonald et al., 2012) or NCBI (Federhen, 2011), and those taxonomic classifiers have shown to work well for 16S rRNA sequencing (Balvočiūtė et al., 2017). Then, basic analysis including community richness, diversity, and major microbiota composition are analyzed and reported. Based on unique experimental design, advanced analyses will be performed depending on the bioinformatics. The continued development of machine learning algorithms is being developed and applied to the microbiome for deep analysis to extract further data. Overall, deep microbiome analysis focuses on within community or between multiple communities, and how the microbiome is influenced by various external factors such as vaccination, diet, body weight, etc. In order to find ecological interactions among bacteria within a community, network analysis is the most popular method. It is becoming increasingly clear that understanding microbiome interactions is essential for understanding microbiome function (Layeghifard et al., 2017). A key feature of network theory suggests that architectural features of networks appear to be universal to most complex systems, including microbiomes, molecular interaction networks, computer networks, microcircuits, and social networks. A wide range of methods have been used to construct ecological networks based on microbiome data. These approaches vary in their efficiency, accuracy, speed, and computational requirements, and span from simple pairwise Pearson or Spearman correlation measures, to more complex multiple regression and Gaussian graphical models. A study, using co-occurrence analysis by calculating all possible Spearman's rank correlation coefficients, found "core" community structures formed between taxa in both healthy and diseased airways (Einarsson et al., 2019). There are also many

analytical methods for the comparison of sequenced, multiple microbial communities. A classification in machine learning and statistics could be performed for identifying where a set of categories belongs, on the basis of a training set of data (those sequenced and targeted communities). In other words, the listed microbes of classification could be used to predict BRD since they could be used as the biomarkers or signatures to distinguish healthy and BRD groups. To perform this classification, there are many machine learning models including Random Forest (Breiman, 2001), Support Vector Machine (SVM) (Cortes et al., 1995), Linear discriminant analysis Effect Size (LEfSe) (Segata et al., 2011), etc. Random Forest is the most popular robust machine learning technique for classification. It can deal with binary, categorical and numerical features, works significantly faster than training speed with high dimensional data, and handles well in class population unbalanced data sets. Moreover, regression can also be performed in Random Forest algorithm. Additionally, there is an updated Random Forest method, area-under-the ROC curve (AUC) of the Random Forest (AUCRF), that has been published (Calle et al., 2011).

As we know, microbiota from the URT could move into and colonize the lung via respiration and microaspiration (Venkataraman et al., 2015). For the respiratory microbiome, detection of microbial movement/dispersion from the source to sink environment community usually uses the island model (Dickson et al., 2014b). This viewpoint considers source ecological communities as dynamic assemblages of microbes whose presence, absence, and relative abundance in the sink environment are administered by random dispersal, speciation, extinction, and stochastic birth and death events (Hubbell, 2001). Some models, including the neutral model and source tracker, are applied to characterize the spatial movement of the respiratory microbiome. Source tracker applies a Bayesian approach to determine the relative contributions

of one or more sources to a particular sink microbiota (Knights et al., 2011). The neutral model assesses the microbial drift from one source to one sink (Venkataraman et al., 2015). Moreover, the association between microbial abundance with phenotypes (body weight, diet, disease, age, environment, etc.) or genotypes is also conducted and combined with the above methods to determine the microbial shifts between case and control. When meeting a condition in which multiple factors contribute to bacterial changes, using statistical technology (i.e., multivariate model) to identify which is the most effective factor is another developing method. For example, Wang et al. used Permutational multivariate analysis of variance (PERMANOVA) to screen the most important factors shaping the dynamics of the swine gut microbiome (Wang et al., 2019). Additionally, some analyses such as the Procrustes statistic and multiple co-inertia analysis are approaches to integrate multi-omics datasets. In BRD studies, we can use these algorithms to explore the mechanisms of BRD development, and classify the factors leading to BRD.

Biogeography of the bovine respiratory tract

The biogeography of the respiratory tract is discussed in reference with the URT and the LRT anatomy due to the differences in physiological conditions. The URT consists of the nostrils, oral cavity, and the pharynx (the nasopharynx and oropharynx), whereas the LRT includes the trachea, bronchus, bronchioles, alveolar ducts, alveolar sacs, and alveoli. Considering ruminants, the tracheal bronchus specifically arises cranial to the tracheal bifurcation. The primary function of the respiratory tract in physiology is gas exchange (oxygen and carbon dioxide) from the blood. For this purpose, the airway must filter, warm, and humidify inhaled air and, while doing so, prevent the establishment of noxious infectious agents that may gain access to the system and threaten function and life. Thus, the pH and temperature gradually increase along the respiratory tract, while the partial pressures of oxygen (pO_2) and carbon

dioxide (pCO_2) have opposing slopes that are regulated by environmental air conditions and gas exchange at the surface of the lungs (Figure 3). Due to the connection with the external environment and direct dispersion along mucosal surfaces, the microbiome harbors on the URT and further transmits to the LRT. However, the niche-specific microbial communities along the respiratory tract were selectively grown due to the niche physiological parameters (e.g., temperature, pH, ventilation, etc.) (Man et al., 2017). Therefore, understanding the biogeography of the respiratory microbiome provides evidences of the complex respiratory ecosystem.

The URT contains several distinct anatomical structures that have different physiological conditions and have greater prevalence to generate contact with the pen environment (e.g., diet, water, feces, and urine) and other cattle. The nasal cavity connects with the external environment and is lined with a skin-like keratinized squamous epithelium, including serous and sebaceous glands, the latter of which produces sebum, which leads to the enrichment of lipophilic skin colonizers. The genera associated with common BRD pathogens such as *Mycoplasma*, *Moraxella*, *Pasteurella*, and *Mannheimia* are observed in the nostrils of both healthy and BRD cattle (Nicola et al., 2017). Other dominant genera in the nasal cavity, including *Psychrobacter*, *Aggregatibacter*, *Sphingomonas*, *Corynebacterium*, and *Coprococcus*, are also reported in cattle (McDaneld et al., 2018; Nicola et al., 2017). The nasopharynx, whose microbial community has been widely investigated, is the space at the back of the nose and connects to the oral cavity. Its composition of bacterial communities in healthy individuals is more diverse than in the nasal cavity, and demonstrates considerable overlap with nasal samples (Man et al., 2017). The four common BRD pathogens previously mentioned have been observed in nasopharyngeal samples from healthy and BRD animals (Holman et al., 2015; Lima et al., 2016; Zeineldin et al., 2017b). Other reported dominant genera include *Pseudomonas*, *Psychrobacter*, *Actinobacillus*,

Clostridium, *Acinetobacter*, *Bacillus*, *Proteus*, *Bifidobacterium*, *Rathayibacter*, *Cellulomonadaceae*, *Corynebacterium*, *Jeotgalicoccus*, and *Planomicrobium* (Gaeta et al., 2017; Holman et al., 2017; Timsit et al., 2018; Zeineldin et al., 2017b). The temporal changes of nasopharyngeal microbiota have also been reported. A previous study confirmed that within several days of transport to the feedlot, the nasopharyngeal microbiota changed significantly, increasing in both phylogenetic diversity and richness (Holman et al., 2017). In addition, the oral cavity and oropharynx become part of the airway when the oral cavity is used as an additional opening of the respiratory system. There were very few studies about the oral microbiome in cattle. The first study to investigate the microbiomes of bovine oral health and periodontitis found that the most prevalent bacterial microbiotas in cattle considered healthy were *Pseudomonas*, *Burkholderia* and *Actinobacteria*, whereas *Prevotella*, *Fusobacterium*, and *Porphyromonas* were significantly reported in diseased subjects (Borsanelli et al., 2018). There are still no published studies of the microbiome in the oropharynx of cattle. In humans, bacterial communities within the nasopharynx and oropharynx are distinct (Stearns et al., 2015), and the oropharynx contains more diverse microbiota than the nasopharynx (Charlson et al., 2010). The oral and oropharyngeal microbiome will be a new direction for research of healthy and BRD associated lung microbiomes since they are important sources for the lung (Venkataraman et al., 2015). This is especially relevant in cattle, because ruminating affects the oral microbiome, and subsequently the lung microbial structure.

The LRT is comprised of the trachea and lungs. The passageway within the lungs that contains bronchi (primary, second, tertiary), bronchioles, alveolar ducts, and alveolar sacs shapes along the respiratory tree (Man et al., 2017). The airways after the walls of the bronchioles, alveolar ducts, and alveolar sacs have club cells in their epithelial lining. Until now, limited

research has been conducted that separates the LRT to the trachea and lung in bovine to investigate the LRT microbiome. However, there are some studies that have started to investigate the LRT microbial composition and structure. In the LRT of cattle considered healthy, the genera *Mycoplasma*, *Moraxella*, *Pasteurella*, *Mannheimia*, *Bacteroides*, and *Clostridium* were observed in both TTA and BAL samples (Klima et al., 2019; Nicola et al., 2017; Zeineldin et al., 2017a). Other bacteria such as *Bibersteinia* and *Prevotella* were also observed in BAL fluids (Zeineldin et al., 2017a). Moreover, spatial variation may result in a misunderstanding of the lung microbiome. Research in sheep has found that brushing samples of microbiota at different anatomical locations in the lung showed significant differences (Glendinning et al., 2016). In humans, a previous study confirmed that spatial variation in lung microbiota across individuals was significantly larger than within individual variation, and BAL was found as an acceptable method of sampling the microbiome (Dickson et al., 2015b). Therefore, the notion that local physiological conditions can selectively grow microbiota has developed. Future studies are needed to investigate the homeostasis of the respiratory microbiome and host.

The spatial movements or dispersal of microbiome within the respiratory tract is a new research direction and reflects the health status of animals due to anatomical connections (Venkataraman et al., 2015). In healthy individuals, bacteria enter the lungs through an active and continuous process by inhalation of air, direct mucosal dispersion and micro-aspiration from the URT (Figure 3) (Dickson et al., 2014a). Until now, no studies focusing on cattle respiratory microbiome dispersal have been conducted. In healthy humans, the lung microbiome is more influenced by microbial immigration and elimination (the adapted island model) than by the effects of local growth conditions on bacterial reproduction rates (Dickson et al., 2015b). The composition of healthy lungs' microbiome fit a neutral model when using oral microbiota as

source, which meant the oral microbiota is one of the major sources for the human lung microbiome (Bassis et al., 2015; Venkataraman et al., 2015). Moreover, a study found that 75% of the OTUs from the oral cavity fell within the neutrally distributed taxa in the upper GI tract in humans (Venkataraman et al., 2015). Therefore, especially for ruminants, investigation of microbial movement within the airway affected by external sources is important and complex. Due to the specific ruminating activity in cattle, where the oropharynx is exposed to ruminal contents on a frequent basis, one would anticipate that the lung microbiota would similarly reflect this influence (Glendinning et al., 2017). The lungs contain several rumen-associated bacteria, which may indicate that there is a certain degree of micro-aspiration of ruminal contents in lambs (Glendinning et al., 2017). The shift in gut microbiota that results from dietary changes during the early post-weaning period may also contribute to changes in the respiratory microbiome. Feeding weaned calves selenium-biofortified alfalfa hay for nine weeks in a preconditioning program, prior to feedlot entry, favorably altered nasal microbial communities (Hall et al., 2017). Simultaneously, the cattle were living in an environment with abundances of microbiota that might be a significant factor for the URT microbiota and subsequently influence the lung microbiota. A study confirmed that the nasal and oral microbiota of dairy farmers showed high microbial diversity with hundreds of unique genera that reflected environmental and occupational exposures compared to non-farmers living in an urban area (Shukla et al., 2017). Another potential source is the direct inhalation of ambient air, however, its mechanism was not clear until recently (Man et al., 2017). Finally, those dynamic movements are relevant for our understanding of pathogenesis in lung health and disease (Segal et al., 2014). Moreover, there are multiple factors that affect the URT microbiota and eventually microbial shifts in newly weaned calves, including antibiotics, external environments, stress and host immunity (Man et

al., 2017; Zeineldin et al., 2019). Recently, a study confirmed that a single injection of oxytetracycline and tulathromycin led to significant changes in the nasopharyngeal and fecal microbiota during the first five days after treatment, and increased the relative abundance of several antibiotic resistance determinants in the fecal and nasopharyngeal microbiome at either day 12 or 34 (Holman et al., 2019). Therefore, investigation of microbial movement within the airway would be a complex process and great direction for BRD research.

Association between respiratory microbiome and BRD

Disease is one of the main factors influencing the respiratory microbiome. Many studies have confirmed that the alteration of the respiratory microbiota can be observed in BRD plagued calves. For example, the nasopharyngeal microbiota in BRD feedlot calves was distinct from pen matched healthy calves (Zeineldin et al., 2017b). Additionally, a distinct longitudinal shift of microbial composition of the nasopharynx from arrival in the feedlot (incubation phase) to BRD appearance was observed (McMullen et al., 2018). Furthermore, bacterial OTUs associated with BRD including *Fusobacterium*, *Mycoplasma*, *Trueperella*, and *Bacteroides*, and an OTU not yet associated with BRD (*Leptotrichiaceae*), had greater relative abundances and frequencies in lung tissue samples collected from BRD calves (Johnston et al., 2017). Although these reports support the hypothesis that microbial dysbiosis relates to cattle health, we still do not know the accuracy of the association between the microbiome and BRD despite the microbial changes found. A key unknown factor is the trigger and outcome. In other words, whether the pathogens and the microbiota are the inducers of inflammation and onset of BRD, or whether host inflammation leads to the alteration of microbial structure by selectively overgrowing pathogenic microbes thriving in a more inflammatory milieu (Hakansson et al., 2018). Therefore, integration of the

mechanism of microbial dysbiosis or host immunity developed by causal agents is important for clarity of the association of the respiratory microbiome and BRD.

Understanding of the physiological functions of the healthy airway and resident microbiome colonization helps us to identify BRD pathogenesis. In healthy cattle, a mucosal layer covers the respiratory tract and provides immune and mechanical protection for maintaining homeostasis under the interaction of the interface of the host, microbes, and the external world (Ackermann et al., 2010; Hakansson et al., 2018; Roy et al., 2014; Zeineldin et al., 2019) (Figure 3). The mucus consisting of antimicrobial peptides, immunoglobulins, glycoproteins, mucins, polysaccharides, ions, cells, and bacteria maintains harmonizability. In addition, mucus in the URT offers protective barriers against pathogens and toxins from the external environment (Osman et al., 2018). The dynamic mucociliary transport moves mucus and its contents with small particle size (infectious agents and bacteria, etc.) toward the nasopharynx or oropharynx to be swallowed, which helps prevent foreign objects from entering the lungs during breathing (Erickson et al., 2015). The nostrils, nasopharynx and trachea are connected with respiratory epithelium containing goblet cells containing columnar epithelial cells for mucous production. The direction of the bronchioles in the epithelium gradually shifts toward a cuboidal epithelium with some cilia and club cells, producing glycosaminoglycans and secretory proteins for lung maintenance and host defense. The surface of epithelial cells of the alveoli consist of two types: the type I cells that are responsible for gas exchange and barrier function, and type II pneumocytes producing lipid-rich surfactant with high bacteriostatic capacities. These mechanical defense mechanisms are complemented with antimicrobial peptides, produced by both types of pneumocytes, whose production is increased during inflammation, and with the presence of resident lung leukocytes, in particular activated macrophages and dendritic cells

(Dickson et al., 2015a). Alveolar epithelial cells also produce chemokines and cytokines that recruit and activate immune cells in damaged or infected areas (Wong et al., 2013). Surfactant proteins A and D in type II and clara cells potentially have antimicrobial roles and immunomodulatory roles by binding and inactivating microbial agents (Ackermann et al., 2010). Moreover, the airway microbiota and their metabolites can interact with the host to protect against pathogen invasion/overproduction. Steed et al. reported a microbial metabolite (desaminotyrosine) that can protect the host by amplifying type I IFN signaling (Steed et al., 2017). In addition, other factors, such as the ion, chloride secretion and sodium uptake, are necessary to maintain normal regulation. Altogether, the microbiome adapts to a state of microbial symbiosis and homeostasis with the host mucosal surface and immune system.

Newly weaned calves experience multiple stressors resulting in sub-health, possible dysbiosis of the respiratory ecosystem, and BRD infection (Hall et al., 2017; Timsit et al., 2016a; Timsit et al., 2017; Zeineldin et al., 2017b). At feedlot arrival, the homeostasis of microbial communities in healthy cattle prevents pathogens from establishing infection on mucosal surfaces through the consumption of all presented nutrients, the adjustment of the local niche environment and microbial composition, the occupying of receptor sites, and the regulation of mucosal inflammation (Ackermann et al., 2010; Man et al., 2017; Zeineldin et al., 2019). A previous study confirmed that the URT microbiota in healthy cattle rapidly changed from weaning to arrival at the feedlot and from arrival to day 40 (Timsit et al., 2016b), indicating that the respiratory ecosystem responded to new challenges in the feedlot. Likewise, viral and bacterial pathogens invading over breakthrough points leads to microbial dysbiosis, damage of airway tissue and the subsequent infections of BRD after feedlot arrival due to respiratory ecosystem response. The first step for dysbiosis is the colonization of pathogens into the URT,

and the subsequent changing of the structure of the URT microbiome (Bogaert et al., 2004), and then proliferation and infection of the lungs. Investigation of this first colonization of pathogens in the URT would benefit disease prediction and prevention. The pattern-recognition receptors in the URT that are expressed by the mucosal epithelium and immune cells (e.g., dendritic cells, macrophages, and neutrophils) can recognize pathogens or other noxious substances and then provide signals to regulate the acquired immune responses (Osman et al., 2018). Increasing pathogen loads cause URT immune responses, alteration of the niche environment and subsequent disequilibrium. Regarding the first step of dysbiosis in increasing pathogens and host inflammation, the host state is in the 'pre-BRD' state in which microbial communities are in an unstable state and reach the breakthrough point. The mucosal barrier function responds to respiratory ecosystem dysbiosis and pathogen invasion by secreting signaling molecules (e.g., inflammatory cytokines and chemokines) in mucus production, and the stimulation of an immune response in local niches (Zeineldin et al., 2019). This pre-BRD state can be considered a reversible phase. To resist pathogen invasion, protect the host, and reestablish the barrier function, inflammatory events are activated until risk signals disappear, and reestablishment of damage can start to occur (Hakansson et al., 2018). Alternatively, the onset of BRD occurs when cattle in the unstable pre-BRD state are susceptible to failure as a result of pathogens. Then, detectable mucosal damage accompanied by a deficiency of the mucosal barrier is usually found in the respiratory tracts of BRD calves. A previous study found that the pathogen *M. haemolytica* invades differentiated bovine bronchial epithelial cells by transcytosis and undergoes rapid intracellular replication prior to BRD causing extensive cellular damage (Cozens et al., 2019). Kiser et al. also found the pathogen leads to lung lesions in steers exhibiting subclinical BRD (Kiser et al., 2017). However, until now, the mechanism of epithelial damage by BRD has not

been clear. One of the difficulties is the complex interaction of multiple viral and opportunistic bacterial pathogens. Citing *M. haemolytica* as an example, it is a commensal in the URT of healthy cattle; however, a sudden explosive proliferation associating with stress and viral infection occurs in the URT of susceptible animals (Singh et al., 2011). One specific strain of *M. haemolytica* adheres to and colonizes bovine bronchial epithelial cells, and subsequently forms foci of infection through damaging tight-junction integrity, transcytosis and rapidly replicating intracellularly (Figure 4) (Cozens et al., 2019). During the invasion process, *M. haemolytica* stimulates host epithelial cells producing proinflammatory mediators such as cytokines (IL-1β, IL-6, TNF-α) and the chemokine (CXCL8). The generation of proinflammatory mediators and the release of lipopolysaccharide together affect the migration of neutrophils into the lungs, and these immune cells are mainly responsible for the tissue damage associated with BRD (Kiser et al., 2017; Zecchinon et al., 2005). In humans, the summary of how respiratory viruses interacted with bacteria generating damage peripheral the bronchi and bronchioles is reported (Griffiths et al., 2017). The presence of viruses may change the microenvironment of mucosal surfaces and bacterial structure, leading to reduced mucociliary function and cilia damage. Additionally, viral infection could reduce the concentration of antimicrobial peptides, bacterial adherence and invasion by adjusting the host immune system (Bosch et al., 2013). Segal et al. observed that enhanced expression of inflammatory cytokines (Th17 immune activation) associated with bacteria in the human LRT, indicating a role for the aspiration-derived microbiota in regulating the basal inflammatory status at the pulmonary mucosal surface in humans (Segal et al., 2016). Host defense and clearance of the viral infection is facilitated by a balance of neutralizing antibodies of the humoral immune response and cytotoxic T cells of the cell-mediated immune response. Undeniably, bovine airway viruses may damage ciliated host cells and the epithelial

layer by disarranging host cellular functions and/or killing infected epithelial cells, resulting in reduced mucociliary velocity (reduction of bacterial clearance) and consequently exposing the respiratory tracts basement membrane (Lima et al., 2016; Zeineldin et al., 2019). Thus, a changed niche environment leads to a compromised host immune response to secondary bacterial infection by facilitating adhesion and the colonization of bacterial pathogens (Bosch et al., 2013; Czuprynski et al., 2004).

Microbial drifts may be another way to associate the microbiome with the status of BRD. Until now, the complex mechanisms involving microbial drifts affected by BRD have not been sufficiently analyzed. However, achievements of the interaction within the gut-lung axis in other species provides an alternative way to study the microbial drift associated with BRD. In lambs, lungs were reported to harbor several rumen-associated bacteria which may indicate that there is a certain degree of microaspiration of ruminal contents (Glendinning et al., 2017). While the mechanistic pathways by which the gut microbiota affects the respiratory microbiota have not been fully elucidated in cattle, the roles of micro-aspiration, inhalation of bacteria, and direct mucosal dispersion have been well described in humans (Bassis et al., 2015). In humans, the neutral model can estimate microbial drifts from the URT to the lungs successfully in healthy individuals, but fails in patients with cystic fibrosis (Bassis et al., 2015; Venkataraman et al., 2015). In addition, detecting determinants that increase host immune function is a new research guidepost for the association of BRD and the gut microbiome. Studies in mice provide strong evidence for the hypothesis that gut bacterial community composition can profile immune function to protect against allergic sensitization. Trompette et al. and their colleagues concluded that feeding a diet with high fermentable fiber altered the ratio of gut *Firmicutes* to *Bacteroidetes,* increased levels of circulating short-chain fatty acids, promoted TH2 cell effector

function through the alteration of dendritic cell capacity, and protected against allergic inflammation in the lung (Trompette et al., 2014). Together, microbial drift can integrate more niches and give more insights into the pathogenesis of BRD.

Integrating the findings of phenotypic features of disease subjects and respiratory microbiota through multi-omics analysis (e.g., metagenomics, metatranscriptomics and metabolomics) can help us to understand BRD development. The integration of metagenomics analysis of taxa of interest and their gene functions to infer the biological higher-level functions encoded by microbiota members has been reported in humans (Dai et al., 2019; Segal et al., 2016). In cattle, a report suggests that the host's transcriptional responses are different based upon the invading pathogens and their binding receptors (Behura et al., 2017). Altogether, since the triggers of BRD development, the complex processes of pathogen interaction, the resident microbiome, and host immunity have not been identified, there is necessary exploration for BRD pathogenesis to maintain healthy cattle.

Summary

The rapid development of sequencing techniques and machine learning or data science allows us to deeply characterize the biogeography of microbial communities in the respiratory system between healthy cattle and those infected with BRD. Despite significant efforts of interpreting microbial composition, this research field is only at its initial stage. The complexity of the respiratory ecological system and multiple BRD pathogenic factors limits our understanding of mechanism such as microbial colonization and symbiosis, and the associations between the respiratory microbiome and disease. While many studies have analyzed the differences of the respiratory microbial composition in healthy or BRD bovine, the dynamic microbial movements within the airway and host-microbiota interactions have not been

identified. Furthermore, characterizing the function of the microbiome is the next step. Thus, advanced multi-omics techniques will provide key insights in to the respiratory microbiota and its interaction with epithelial cells for health and BRD development. Future studies about BRD should consider more interactions of the viral and microbial communities, and the association of microbial dispersion and BRD status, to determine which factors contribute to pathogen colonization. Most progress can be expected from longitudinal studies consisting of large cohorts, in which the microbiota of healthy individuals and individuals who have an increased risk of infectious respiratory diseases is longitudinally characterized. In parallel, multi-omics (for example, metatranscriptomics and metabolomics) and clinical data should be integrated to study the crosstalk between the host and microbiome functions. Consequently, advances in bioinformatics will be required to appropriately combine and analyze multiple high-dimensional datasets. Methods to analyze complex combinatorial data sets are sparse now, but the field is rapidly progressing by applying machine-learning algorithms and time-resolved data modeling. A multidisciplinary approach to extract patterns and associations from BRD studies could culminate in individualized risk assessment and personalized preventive medicine. The respiratory microbiome opens up new possibilities for therapeutic and rapidly detective approaches for BRD or other respiratory diseases. Deep interpretation of the respiratory microbiome will benefit long-term animal health, save costs and increase meat sources for humans.

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Figures

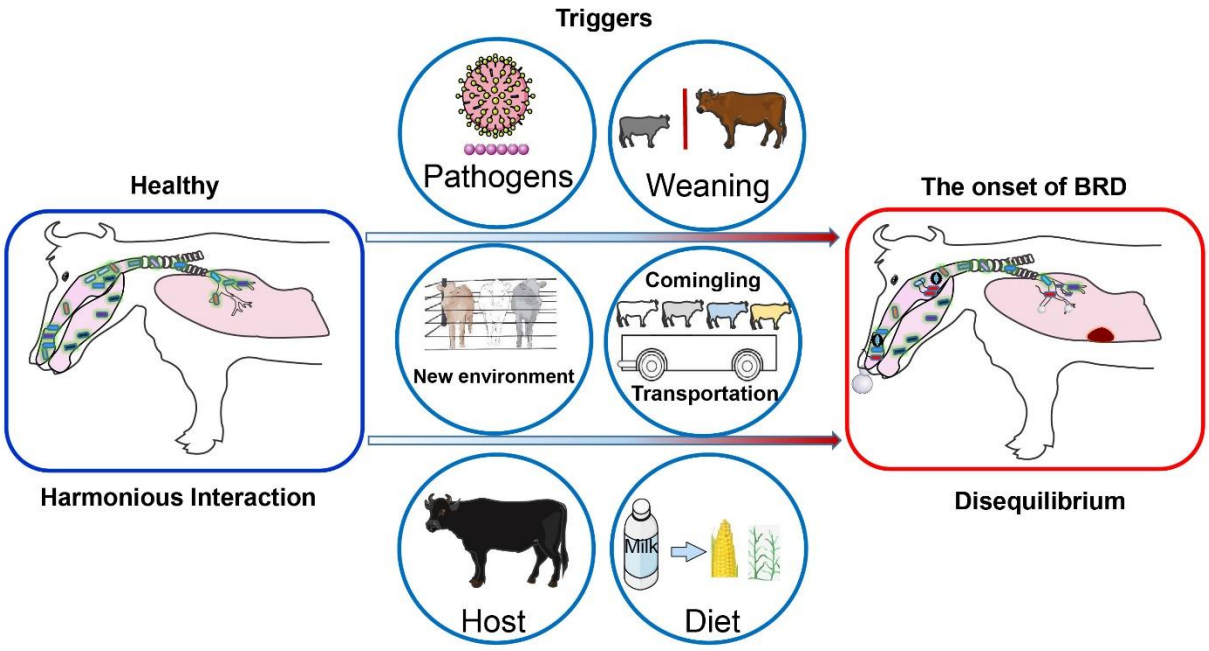

Figure 1. The triggers affecting the healthy respiratory ecosystem and leading to the onset of bovine respiratory disease (BRD) in newly weaned cattle. The bovine respiratory ecosystem has increased risk to be at disequilibrium and show BRD signs when hosts are affected by pathogens, the environment and feeding strategies (e.g., weaning, comingling, transportation and dietary changes etc.).

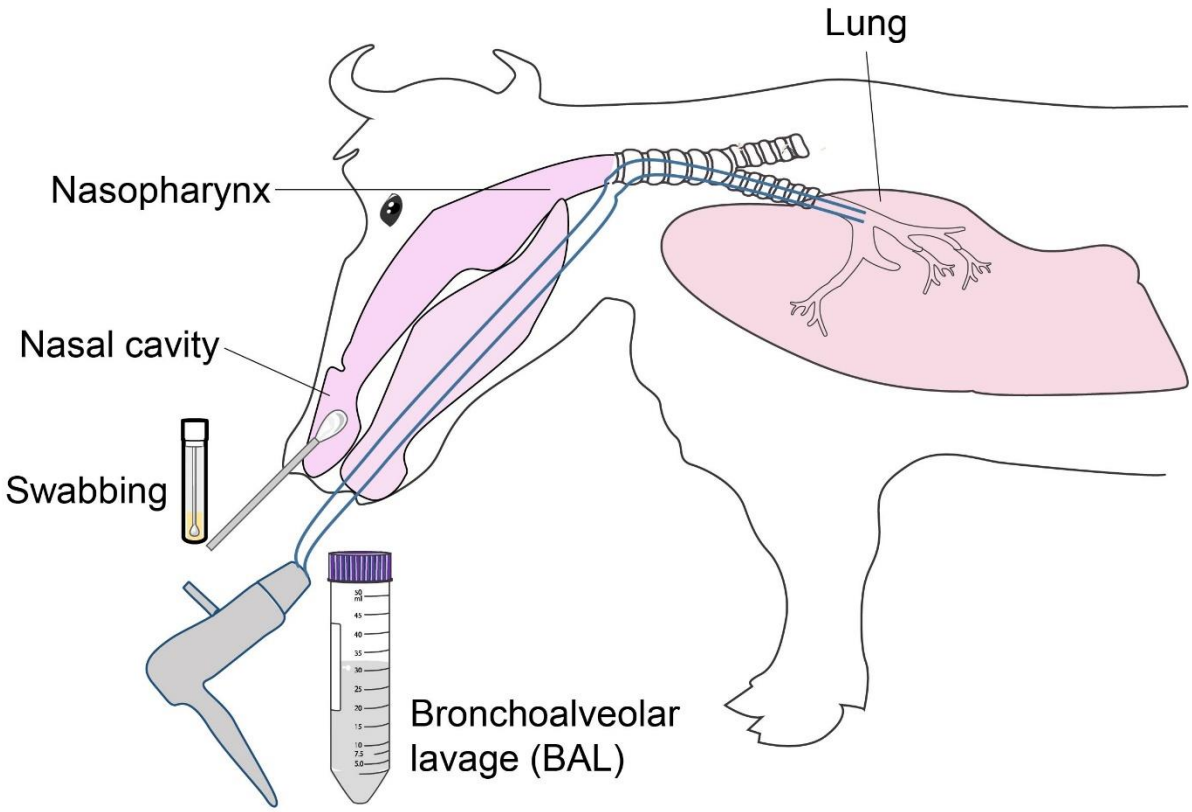

Figure 2. The sampling techniques for bovine respiratory niches

The common bovine sampling niches, including the nasal cavity, nasopharynx and lungs, are displayed. Regarding the anatomy of the upper and lower respiratory tract (URT and LRT), the sterile swabbing approach is popular for collecting microbiome samples in the URT, while the bronchoalveolar lavage (BAL) is a more useful approach for the LRT samples. Usually, short swabs (17 cm length) are used for nasal or oral samples, and long swabs (84 cm length) can reach the nasopharynx for collection of microbial samples. All these swabs are stored in transport medium for down-stream analysis. BAL is a medical procedure in which a bronchoscope is passed through the mouth or nose into the lungs for sampling the lower generation bronchi and alveolar spaces with minimal host invasion.

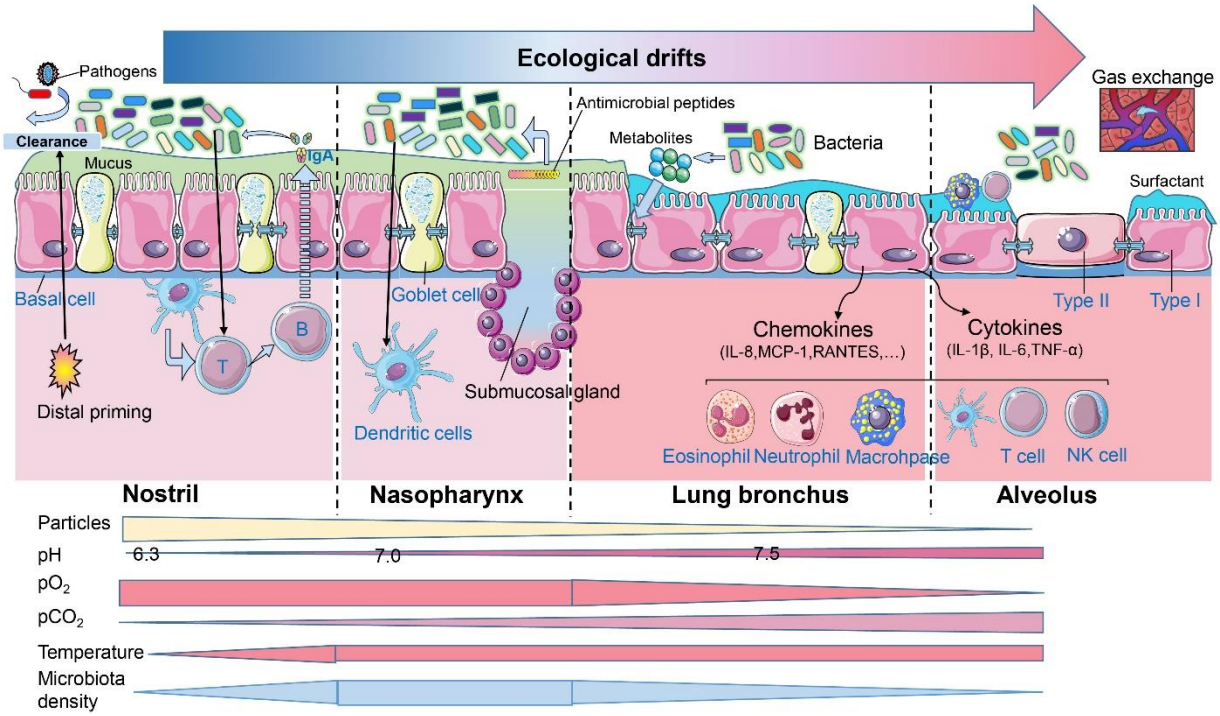

Figure 3. The harmonious interaction of the respiratory ecosystem in healthy cattle

The complex respiratory ecosystem in healthy cattle is harmonious and contains niche specific environmental properties, microbiota immigrations and host-microbiota interactions, which could resist pathogen colonization to some extent. The physiological gradients exist along the nasal cavity, nasopharynx, trachea and the lungs. Lower temperature is reported in the nostrils while the lungs reach to body temperature. The respiratory temperature can be affected by various factors such as diet changes, handling stress and disease. The partial pressures of oxygen (pO_2) and carbon dioxide (pCO_2) have opposing slopes that are regulated by ventilation and gas exchange at the epithelial surface of the airway (West, 1978). Concerning respiration and microaspiration, micro-particles from the external environment enter the respiratory tract and move to lungs. Large particles (over 10 μm in diameter) are deposited in the upper respiratory tract (URT), whereas small particles (smaller than 1 μm in diameter) can reach the lungs. The specific environment in each niche grows the microbial communities selectively along the respiratory tract. Therefore, high microbial density can be found in the URT, which is also summarized in the human airway (Man et al., 2017). Equilibrium between the host and the microbiome is reached when the microbial community is at balance.

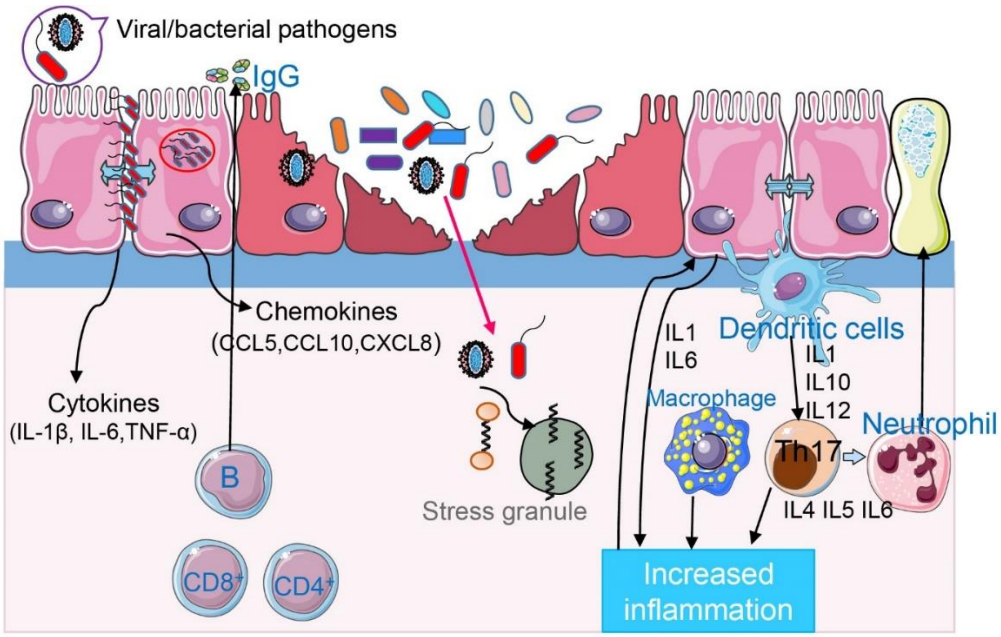

Figure 4. The process of respiratory pathogens invading and host response

Dysbiosis is due to the colonization of pathogens into the upper respiratory tract (URT), shifting the structure of the URT microbiome (Bogaert et al., 2004), and then proliferating and infecting the lungs. In the pre-BRD state, the mucosal barriers function responds to bacterial dysbiosis and colonization of pathogens across the epithelium by releasing proinflammatory cytokines and chemokines in mucus production, and activates the local immune cells (Hakansson et al., 2018). The onset of disease occurs when the unstable pre-BRD state suffers a decline, or a downward transition, into the clinical deterioration state. Then, detectable mucosal damage accompanied by a deficiency of the mucosal barrier is usually found in the respiratory tracts of BRD calves. One specific strain of *M. haemolytica* adheres to bovine bronchial epithelial cells, and subsequently forms foci of infection through damaging tight-junction integrity, transcytosis and rapidly replicates intracellularly, which increases IL-1β, IL-6, TNF-α and CXCL8 (Cozens et al., 2019). Moreover, viral invasion causes increased colonization of the bacterial pathogens, diminished barrier function allowing pathogen invasion, and a cascade of epithelial changes and host innate immune responses. The host can detect RNA and form stress granules during the innate response against respiratory syncytial virus infection (Griffiths et al., 2017). During replication, respiratory viruses trigger an inflammatory process with the induction of cytokine and chemokine production. Dendritic cells are directed to secondary lymphoid organs after capturing viral antigens, where they stimulate the lymphoid cells, protagonists of the specific immune response. In diseased state individuals, the production of IFNs is reduced, allowing greater viral replication. There is a deviation from the lymphoid profile to helper T lymphocyte 17 (Th17), promoting lower antiviral response and increased inflammation, with bronchial hyperreactivity and increased production of mucus, generating clinical signs (Segal et al., 2016). Cytokines within the lung can regulate host responses to infection or injuries, resulting in repair of damaged tissue, and return to homeostasis (Atamas et al., 2013). However, hyperactivity of cytokines leads to airway remodeling and fibrosis. Therefore, damaged lungs are often observed in BRD calves.

Chapter III. Biogeography of Bovine Respiratory Microbiome

Abstract

Bovine respiratory disease (BRD) is considered the most common and costly disease in the North American beef cattle industry, with a multifactorial etiology. The bovine respiratory microbiome has been implicated in the onset of BRD, but the microbial composition of the bovine upper and lower respiratory microbiomes are still largely unknown. To fill this knowledge gap, we collected nasal swabs (NS), nasopharyngeal swabs (NPS), and bronchoalveolar lavages (BAL) from 48 weaned calves in a test trial (animal trial 1), and then a second animal trial (the validation trial) was performed to validate the discoveries. The microbiotas of all samples from both the test and validation trials were characterized by sequencing the V4 region of the 16S rRNA gene via the Illumina Miseq platform. Significant differences in microbial diversity and structure were observed among NS, NPS, and BAL sampling sites. Random Forest algorithm was performed to find the signature microbiota for these three sampling niches. *Moraxellaceae* appeared to be NS specific. Several bacterial features, including F8, F10, F18, F2, and F32 in this family, were detected and over-represented in NS. Features associated with common BRD pathogens were also observed and enriched in the NS microbiome, including *Pasteurellaceae* (F5) and *Mycoplasma* (F38). However, other major BRD pathogen-related microbiota (e.g., *Mycoplasma hyorhinis* (F1)) was signature of the NPS microbiome. The BAL microbiome was over-represented by bacteria such as *Microbacteriaceae* (F6), and *Prevotella ruminicola* (F60 and F286). Although respiratory microbiota were niche specific, the top bacterial features were detected in all three niches. Based on correlation analysis, NS demonstrated a high correlation with NPS ($r=0.63$, $p<0.001$), while NPS was fairly correlated with the lung microbiome ($r=0.52$, $p<0.001$). Similar outcomes were found in the

validation trial. In conclusion, while significant differences surrounding microbial community structure and predominant bacteria among NS, NPS, and BAL niches were observed, we confirmed there still exists a strong microbial association between adjacent niches in the bovine respiratory tract. This study indicates that nasal swabbing could be a potentially effective approach to investigate the lung microbiome and bovine respiratory disease.

Key words: bovine respiratory microbiome, alpha diversity, biogeography, nasal microbiome, disease, Next-Generation sequencing, cattle

Introduction

Bovine respiratory disease (BRD) is one of the most problematic diseases throughout the beef industry, especially in newly weaned and transported feeder cattle, which generates high morbidity, mortality and economic loss (Nicola et al., 2017). Previous research using culture-dependent approaches has identified the most common bacterial pathogens, including *Mycoplasma bovis*, *Mannheimia haemolytica*, *Histophilus somni*, and *Pasteurella multocida* (Holman et al., 2015; Klima et al., 2014; Taylor et al., 2010).

Microbiota colonized in the upper respiratory tract (URT) could potentially contribute to the lung microbiota. In healthy humans, bacteria enter the lungs through an active and continuous process by inhalation of air, direct mucosal dispersion and micro-aspiration from the URT (Dickson et al., 2014). Investigation of the biogeography of bovine respiratory microbiome is important to better understand the pathogenesis of BRD and develop more precise sampling techniques. To date, the nasopharynx has been used as the most popular sampling site for BRD research, and several studies have explored the nasopharyngeal microbiota and their relationship with BRD (Holman et al., 2015; Holman et al., 2017; Timsit et al., 2016). A recent study confirmed that the nasopharyngeal bacterial community is most similar to the lung microbiota in clinically healthy cattle (McMullen et al., 2020). However, it has never been definitively shown that the nasopharynx could act as a source for the BRD pathogens that cause lung infections. Airway physiology (e.g. discharge, breath rhythm etc.) is known to be changed by the onset and development of airway disease (Dickson et al., 2016). The dissimilarities and relationship of microbial communities between the upper and lower airways should be studied more deeply in both disease and healthy calves. Moreover, the nasal cavity, one of the main URT microbial niches, connects to both the external environment and the respiratory system, could also play

important roles in BRD research. The genera associated with common BRD pathogens such as *Mycoplasma*, *Moraxella*, *Pasteurella*, and *Mannheimia* were observed in the nostrils of both healthy and BRD cattle (Nicola et al., 2017). Therefore, comprehensive characterization of the microbial composition in the bovine respiratory tract, including the upper and lower airway, is necessary for the research and development of modern prediction and diagnosis strategies concerned with BRD so that we can focus on the niched-based communities (e.g. nasal cavity) where sampling difficulty is minimized and both high accuracy and throughput can be achieved. In this study, the primary goal was to determine the dissimilarities and connections of the microbial composition of nasal swabs (NS), nasopharyngeal swabs (NPS) and the lung microbiome.

Materials and Methods

Animals and samples collection

Animal trial 1 (test trial)

The use of animals for this study was approved by the University of Arkansas Animal Care and Use Committee (Protocol # 16024). This animal trial was conducted from June to July 2016. A total of forty-eight newly weaned Angus beef calves used in this study were fed in the West Texas A&M University Research Feedlot (WTAMU), USA. Upon arrival to the feedlot (d0), all calves, including only bulls and steers, were given access to hay and water and rested overnight in holding pens. The following morning (d1), calves were stratified by weight, and allocated randomly to different pens. In addition, on d1, calves were vaccinated against clostridial toxins, and bovine respiratory viruses (using a commercially available modified-live multivalent vaccine), given an anthelmintic, and castrated (if necessary). Calves were sorted 12/pen. Calves were fed a diet that met their nutritional requirements.

In this study, all calves including BRD and healthy calves were sampled at feedlot arrival and the onset of BRD using nasal swabs (NS), nasopharyngeal swabs (NPS), and bronchoalveolar lavages (BAL). NS were collected by swirling two Puritan Opti-Swabs (Puritan Medical Products Co. LLC, Guilford, Maine) in the mid-nare region until saturation. NPS were collected by inserting a double guarded culture swab (Jorgensen Labs, Loveland, Colorado) up the nares until reaching the nasopharynx where the swab was advanced through the guard, rotated against the nasopharyngeal mucosa, then retracted back into the guard and removed from the nares. For retrieving a BAL sample, a bal-240 tube (MILA International, Florence, KY) was passed through the nares, guided through the larynx into the trachea, and advanced until resistance was met. Sterile 0.9% saline was administered in aliquots of 60 ml (up to 240 ml) and aspirated. Then, all samples were transported with dry ice and stored at -80 °C for further analysis.

Animal trial 2 (validation trial)

The use of animals for this study was approved by the University of Arkansas Animal Care and Use Committee (Protocol # 19071). To validate the discoveries from animal trial 1, a total of 117 calves (63 from Arkansas research units, and 54 from the auction market) were selected. When these calves arrived at the WTAMU in May 2020, cattle were housed in pens (by sources) and had access to 0.5% body weight (BW) coastal Bermuda grass hay and 0.5% BW of the WTAMU standard starter ration, and water *ad libitum* until next morning. All calves were weighted, and received a clostridial vaccine (Cavalry 9, Merck, Madison, NJ), an anthelmintic (ivermectin and albendazole), and a growth promoting implant (Component E-S with Tylan, Elanco Animal Health, Greenfield, IN). No calves were given respiratory vaccines at initial

processing. Cattle were subsequently allotted to pens using randomization sheets created beforehand, and were offered a total mixed ration twice per day during the feedlot phase.

All animals were sampled with NS using the same methods and swabs as previously described in animal trial 1. Thirty-six calves, eighteen from University of Arkansas units and eighteen from the auction market, were selected based on sources and pens, were used in the intensive study to collect additional NPS and BAL at the feedlot arrival and the onset of BRD. These calves were allocated to six pens, with three auction market calves and one Arkansas calf from each of the three research units in each pen, totaling six intensive calves per each of the six pens. The sampling technique of NPS and BAL collection was the same as in animal trial 1. All samples were transported to our lab with dry ice and stored at -80 °C for further analysis.

DNA extraction and Next-generation sequencing

DNA was extracted using the DNeasy PowerLyzer PowerSoil Kit (Qiagen, Germantown, MD). Sterile Opti-Swab Amies buffer was taken through the extraction process representing a negative control. DNA standards (ZymoBIOMICS Microbial Community, Zymo Research, Tustin, CA) were used as a positive control. The V4 region of the 16S rDNA gene was amplified and sequenced on an Illumina MiSeq 2 × 150 platform. From each sample, a 10 ng/μL DNA aliquot was used to construct a sequencing library targeting the V4 region of 16S rRNA. All samples were amplified with dual-index primers via PCR, and amplicons were normalized using a SequalPrep^{TM} Normalization kit (Life Technologies Corp., Grand Island, NY). PCR amplicons from each sample were possessed with specific barcode sequences to differentiate them within the pooled library. A 5 μL aliquot of each normalized sample was combined to generate one pooled library for further assays. Both library concentration and exact product size were measured using a KAPA Library Quantification Kit (Kapa Biosystems, Woburn, MA) through

the use of a quantitative PCR Eppendorf Realplex^4 (Eppendorf, Hamburg, Germany) assay and an Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA), respectively. Based on the qPCR and Bioanalyzer results, the pooled library was subsequently diluted to 2 nM prior to sequencing. Next-generation sequencing was performed on an Illumina MiSeq sequencer (Illumina, San Diego, CA) located in the Biomass Research Center of the University of Arkansas Division of Agriculture.

Bioinformatics and statistics

The software package QIIME2 (version 2020.6) (Bolyen et al., 2019) was applied to analyze the next-generation sequencing data from the Illumina MiSeq platform. After all fastq reads from two trials were imported together into QIIME2, the Deblur program were used to process the raw reads. Deblur, a novel sub-operational-taxonomic-unit approach, uses error profiles to obtain putative error-free sequences, resulting in high quality sequence variant data (Amir et al., 2017). After the quality filtering step completes, high quality reads were normalized to minimize the effects of sequencing depth on alpha and beta diversity measures. The Bray-Curtis and Jaccard distance metrics were calculated to investigate the dissimilarities in community structure. The ANalysis Of SIMilarity was employed to compare the significance of beta diversity. Then, clean reads were classified using Greengenes references database (13-8 version) classifies with 99% similarity. A feature table was generated using QIIME2 command. The high quality sequences were generated using this pipeline based on the correction of eight bacterial taxa from the mock communities with great relative abundance as expected.

Random Forest was used to identify the top microbiome features for each niche. The plot of variable importance was generated by the importance scores (Mean Decrease Accuracy) of features. In this study, the top 25 features were considered important predictors. R package

'RandomForest v.4.6-7' was used to perform the random forest process. The 'importance' and 'proximity' parameters were set 'True', and 'ntree' was set 10000 in the model. The boxplots of relative abundance of optimal predictors were performed by using the R ggplot2 package (v.3.0) and labeled p-values of Wilcoxon test.

Results

Metadata description

A total of 48 calves were included in this test trial. Nasal swabs (NS), nasopharyngeal swabs (NPS), and bronchoalveolar lavage (BAL) were collected from all animals during feedlot phase. We next validated the biogeography of the bovine respiratory microbiome in a second animal trial (i.e., the validation trial) that included 117 calves and sample collection as previously mentioned in the above section. In total, 553 samples (222 in the test study, and 331 in the validation trial) were sequenced and the raw Illumina sequencing files were processed together in QIIME2, resulting in 5,616,577 clean reads, with an average of 24,852 sequences per sample.

Differences of microbial structure between sampling niches

We first examined the biogeography of the bovine respiratory microbiome at the community level. Significant differences in alpha diversity measures were observed between the three niches. BAL had the greatest microbiome diversity (Shannon index and the numbers of observed features) followed by NS, while NPS microbiomes possessed the least microbial diversity (Figure 1 A-B). Principal coordinate analysis (PCoA) based on Bray-Curtis distance showed distinct clusters, indicating that the bovine respiratory system harbors different microbiomes in different niches (Figure 1C, ANOSIM, R-value: NS-NPS=0.42, NS-BAL=0.54, and NPS-BAL=0.29, $P<0.05$). Consistently, the PCoA plot based on Jaccard distance also

demonstrated significant differences in microbiome structure between the three respiratory niches (Figure 1D, ANalysis Of SIMilarity, ANOSIM, R-value: NS-NPS=0.36, NS-BAL=0.67, and NPS-BAL=0.50, $P<0.05$).

In the validation trial, dissimilarities of community structure among sampling niches were also observed, consistent with the test trial. Regarding alpha diversity in the validation trial (Figure 1 E-F), patterns among the three sampling niches were synonymous with the test trial, except no differences in the number of observed features between NS and BAL were observed. For beta diversity, distinct clusters among the three niches were also observed based on both Bray-Curtis (Figure 1G, ANOSIM, R-value: NS-NPS=0.54, NS-BAL=0.52, and NPS-BAL=0.28, $P<0.05$) and Jaccard distance (Figure 1H, ANOSIM, R-value: NS-NPS=0.52, NS-BAL=0.59, and NPS-BAL=0.28, $P<0.05$).

The signature microbiome for biogeography of bovine respiratory tracts

In order to identify signature microbiotas that distinguish these three sampling niches (NS, NPS and BAL), random forest was performed, as described previously (Kong et al., 2016; Wang et al., 2019). The top 25 most predictive features in the test trial were listed based on mean decrease accuracy (Figure 2A). Bacterial features associated with common BRD pathogens (e.g., *Mycoplasma* and *Pasteurellaceae*) were among the top features to differentiate the three niches of the bovine airway. Signature features of the NS microbiome have been listed in Figure 2B and Figure S1. *Pasteurellaceae* (F5) and *Mycoplasma* (F38), associated with common BRD pathogens, were enriched in NS (Figure S1). *Moraxellaceae* appeared to be NS specific. Several bacterial features, including F8 (Figure 2B), F10, F18, F2 and F32 (Figure S1) in this family, were detected and over-represented in NS. *Streptococcus* (F16) and *Corynebacterium* (F33) were another NS signature bacterium. Interestingly, some features associated with the gastrointestinal

tract were also enriched in the NS microbiomes. For example, *Carnobacteriaceae* (F24), *Turicibacter* (F70) and *Weeksellaceae* (F22) were observed in the NS samples (Figure S1). Other major BRD pathogen-related microbiota (e.g., *Mycoplasma hyorhinis* (F1)) was signature of NPS microbiome (Figure 2C). BAL microbiome was over-represented by bacteria such as *Microbacteriaceae* (F6) (Figure 2D), *Bacteroidales* (F182, F350, F454 and F260), *Prevotella ruminicola* (F60 and F286), and *Succiniclasticum* (F153) (Figure S1). The distribution of other signature microbiota are demonstrated in Figure S1.

Next, the niche-associated signature microbiota in the validation trial were also identified using same the machine-learning algorithm. The accuracy of Random Forest model for both the test and validation trials were 85.1% and 87.6%, suggesting highly accurate predictive models. Identical signature microbiomes (11 of 25) for each niche were observed (Figure 2 A&E). For example, *Moraxellaceae* (F8, F10, F5, F18, F2, F15), *Streptococcus* (F16) and *Carnobacteriaceae* (F24) were also identified as NS signature profiles (Figure 2F, Figure S2), and *Mycoplasma hyorhinis* (F1) was identified as a signature of NPS microbiome as well (Figure 2G), while *Microbacteriaceae* (F6) was also as BAL signature (Figure 2H). Other niche-associated signatures in our validation animal trial are shown in Figure S2.

The major bacterial taxa within bovine respiratory tracts

At the phylum level, the dominant phyla across all samples were *Proteobacteria*, followed by *Tenericutes*, *Firmicutes*, *Bacteroidetes*, and *Actinobacteria*. Those five phyla accounted for 96% of total sequences (Figure S3). Regarding both the test and validation trials, NS showed high relative abundance of the phylum *Proteobacteria*, while NPS had high abundances of *Tenericutes* and *Firmicutes*, and *Bacteroidetes* were enriched in BAL.

We next sorted the differences and similarities of genus composition in the bovine respiratory tracts. In animal trial 1, NS had high abundances of *Aggregatibacter*, *Enhydrobacter*, and *Streptococcus* compared to NPS and BAL (Figure 3A). The genus *Mycoplasma* was enriched in NPS (41.75%) higher than NS (9.53%) and BAL (17.75%), while *Histophilus* was greater in NPS (5.13%) compared to NS (1.13%) and BAL (0.68%) (Figure 3 B, E). *Moraxella*, *Mannheimia* and *Pasteurella* were more abundant in the upper airway (both NS and NPS) (Figure 3 C-D, Figure S4 A). Genus *Prevotella* had the highest abundance in BAL, followed by NS and NPS (Figure 3A, Figure S4 B). In the validation trial, the genus composition produced similar patterns for all the above-mentioned genera, besides *Mannheimia*, with similar abundances in NS, NPS and BAL (Figure 3 F-J, Figure S4 E-H).

The microbial connection within bovine respiratory tracts

To better understand microbial composition and connections between sampling niches, the top 20 bacterial features were compared. Moreover, the top bacterial features, although also niche specific, were detected in all three niches. They were differentially distributed between the three niches, as shown in the stacked bar chart (Figure 4A). *Mycoplasma* (F3) had similar abundances between three niches. Features related to *Moraxellaceae* (e.g. F2, F5, F8) and *Streptococcus* (F16) were specifically enriched in NS. Although *Mycoplasma hyorhinis* (F1) and *Histophilus somni* (F11) had greater abundance in NPS, they were also detected in NS and BAL. *Mannheimia* (F4) and *Pasteurella* (F13) were overrepresented in the upper airway. The abundance of *Mycoplasma* (F7) was high in the NPS and BAL. Moreover, we validated the findings of these major features in the validation trial (Figure 4F).

Then, regarding the frequencies and abundances of bacteria in the three sampling niches, the predictability of the lung microbiome by using the upper airway microbiota was determined.

We assessed the extent to which the upper airway microbial community could reflect the lung microbiome by measuring the Pearson correlation between sampling niches, thus accounting for both rank order of features and the magnitude of relative abundances between sampling sites being compared. Two adjacent niches in anatomy were highly correlated (Figure 4 B-D). NS had high correlation with NPS ($r=0.63$, $p<0.001$), while NPS was correlated with the lung microbiome ($r=0.52$, $p<0.001$). As expected, the correlation between NS and BAL was modest ($r=0.35$, $p<0.001$). Furthermore, these correlations were repeatedly observed in the validation trial (Figure 4 F-H).

Discussion

This study described the microbial composition in the bovine upper and lower airway, compared the differences of respiratory microbiota in three niches, and measured the connections along respiratory communities. We observed the different community diversities and structures among different respiratory niches, driven by the major microbiota. The presence of major bacterial features and strong correlations among all three sampling niches provide the possibility that using upper airway sampling could replace lung sampling for studies of the bovine respiratory microbiome. Our study indicated that nasal swabbing is an effective approach to reflect the lung microbial community.

Investigations of microbial differences between the upper and lower respiratory tract have been reported in cattle (Nicola et al., 2017; Timsit et al., 2018; Zeineldin et al., 2017a). However, these previous studies were limited in comparing the upper airway microbiota (either nasal or nasopharynx) to trans-tracheal aspirations or bronchoalveolar lavages. A recent study compared microbial composition of respiratory location from 15 healthy feedlot steer cattle (McMullen et al., 2020), but the lack of samples from BRD calves and other time points (e.g.

feedlot arrival) in feedlot lead to a lack of representation for whole the feedlot phase. Regarding the temporal dynamics of the microbial shifts after entry to the feedlot, as well as differences between BRD and healthy calves (Holman et al., 2019; Holman et al., 2015; Timsit et al., 2016; Zeineldin et al., 2017b), a study with a higher combination of samples from disease and healthy calves at different time points could give more general insights for the biogeography of the bovine respiratory microbiome, performed in this study.

Distinct microbial composition and structure among nasal cavity, nasopharynx, and lung sampling sites were observed in this study. A meta-analysis study reported the significant diversity and richness between the upper and lower respiratory tracts using data from previous studies (Zeineldin et al., 2020a), which is similar with our study. As we know, the physiological and biochemical differences along the respiratory tract are caused by the pH, oxygen, temperature, humidity, epithelial cell types, mucus thickness and immune cells (de Steenhuijsen Piters et al., 2015; Dickson et al., 2016; Man et al., 2017). Therefore, niche-specific microbial communities along the respiratory tract were selectively grown, which could lead to the differences of predominant microbiota in each niche.

Moraxella were more abundant in the upper airway (both NS and NPS), which is consistent with previous research (McMullen et al., 2020; McMullen et al., 2019; Nicola et al., 2017). In this study, several features related to *Moraxella* including F8, F10, F18, F2 and F32 were identified as nasal signatures. It was reported that *Moraxella boevrei* (F10) and *Moraxella bovis* (F32) were related with BRD and infectious bovine keratoconjunctivitis (Angelos, 2010; McMullen et al., 2019). Hence, those niche specific signatures could be also related with BRD. We also observed that *Streptococcus* (F16) and *Corynebacterium* (F33) were other NS signature bacterium in cattle. *Streptococcus* was reported as the dominate genus in oral microbiota

(McMullen et al., 2020) and *Corynebacterium* was found in the nasal cavity as a dominating microbial member (Nicola et al., 2017). Therefore, the microbial shifts between the mouth and nostrils in cattle would be a question for future research.

Genus *Mycoplasma* had the highest abundance in nasopharynx, then lung and nasal cavity. This finding is similar with several studies that have reported *Mycoplasma* as one of the most commonly associated genera in the nasopharynx (McMullen et al., 2019; Stroebel et al., 2018; Timsit et al., 2018; Zeineldin et al., 2017b). F1 (NCBI blast: *Mycoplasma dispar*), as nasopharynx signature in our results, was identified as commensal bacteria in healthy feedlot cattle (Timsit et al., 2018). In general, genus *Mycoplasma* has a high affinity to attach to the epithelial cells of the bovine respiratory tract through adhesion proteins (McMullen et al., 2020). F1 may elicit a mild cellular response, which answered why it highly exists in the nasopharynx and was detected in the nostrils and lungs too. It would be necessary to understand how *Mycoplasma dispar* is associated with other commensal bacteria and BRD pathogens, which is helpful for understanding microbial colonization in the respiratory tract. It would be valuable to clarify the reason why the nasopharynx is the priority location colonized by *Mycoplasma*.

In the lung microbiome, excluding *Mycoplasma,* we observed that the second dominate genera is *Prevotella* and several features associated with it were identified as lung signatures in both the test and validation trials, and exhibited presence in the upper airway. It is well known that *Prevotella* is a common bacterium in the rumen. Regarding the ruminating behaviors in cattle, tracking the species belong to this genus may be a potential way to understand microbial movement. Furthermore, another feature (*Microbacteriaceae* (F6)) with high abundance in NPS and BAL was identified as a lung microbial biomarker. It was reported as a dominate bacterium in the nasopharynx and its relative abundance was significantly affected by antibiotic treatment

and BRD (Holman et al., 2019; Zeineldin et al., 2020b). Thus, the lung microbiome is associated with other niches and could be affected by factors including disease.

Although we found the signatures to distinguish these three sampling sites, the overlaps of the relative abundance and presence of respiratory microbiota were also observed. For example, *Mycoplasma* (F3) had similar abundances in the upper and lower airways, and other top bacterial features were detected in all three niches. The association between the presence of particular species of the microbiome in the upper and lower airways also was confirmed by another study (Zeineldin et al., 2017a). Based on correlation analysis, adjacent niches demonstrated high correlation, possibly explained by higher rates of mucus exchange. Lower correlation and fewer similarities in the microbiome structures between the nostril and the lungs might be due to the remarkable differences in the anatomy, ecosystem and distances separated by the larynx. However, it is notable that nasal microbiome could reflect the nasopharynx well, and the NPS microbiome could be a mirror of the lung community. Overall, although distinct differences of the NS, NPS, and BAL microbiotas were observed, many BRD associated bacterial species were found in all the three sampling sites, despite having different relative abundance. Our study shows distinct yet interconnected bovine respiratory microbiome in the three niches, consistent with previous reports (McMullen et al., 2018; Nicola et al., 2017).

A limitation of this study is the lack of oral samples. In humans, the oropharynx has been suggested as a major source contributing to the lung microbiome (Venkataraman et al., 2015). However, a recent finding suggested that the oral microbiotas (specifically the oropharynx) have less compositional overlap with the lung than the nasopharynx in healthy cattle (McMullen et al., 2020). Another study in lambs found that the lung microbiota is dissimilar to the upper aerodigestive tract due to physiological and anatomical differences between sheep and humans

(Glendinning et al., 2017). As high abundances of *Prevotella* in the lung were found in our study, the association between the oropharynx and/or rumen and lung is necessary for further investigation.

Conclusions

In this study, the nasal, nasopharynx and lung microbiome were characterized, and the signature microbiota for each niche were identified. While the significant differences of microbial community structure and predominant bacteria among NS, NPS, and BAL sites were observed, we confirmed strong microbial associations between adjacent niches in the bovine respiratory tract. This study indicates that nasal swabbing could be an effective approach to investigate the lung microbiome and bovine respiratory disease.

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Tables and Figures

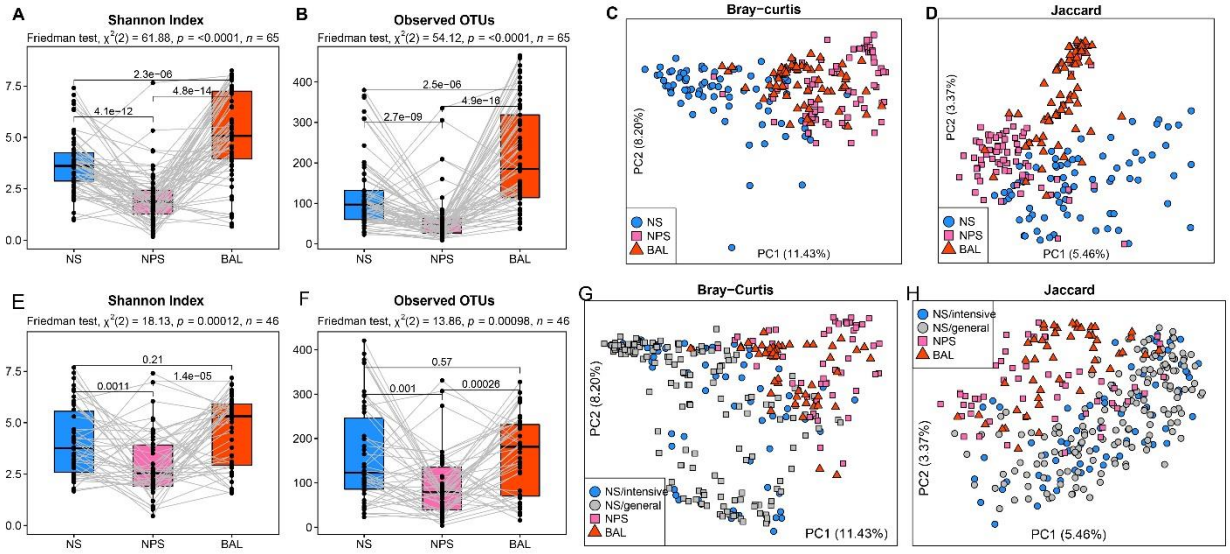

Figure 1 The biogeography of the bovine respiratory microbiome
The top panel of Figure 1 were from the test trial. (A) Community diversity in the test trial, (B) Richness in the test trial. The numbers over the bars are p values calculated by the Wilcoxon test. Connected points represent different sampling niches from the same animal. Principal coordinate analysis (PCoA) plots based on the Bray-Curtis (C) and Jaccard (D) distances. Each point represents one respiratory microbiome from different niches (NS: dodgerblue circle, NPS: hot pink square, and BAL: red triangle). The bottom panel showed same parameters for the validation trial. The gray and blue color for NS in H (the validation trial) separates the 36 intensive calves and others (labeled as general).
Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL).

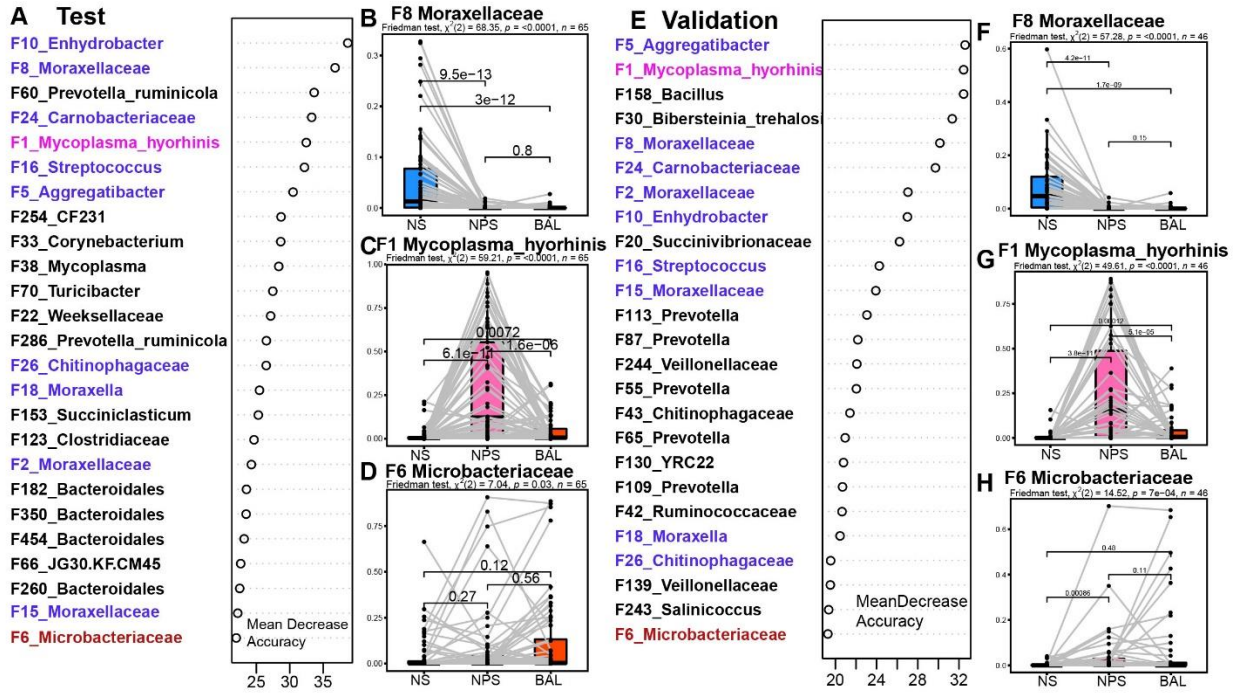

Figure 2 Microbial signatures differentiating the three sampling niches

The left panel (A: Top 25 Features classified by Random Forest to differentiate the three niches; B, C, D represent the signature microbiota for each niche) is for the test trial. The numbers over the bars are p values calculated by the Wilcoxon test. Connected points represent different sampling niches from the same animal. E-H in the right panel were the outputs of Random Forest from validation trial. The blue color features in A and E represent the same signature microbiota for NS in both trials, while the hot pink color is shared signature for NPS and common red feature is for BAL.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL).

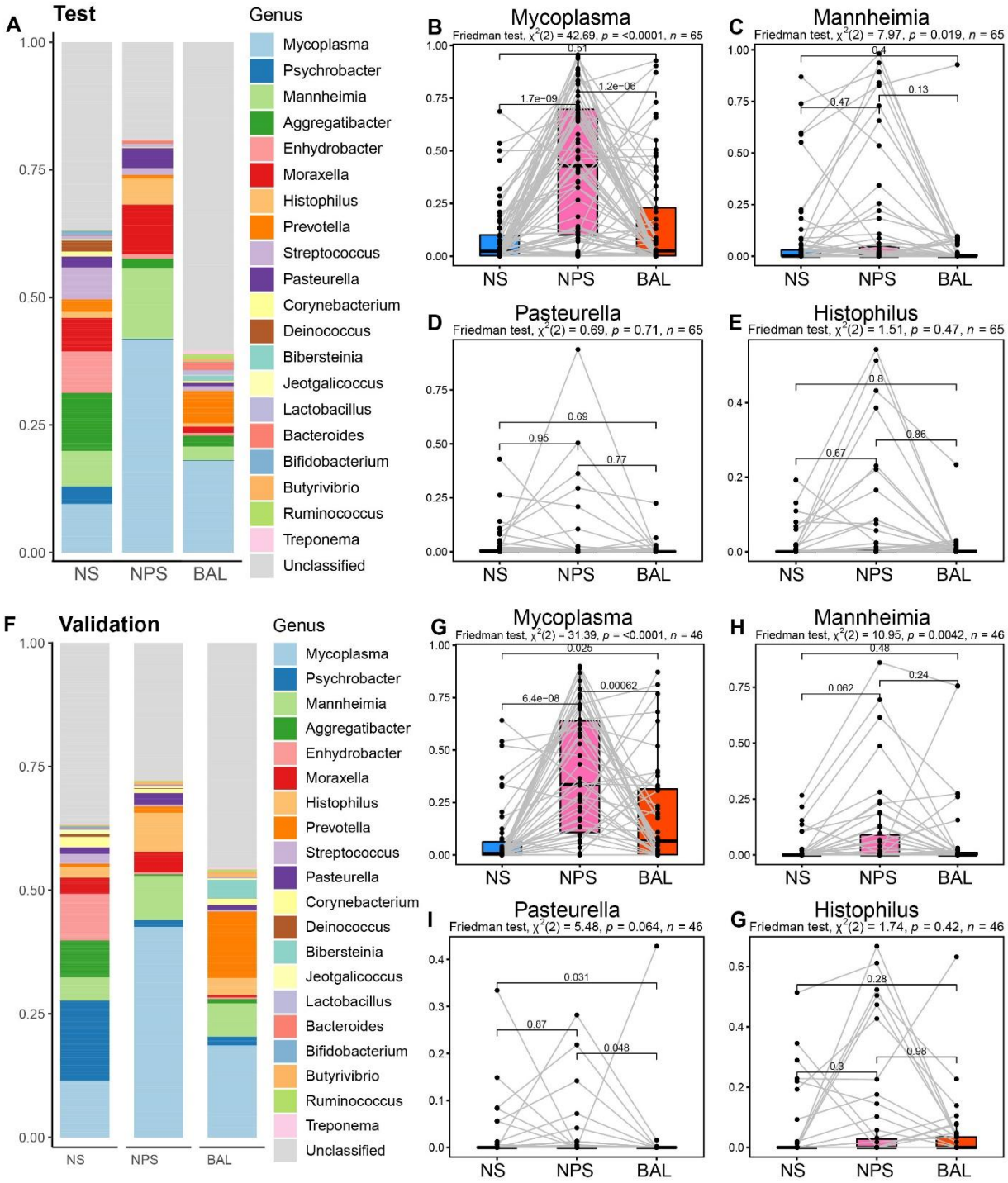

Figure 3 Genus composition in bovine respiratory community

The top panel (A: Top 20 genera in three niches; B, C, D, E showed relative abundances of main BRD pathogens) is for the test trial. The numbers over the bars are p values calculated by the Wilcoxon test. Connected points represent different sampling niches from the same animal. F-J in the bottom panel were the outputs from the validation trial.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL).

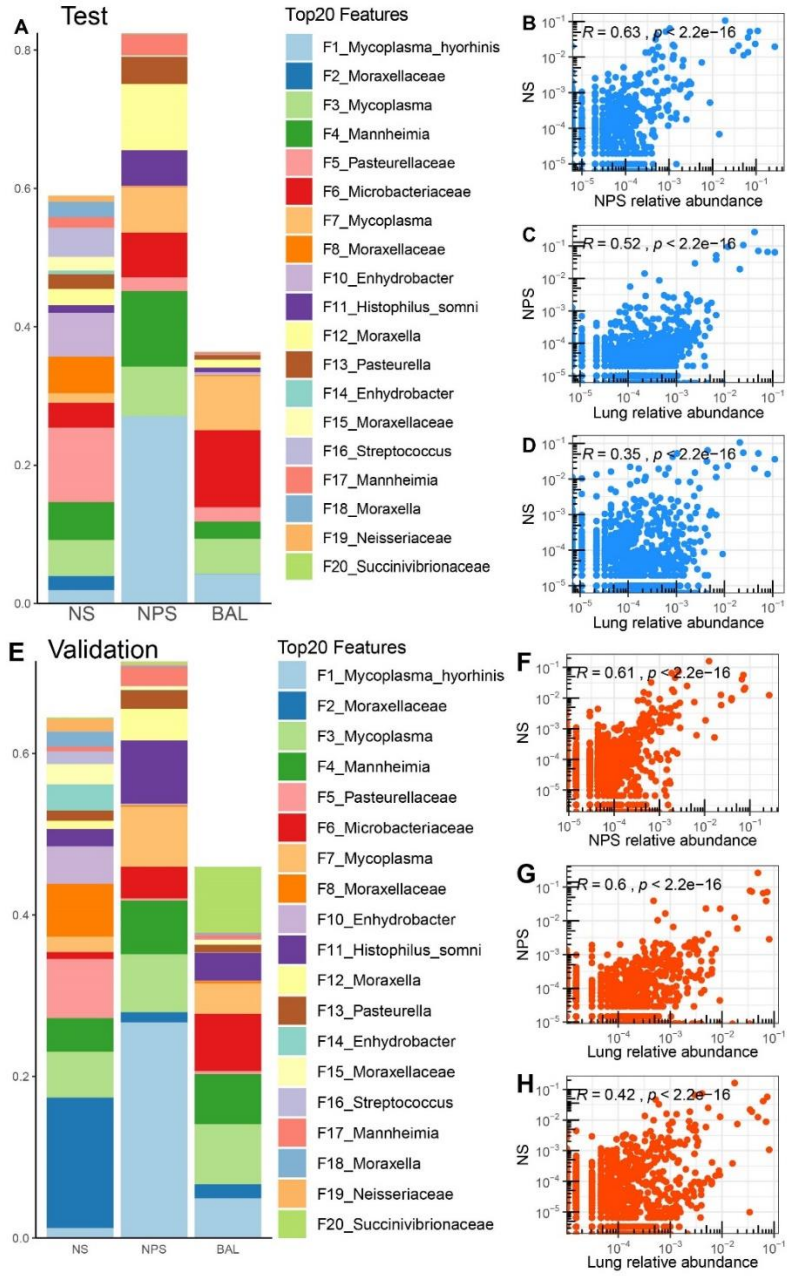

Figure 4 The top 20 microbial features in the three niches and correlation between sampling niches

The top panel (A: Top 20 feature in three niches; B, C, D showed correlation between sampling niches) is for the test trial. Each dot corresponds to the average relative abundance of a feature across all animals for each of respiratory sampling regions (NS, NPS, and BAL). To measure correlation, Pearson's r was calculated based on features' abundances of two niches. The blue marks on the x axis (vertical lines) or y axis (horizontal lines) margins represent microbial features with zero relative abundance at one sampling niche but non-zero abundance at the other. E-H in the bottom panel were the same pattern of outputs from the validation trial.
Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL).

Supplemental Materials

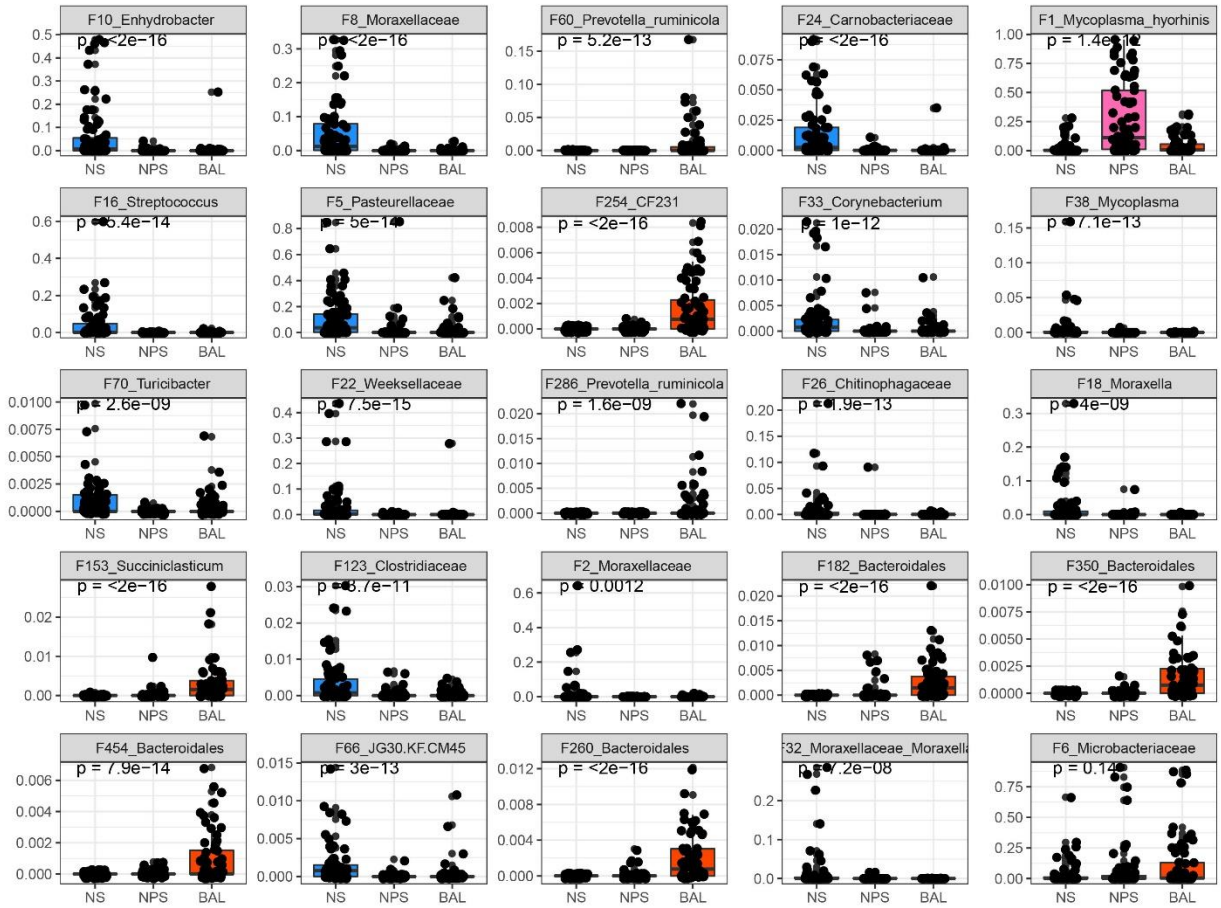

Figure S1 Signatures classified by random forest to differentiate NS, NPS, and BAL in the test trial. The numbers over bars are p values from Kruskal–Wallis test.
Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL).

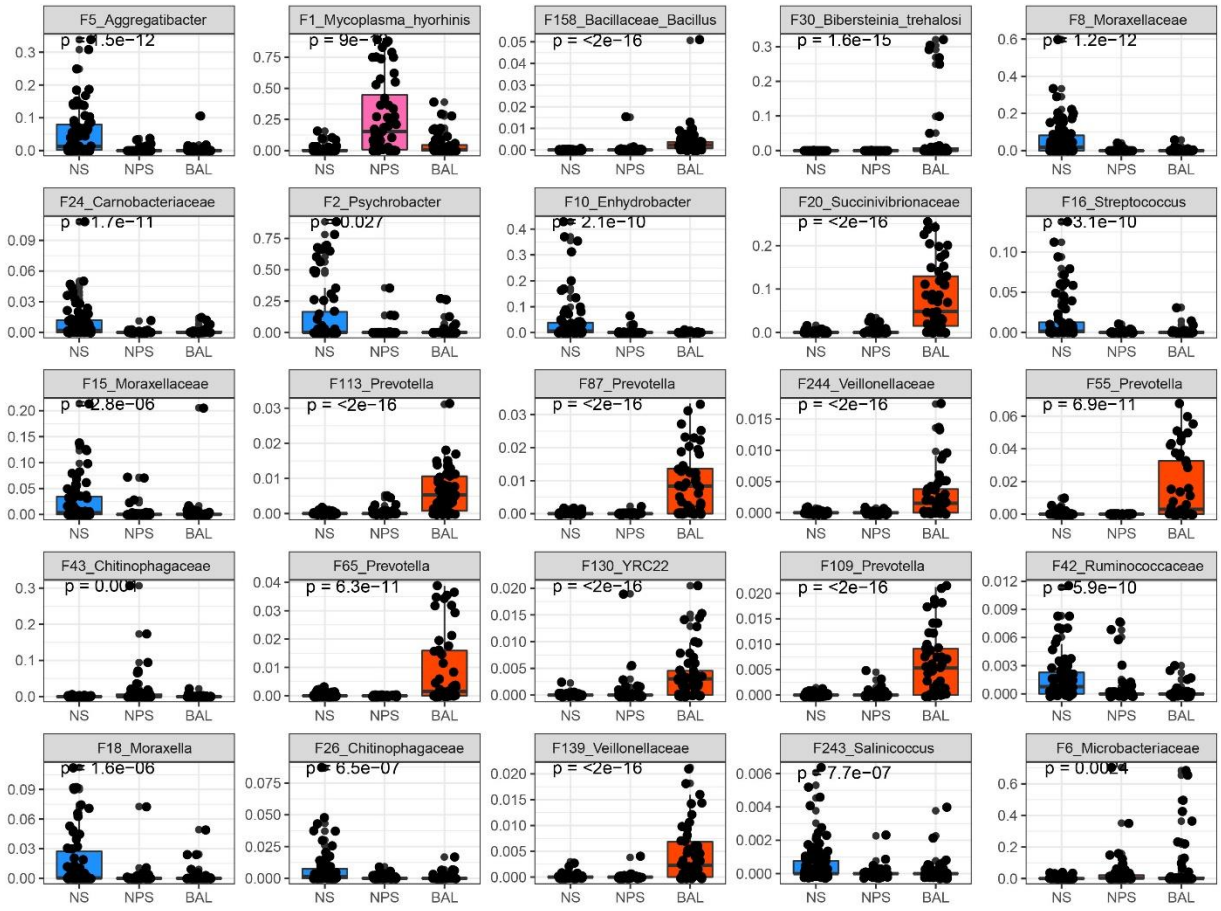

Figure S2 Signatures classified by random forest to differentiate NS, NPS, and BAL in the validation trial. The numbers over bars are p values from the Kruskal–Wallistest. Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL).

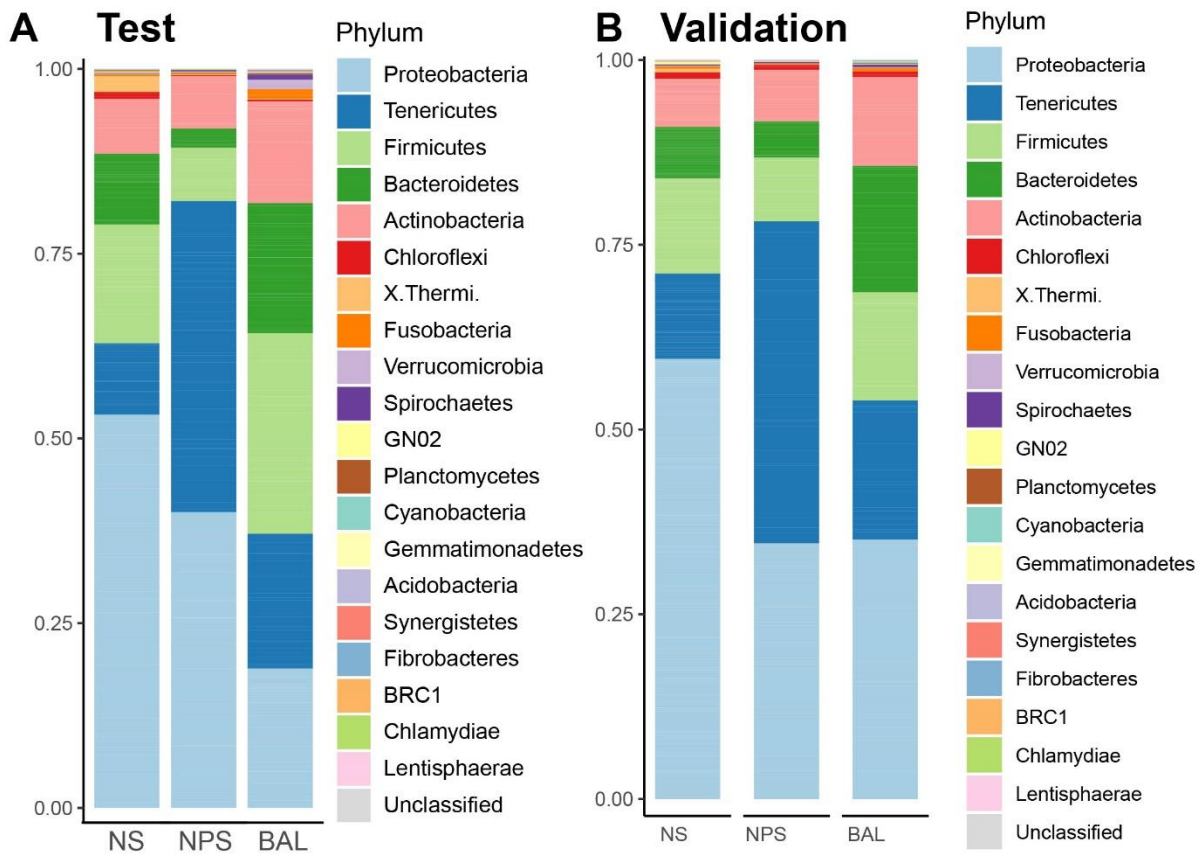

Figure S3 Microbial composition in the bovine respiratory tract at phylum level in the test and validation trials.
Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL).

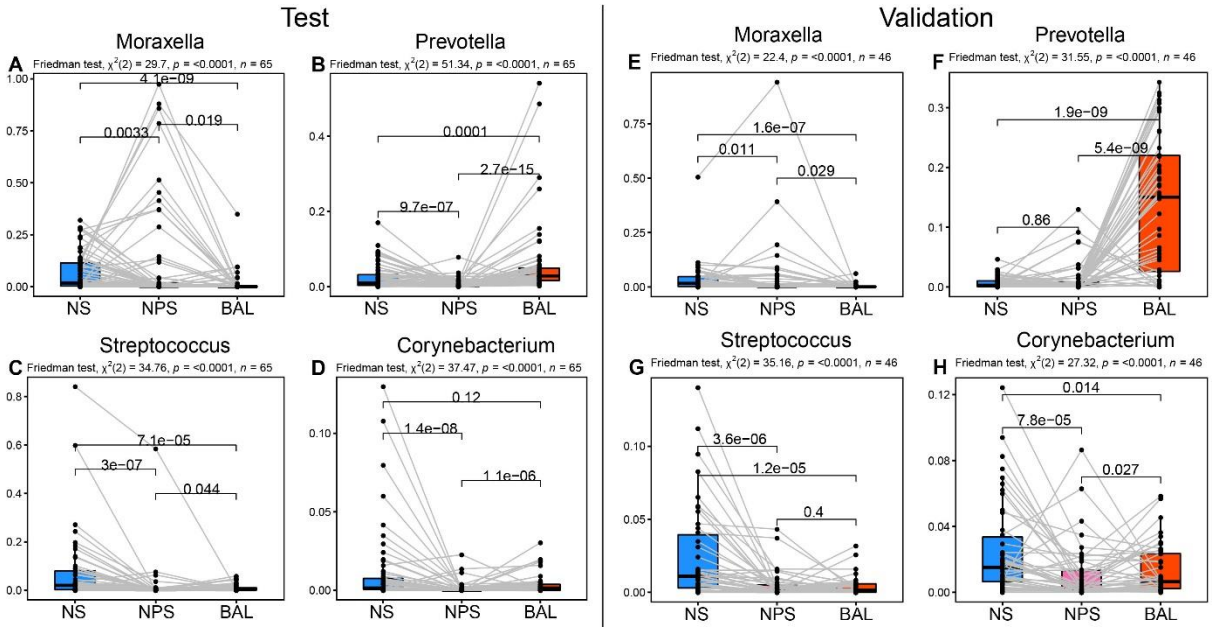

Figure S4 Main genera distribution in NS, NPS, and BAL in the test and validation trials. The numbers over bars are p values from the Wilcoxon test.
Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL).

Chapter IV. Investigation of the Relationship between Microbiome and Bovine Respiratory Disease

Abstract

Bovine respiratory disease (BRD) is generated by a multifactorial onset. The bovine respiratory microbiome has been implicated in the onset of BRD, but the temporal dynamics and spatial variation of the bovine respiratory microbiome and their correlation with BRD are still largely unknown. To fill this knowledge gap, we collected nasal swabs (NS), nasopharyngeal swabs (NPS), and bronchoalveolar lavage (BAL) fluid from 48 weaned calves when they arrived at a feedlot and when they had signs of BRD. A second animal (validation) trial was performed to improve the discoveries. The microbiotas of all the airway samples (both the test and validation trials) were characterized by the 16S rRNA gene technology. The differences of microbial composition of three sampling niches were affected by BRD. The respiratory microbiomes of all the three niches were able to predict the onset of BRD using Random Forest model with over 90% of accuracy. Significant shifts were observed from feedlot arrival and the onset of BRD in healthy and BRD calves. From arrival to the onset of BRD, consistent temporal changes in the relative abundance of several taxa (e.g. *Mycoplasma* (F7), *Histophilus somni* (F11), *Corynebacterium* (F34) and *Bifidobacterium pseudolongum* (F31)) were observed in all three niches. The signature microbiome including F7 and *Mycoplasma hyorhinis* (F1) that were enriched in BRD calves can be used to diagnose BRD. Our study suggests that the nasal microbiome could be used as a rapid and non-invasive method to predict and diagnose BRD. Our data also revealed temporal dynamics of the bovine respiratory microbiome from arrival to a feedlot to the onset of BRD, which provides insight into the pathobiology of BRD.

Key words: Bovine respiratory disease, respiratory microbiome, bacterial pathogens, Mycoplasma, cattle, random forest, Mycoplasma

Introduction

Bovine respiratory disease (BRD) is one of the most common causes of morbidity and mortality in feedlot cattle (Holman et al., 2015b; Nicola et al., 2017). Despite the recent advances in vaccine and antimicrobial technologies for bacterial pathogens, there does not appear to be many improvements in health outcomes, as morbidity and mortality rates have not decreased over the last 20 years (Lima et al., 2016). The reason might be due to the complex interactions of bacterial and viral pathogens under the effect of many factors, such as management, host, and environment (Nicola et al., 2017). Previous research using culture-dependent approaches has identified the most four common bacterial pathogens (Holman et al., 2015b; Klima et al., 2014; Taylor et al., 2010); however, these opportunistic pathogens can also be isolated from healthy cattle (Lima et al., 2016). It is well known that stress and viral infection cause rapid growth of these opportunistic pathogens, and subsequently lead to suppression of the host immune system and disequilibrium of the bovine respiratory microbiome (Hakansson et al., 2018). Although respiratory microbiome relates to cattle health, we still do not understand the accurate association between the microbiome and BRD. Therefore, a deep characterization of the bovine respiratory microbiome, including those uncultured bacteria, is critical to better understanding the etiology of BRD.

The development of next-generation sequencing technologies has allowed us to understand the bovine airway microbiome with unprecedented depth. Several studies have explored the nasopharyngeal microbiota and their relationship with BRD (Holman et al., 2015a; Holman et al., 2017a; Holman et al., 2015b; Timsit et al., 2016b; Zeineldin et al., 2017). Significant changes in the nasopharyngeal microbiota during the first 60 days in the feedlot were observed (Holman et al., 2015a; Timsit et al., 2016b). In addition, decreased bacterial diversity in the nasopharynx was found in calves diagnosed with BRD (Holman et al., 2015b; Timsit et al., 2018) at both feedlot

entry (day 0) and over 60 days after placement. Indication that the nasopharyngeal microbiota at entry into a feedlot could play a key role in the pathophysiology of BRD has been noted (McDaneld et al., 2018; Zeineldin et al., 2017). Despite these advances in bovine nasopharyngeal microbiota characterization, little is known about the microbiota in other niches of the bovine respiratory system (McDaneld et al., 2018; Nicola et al., 2017). A systemic and comprehensive study of the biogeography of the bovine respiratory microbiome and its relationship with BRD is lacking and greatly needed. The objectives of this study were to characterize the spatial composition and temporal dynamics of the bovine respiratory microbiome in three niches of the respiratory system: nasal cavity, nasopharynx, and lungs, to determine their relationships with the onset of BRD.

Materials and Methods

Experimental design, animals, and samples collection

Animal trial 1 (test trial)

A longitudinal and cross-sectional design was used in this study. The weaned calves were monitored for clinical BRD symptoms each day after they arrived at the feedlot (longitudinal comparison), and clinically healthy control calves were assigned when others were diagnosed with BRD (cross-sectional comparison). In the morning (d1) after arrival to the feedlot (d0), samples of nasal swabs (NS), nasopharyngeal swabs (NPS) and bronchoalveolar lavages (BAL) were collected from all selected calves including nine absolutely healthy calves (control $_{arrival}$) and additional calves that developed BRD later (BRD $_{arrival}$), used to classify the respiratory microbiota for BRD prediction. Then, calves were monitored for clinical respiratory disease every day until day 30 after feedlot arrival for measurement of microbial changes in sick animals from feedlot arrival to the onset of BRD. When calves were diagnosed with BRD, NS, NPS and BAL samples were collected and labeled “BRD”. Simultaneously, selected clinically healthy calves penned

together with the animals diagnosed with BRD, labeled “Control”, were sampled (Figure S1 A). Interestingly, several clinically healthy calves were diagnosed with BRD and sampled after they were previously sampled as Control, and were used for down-stream analysis. The animal feeding and sample (NS, NPS, and BAL) collection processes were previously described in Chapter III.

Clinical diagnosis of BRD was made by trained feedlot personnel. If any animal exhibited at least two symptoms consistent with BRD (depression, nasal discharge, ocular discharge, cough, gaunt appearance, or inappetence), it was moved to a hospital facility, and rectal temperature was recorded. Based on rectal temperature, a calf was placed in 1 of 2 BRD diagnostic categories: 1. if rectal temperature is ≥ 40 ℃, the animal is designated as “febrile” BRD; 2. if rectal temperature is ＜ 40 ℃, the animal is designated as “non-febrile” BRD. Regardless of designation, each BRD calf received the same antimicrobial treatment regimen. After BRD diagnosis, the calves were sampled firstly and subsequently administered ceftiofur crystalline free acid (Excede, Zoetis, Kalamazoo, MI) at 6.6 mg/kg body weight for first treatment. If a calf was diagnosed with BRD again, a second antimicrobial regimen was administered, which consisted of florfenicol (Nuflor, Merck Animal Health, Summit, NJ) at 40 mg/kg body weight. Upon a third BRD diagnosis, a final antimicrobial treatment was with oxytetracycline (4.4 mg/kg body weight; Norbrook Inc., Lenexa, KS).

Animal trial 2 (validation trial)

The experimental design was synonymous with the test trial. Animal managements and sample collection were also described in Chapter III. When 117 newly weaned calves (63 from Arkansas research units (20 from Batesville, 23 from Fayetteville, and 20 from Hope), and 54 from the auction market) arrived to the WTAMU feedlot, all calves were sampled with nasal swabs (NS) on d1. Thirty-six calves, eighteen from University of Arkansas units and eighteen from the auction

market, were selected based on sources and pens, were used in the intensive study to collect additional NPS and BAL at the feedlot arrival and the onset of BRD. These calves were allocated to six pens, with three auction market calves and one Arkansas calf from each of the three research units in each pen, totaling six intensive calves per each of the six pens. All the calves were monitored for BRD symptoms each day by trained personnel until day 30 after arrival in to the feedlot, and 59 of 117 calves (31 from Arkansas research units (12 from Batesville, 13 from Fayetteville, and 6 from Hope), and 28 from the auction market) were diagnosed BRD during feedlot. Samples were collected from the onset of BRD calves (Figure S1 B), and simultaneous sampling of selected clinically healthy calves followed the same pattern of the BRD animals sampling at feedlot arrival (control $_{arrival}$) and onset of disease (Control). In addition, while NS were collected from Control and BRD calves during the onset of BRD, only intensive calves were additionally sampled with NPS and BAL in BRD calves.

BRD case definition followed the Clinical Illness Score (CIS, Table S1) protocol at the WTAMU. If a score was ≥ 2 (and/or 2 or more clinical signs of respiratory disease), calves would be pulled from the pen and examined by taking a rectal temperature in the holding facility. If the rectal temperature ≥ 40°C and/or if score was ≥ 3, calves would be clinically defined as BRD inflicted and were subsequently treated with antimicrobials by using florfenicol (Merck) for first treatment, tildipirosin (Merck) for second treatment, and enrofloxacin (Bayer, Barmen, Germany) for third treatment.

Next-generation sequencing and Bioinformatics

The DNA extraction and sequencing were all described in Chapter III. The raw Illumina files from the test and validation trials were processed together in QIIME2 as described in Chapter III.

Statistics

The alpha diversity (Shannon Index, and the number of observed features) and beta diversity for all four healthy statuses (control $_{arrival}$, BRD $_{arrival}$, BRD and Control) were visualized in R. The Bray-Curtis and Jaccard distance metrics were calculated to investigate the dissimilarities in community structure. The ANalysis Of SIMilarity was employed to compare the significance of beta diversity. The top 500 features were used to classify the predictors using the R package 'AUCRF' (v.1.1) (Calle et al., 2011). A leave-one-subject out method was used in the AUCRF model, and 10-fold cross-validation (AUCRFcv) was set to estimate the prediction error of the model and repeatedly run twenty times to minimize the error. Thus, the produced model predicted the left-out subject, and results were plotted as Receiver Operator Characteristic curves using the pROC package (v.1.13). The optimal predictors of AUCRF were listed based on their mean decrease accuracy (MDA). The boxplots of relative abundance of optimal predictors were performed by using the R ggplot2 package (v.3.0) and labeled p-valued of the Wilcoxon test.

Results

Microbial composition associated with healthy status

Firstly, to examine the microbial differences caused by healthy status, the genus bar chart was created (Figure 1). At feedlot arrival, the abundances of most genera were similar between healthy control $_{arrival}$ and BRD $_{arrival}$. However, some genera were different. *Streptococcus* was specifically enriched in NS BRD $_{arrival}$ (9.40%) compared to NS control $_{arrival}$ (4.29%). *Mannheimia* in NS and NPS microbiome was enriched in the control $_{arrival}$ (10.72% and 18.07) compared to the BRD $_{arrival}$ (1.14% and 8.14%). *Moraxella* had similar pattern with *Mannheimia*.

The genus *Mycoplasma* were overrepresented in control $_{arrival}$ of BAL samples (13.09%) compared to BRD $_{arrival}$ of BAL (1.37%).

With increasing time at the feedlot, both healthy control and BRD calves had significant microbial changes. For longitudinal changes from feedlot arrival to the onset time of BRD, some genera were increased with time in both clinically healthy and BRD calves. For example, *Mycoplasma* increased from control $_{arrival}$ (NS: 4.07%, NPS: 30.30%, BAL: 13.09%) to Control (NS: 11.53%, NPS: 51.47%, BAL: 19.78%), and from BRD $_{arrival}$ (NS: 5.22%, NPS: 42.04%, BAL: 7.37%) to BRD (NS: 13.15%, NPS: 38.63%, BAL: 26.19%). *Histophilus* demonstrated a similar pattern. Other genera only increased with time in feedlot for BRD calves. Genus *Mannheimia* only increased from BRD $_{arrival}$ (NS: 1.14%, NPS: 8.14%, BAL: 1.04%) to BRD (NS: 9.35%, NPS: 17.55%, BAL: 5.11%).

Comparing the differences between the onset of BRD and Control, we found complex genus changes through observing all respiratory niches. *Moraxella* and *Streptococcus* were enriched in the upper airway (NS and NPS) of BRD calves, while no remarkable differences between Control and BRD in BAL samples were observed. For *Mannheimia* and *Pasteurella*, they were higher in NPS (17.55%, 7.34%) and the lung (5.11%, 1.05%) microbiome from BRD calves compared with Control (NPS: 12.37%, 0.89%; BAL: 1.60%, 0.49%). In NS and lung microbiome, *Histophilus* were enriched in BRD calves, but was higher in Control for the NPS microbiome.

In the validation trial, healthy calves had more *Moraxella* compared to BRD calves at feedlot arrival. Compared to the test trial, *Mannheimia* were only increased in NPS BRD $_{arrival}$. For longitudinal changes, *Mycoplasma*, *Histophilus* and *Mannheimia* in NS, NPS and BAL increased positively with time in the feedlot, synonymous changes to the test trial.

Bovine respiratory microbiome signatures predicting the onset of BRD

To determine if the bovine respiratory microbiome could be used to predict the onset of BRD, we analyzed the d1 (arrival) bovine respiratory microbiome from nine calves that had no clinical signs of BRD throughout the study period (healthy control $_{arrival}$) and 20 calves that developed BRD after arrival (BRD $_{arrival}$). No significant differences in alpha diversity were observed between control $_{arrival}$ and BRD $_{arrival}$ calves in any of the three niches (Figure S2). Moreover, there were also no significant difference in community structure based on beta diversity (Figure S3). We observed the same results in the validation trial.

We next developed a random forest machine-learning model to identify the microbiota differentiating control $_{arrival}$ and BRD $_{arrival}$. The optimal model was developed based on the maximum area under the curve (AUC) using the AUCRF algorithm. All the three niches of the bovine respiratory microbiome were able to accurately predict whether or not certain calves will develop BRD, with 30, 3 and 9 features yielding an AUC of 0.995 (NS, specificity=0.952, sensitivity=1.00), 0.956 (NPS, specificity=1.00, sensitivity=0.889) and 0.944 (BAL, specificity=0.762, sensitivity=1.00), respectively (Figure 2 A). In the validation trial, although AUC decreased slightly compared to the test trial, the accuracy of the random forest model was still notable (Figure 2 B).

The 30 features that distinguished healthy $_{arrival}$ calves from those that developed BRD after arrival to the feedlot in the NS microbiome included *Enhydrobacter* (F23), and several GI-tract features such as *Bacteroides* (F81), *Prevotella copri* (F46), and *Prevotella stercorea* (F216) (Table S2, Figure S4). These bacteria were significantly more abundant in healthy calves (Figure S4). Several features including *Enhydrobacter* (F10), *Lactobacillus reuter* (F102, F192),

Lactobacillus (F10) and *Jeotgalicoccus psychrophilus* (F29) were overrepresented in the NS of calves that developed BRD.

Among the 30 predictive features in the NPS microbiome (Table S3), two features of *Prevotella copri* (F46) and *Faecalibacterium prausnitzii* (F327) were more abundant in healthy calves, whereas *Jeotgalicoccus psychrophilus* (F29) and *Streptococcus* (F49) indicated higher abundances in morbid animals (Figure S6).

In the BAL microbiome, bacterial features related to pathogens including *Mycoplasma hyorhinis* (F1) and *Mannheimia* (F17) were listed (Table S4). *Mycoplasma hyorhinis* (F1) and *Streptococcus luteciae* (F37) were enriched in lung microbiome of morbid calves (Figure S8). Interestingly, F1 and F37 also enriched in the NS and NPS microbiomes of calves that developed BRD later in the test trial (Figure 2 C-D).

In the second animal trial, five features of the top 30 features that differentiated NS control $_{\text{arrival}}$ and BRD $_{\text{arrival}}$ were shared with the test trial (Table S2), with high AUC (Figure 2 B). Some gut microbiotas were still higher in healthy calves, such as *Prevotella* (F65) (Figure S5). NPS signature microbiotas in the validation trial were not found highly consistent with the test trial (Table S3), and the distribution of features was shown in Figure S7. For the BAL microbiome, shared features between the test and validation trials were found (Table S4), and the same trends of microbial abundance (e.g. F1) were found (Figure 2 C, Figure S8-S9).

Longitudinal changes of bovine respiratory microbiota in healthy calves after feedlot entry

We compared the microbiota changes between healthy control $_{\text{arrival}}$ and Control for any implications that longitudinal changes of the respiratory microbiome in healthy calves altered in the feedlot. No differences of alpha diversity were observed (Figure S2) due to animals' individual variation. Regarding beta diversity, while no differences were observed based on

Bray-Curtis distance (ANOSIM, NS: R=0.11, *P*<0.120; NPS: R value=0.20, *P*=0.015; BAL: R value=0.07, *P*=0.261), distinct clusters were found based on Jaccard distance (ANOSIM, NS: R=0.88, *P*<0.001; NPS: R value=0.70, *P*=0.001; BAL: R value=0.88, *P*=0.045) (Figure S10). In the validation trial, significant decreases for alpha diversity, including the Shannon index and the number of observed features, were observed in the NS microbiome of control $_{arrival}$ and Control calves (Figure S2). We also found significant shifts of the NS microbiome based on Bray-Curtis distance (ANOSIM, NS: R=0.54, *P*=0.001) and Jacccard distance (ANOSIM, NS: R=0.41, *P*=0.001) (Figure S10).

To define the longitudinal changes of the microbiota from control $_{arrival}$ to Control, random forest was performed. High AUCs were found and a set of microbial features were listed based on mean decrease accuracy (Figure 3). *Bifidobacterium pseudolongum* (F31) was a found as a shared feature to differentiate microbial dynamics in NS, NPS and BAL from healthy calves (Figure 3 C-D, Table S5). Its abundance in NS and NPS microbiomes decreased with animal time in feedlot, while it did not significantly change in BAL (Figure S11, S12, S13, and S14). Another bacterium (*Mycoplasma* (F7)) shared in NPS and BAL increased in Control compared with control $_{arrival}$ (Table S5, Figure S13, S14).

To validate temporal dynamics of the microbiome in healthy calves, we measured the NS microbiome in the validation trial, with 182 features yielding AUC of 0.992 (Figure 3B). Among top 30 features, nine of them were the same with the test trial, including F31, *Streptococcus luteciae* (F37), *Jeotgalicoccus psychrophilus* (F29, F221), *Corynebacterium* (F96), *Moraxellaceae* (F8), *Trichococcus* (F73), and *Veillonella* (F136) (Figure 3 C-D). Those shared bacteria decreased abundances in Control compared to healthy $_{arrival}$ (Figure S11, and S12). In

addition, we found features related with BRD pathogens, such as *Mycoplasma* (F3) and *Mannheimia* (F4), increased abundances with time in feedlot (Figure S12).

Longitudinal changes of bovine respiratory microbiota upon the onset of BRD

The next question is how the bovine respiratory microbiome changes before and upon the onset of BRD. To address this question, we analyzed the respiratory microbiome from 20 calves when they arrived at the feedlot ($BRD_{arrival}$) and when they were diagnosed with BRD (BRD_{onset}). There was a trending decrease in the Shannon index in the NS microbiome in these calves with the onset of BRD (p=0.062), and significant decrease of the number of observed features in BRD calves (p=0.024) (Figure S2 A-B). No significant changes in the alpha diversity of any NPS and BAL samples before and during the onset of BRD were observed (Figure S2 E-F, I-J). Significant changes in the microbial community membership and structure between $BRD_{arrival}$ and BRD were observed, as shown on the PCoA plots based on Bray-Curtis distance (ANOSIM, NS: R=0.36, P<0.001; NPS: R value=0.06, P=0.04; BAL: R value=0.08, P=0.02) and Jaccard distance (ANOSIM, NS: R=0.58, P<0.001; NPS: R value=0.19, P=0.04; BAL: R value=0.06, P=0.03) (Figure S15). We validated the changes of alpha and beta diversities in a second animal trial. The alpha diversities and richness decreased in the NS and BAL microbiome from BRD calves compared to the lung microbiome in $BRD_{arrival}$ (Figure S2). For beta diversity, significant changes between $BRD_{arrival}$ and BRD were also observed based on Bray-Curtis distance (ANOSIM, NS: R=0.47, P<0.001; NPS: R value=0.32, P=0.001; BAL: R value=0.32, P=0.001) and Jaccard distance (ANOSIM, NS: R=0.51, P<0.001; NPS: R value=0.48, P=0.001; BAL: R value=0.38, P=0.001) (Figure S15).

Random forest models were developed to identify bacterial features that changed significantly before ($BRD_{arrival}$) and upon the onset of BRD (BRD_{onset}) in NS, NPS, and BAL.

All three niches demonstrated high accuracy to identify the signature microbiota to differentiate these two healthy statuses (Figure S16). In the test trial, three features (*Mycoplasma* (F7), *Histophilus somni* (F11), *Corynebacterium* (F34)) were shared among the top 30 features of NS, NPS and BAL microbiomes (Figure 4 A, B, C). They showed consistent dynamics among the three niches upon the onset of BRD: F7 and F11 increased in all the three niches upon the onset of BRD (Figure 5 A, C), while F34 decreased in BRD for all three sites (Figure 5 E, Fig S17, S19, and S21). These AUCRF models accurately classified NS, NPS, and BAL samples in $BRD_{arrival}$ vs. BRD_{onset} (Figure S16). For the nasal microbiome, thirty-two microbiota were able to differentiate the d1 vs. BRD samples, including *Streptococcus luteciae* (F37), *Bifidobacterium pseudolongum* (F31), *Jeotgalicoccus psychrophilus* (F29), *Streptococcus* (F16), *Faecalibacterium prausnitzii* (F155), and *Facklamia* (F127, F368, F142) with an AUC of 0.989. These bacteria all decreased upon the appearance of BRD clinical symptoms (Figure 4 A, Figure 5 E-H, Figure S17). In the NPS microbiome, a total of 44 features were needed to obtain the greatest AUC of 0.998. Another *Mycoplasma* (F3) identified as a NPS signature increased significantly with BRD in all three niches (Figure 4 B, Figure 5 B, and Figure S19). We also observed F29 and F31 in NPS decreased in relative abundance with BRD appearance (Figure 4 B, Figure 5 F-G, and Figure S19). In the BAL microbiome, with the exception of shared F7 and F11 increasing in abundances during the onset of BRD, another BRD pathogen *Mannheimia* (F4) also increased upon BRD in the lung, and it consistently increased in NS and NPS (Figure 4 C; Figure 5 D; Figure S21). Moreover, F37, which decreased abundance with BRD_{onset}, was also identified as a BAL signature (Figure 4 C; Figure 5 H; Figure S21).

We validated the longitudinal dynamics of microbiota in the three niches of BRD calves, and found that the respiratory microbiota could distinguish $BRD_{arrival}$ and BRD_{onset} very

accurately (Figure S16 B). The signature microbiota including F7, F11, F34 were also classified as signatures (Figure 4 D, E, F), and showed similar changes with the test trial (Figure 5 A, C, E; Figure S18, S20, S22). Others common signature bacteria, such as F29, F31, F37, F4 and F3, were also decreased or increased abundance with BRD development, synonymous patterns with the test trial (Figure 5, Figure S18, S20, S22).

Bovine respiratory microbiome differentiating healthy calves from those with BRD

To determine if the bovine respiratory microbiome could accurately diagnose BRD, we compared NS, NPS, and BAL microbiomes from calves when they were diagnosed with BRD (BRD $_{onset}$) with those from their pen mates as healthy Controls without any visible BRD clinical signs (Control $_{onset}$). Significant differences in community membership between the asymptomatic healthy control and BRD calves were observed as shown by the PCoA plot based on Jaccard distance in NS or NPS, but not in BAL (ANOSIM, NS: R=0.23, *P*=0.001; NPS: R value=0.34, *P*=0.001; BAL: R value=-0.02, *P*=0.691, Figure S23). We did not detect any significant difference in community structure between BRD vs. Controls in any of the niches (ANOSIM, NS: R=0.07, *P*=0.033; NPS: R value=0.02, *P*=0.214; BAL: R value=-0.01, *P*=0.577) based on Bray-Curtis distances (Figure S23).

Microbial features differentiating BRD from controls in NS, NPS, and BAL were also identified by AUCRF (Table S6 and S7). The high AUCs of the random forest models generated based on the microbiome in the diagnosis of BRD were 0.927, 0.962, and 0.944 for NS, NPS, and BAL microbiomes, respectively (Figure S24). In the NS microbiome, the top 30 features included many GI-tract associated features such as *Faecalibacterium prausnitzii* (F327, F155), *Lactobacillus reuteri* (F102), *Lactobacillus* (F99), *Lachnospiraceae* (F275, F503) and *Prevotella copri* (F46) (Table S6). These features were more abundant in the healthy Control compared to

BRD calves (Figure S25). A feature related to BRD pathogen (*Mycoplasma* (F3)) was enriched in the NS microbiome BRD calves (Figure 6 B, Figure S25) and other niches (Figure 6 B). Interestingly, many of these NS signature were also among the top 30 features in the NPS, and the pattern of their abundances were opposite with NS (Table S7). For example, F46, F275, F99, F102 F155 and F327 had higher abundance in NPS of BRD calves than those in healthy Control calves (Figure S27). While *Mycoplasma* (F7) was enriched in the NPS microbiome from healthy calves, it had high abundance in the NS and lung microbiomes from BRD calves (Figure 6 C, Figure S27). In BAL samples, *Mycoplasma hyorhinis* (F1) was overrepresented in BRD calves, and other niches showed the same trend but no statistical significance (Figure 6 A, Figure S28).

In the validation trial, we classified NS signatures to differentiate healthy Control and BRD $_{onset}$ calves, with an AUC of 0.831 (Figure S24 B). Two shared features were found between validation and test animal trials (Table S6). Among top 30 features (Table S6), the important features including *Mycoplasma* (F7), *Mannheimia* (F4), *Jeotgalicoccus psychrophilus* (F29), and *Streptococcus* (F16) were identified. However, F4 and F7 had high relative abundance in NS Control than NS BRD, which showed different patterns compared with the test trial (Figure 6 C-D, Figure S26). Their abundances are shown in Figure S26.

Discussion

This is a comprehensive study to characterize the bovine respiratory microbiomes in three niches and their longitudinal and cross-sectional changes associated with BRD. Previous studies mainly focused on the pathogens in the upper bovine airway, especially the nasopharynx (Gaeta et al., 2017; Holman et al., 2017a; Holman et al., 2019; Holman et al., 2015b; Holman et al., 2017b; Lima et al., 2016; McDaneld et al., 2018, 2019; McMullen et al., 2019; Timsit et al., 2018; Timsit et al., 2016b; Timsit et al., 2017; Zeineldin et al., 2017). These recent studies

characterized either the upper or lower bovine airway microbiome with regard to BRD, which remarkably improved our understanding of the biogeography of the bovine respiratory microbiome. However, limited information regarding nasal and lung microbiomes affected by BRD has been reported. In addition, whether NS could be used to replace NPS or BAL as a rapid and non-invasive method to study the bovine respiratory microbiome is unknown. In this study, we demonstrated that microbiomes of all three niches of within the bovine airway (i.e. NS, NPS, and BAL) were associated with BRD, although different community structure for each niche was observed. Many signature microbiota for the onset of BRD or clinical signs of BRD were identified.

Prediction of the BRD using nasal swabs

Predicting the likelihood of BRD is important for animal health and management. We confirmed a high accuracy of the respiratory microbiome for this prediction (AUC: NS 0.99, NPS 0.96, BAL 0.94), which provides insight that using the NS microbiome to predict the onset of BRD is rational. The NS signature, *Mycoplasma hyorhinis* (F1), and *Streptococcus luteciae* (F37), consistently increased in abundances in the NS, NPS and BAL of BRD $_{arrival}$ calves compared to healthy $_{arrival}$ calves, which reflects the possibility of using the NS microbiome to predict BRD. *Streptococcus* is a common COPD lung pathogen and a cause of pneumonia (Pragman et al., 2018). *Mycoplasma hyorhinis*, considered as commensal microbiota of the upper airway, could cross-interact with other pathogens (e.g. virus or *Mycoplasma hyopneumoniae*) and increase the chances of swine pneumonia in several studies (Pereira et al., 2017; Roos et al., 2019). BRD $_{arrival}$ represents the pre-disease state, which is a partial loss of mucosal barrier function, bacterial dysbiosis, pathogen colonization on the epithelium, and activation of the immune response (Burns et al., 2016). The increases of potential pathogens in BRD $_{arrival}$ lungs

may reflect the decline of the pre-disease state. Thus, these two bacteria could serve as potential NS biomarkers to predict BRD.

Several features including *Lactobacillus reuter* (F102, F192) and *Lactobacillus* (F10) were overrepresented in the NS of calves that developed BRD. The *Lactobacillus* features that increased in the BRD $_{\text{arrival}}$ calves can prevent and treat chronic airway diseases through their capacity to modulate the epithelial barrier function and their mode of interaction with the immune system (Martens et al., 2018). A previous study reported that *Lactobacillus reuter* administration could attenuate the allergen-induced airway hyperresponsiveness and inflammation (Forsythe et al., 2007). We also identified other NS microbiome biomarkers for BRD including several GI-tract features such as *Prevotella copri* (F46), and *Prevotella stercorea* (F216), which were significantly more abundant in the healthy calves. Another gut feature, *Prevotella* (F65) identified as signature in the validation trial, was enriched in the NS of healthy calves. The proportions of *Prevotella*, commensal members in the upper respiratory tract, may alter the ecosystem of the infected host through their effects on the microbiota, mediated by their ability to adhere to various bacteria (Kim et al., 2018; Larsen, 2017). In addition, gut microbiota identified as signatures were not unlikely. A previous study found that rumen-associated bacteria were detected in lung fluids, indicating that rumen microbiota could be a source for the respiratory community (Glendinning et al., 2017). Therefore, the gut-airway axis in beef cattle may exist and, subsequently, may be influenced by disease.

Longitudinal dynamics of respiratory microbiome in the healthy calves

The healthy cattle in this study were exposed to the same management and environmental challenges compared to the BRD afflicted calves. The longitudinal dynamics of the respiratory microbiome in the healthy calves were anticipated to be a correction for the changes in

pathogens and commensal bacteria of BRD calves, allowing us to understand the BRD pathogenesis associated with the airway microbiome. In correlation with other studies on nasopharyngeal microbiota differentiation after feedlot entry (Holman et al., 2015b; Zeineldin et al., 2017), we found significant membership changes of nasal, nasopharyngeal and lung microbiome in the healthy calves from the test trial, and a vast decrease of diversity and richness of the nasal microbiome in the validation trial. The differences in results for alpha diversity changes in the test and validation trials could be caused by the uniqueness of each individual. In the test trial, healthy $_{arrival}$ samples were collected from absolutely healthy calves, while we collected samples from the same animals considered as healthy control at feedlot arrival and BRD onset in the validation trial. In future studies, the dynamics of the respiratory microbiome need to investigate the differences in absolutely and clinically healthy calves in feedlot.

Due to a high AUC of random forest models and many shared signature microbiota for the nasal microbiome in both the test and validation trials, the dynamics of the respiratory microbiome in healthy control calves could give possible implications, although healthy arrival and control were from different calves in the test trial. Secondly, decreases in these shared nasal signatures with increasing time in the feedlot provide evidence that the nasal microbiome community is easily influenced by the external environment (e.g. diet, living environment, comingling, etc.) (Holman et al., 2019; Zeineldin et al., 2019). In addition, after feedlot arrival, an equilibrium of the respiratory microbiome may be disrupted in the feedlot, leading to dysbiosis due to an increase of pathogens, regarding the significance of the temporal dynamics in healthy calves.

By performing random forest, the signature *Bifidobacterium pseudolongum* (F31) shared feature to differentiate the longitudinal dynamics in NS, NPS and BAL from healthy calves, and

its abundance in NS and NPS microbiome decreased with time in feedlot but no changes in BAL. *Bifidobacterium pseudolongum* is a commensal microorganism found in the gastrointestinal tract that can exert beneficial effects through various mechanisms including pathogen exclusion and immune modulation (Ruiz et al., 2016). A study reported that it could exert a protective effect against influenza infection in mice (Zhang et al., 2020). Therefore, decreases of *B. pseudolongum* in the upper airway communities of healthy calves from arrival to the feedlot to the onset of BRD could be generated by challenges or stresses from new management and environmental factors. Other commensal bacteria, including *Streptococcus luteciae* (F37), *Jeotgalicoccus psychrophilus* (F29) and *Corynebacterium* (F96), decreased abundances with time in feedlot, which also reflects that the respiratory microbiome in healthy calves changes to adapt the new environment. Another signature bacteria (*Mycoplasma* (F7)) related with BRD was increased in clinically healthy control calves than feedlot arrival, which indicates that those healthy control calves are not absolute health and it is one of the reasons for the difficulty in the identification of BRD pathogens. Overall, consistent changes of airway microbiome not only imply the importance of the upper airway microbiome, but also indicates that microbial symbiosis relates to health and disease.

Longitudinal dynamics of respiratory microbiome in the BRD calves

The microbiome heterogeneity over disease development aids in understanding BRD pathogenesis. In our study, significant longitudinal changes in the community structure and membership of the airway microbiome were found. It is not surprising that the impacts of the respiratory physiological environment from the latent period to BRD appearance resulted in dynamic microbial change. A previous study confirmed that nasopharyngeal microbial structure in the weeks following entry is associated with the development of BRD (Timsit et al., 2016a).

This study confirmed that two shared features (*Mycoplasma* (F7) and *Histophilus somni* (F11)) were among the top 30 features of NS, NPS and BAL microbiomes, and they showed consistent dynamics among the three niches upon the onset of BRD: F7 and F11 increased in all the three niches upon the onset of BRD. F7 is associated with *Mycoplasma bovis* based on NCBI BLAST. *Mycoplasma bovis* and *Histophilus somni* are common BRD pathogens (Holman et al., 2015b; Klima et al., 2014; Taylor et al., 2010). The consistent increases of these two bacteria upon the onset of BRD indicate not only their involvement in the pathobiology of this disease but also nasal swabbing could be a technology to reflect airway microbiome changes. Compared to longitudinal changes of these pathogens in healthy calves during the feedlot period, BRD onset showed more abundant pathogens in the lung community, which indicates that increasing pathogens could lead to dysbiosis and cause subsequent BRD infection.

Decreased abundances of microbiota associated with F31, *Streptococcus* (F37 and F16) *Corynebacterium* (F34) and *Facklamia* (F127, F368 and F142) were detected, especially in nasal swabs. *Corynebacterium* (*pseudodiphtheriticum* species), a normal bacteria of the upper respiratory tract, appears to have the ability to regulate the innate immune response and thus enhance resistance to bacterial and viral infections in mice (Kanmani et al., 2017). *Facklamia* was observed in nasopharyngeal swabs in healthy horses with higher relative abundance than in horses with respiratory disease (Bond et al., 2017). The reduction of these commensal residents indicated the airway microbiome dysbiosis, which provided an opportunity/environment for the pathogens to invade and proliferate in the lung. Furthermore, compared to microbial dynamics in healthy control calves from arrival in the feedlot to BRD onset, more commensal microbiota in BRD calves decreased abundances in this study. It is necessary to investigate the differences of airway microbiota in between BRD and clinically healthy calves.

Respiratory microbiome associated with BRD diagnosis

The differences in respiratory microbiome structure between healthy calves and those with BRD have been controversial. No apparent separation of community structure between BRD and Control in NS and BAL was observed in our study, consistent with a report where no correlation between clinical symptoms and microbial composition was detected in NS and trans-tracheal aspiration samples (Nicola et al., 2017). Several studies showed that BRD calves had distinct membership of the respiratory microbiome as compared with healthy subjects (Holman et al., 2017b; McDaneld et al., 2018; Zeineldin et al., 2017), and we also found significant differences in membership of the upper airway community between the asymptomatic healthy control and BRD calves.

The microbial biomarkers for BRD diagnosis classified by AUCRF included several GI-tract bacteria associated with *Lactobacillus, Faecalibacterium prausnitzii* and *Prevotella copri*, which were more abundant in healthy calves. *Lactobacillus* is a lactic acid-producing bacterium. In a murine model of asthma, administration of *Lactobacillus* inhibits airway disease (Feleszko et al., 2007). In cattle, *Lactobacillus* strains are shown to adhere to respiratory epithelial cells and suppress the BRD pathogen *Mannheimia haemolytica* (Amat et al., 2017). *Faecalibacterium* can generate short-chain fatty acids that play an important role in asthma protection (Peccia et al., 2016). When mice with airway disease were challenged, those whose guts were enriched with *Faecalibacterium* after fecal matter transplantation were associated with asthma protection, with significant reductions in airway inflammation (Arrieta et al., 2015). *F. prausnitzii* could induce a tolerogenic cytokine profile to play its anti-inflammatory charterers (Lopez-Siles et al., 2017). Therefore, we hypothesized that the enriched members of gut microbiota in the healthy-control group could protect microbial dysbiosis and the calves from the onset of BRD.

We also observed BRD pathogens were significantly abundant in the lung microbiome of BRD calves, such as *Mycoplasma* (F7, *M. bovis*), and some species with *Mycoplasma* (F1 and F3; *M. dispar* and *M. bovrhinis*) showed high abundances in BRD calves without statistical significances. *Mycoplasma spp.* have caused pneumonia in calves when introduced either into the lower airway or via nasal inoculation, which could explain similar changes of NS and BAL in our study. In general, *Mycoplasma* donate their pathogenicity through inhibition of the mucocilliary transport mechanism, causing some degree of humoral and cell-mediated immunosuppression in calves, and attaching to ciliated epithelium above the level of alveolar macrophages to avoid phagocytosis (Maunsell et al., 2009). A previous study indicated that *M. bovis* inoculation caused lung lesion and BRD clinical signs including abnormal respiration and depression (Godinho et al., 2005). Another pathogen *Histophilus somni* (F11) showed high frequency and abundance in the respiratory tract of BRD calves, especially in the lung, although it was not identified as a signature for diagnosis. *H. somni* is an opportunistic pathogen responsible for respiratory disease since it can secret immunoglobulin-binding protein A which causes dramatic retraction and rounding of bovine alveolar type 2 (BAT2) epithelial cells (Zekarias et al., 2010). *Mannheimia* (F4) had similar pattern with F11 in this study. *M. haemolytica* could defend against phagocytosis, produce exotoxin lethal, colonize in the upper respiratory tract and then convert/or overgrow under stimuli to a pathogenic serotype (A1) with stronger virulence (Rice et al., 2007). Therefore, these bacterial pathogens may work solely or in conjunction with one another to develop BRD. The opposite pattern of BRD pathogens (e.g. *Mycoplasma*) in NPS, could give insight that microbial movement within the respiratory tract is related with BRD status. Collectively, those microbial biomarkers identified by Random Forest could be used to diagnose BRD.

The limitations and strengths

A limitation of this study may be the absence of sampling healthy controls at arrival in the test trial. Even though nine absolutely healthy calves were sampled at arrival, the individual differences may cause microbiome variation. Additionally, samples were not collected from nine healthy calves on the day of BRD diagnosis in the other calves. Despite the limitations of our study, the niche-specific signatures could be used to predict and diagnose BRD, and shared signatures between the test and validation trials confirmed the accuracy and repeatability of our study. Moreover, the calves in our study were not exposed with BRD vaccinations when they arrived to the feedlot, limiting our knowledge of how antimicrobials affect the respiratory microbiome.

Several strengths supported our study methodology. The validation of a second animal trial provides further insights that the longitudinal and cross-sectional design could be useful to determine the bovine respiratory microbiome and identify BRD pathogens. Through random forest machine learning algorithm, consistent changes of the microbiome in the upper and lower respiratory tract provide the possibility to using an effective approach (nasal microbiome) to study bovine respiratory disease. Invasive sampling (i.e., BAL) for respiratory disease research is not effective and may have unintended consequences; however, we confirmed that using nasal swabs is an accurate, less invasive approach. Finally, the Deblur program was used to handle reads at the single nucleotide levels, allowing for a more in-depth and accurate analysis of the different niches of microbiota comparing to the standard methods of binning sequences into operational taxonomic units by 97% similarity.

Conclusion

In summary, it is our assertion that the spatial composition and temporal and dynamics of the bovine respiratory microbiome are associated with BRD. Our data shows that when there is an increase in the presence of pathogens (e.g. *Mycoplasma* (F7) and *Histophilus somni* (F11)) leading to BRD, there is, at the same time, a decrease in the presence of beneficial probiotic or commensal bacteria. Compared to clinically healthy calves, significantly higher abundances in pathogens lead to dysbiosis of the respiratory system and produce lung infections of BRD. Across three sampling niches, high accuracy of prediction model and shared signatures with consistent changes provide insight that the nasal microbiome is able to predict and diagnose BRD. Nasal swabs may be used in future research as a non-invasive, more effective technology for studying bovine respiratory disease, and the statistical models (AUCRF) are useful tools for disease signature recognition.

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Tables and Figures

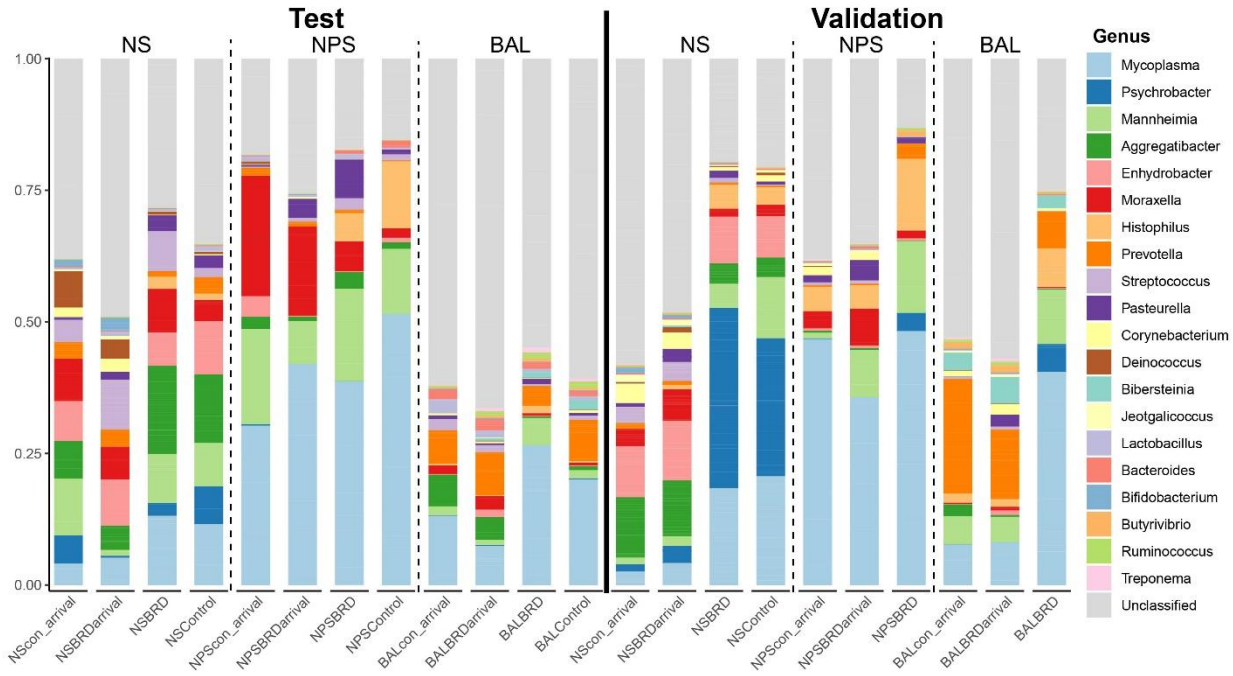

Figure 1 Microbial composition at genus level
Each bar shows the relative abundance of the average sample collected at different airway niches of the healthy statuses
Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot; Control: clinically healthy cattle samples were collected samples as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

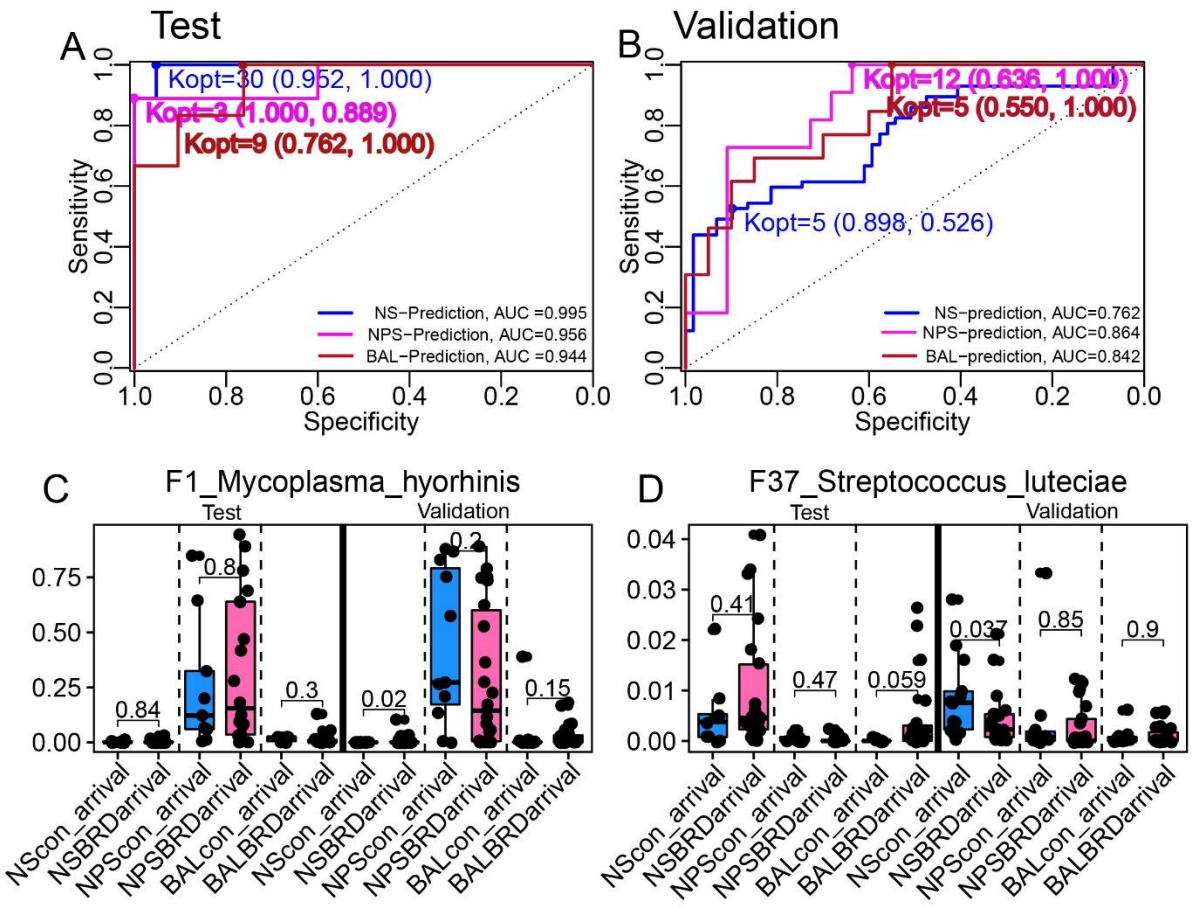

Figure 2 Bovine respiratory microbiome signatures predicting the onset of BRD
(A-B) The area-under-the ROC curve (AUC) of the Random Forest (AUCRF) for healthy control $_{arrival}$ and BRD $_{arrival}$ using NS (blue lines), NPS (hot pink lines), and BAL (red lines) microbiomes, respectively, in the test and validation trials. Kopt represents the number of optimal predictors obtained based on the area-under-the ROC curve (AUC) of the Random Forest (AUCRF). The numbers within parentheses behind Kopt are (specificity, sensitivity). (C and D) Representative features in NS, NPS, and BAL for predicting the onset of BRD. The numbers over the bars are p values calculated by the Wilcoxon test.
Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot.

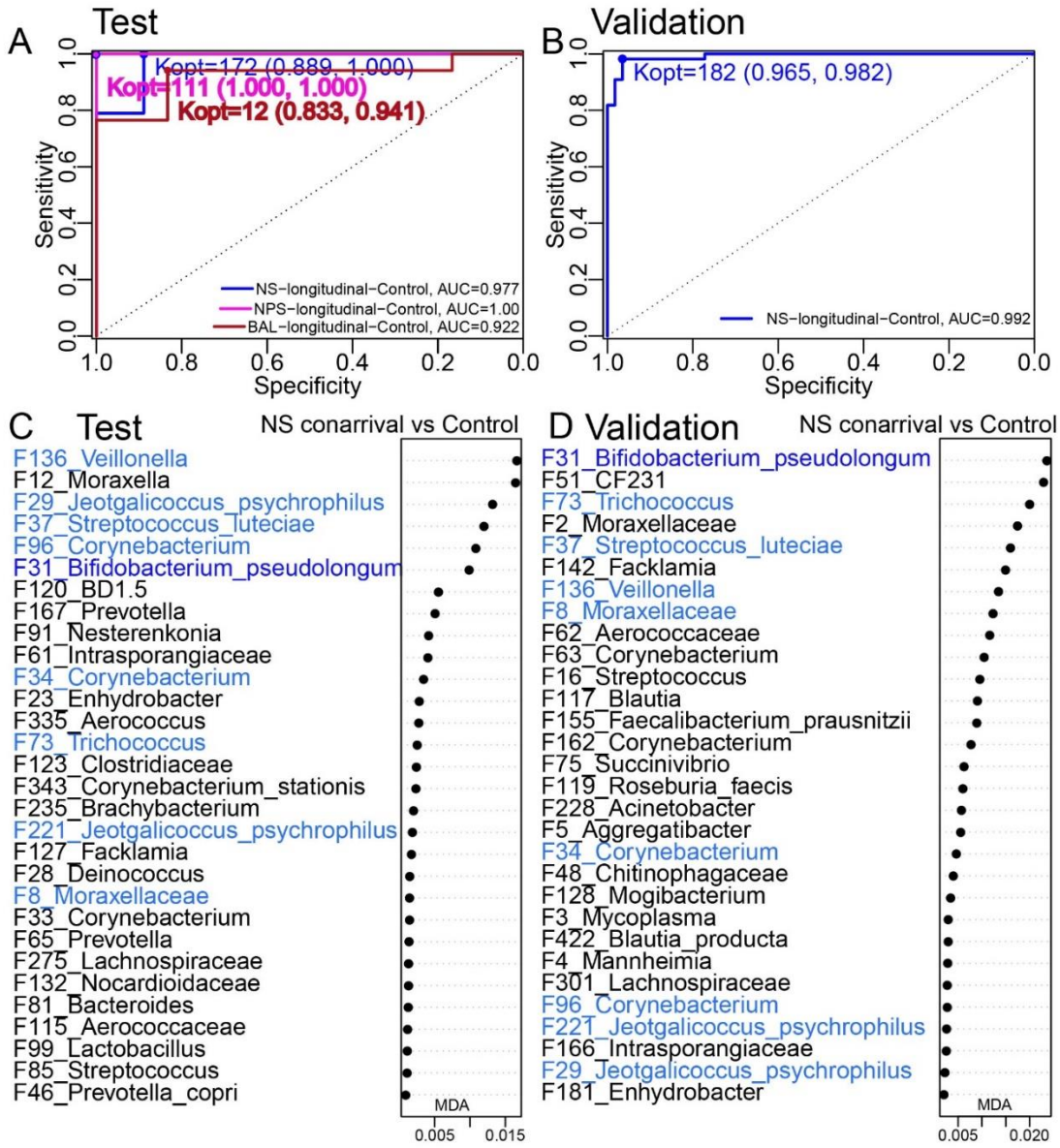

Figure 3 Longitudinal changes of the bovine nasal microbiome in healthy animals from control $_{arrival}$ to Control

(A-B) The area-under-the ROC curve (AUC) of the Random Forest (AUCRF) for healthy control $_{arrival}$ and Control using NS (blue lines), NPS (hot pink lines), and BAL (red lines) microbiome respectively in the test and validation trials. Kopt represents the number of optimal predictors obtained based on the area-under-the ROC curve (AUC) of the Random Forest (AUCRF). The numbers within parentheses behind Kopt are (specificity, sensitivity). (C and D) Top 30 features classified by AUCRF to show the longitudinal microbiome changes of control $_{arrival}$ and Control in NS NPS and BAL. Features are listed based on importance score (Mean Decrease Accuracy, MDA), and features with blue color are shared features among the test and validation trials.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); Control: clinically healthy cattle samples were collected samples as controls for BRD cattle.

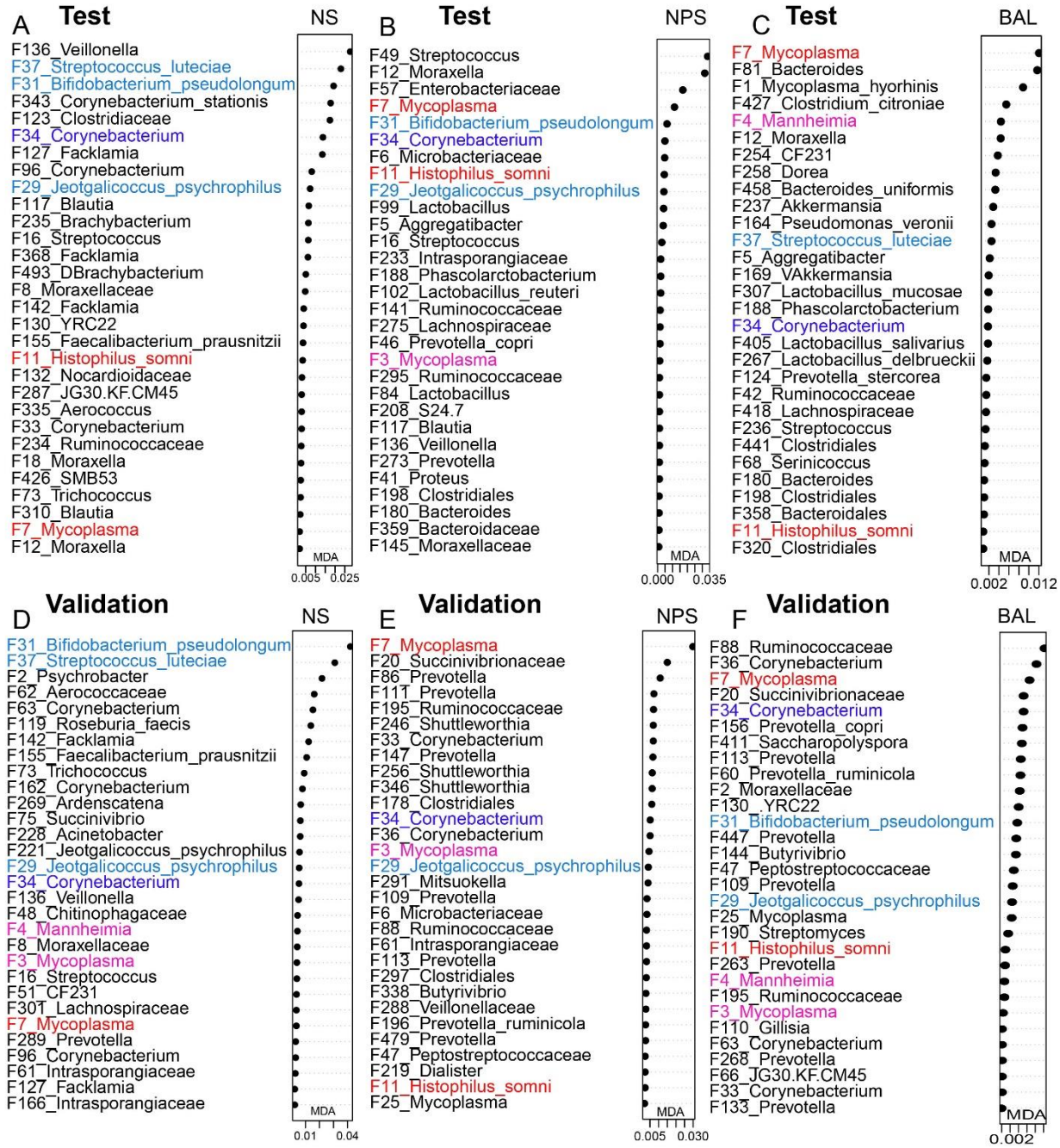

Figure 4 Longitudinal changes of the bovine respiratory microbiome from BRD $_{arrival}$ to BRD Top 30 features classified by AUCRF to show the longitudinal microbiome change of feedlot arrival and BRD in NS NPS and BAL in the test (A, B and C) and validation (D, E, F) trials. Features are listed based on importance score (Mean Decrease Accuracy, MDA), and features with red and blue colors are shared Features among these three niches. While red color represents relative abundances of features increased with BRD development, blue color means features decreased abundances with BRD development.
Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL).

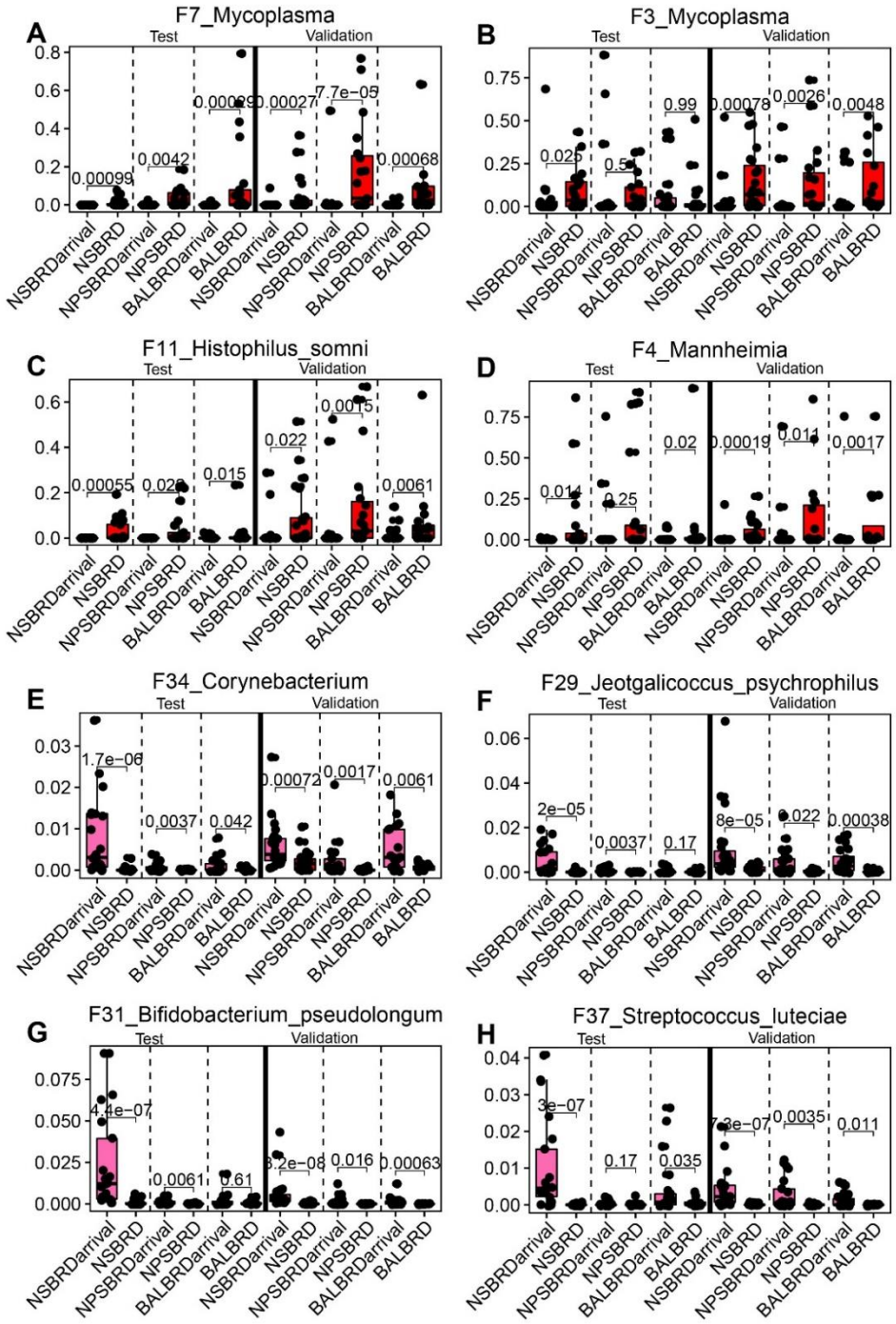

Figure 5 The signature microbiome to differentiate BRD $_{arrival}$ and BRD

(A-G) Representative features in NS, NPS, and BAL for longitudinal changes of BRD $_{arrival}$ and BRD calves from the random forest. The dark horizontal bar represents the median value in each group, while the boxes represent the 25th and 75th percentile values. The numbers over the bars are p values calculated by the Wilcoxon test.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot; BRD: cattle were diagnosed with BRD.

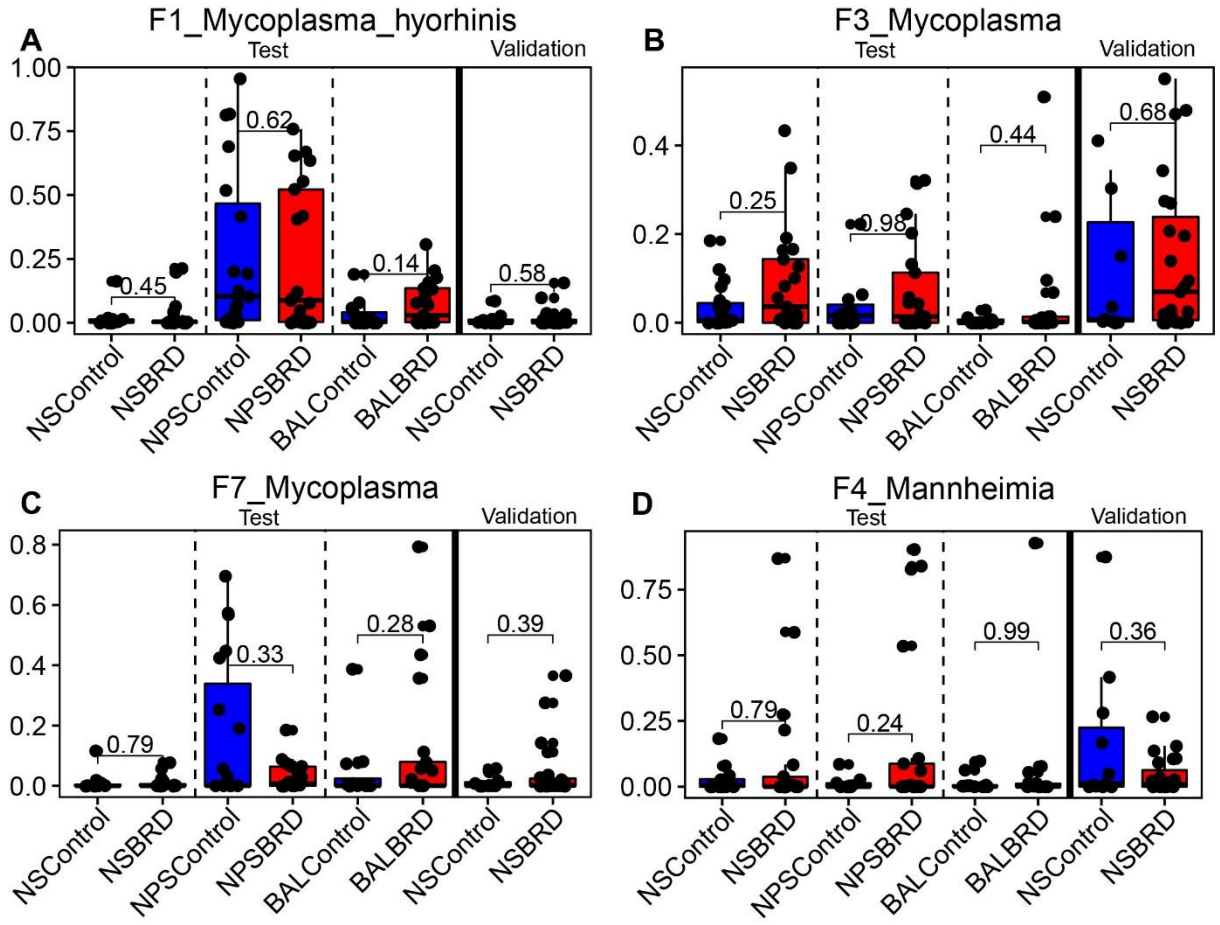

Figure 6 Biomarkers of the bovine respiratory microbiome for diagnosis of healthy Control and BRD calves

(A, B, C, and D) Representative features in NS, NPS, and BAL for diagnosis of healthy control and BRD calves from random forest. The dark horizontal bar represents the median value in each group, while the boxes represent the 25th and 75th percentile values. The numbers over the bars are p values calculated by the Wilcoxon test.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). Control: clinically healthy cattle samples were collected samples as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

Supplemental materials

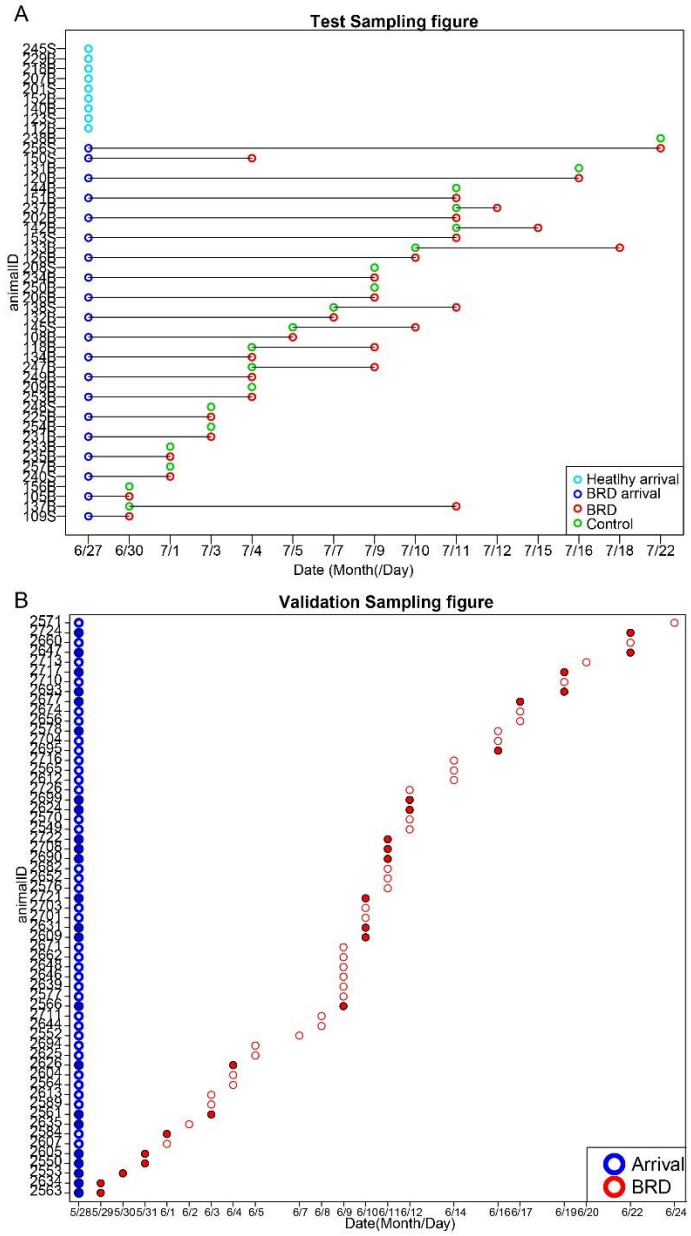

Figure S1 Sampling figure

A and B showed sampling in the test and validation trials, respectively. Calves were monitored for clinical respiratory disease every day until day 30 after arrival at the feedlot. On day 1, nasal, nasopharyngeal and BAL samples were sampled from nine healthy cattle (control $_{arrival}$) that were healthy over the study period and 20 calves (BRD $_{arrival}$) that developed BRD at different time points. These samples were used to identify biomarkers that predict the onset of BRD. The same set of samples were also collected from these 20 calves upon the onset of BRD (BRD $_{onset}$) and their respective healthy pen mates as healthy Controls (Control $_{onset}$). In the second animal trial, we collected samples from same morbid calves at feedlot arrival (Arrival, BRD $_{arrival}$) and the onset of BRD, while healthy calves followed same sampling pattern with morbid calves.

Figure S1 (Cont.)
Health $_{arrival}$: day 1 samples collected from healthy-control $_{arrival}$ (cyan); BRD $_{arrival}$: day 1 samples collected from BRD $_{arrival}$ (blue) calves; BRD: samples were collected when calves were diagnosed with BRD (red); Control: healthy control animals penned with BRD calves sampled simultaneously representing healthy controls (green). Each point represents one animal. Connected points represent the same animal at 2 different time points. Animal ID: S: steer; B: bull.

Table S1 Clinical Illness Score for BRD case definition

Clinical Illness Score	Description	Appearance
0	Normal	Normal
1	Slightly ill	Gaunt, nasal/ocular discharge
2	Moderately ill	Gaunt, nasal/ocular discharge, lags behind other animals in the group, cough, labored breathing
3	Severely ill	Purulent nasal/ocular discharge, labored breathing, not responsive to human approach
4	Moribund	Near death

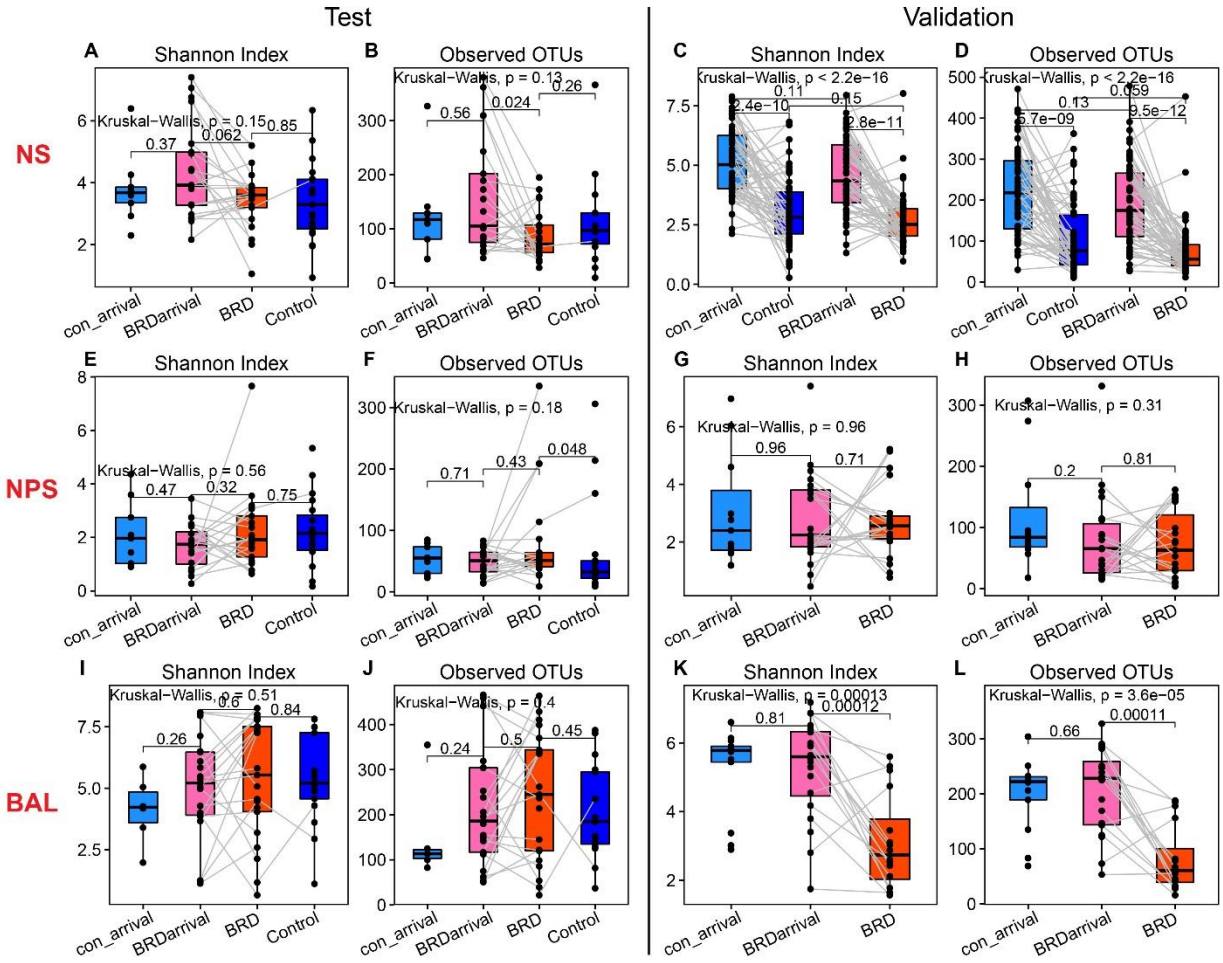

Figure S2 Alpha diversity of respiratory microbiome in different healthy status
(A-B) Shannon Index and the number of observed features of NS, NPS and BAL under 4 healthy status (health control $_{arrival}$ (con_arrival), BRD $_{arrival}$, health Control $_{onset}$ (Control) and BRD $_{onset}$ (BRD)). The number over bars are p values received from Wilcoxon test. Connected points represent different sampling techniques from the same animal. In plots of alpha diversities, the number over bars are p values received from Wilcoxon test. Connected points represent different sampling techniques from the same animal.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot; Control: clinically healthy cattle samples were collected samples as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

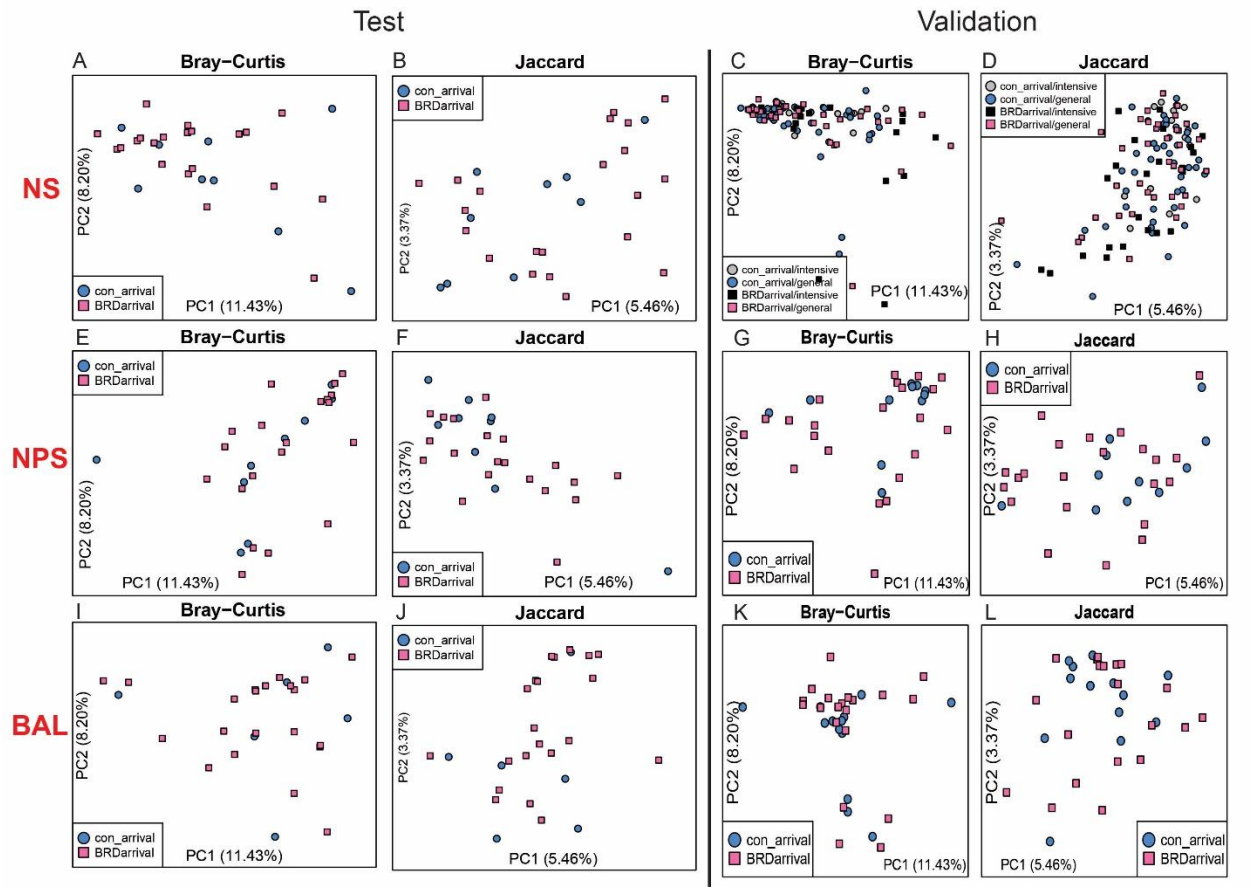

Figure S3 Beta diversity of comparison of BRD $_{arrival}$ and control $_{arrival}$ in NS, NPS and BAL

Principal Coordinates Analysis (PCoA) plot showed the beta diversity of BRD $_{arrival}$ and control $_{arrival}$ based on Jaccard and Bray-Curtis distance. Each point represents 1 sample with control $_{arrival}$ as a blue circle and BRD $_{arrival}$ as a hot pink square

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot.

Table S2 Nasal signatures (NS) to predict the onset of BRD based on Mean Decrease Accuracy (MDA) in the test and validation trials

Test trial	MDA	Validation trial	MDA
F123_Clostridiaceae	11.9	F295_Ruminococcaceae	4.00
F10_Enhydrobacter	9.94	F392_Prevotella	3.61
F167_Prevotella	9.92	F28_Deinococcus	3.54
F46_Prevotella_copri	6.08	F39_Ruminococcaceae	2.83
F23_Enhydrobacter	5.94	F410_Arthrobacter	1.72
F239_Gemmiger_formicilis	3.14	F311_Treponema	1.40
F18_Moraxella	2.89	F5_Aggregatibacter	1.29
F216_Prevotella_stercorea	2.71	F51_CF231	1.27
F460_Roseburia_faecis	2.38	F75_Succinivibrio	0.972
F19_Neisseriaceae	2.13	F73_Trichococcus	0.965
F81_Bacteroides	1.51	F232_Paracoccus	0.965
F234_Ruminococcaceae	1.24	F110_Gillisia	0.956
F53_Megasphaera	1.08	F157_Intrasporangiaceae	0.851
F422_Blautia_producta	0.975	F137_Alysiella	0.823
F102_Lactobacillus_reuteri	0.971	F270_Ruminococcaceae	0.755
F117_Blautia	0.944	F121_JG30.KF.CM45	0.745
F498_Dorea	0.936	F257_Psychrobacter_pulmonis	0.646
F299_Mitsuokella	0.912	F264_Proteiniclasticum	0.639
F166_Intrasporangiaceae	0.880	F117_Blautia	0.609
F142_Facklamia	0.859	F65_Prevotella	0.607
F26_Chitinophagaceae	0.820	F500_Pseudomonas	0.597
F15_Moraxellaceae	0.803	F128_Mogibacterium	0.571
F192_Lactobacillus_reuteri	0.788	F353_Planomicrobium	0.555
F232_Paracoccus	0.653	F310_Blautia	0.543
F99_Lactobacillus	0.642	F136_Veillonella	0.479
F339_Bacteroidales	0.511	F53_Megasphaera	0.469
F465_Blautia	0.507	F294_CF231	0.466
F423_Prevotella	0.472	F422_Blautia_producta	0.456
F39_Ruminococcaceae	0.460	F62_Aerococcaceae	0.443
F29_Jeotgalicoccus_psychrophilus	0.421	F252_Corynebacterium	0.435

Table S3 Nasopharyngeal (NPS) signatures to predict the onset of BRD based on Mean Decrease Accuracy (MDA) in the test and validation trials

Test trial	MDA	Validation trial	MDA
F49_Streptococcus	14.8	F66_JG30.KF.CM45	7.02
F327_Faecalibacterium_prausnitzii	11.6	F51_CF231	4.98
F46_Prevotella_copri	9.49	F449_Aerococcaceae	4.65
F57_Enterobacteriaceae	6.10	F75_Succinivibrio	4.52
F239_Gemmiger_formicilis	4.66	F166_Intrasporangiaceae	4.16
F84_Lactobacillus	3.70	F301_Lachnospiraceae	3.61
F216_Prevotella_stercorea	3.34	F353_Planomicrobium	3.04
F23_Enhydrobacter	2.96	F64_JG30.KF.CM45	2.99
F102_Lactobacillus_reuteri	1.52	F157_Intrasporangiaceae	2.76
F117_Blautia	1.45	F79_Dietzia	2.63
F28_Deinococcus	1.14	F110_Gillisia	2.45
F89_Prevotella_copri	1.02	F305_Ruminococcaceae	2.04
F426_SMB53	1.01	F326_Actinomycetales	2.03
F108_Lactobacillales	0.995	F242_Facklamia	1.93
F19_Neisseriaceae	0.942	F6_Microbacteriaceae	1.82
F21_Moraxellaceae	0.818	F207_Planomicrobium	1.71
F126_Pasteurellaceae	0.752	F91_Nesterenkonia	1.63
F462_Prevotella	0.666	F230_5.7N15	1.57
F78_Weeksellaceae	0.660	F192_Lactobacillus_reuteri	1.49
F65_Prevotella	0.660	F464_JG30.KF.CM45	1.37
F26_Chitinophagaceae	0.590	F71_Bacillales	1.21
F442_Blautia_obeum	0.551	F430_Chryseobacterium	1.18
F498_Dorea	0.437	F276_Mogibacterium	1.08
F56_Ureaplasma	0.222	F72_Planomicrobium	1.05
F99_Lactobacillus	0.217	F257_Psychrobacter_pulmonis	0.992
F64_JG30.KF.CM45	0.205	F264_Proteiniclasticum	0.947
F29_Jeotgalicoccus_psychrophilus	0.204	F471_Ruminococcaceae	0.910
F448_Prevotella	0.198	F222_Oscillospira	0.814
F123_Clostridiaceae	0.168	F94_Dietzia	0.757
F335_Aerococcus	0.119	F142_Facklamia	0.744

Table S4 BAL signatures to predict the onset of BRD based on Mean Decrease Accuracy (MDA) in the test and validation trials. Features labeled with red color represent shared signatures between the test and validation trials.

Test trial	MDA	Validation trial	MDA
F1_Mycoplasma_hyorhinis	3.35	F185_Veillonellaceae	10.4
F43_Chitinophagaceae	2.51	F175_Prevotella	9.23
F112_Enterobacteriaceae	1.58	F178_Clostridiales	8.47
F79_Dietzia	1.52	F147_Prevotella	7.25
F104_Enterobacteriaceae	0.90	F55_Prevotella	7.04
F99_Lactobacillus	0.75	F196_Prevotella_ruminicola	5.94
F273_Prevotella	0.75	F240_Clostridiales	5.68
F231_Collinsella_aerofaciens	0.53	F65_Prevotella	4.56
F37_Streptococcus_luteciae	0.49	F60_Prevotella_ruminicola	4.08
F100_Listeria	0.48	F133_Prevotella	3.99
F153_Succiniclasticum	0.48	F20_Succinivibrionaceae	3.83
F17_Mannheimia	0.42	F390_Prevotella	3.61
F385_Clostridiales	0.37	F347_CF231	3.46
F148_Oscillospira	0.35	F429_Prevotella	2.83
F128_Mogibacterium	0.35	F179_Prevotella	2.80
F152_Peptostreptococcaceae	0.31	F332_YRC22	2.79
F23_Enhydrobacter	0.30	F113_Prevotella	2.60
F336_Christensenellaceae	0.28	F450_Prevotella	2.17
F283_Bacteroidales	0.23	F246_Shuttleworthia	2.14
F102_Lactobacillus_reuteri	0.22	F85_Streptococcus	2.11
F328_Ruminococcus	0.21	F111_Prevotella	1.58
F60_Prevotella_ruminicola	0.18	F75_Succinivibrio	1.22
F67_Staphylococcus	0.16	F293_Prevotella	1.18
F170_Prevotella	0.16	F251_Prevotella	1.11
F201_Turicibacter	0.14	F460_Roseburia_faecis	1.06
F199_Streptococcus_agalactiae	0.12	F1_Mycoplasma_hyorhinis	0.993
F164_Pseudomonas_veronii	0.11	F195_Ruminococcaceae	0.953
F198_Clostridiales	0.11	F88_Ruminococcaceae	0.937
F250_Oscillospira	0.11	F47_Peptostreptococcaceae	0.923
F236_Streptococcus	0.11	F68_Serinicoccus	0.859

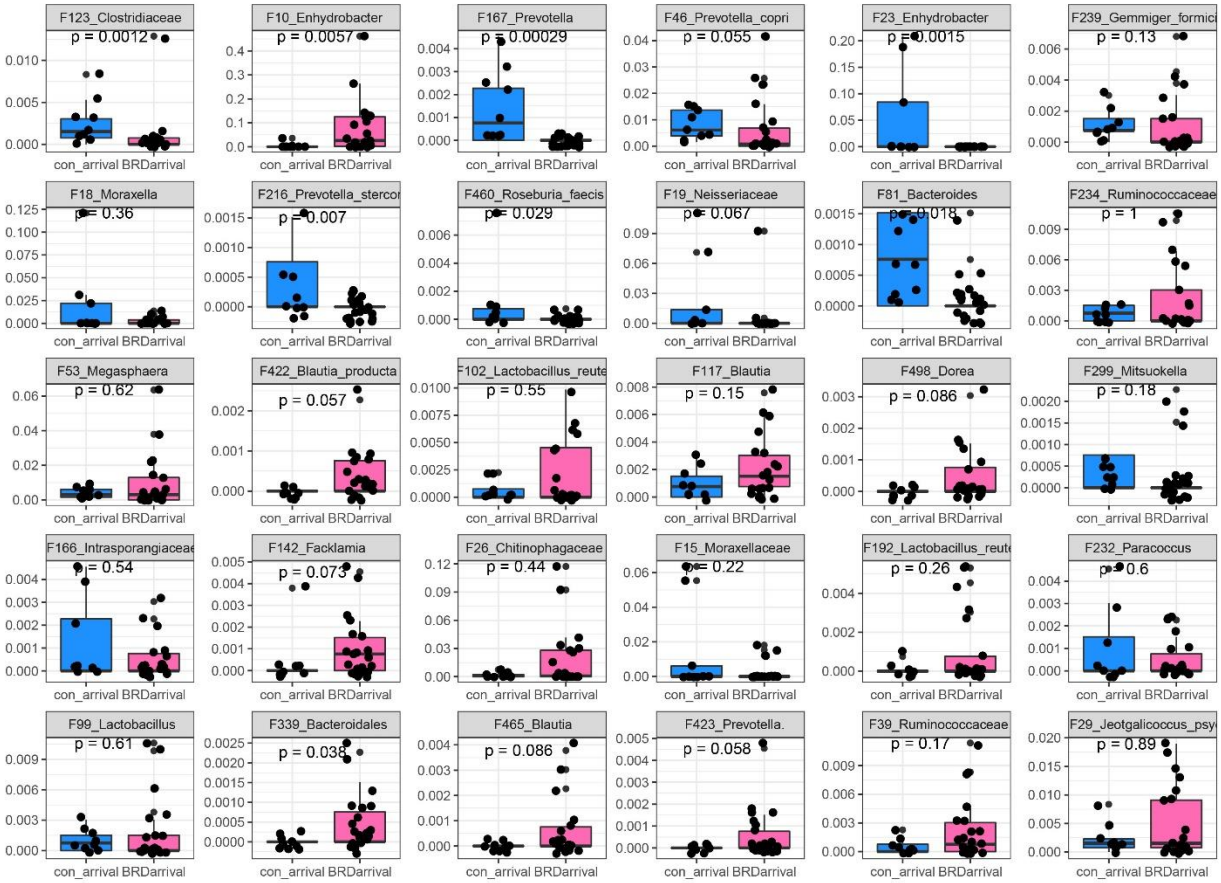

Figure S4 NS microbiome to differentiate healthy control $_{arrival}$ and BRD $_{arrival}$ in the test trial

The NS signatures obtained from the AUCRF algorithm differentiate healthy control $_{arrival}$ and BRD $_{arrival}$. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; Nasal swabs (NS); control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot.

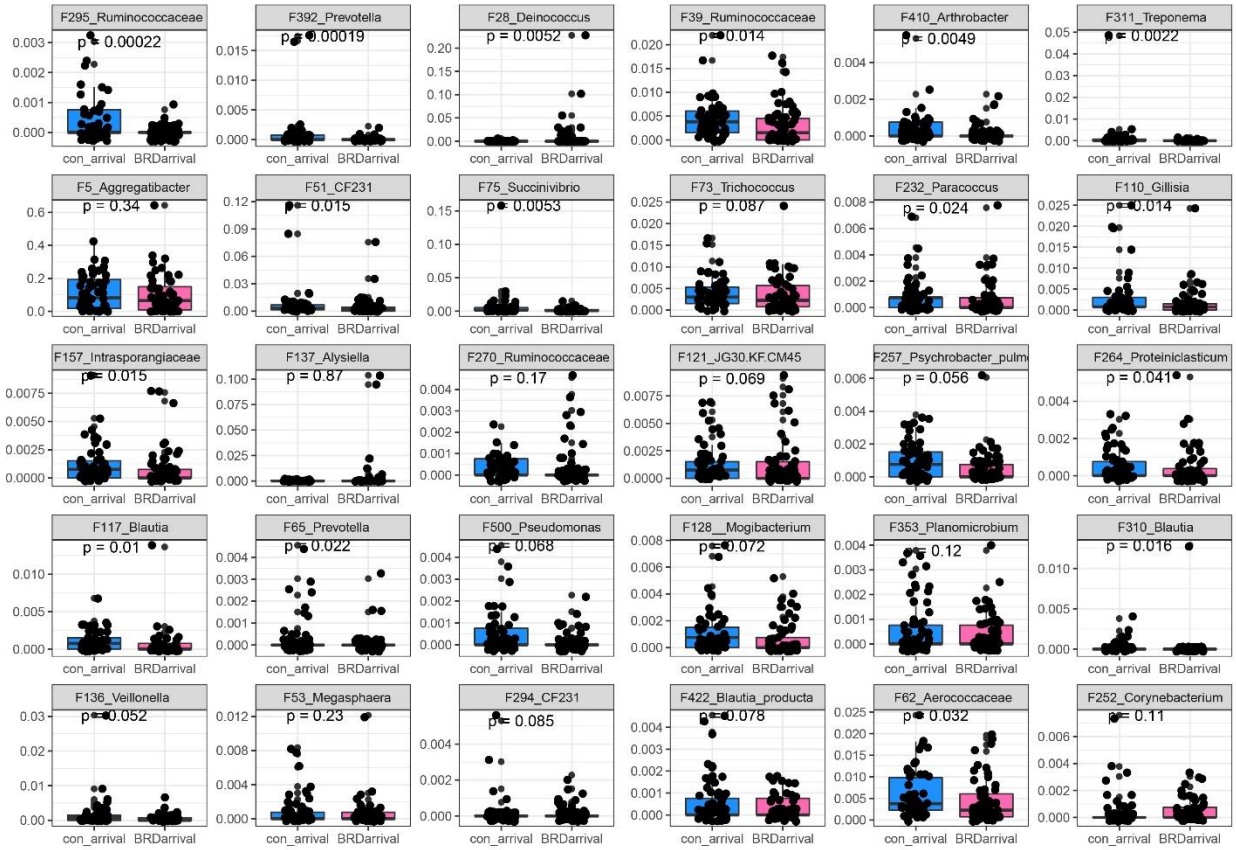

Figure S5 NS microbiome to differentiate the healthy control $_{arrival}$ and BRD $_{arrival}$ in the validation trial

The NS signatures obtained from the AUCRF algorithm differentiate healthy control $_{arrival}$ and BRD $_{arrival}$. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; Nasal swabs (NS); control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot.

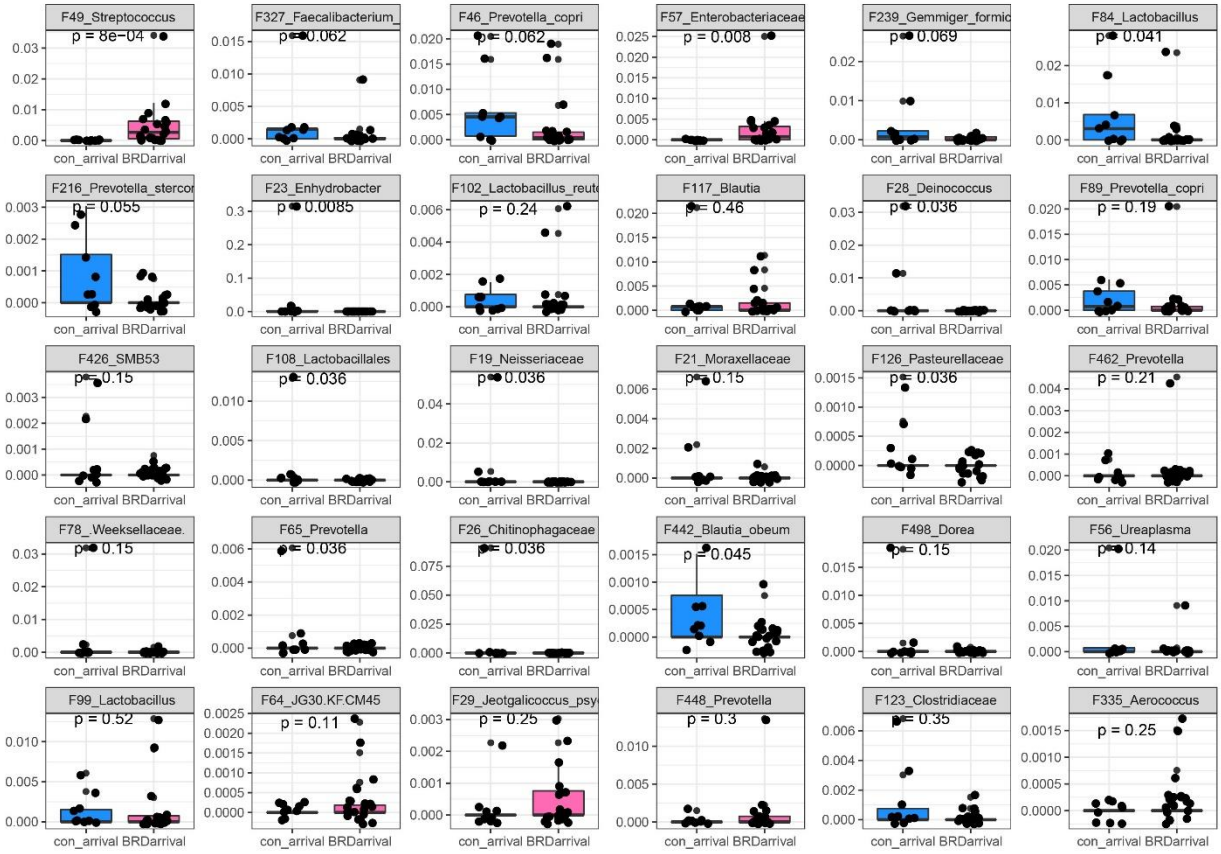

Figure S6 NPS microbiome to differentiate the healthy control $_{arrival}$ and BRD $_{arrival}$ in the test trial

The NPS signatures obtained from the AUCRF algorithm differentiate healthy control $_{arrival}$ and BRD $_{arrival}$. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; nasopharyngeal swab (NPS); control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot.

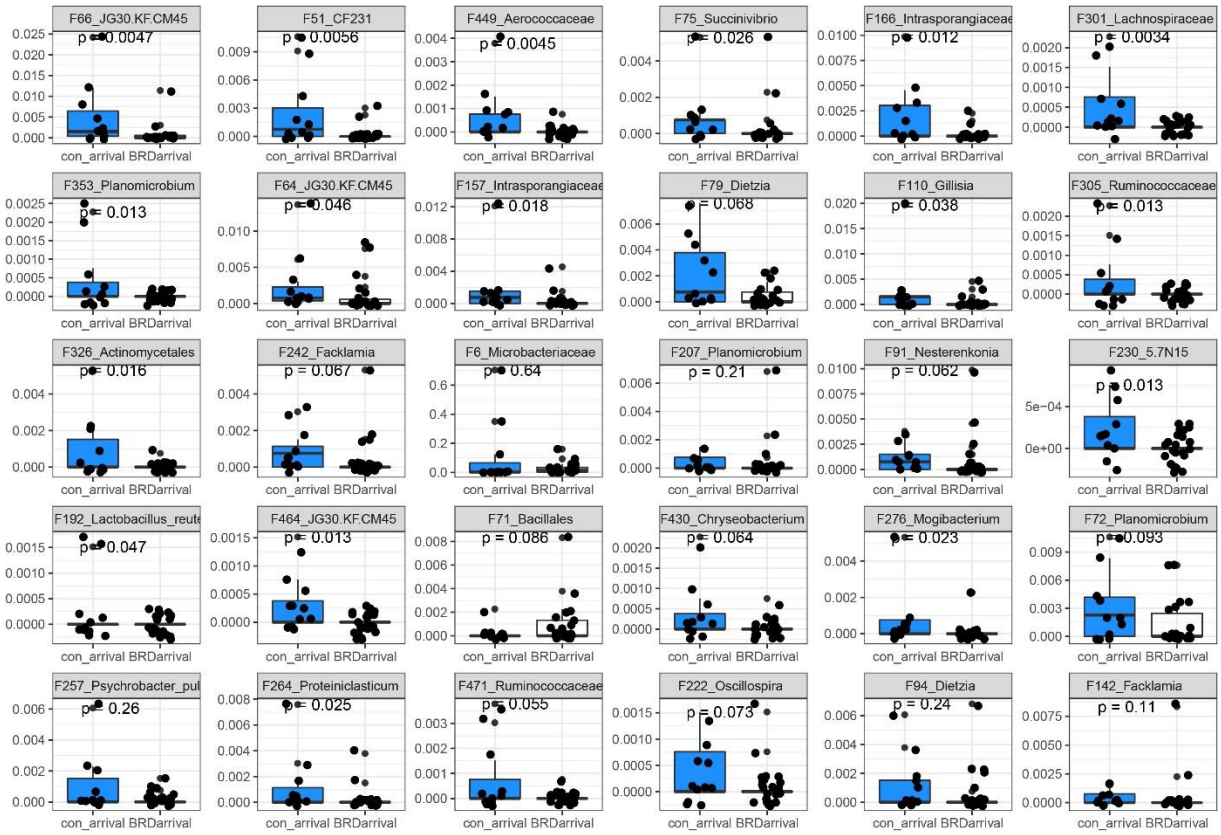

Figure S7 NPS microbiome to differentiate the healthy control $_{arrival}$ and BRD $_{arrival}$ in the validation trial

The NPS signatures obtained from the AUCRF algorithm differentiate healthy control $_{arrival}$ and BRD $_{arrival}$. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; nasopharyngeal swab (NPS); control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot.

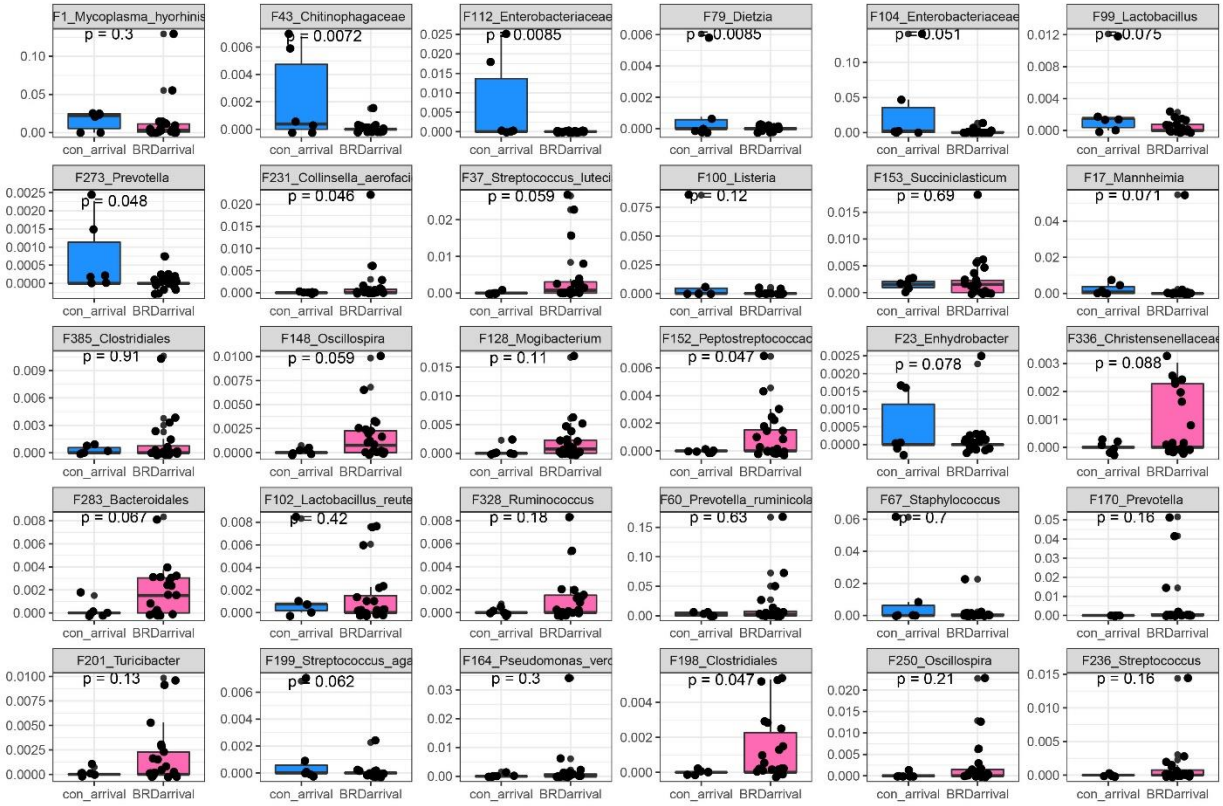

Figure S8 BAL microbiome to differentiate the healthy control $_{arrival}$ and BRD $_{arrival}$ in the test trial

The BAL signatures obtained from the AUCRF algorithm differentiate healthy control $_{arrival}$ and BRD $_{arrival}$. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; bronchoalveolar lavage (BAL); control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot.

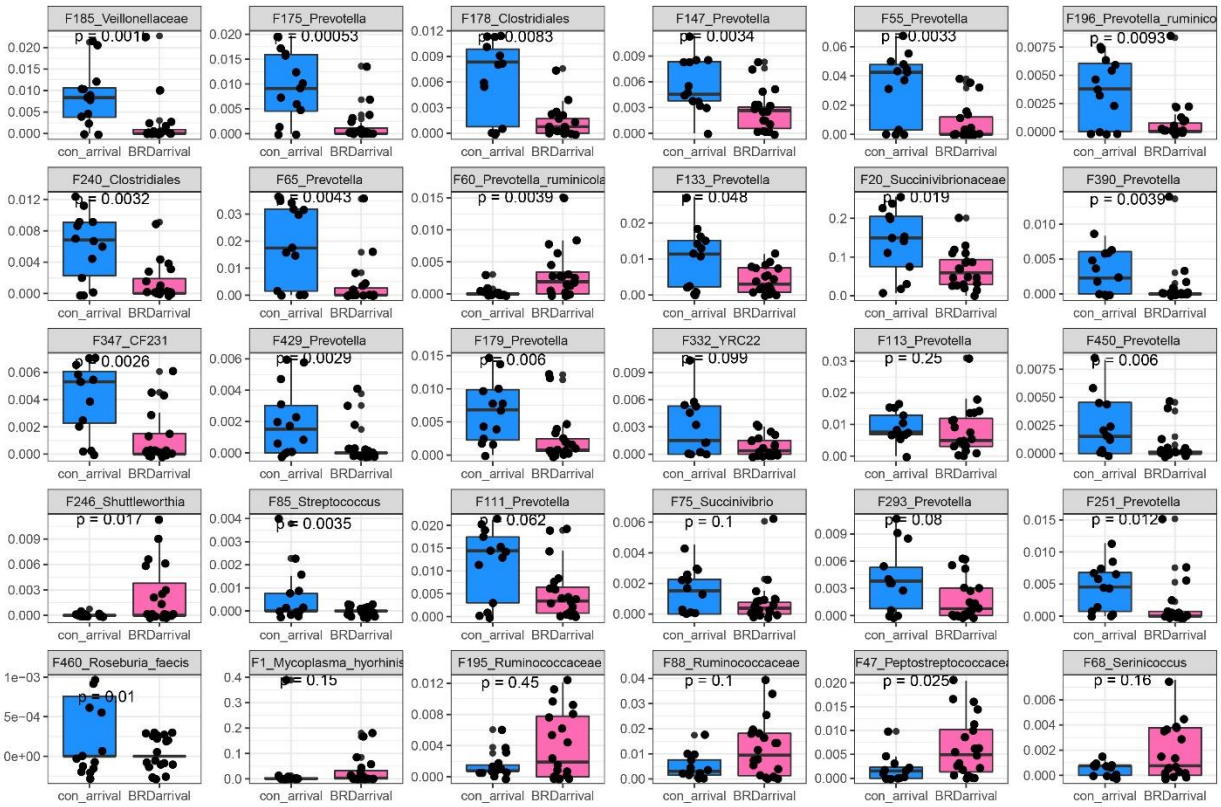

Figure S9 BAL microbiome to differentiate the healthy control $_{arrival}$ and BRD $_{arrival}$ in the test trial

The BAL signatures obtained from the AUCRF algorithm differentiate healthy control $_{arrival}$ and BRD $_{arrival}$. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; bronchoalveolar lavage (BAL); control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot.

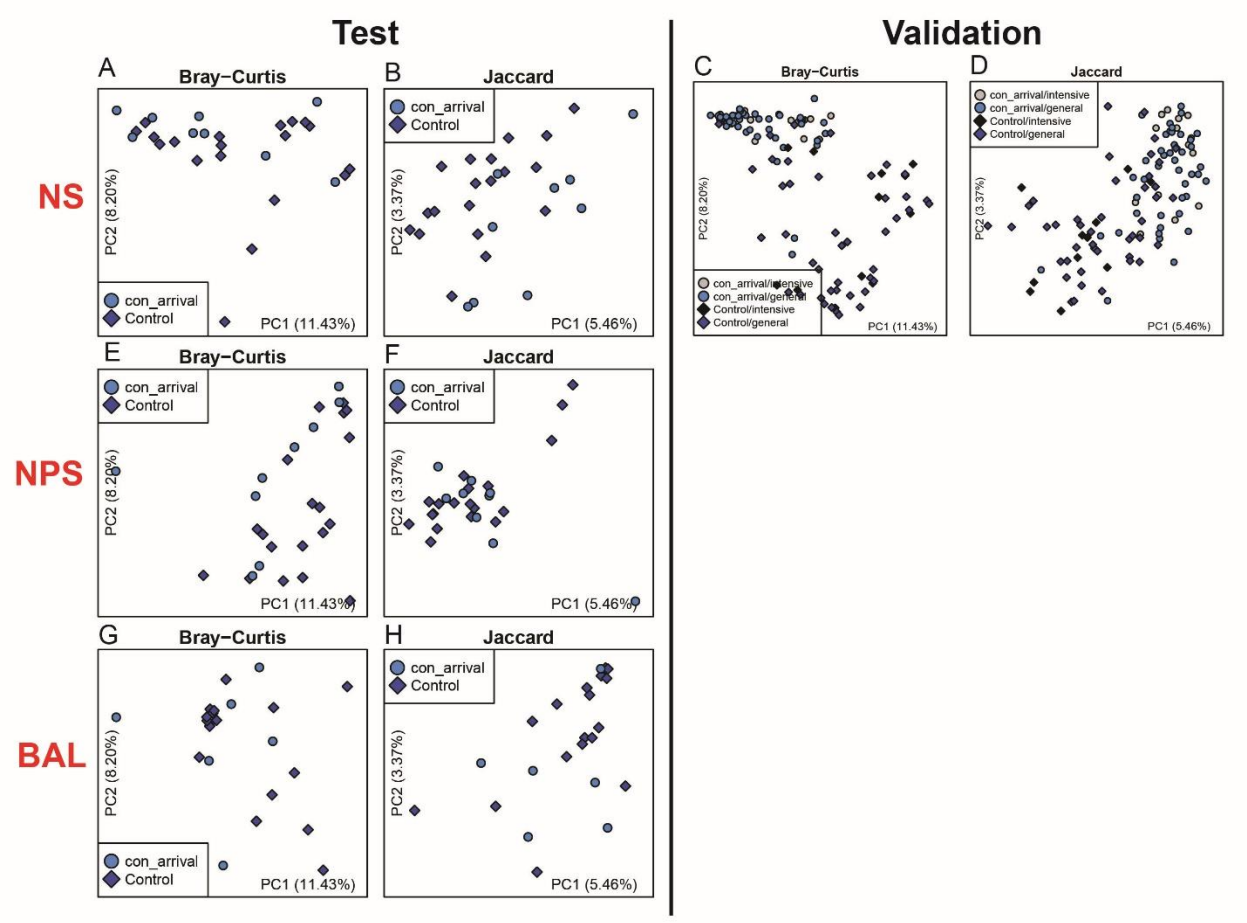

Figure S10 Beta diversity of comparison of control $_{arrival}$ and Control in NS, NPS and BAL

Principal Coordinates Analysis (PCoA) plot showed the beta diversity of BRD $_{arrival}$ and BRD based on Jaccard and Bray-Curtis distance. Each point represents 1 sample with BRD $_{arrival}$ as a dodgerblue circle and BRD as a blue diamond square.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); Control: clinically healthy cattle samples were collected samples as controls for BRD cattle.

Table S5 Nasopharyngeal (NPS) and BAL signatures to predict the NPS control $_{arrival}$ vs Control based on Mean Decrease Accuracy (MDA) in the test trial. Features labeled red color represent shared bacteria among niches having increased abundances in feedlot, while features labeled blue are shared bacteria with decreased abundances in feedlot.

NPS signatures in the test trial	MDA	BAL signatures in the test trial	MDA
F46_Prevotella_copri	23.5	F39_Ruminococcaceae	23.5
F117_Blautia	20.0	F99_Lactobacillus	20.0
F53_Megasphaera	19.9	F17_Mannheimia	19.9
F327_Faecalibacterium_prausnitzii	14.0	F336_Christensenellaceae	14.0
F89_Prevotella_copri	8.65	F105_JG30.KF.CM45	8.65
F239_Gemmiger_formicilis	8.29	F43_Chitinophagaceae	8.29
F478_Ruminococcus_gnavus	7.75	F112_Enterobacteriaceae	7.75
F12_Moraxella	7.57	F85_Streptococcus	7.57
F84_Lactobacillus	5.14	F23_Enhydrobacter	5.14
F7_Mycoplasma	4.98	F64_JG30.KF.CM45	4.98
F216_Prevotella_stercorea	4.69	F30_Bibersteinia_trehalosi	4.69
F234_Ruminococcaceae	4.52	F104_Enterobacteriaceae	4.52
F155_Faecalibacterium_prausnitzii	4.22	F199_Streptococcus_agalactiae	4.22
F241_Ruminococcus.	3.65	F101_Enterobacteriaceae	3.65
F118_Bacteroides_fragilis	3.39	F152_Peptostreptococcaceae	3.39
F188_Phascolarctobacterium	3.10	F262_Clostridiales	3.10
F208_S24.7	3.08	F22_Weeksellaceae	3.08
F85_Streptococcus	2.87	F59_Streptococcus	2.87
F503_Lachnospiraceae	1.77	F7_Mycoplasma	1.77
F275_Lachnospiraceae	1.66	F385_Clostridiales	1.66
F81_Bacteroides	1.56	F502_Dorea	1.56
F442_Blautia_obeum	1.46	F67_Staphylococcus	1.46
F273_Prevotella	1.31	F31_Bifidobacterium_pseudolongum	1.31
F99_Lactobacillus	1.21	F47_Peptostreptococcaceae	1.21
F225_Clostridium_perfringens	1.05	F210_Trueperella	1.05
F123_Clostridiaceae	1.01	F125_Ruminococcaceae	1.01
F119_Roseburia_faecis	0.875	F303_Prevotella	0.875
F462_Prevotella	0.820	F5_Aggregatibacter	0.820
F61_Intrasporangiaceae	0.763	F203_Bacteroidaceae	0.763
F31_Bifidobacterium_pseudolongum	0.756	F237_Akkermansia	0.756

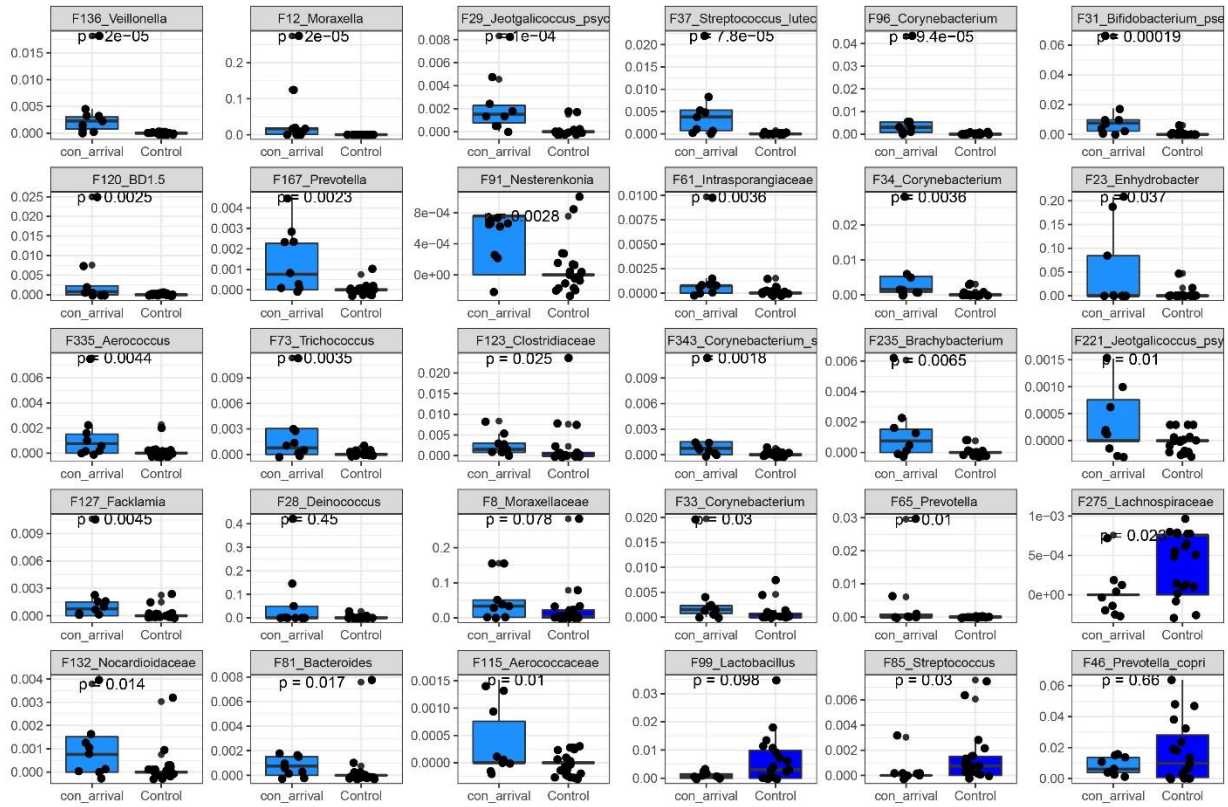

Figure S11 NS signatures boxplots to distinguish control $_{arrival}$ and Control in the test trial

The NS signatures obtained from the AUCRF algorithm differentiate control $_{arrival}$ and Control. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; Nasal swabs (NS); control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); Control: clinically healthy cattle samples were collected samples as controls for BRD cattle.

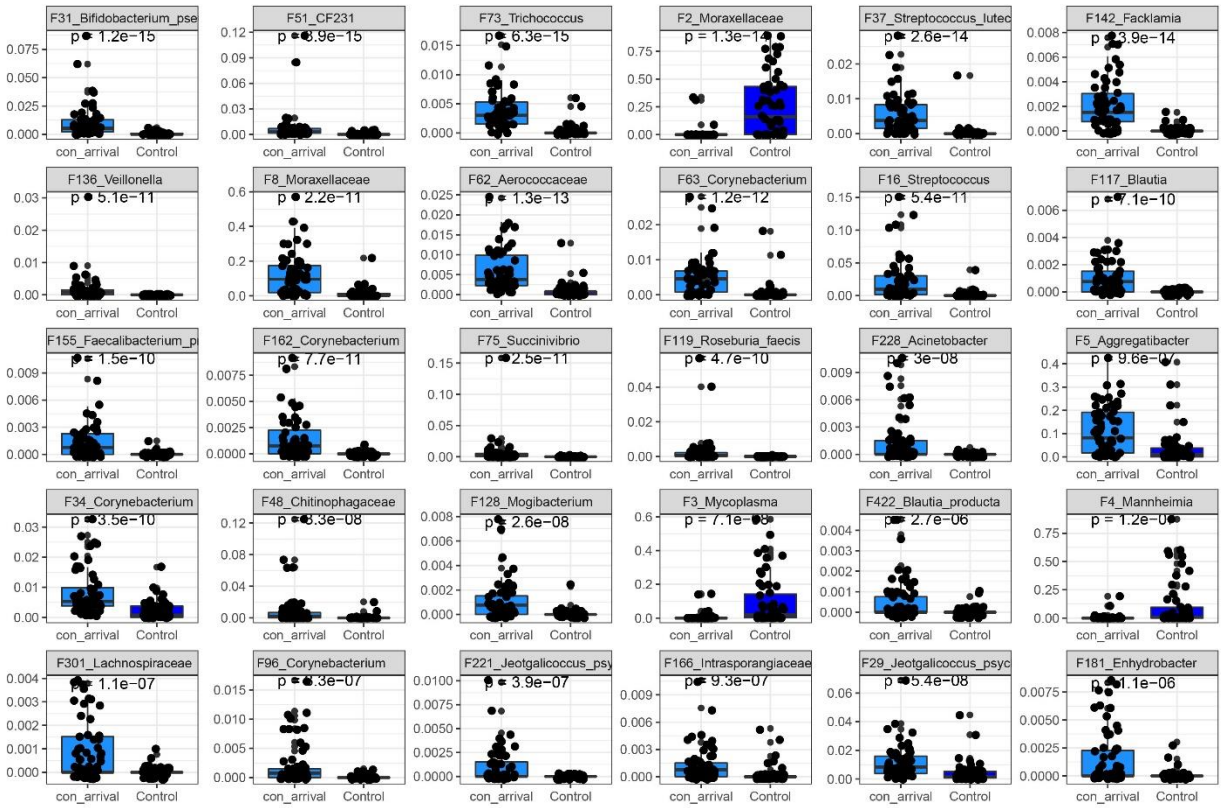

Figure S12 NS signatures boxplots to distinguish control $_{arrival}$ and Control in the validation trial

The NS signatures obtained from the AUCRF algorithm differentiate control $_{arrival}$ and Control. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; Nasal swabs (NS); control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); Control: clinically healthy cattle samples were collected samples as controls for BRD cattle.

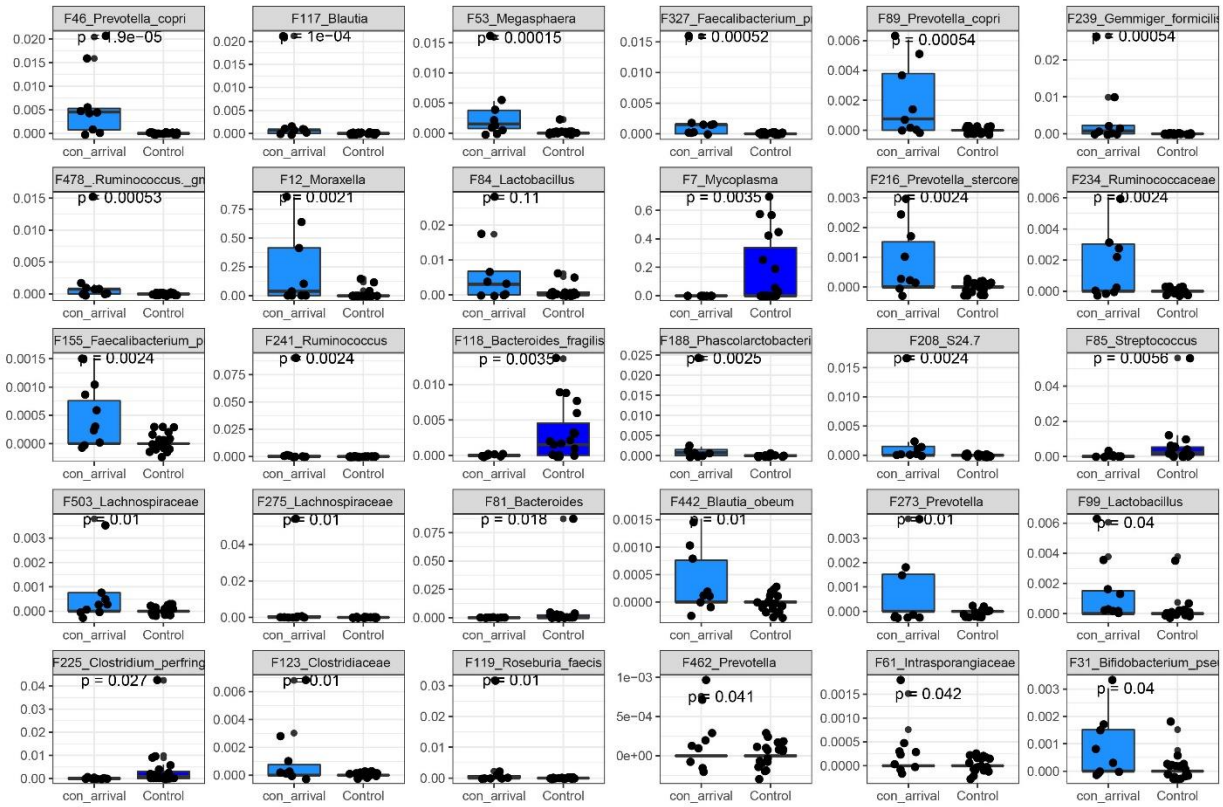

Figure S13 NPS signatures boxplots to distinguish control $_{arrival}$ and Control in the test trial

The NPS signatures obtained from the AUCRF algorithm differentiate control $_{arrival}$ and Control. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; nasopharyngeal swab (NPS); control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); Control: clinically healthy cattle samples were collected samples as controls for BRD cattle.

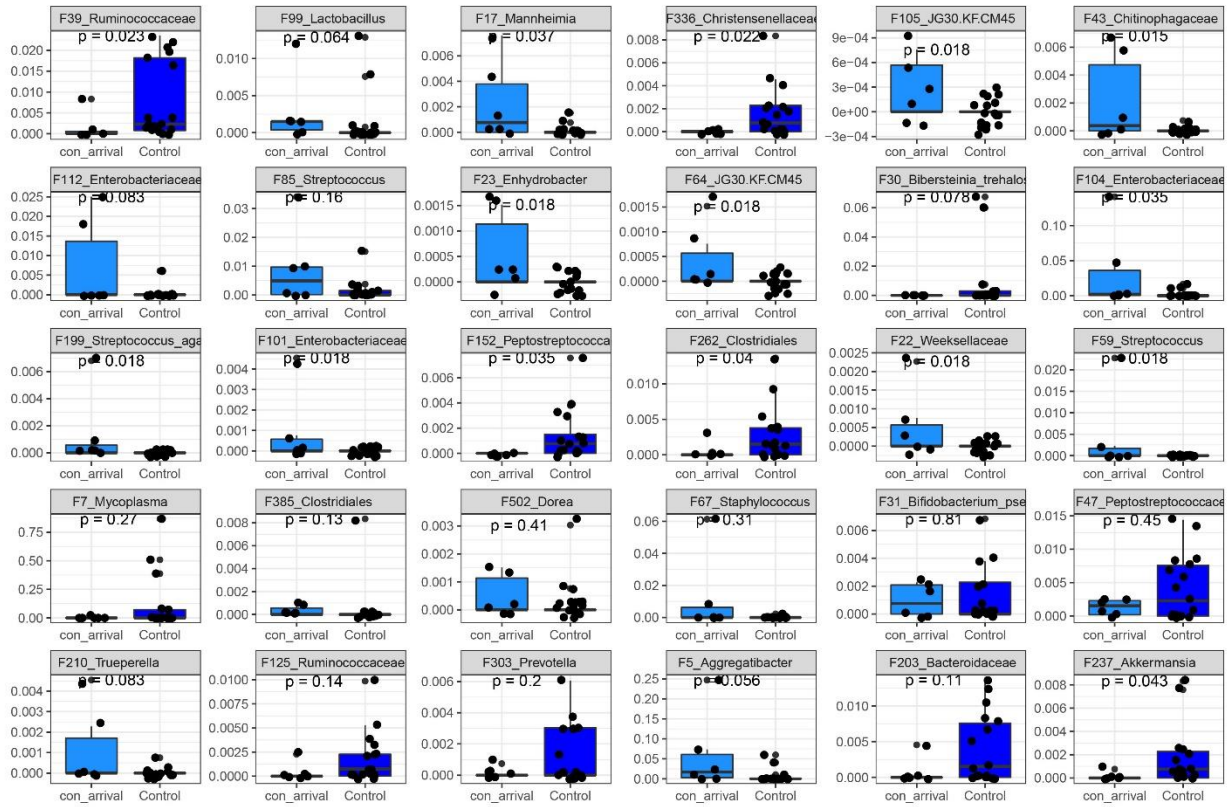

Figure S14 BAL signatures boxplots to distinguish control $_{arrival}$ and Control in the test trial

The BAL signatures obtained from the AUCRF algorithm differentiate control $_{arrival}$ and Control. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; bronchoalveolar lavage (BAL); control $_{arrival}$: healthy cattle samples were collected at arrival to the feedlot (con_arrival); Control: clinically healthy cattle samples were collected samples as controls for BRD cattle.

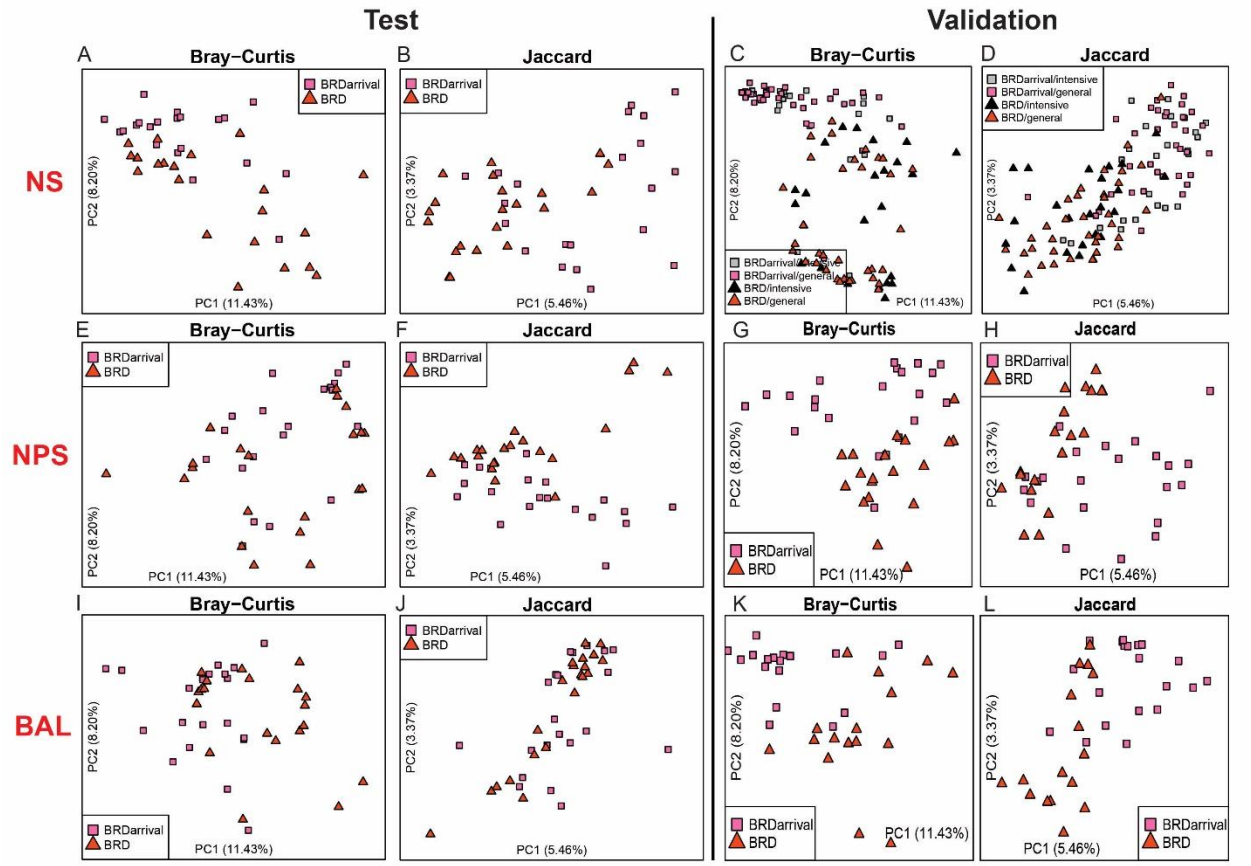

Figure S15 Beta diversity of comparison of BRD $_{arrival}$ and BRD in NS, NPS and BAL

Principal Coordinates Analysis (PCoA) plot showed the beta diversity of BRD $_{arrival}$ and BRD based on Jaccard and Bray-Curtis distance. Each point represents one sample with BRD $_{arrival}$ as a hot pink square and BRD as a red triangle.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot; BRD: cattle were diagnosed with BRD.

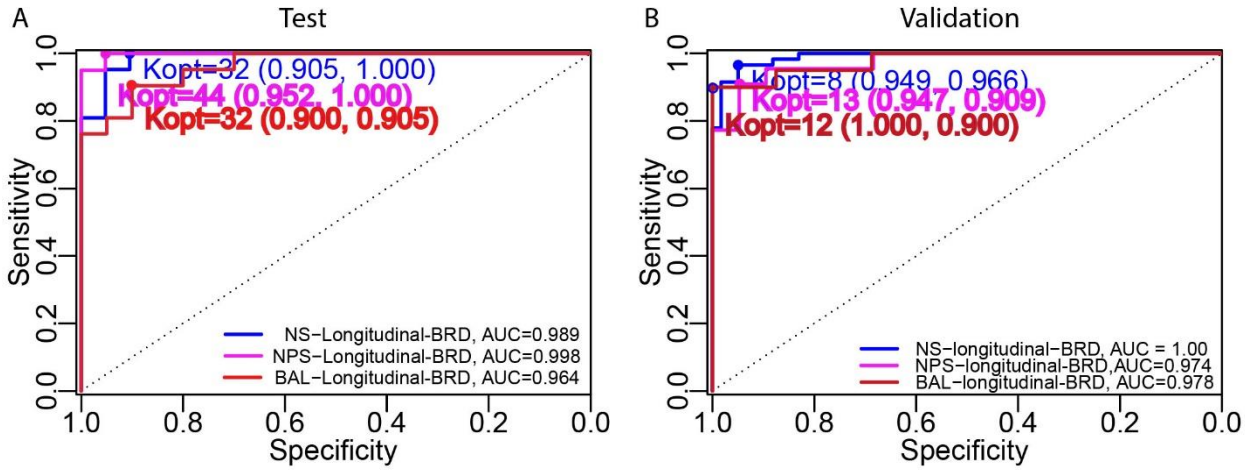

Figure S16 AUC curve of random forest comparing BRD $_{arrival}$ and BRD
(A) The area-under-the ROC curve (AUC) of the Random Forest (AUCRF) for BRD $_{arrival}$ and BRD using NS (blue lines), NPS (hot pink lines), and BAL (red lines) microbiome respectively. Kopt represents the number of optimal predictors obtained based on AUCRF. The numbers within parentheses behind Kopt are (specificity, sensitivity).
Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot; BRD: cattle were diagnosed with BRD.

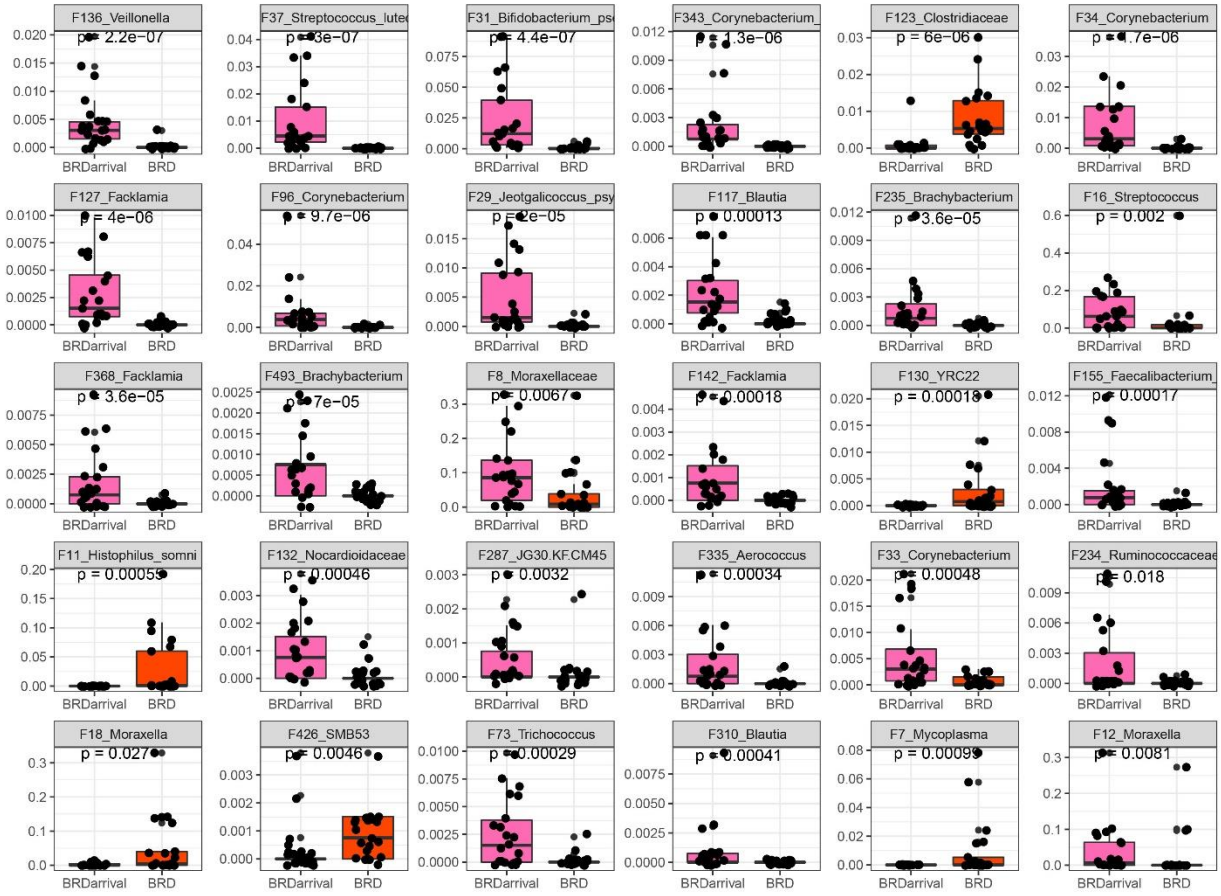

Figure S17 NS signatures boxplots to distinguish BRD $_{arrival}$ and BRD in the test trial

The NS signatures obtained from the AUCRF algorithm differentiate BRD $_{arrival}$ and BRD. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; Nasal swabs (NS); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot; BRD: cattle were diagnosed with BRD.

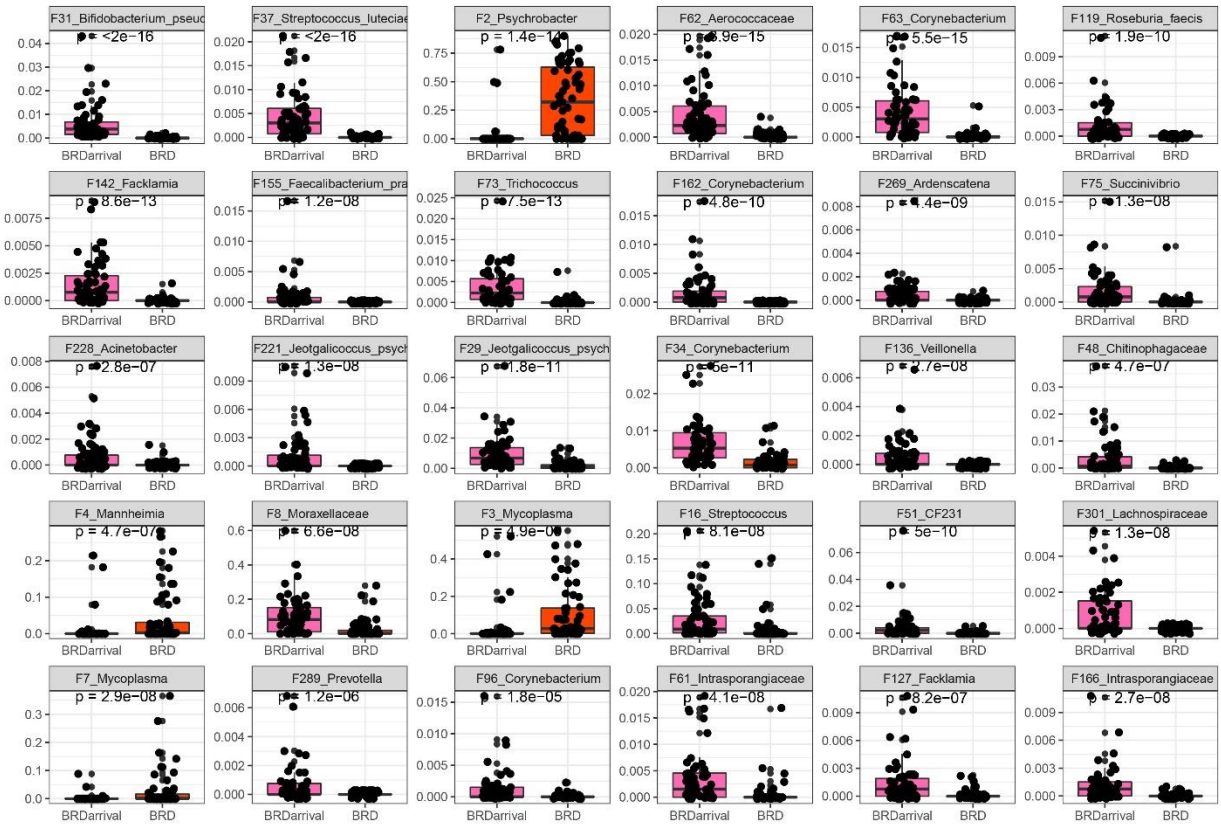

Figure S18 NS signatures boxplots to distinguish BRD $_{arrival}$ and BRD in the validation trial

The NS signatures obtained from the AUCRF algorithm differentiate BRD $_{arrival}$ and BRD. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; Nasal swabs (NS); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot; BRD: cattle were diagnosed with BRD.

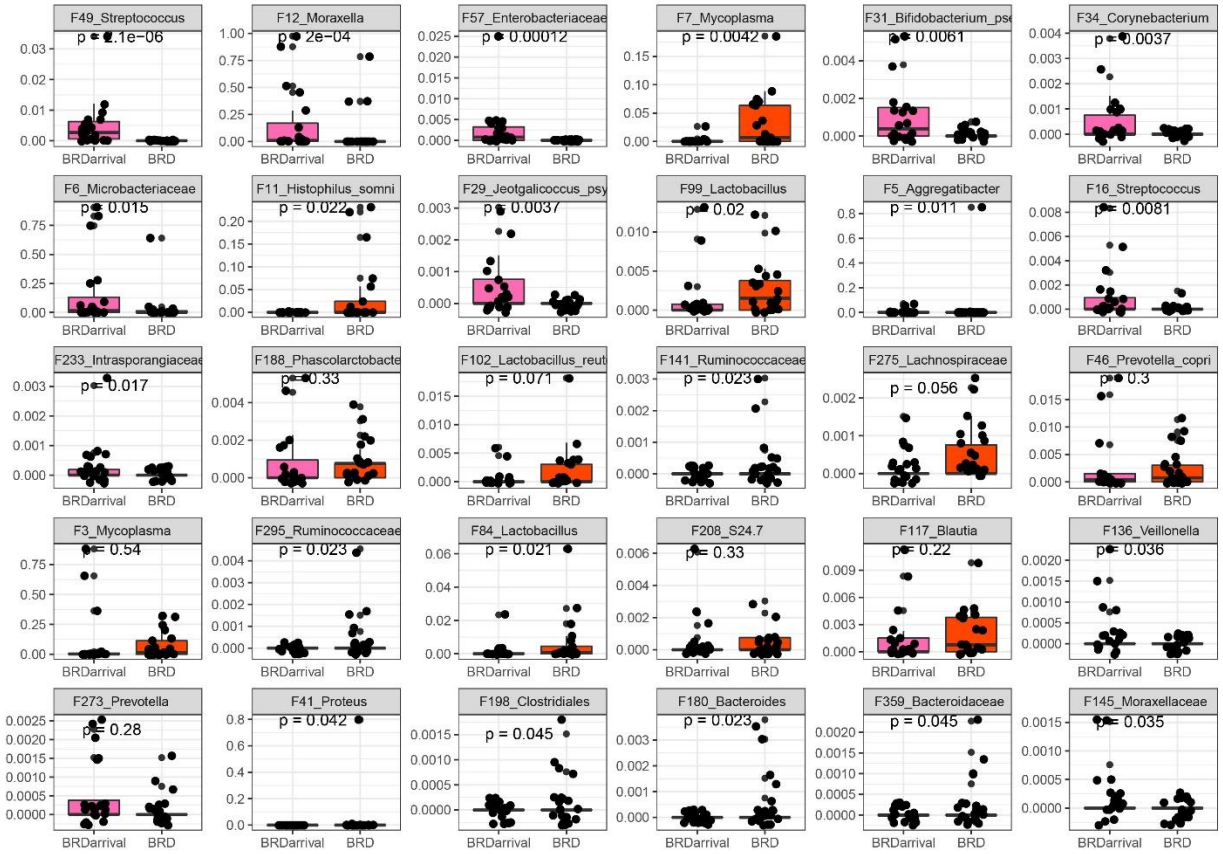

Figure S19 NPS signatures boxplots to distinguish BRD $_{arrival}$ and BRD in the test trial

The NPS signatures obtained from the AUCRF algorithm differentiate BRD $_{arrival}$ and BRD. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; nasopharyngeal swab (NPS); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot; BRD: cattle were diagnosed with BRD.

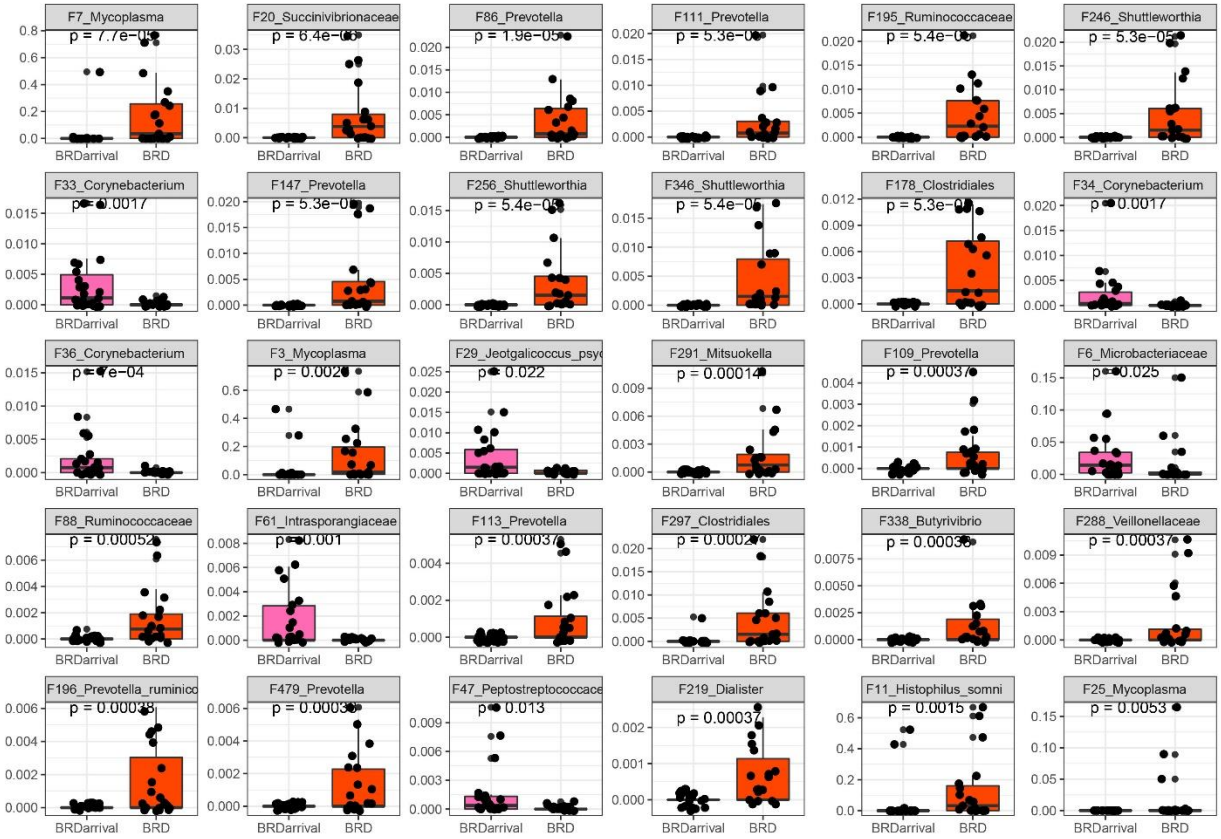

Figure S20 NPS signatures boxplots to distinguish BRD $_{arrival}$ and BRD in the validation trial

The NPS signatures obtained from the AUCRF algorithm differentiate BRD $_{arrival}$ and BRD. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; nasopharyngeal swab (NPS); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot; BRD: cattle were diagnosed with BRD.

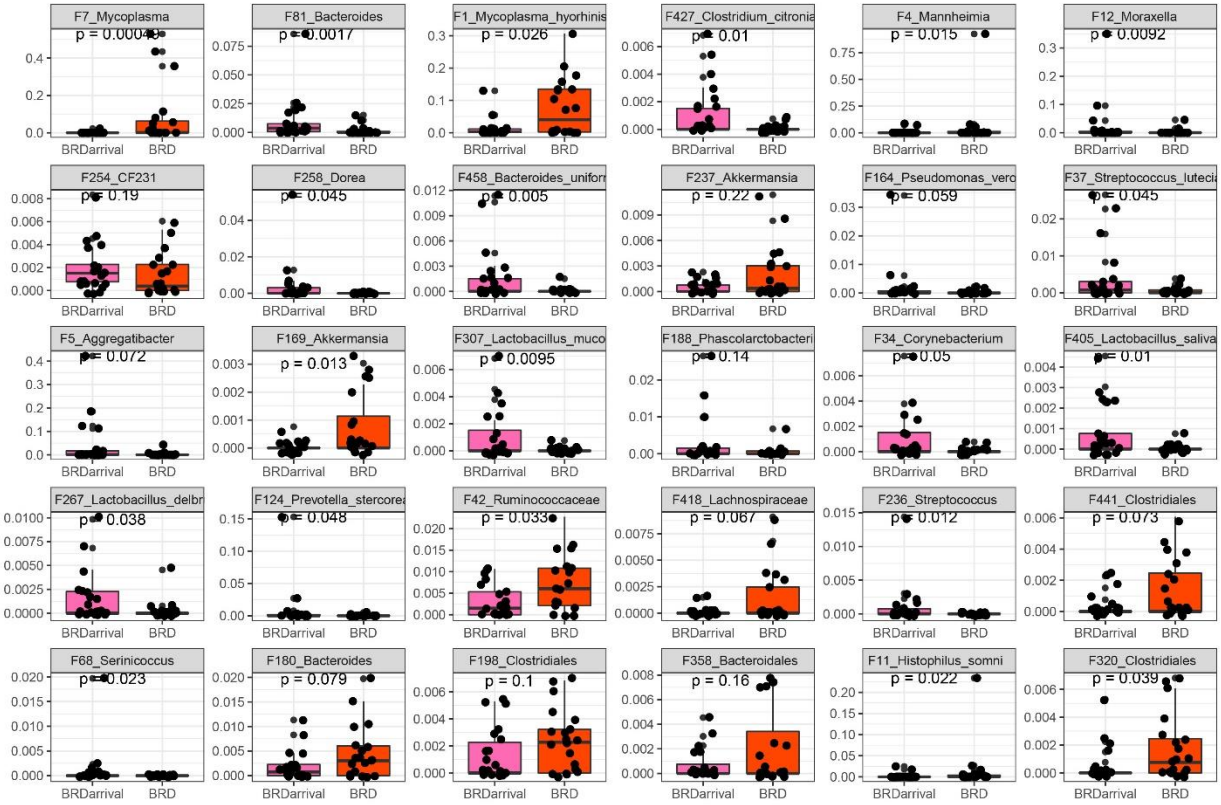

Figure S21 BAL signatures boxplots to distinguish BRD $_{arrival}$ and BRD in the test trial

The BAL signatures obtained from the AUCRF algorithm differentiate BRD $_{arrival}$ and BRD. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; bronchoalveolar lavage (BAL); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot; BRD: cattle were diagnosed with BRD.

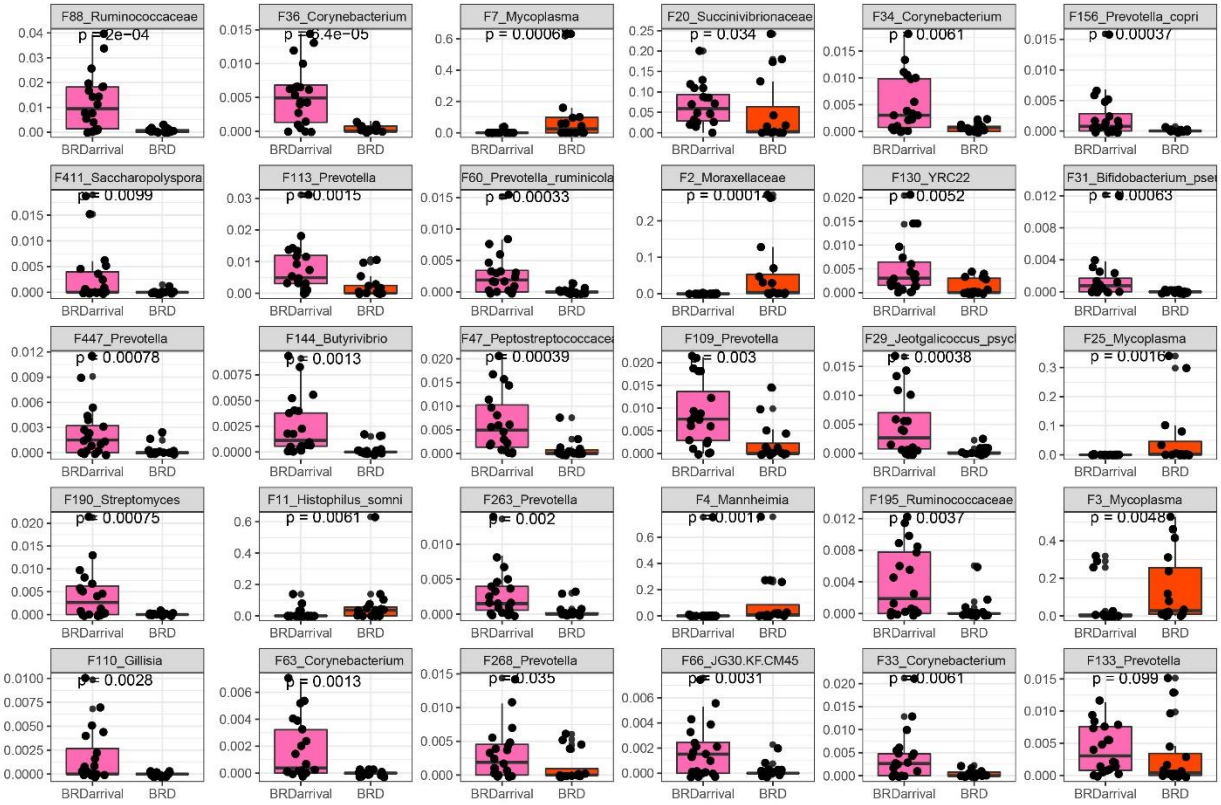

Figure S22 BAL signatures boxplots to distinguish BRD $_{arrival}$ and BRD in the validation trial

The BAL signatures obtained from the AUCRF algorithm differentiate BRD $_{arrival}$ and BRD. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; bronchoalveolar lavage (BAL); BRD $_{arrival}$: cattle developed BRD later and samples were collected at arrival to the feedlot; BRD: cattle were diagnosed with BRD.

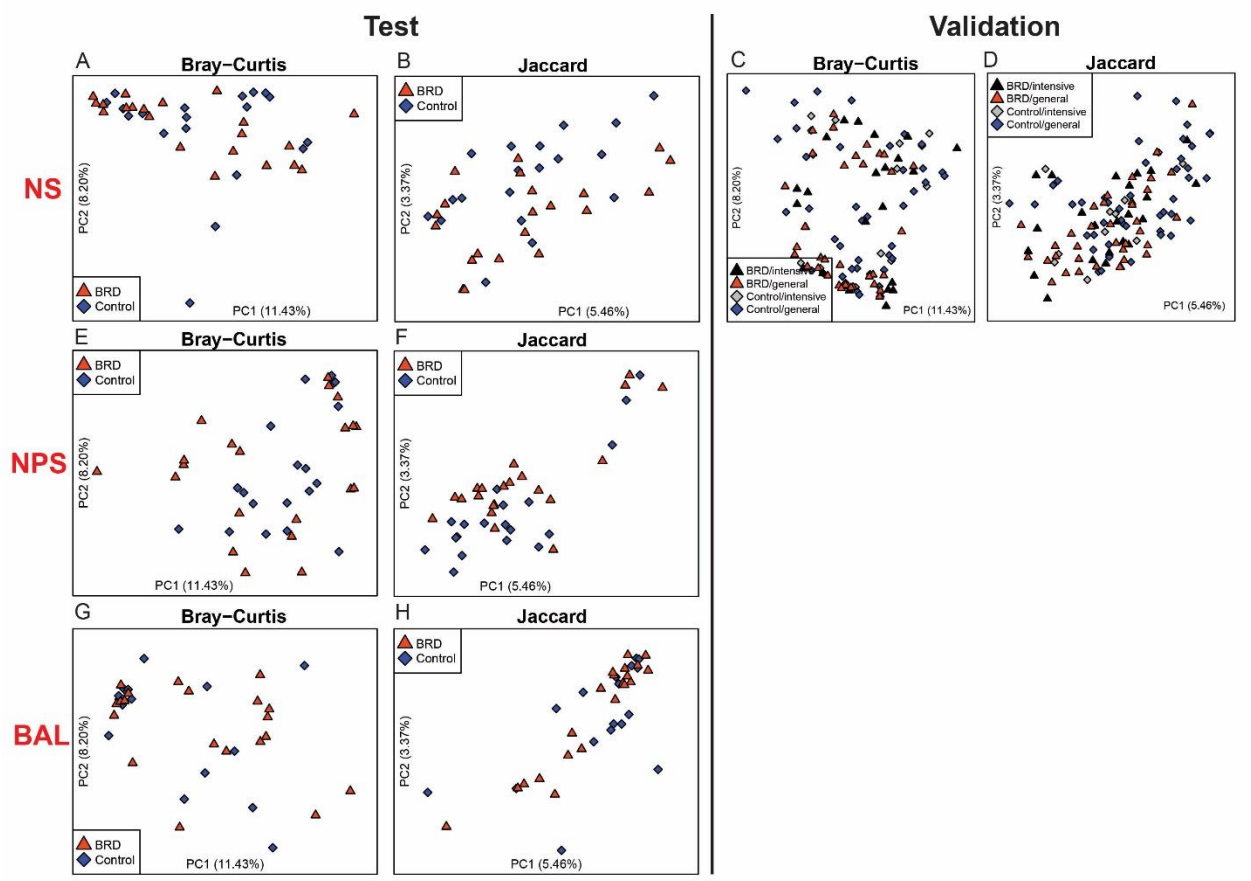

Figure S23 Beta diversity of comparison of BRD and Control in NS, NPS and BAL

Principal Coordinates Analysis (PCoA) plot showed the beta diversity of BRD and Control based on Jaccard and Bray-Curtis distance. Each point represents one sample with BRD as a red triangle and BRD as a blue diamond square.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). Control: clinically healthy cattle samples were collected samples as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

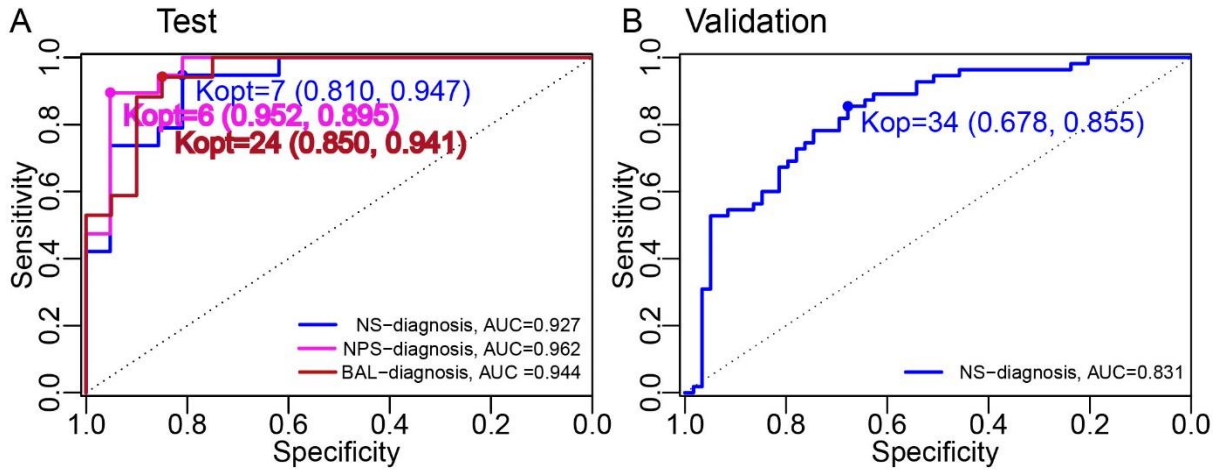

Figure S24 AUC curve of random forest by comparing BRD and Control
(A) The area-under-the ROC curve (AUC) of the Random Forest (AUCRF) for healthy control and BRD using NS (blue lines), NPS (hot pink lines), and BAL (red lines) microbiome respectively. Kopt represents the number of optimal predictors obtained based on AUCRF. The numbers within parentheses behind the Kopt are (specificity, sensitivity).
Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). Control: clinically healthy cattle samples were collected samples as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

Table S6 NS diagnosis signatures to differentiate NS BRD vs Control based on Mean Decrease Accuracy (MDA) in test and validation trial

Test trial	MDA	Validation trial	MDA
F239_Gemmiger_formicilis	16.6	F4_Mannheimia	7.06
F123_Clostridiaceae	16.3	F15_Moraxellaceae	5.58
F99_Lactobacillus	14.4	F68_Serinicoccus	5.25
F327_Faecalibacterium_prausnitzii	9.86	F92_Ruminococcaceae	4.01
F46_Prevotella_copri	8.90	F70_Turicibacter	3.89
F442_Blautia_obeum	8.50	F55_Prevotella	3.85
F273_Prevotella	7.76	F419_RF16	3.82
F234_Ruminococcaceae	7.47	F5_Aggregatibacter	3.12
F102_Lactobacillus_reuteri	5.85	F39_Ruminococcaceae	2.50
F117_Blautia	4.99	F62_Aerococcaceae	2.27
F275_Lachnospiraceae	4.31	F20_Succinivibrionaceae	2.25
F460_Roseburia_faecis	4.22	F61_Intrasporangiaceae	2.15
F367_Clostridium_butyricum	4.16	F132_Nocardioidaceae	1.81
F130_YRC22	3.66	F138_5.7N15	1.59
F134_Clostridium_celatum	2.77	F44_Acinetobacter_lwoffii	1.43
F170_Prevotella	2.02	F29_Jeotgalicoccus_psychrophilus	1.33
F465_Blautia	1.83	F125_Ruminococcaceae	1.33
F18_Moraxella	1.38	F215_Phycicoccus	1.31
F3_Mycoplasma	1.22	F428_CF231	1.18
F306_JG30.KF.CM45	1.02	F420_Prevotella	1.16
F24_Carnobacteriaceae	0.978	F53_Megasphaera	1.12
F53_Megasphaera	0.902	F7_Mycoplasma	1.09
F193_Moraxella	0.815	F179_Prevotella	1.03
F426_SMB53	0.792	F292_Phascolarctobacterium	1.01
F462_Prevotella	0.779	F47_Peptostreptococcaceae	0.988
F155_Faecalibacterium_prausnitzii	0.642	F8_Moraxellaceae	0.965
F6_Microbacteriaceae	0.640	F16_Streptococcus	0.888
F503_Lachnospiraceae	0.606	F107_Mycoplasma	0.787
F2_Moraxellaceae	0.577	F2_Moraxellaceae	0.769
F471_Ruminococcaceae	0.576	F27_Weeksellaceae	0.768

Sup table S7 Nasopharyngeal (NPS) and BAL signatures to differentiate BRD vs Control based on Mean Decrease Accuracy (MDA) in the test trial

NPS signatures	MDA	BAL signatures	MDA
F117_Blautia	21.2	F198_Clostridiales	6.40
F99_Lactobacillus	19.0	F125_Ruminococcaceae	3.74
F234_Ruminococcaceae	18.0	F164_Pseudomonas_veronii	2.97
F46_Prevotella_copri	17.9	F307_Lactobacillus_mucosae	2.66
F275_Lachnospiraceae	15.1	F30_Bibersteinia_trehalosi	2.61
F118_Bacteroides_fragilis	14.8	F203_Bacteroidaceae	1.71
F155_Faecalibacterium_prausnitzii	10.1	F280_Lachnospiraceae	1.55
F208_S24.7	7.28	F255_BF311	1.45
F188_Phascolarctobacterium	6.60	F456_Coprococcus	1.28
F89_Prevotella_copri	5.92	F385_Clostridiales	1.21
F81_Bacteroides	5.56	F100_Listeria	1.12
F53_Megasphaera	5.04	F258_Dorea	1.06
F225_Clostridium_perfringens	4.29	F225_Clostridium_perfringens	0.972
F102_Lactobacillus_reuteri	3.93	F1_Mycoplasma_hyorhinis	0.969
F465_Blautia	3.83	F180_Bacteroides	0.930
F7_Mycoplasma	3.81	F77_Intrasporangiaceae	0.888
F241_Ruminococcus	3.48	F371_Mogibacteriaceae	0.844
F239_Gemmiger_formicilis	3.33	F188_Phascolarctobacterium	0.829
F85_Streptococcus	3.21	F393_Bacteroidaceae	0.814
F505_Actinomyces_hyovaginalis	2.72	F64_JG30.KF.CM45	0.708
F327_Faecalibacterium_prausnitzii	2.51	F263_Prevotellaceae_Prevotella	0.648
F123_Clostridiaceae	2.25	F39_Ruminococcaceae	0.616
F119_Roseburia_faecis	2.21	F113_Prevotella	0.576
F295_Ruminococcaceae	1.77	F329_Peptostreptococcaceae	0.534
F478_Ruminococcus_gnavus	1.50	F104_Enterobacteriaceae	0.530
F267_Lactobacillus_delbrueckii	1.20	F51_CF231	0.485
F503_Lachnospiraceae	1.18	F262_Clostridiales	0.461
F258_Dorea	1.17	F283_Bacteroidales	0.455
F231_Collinsella_aerofaciens	1.12	F377_Prevotella	0.422
F496_Fusobacteriaceae	0.978	F37_Streptococcus_luteciae	0.412

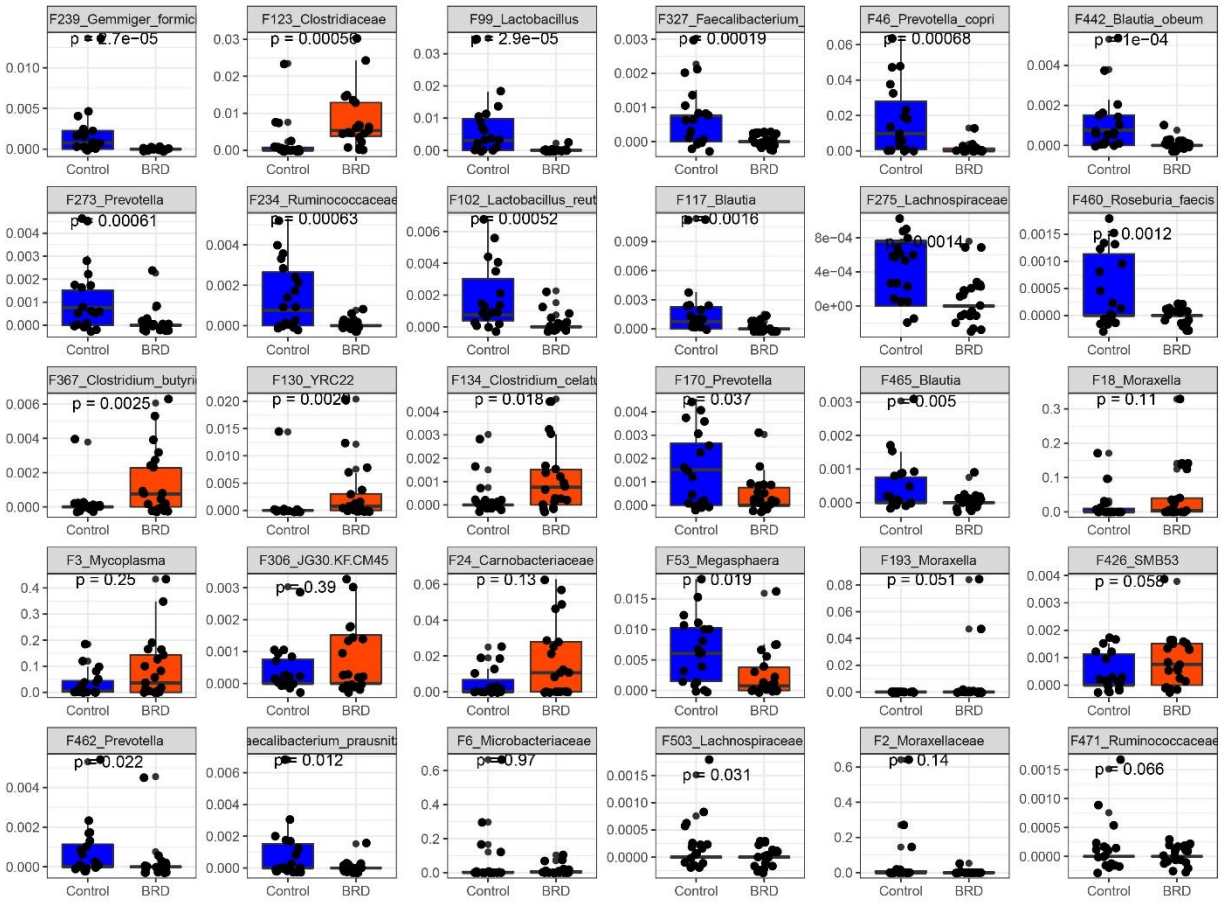

Figure S25 NS signatures boxplots to distinguish BRD and Control in the test trial

The NS signatures obtained from the AUCRF algorithm differentiating BRD and Control. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; Nasal swabs (NS); Control: clinically healthy cattle samples were collected samples as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

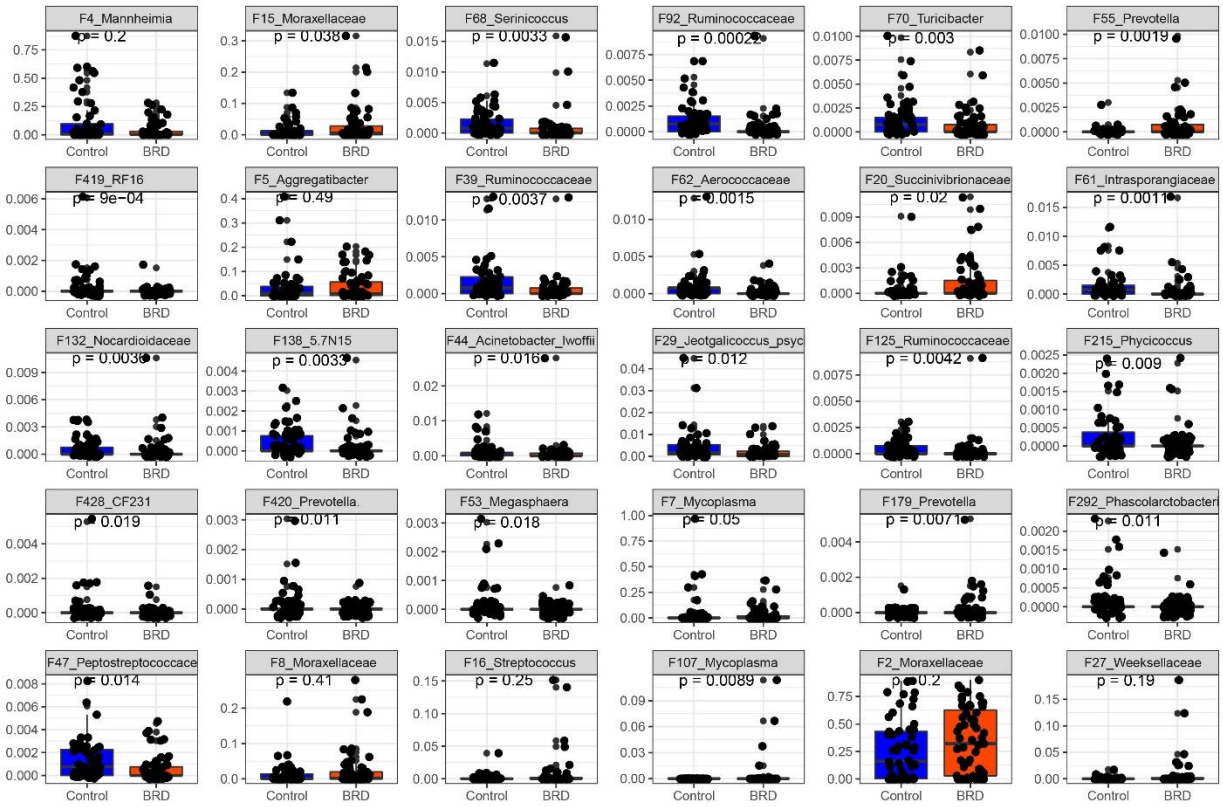

Figure S26 NS signatures boxplots to distinguish BRD and Control in the validation trial

The NS signatures obtained from the AUCRF algorithm differentiating BRD and Control. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; Nasal swabs (NS); Control: clinically healthy cattle samples were collected samples as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

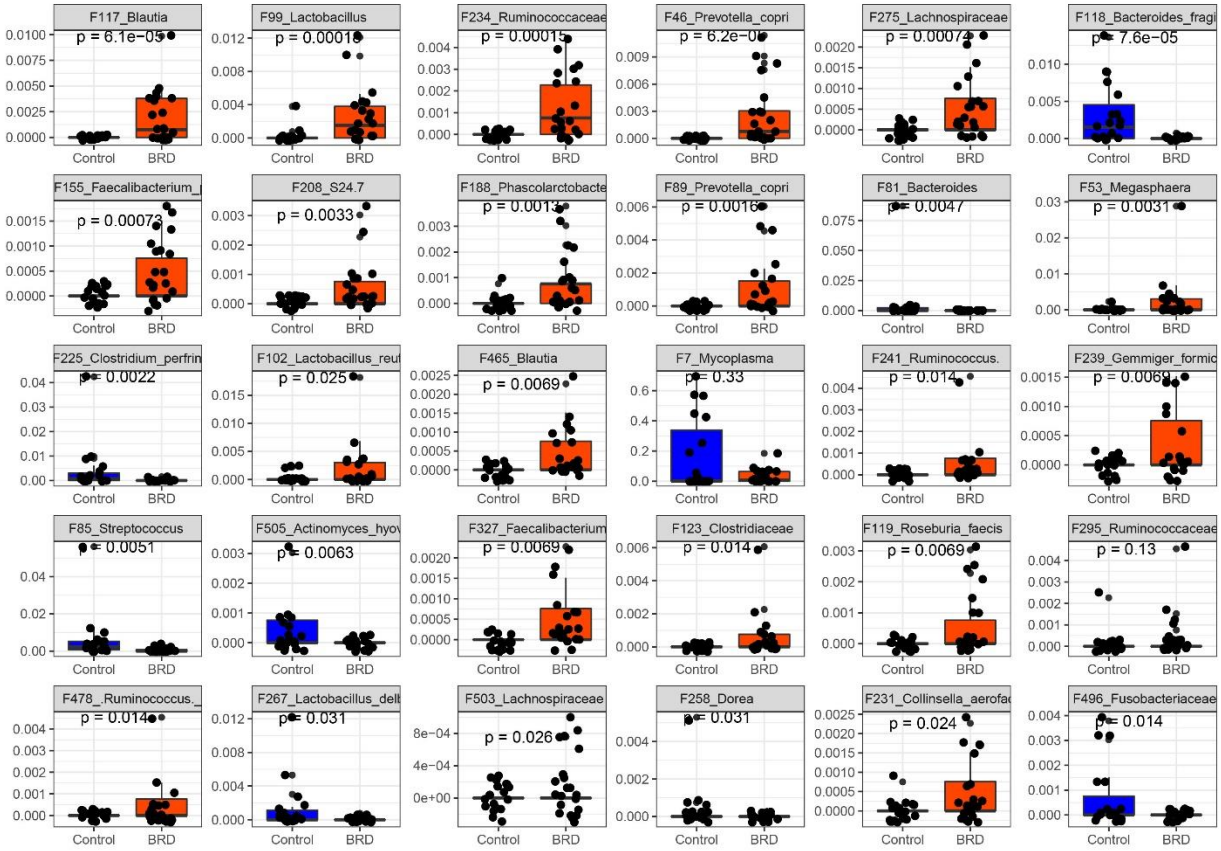

Figure S27 NPS signatures boxplots to distinguish BRD and Control in the test trial

The NPS signatures obtained from the AUCRF algorithm differentiating BRD and Control. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; nasopharyngeal swab (NPS); Control: clinically healthy cattle samples were collected samples as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

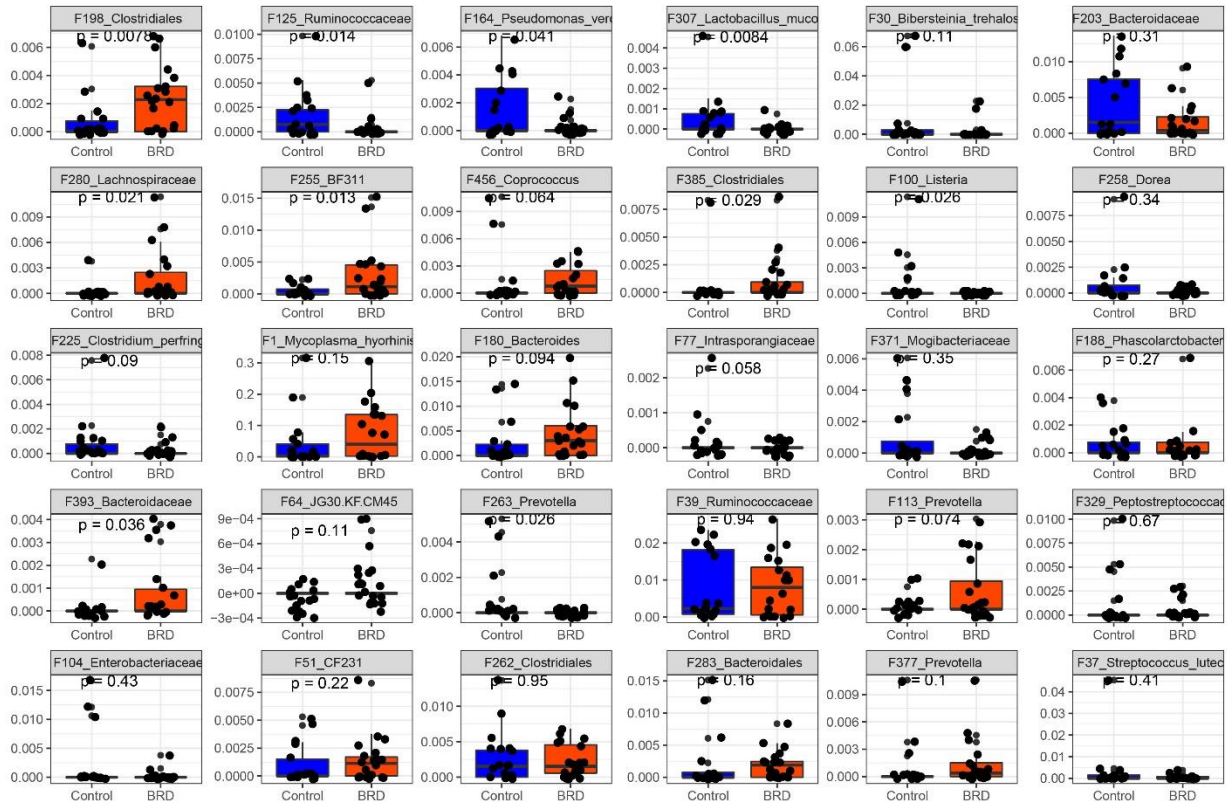

Figure S28 BAL signatures boxplots to distinguish BRD and Control in the test trial

The BAL signatures obtained from the AUCRF algorithm differentiating BRD and Control. The number over bars are p values received from the Wilcoxon test.

Abbreviations: AUCRF: the area-under-the ROC curve (AUC) of the Random Forest; bronchoalveolar lavage (BAL); Control: clinically healthy cattle samples were collected samples as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

Chapter V. Spatial Movement of Microbiome Associated with Bovine Respiratory Disease Status

Abstract

The spatial movement of the microbiome within the respiratory tract is complex and unknown. However, clinical signs are associated with microbial shifts and selective growth in local environments. The spatial movement of the microbiome and its relationship with bovine respiratory disease (BRD) status remains unclear. In this study, we sought to determine how BRD status associates with the spatial dynamics of the bovine respiratory microbiome. The microbial data of the upper (both nasal and nasopharyngeal niches) and lower (BAL, lung) respiratory tracts were obtained from cattle with the same diet and management at different health statuses (healthy $_{arrival}$, BRD $_{arrival}$, BRD and clinically healthy Control) in our previous chapters. The data of the second animal trial were also as a validation for the first animal (test) trial. We found microbial shift patterns from the nostrils to the lungs were affected by disease. Then, correlation of the microbiome between adjacent niches increased in BRD calves compared to healthy calves. Using Source Tracker, more proportions of the lung microbiome from the upper airway were also found in BRD calves. Furthermore, pathogens in BRD afflicted lungs were predicted using their abundances in the nostrils and nasopharynx. Complex microbial interactions within healthy respiratory communities were observed, while dysbiosis with abundant pathogens in diseased calves were found. All discoveries mentioned were additionally identified in the validation animal trial. In summary, the spatial movement of the bovine respiratory microbiome is related to BRD status, and pathogens in the dysbiosis of a BRD ridden respiratory system enter the lung more easily, inducing infection.

Keywords: spatial movement, microbiome, bovine respiratory tract, logistic model, microbiota-microbiota association, BRD, pathogens, airway

Introduction

The respiratory microbiome colonizes in the bovine airway system, and shows niche specific preferences. In healthy individuals, bacteria enter the lungs through an active and continuous process by inhalation of air, direct mucosal dispersion and micro-aspiration from the upper airway tract, and lung microbiome are determined more by the balance of microbial immigration and elimination than by local environment (Dickson et al., 2014a). An island biogeography model of the microbial communities was applied to explore the microbial movement from the upper airway to the lung in healthy humans (Whiteson et al., 2014). However, this theory considers oral microbes as major sources of the lung microbiota, and a variety of other factors including host, environment, diet, antibiotic therapy, etc. could influence microbial immigration. Hence, new algorithms need to be performed to investigate microbial movement. In humans, a study using a neutral model to assess the microbial movements between the upper and lower respiratory tract confirmed that the composition of healthy lungs microbiome fit a neutral model when using oral microbiota as a source (Bassis et al., 2015; Venkataraman et al., 2015). However, a neutral model fails to assess other sources for lung and the respiratory microbiomes in diseased humans. Therefore, spatial dispersals of the airway microbiome could be associated with the health status of the host.

Until now, no studies exist that investigated the spatial dispersion of the bovine respiratory microbiome. Regarding ruminants, the study of microbial movement within the airway and whether it would be affected by disease is important and complex. There are many factors that can influence bovine respiratory microbiome, including diet, ruminating activity, antibiotics, living environment changes for newly weaned calves etc. (Glendinning et al., 2017; Hall et al., 2017; Holman et al., 2019; Man et al., 2017; Shukla et al., 2017; Zeineldin et al.,

2019). However, our samples from previous chapters are capable of investigating the relationship between bovine respiratory disease and spatial movements of the microbiome. In part, this is due to all calves receiving identical diets, and management, while additionally, BRD was the only significant factor to affect nasal, nasopharyngeal and the lung microbiomes. As we know, BRD calves show clinical signs including nasal discharge, coughing, and fever, which could influence local niche environment, mucosal communication and microbial shifts within the airway. Therefore, the microbial immigrations in BRD cattle would be different from with healthy cattle.

Microbial interaction within specific niche community might be another important factor that influence assessment of the spatial microbiome. A complex and harmonious interaction between the host, microbial colonization and local environment within the airways is needed in healthy cattle. In contrast, a disequilibrium related to microbial dysbiosis, mucosal dysfunction as well as acute or chronic inflammation consequently generates opportunity for the development of BRD (Hakansson et al., 2018). Therefore, spatial variation in the airway of healthy cattle is more stable for comparison to diseased cattle. For BRD calves, how the disequilibrium of communities affects microbial movement from the upper respiratory tract to the lung is uncertain. In this study, we compared the differences of biogeographical changes (shifts from nasal cavity to lung) influenced by BRD, and the microbial interaction within niches at different healthy statuses.

Materials and Methods

Animal management, BRD case definition, sample collection, and next-generation sequencing were described in Chapters III and IV. The samples of four status (healthy control $_{arrival}$, BRD $_{arrival}$, BRD and Control) from the test and validation trials were processed together in QIIME2 and analyzed respectively following the patterns described in previous chapters.

Bioinformatics Statistics

The correlation between sampling niches and the logistic model were performed by following methods described for gut microbiota (Yasuda et al., 2015). The direction of beta (β) from the logistic model was used as the type of interaction and attributes included the relative abundances of each bacterial feature. The negative βs represent a bacterial feature may move from sink niche to source niche due to microbiota high in abundance with the source niche. However, in this study, we defined the upper airway as a source environment and lung as a sink environment. Therefore, the direction of the arrows for βs was always from nasal to nasopharynx and to lung, if β was significant.

Source Tracker was used to assess the contribution to the lung microbiota from the upper airway community. The nasal and nasopharyngeal microbiome were sources, while lung microbiome was a sink. In addition, we also measured the microbial movement within the upper airway by using the nasopharynx as a sink and the nasal microbiome as a source. The Gibbs sampling procedure and Dirichlet parameters of Source Tracker were set based on previous reported methods (Knights et al., 2011).

The SparCC algorithm, which is able to determine the correlation from compositional network, was used for co-occurrence network analysis of each niche under different healthy statues. Network plots were created using the abundant bacterial taxa. The network was visualized by using the 'igraph' package in R. Each node represented a feature, and the size of node indicated the relative abundances of bacterium. The thickness of edges connecting nodes (microbial features) were set as a correlation co-efficiency over 0.5 or less than -0.5, and only significant correlations were showed. The color of edges represented the correlation of negative (red) and positive (blue).

Results

Spatial correlation influenced by BRD

General shifts of microbiome from NS to NPS to BAL were observed in individual calves, and the shifts were stable within one healthy status. However, the shifts pattern in different health statuses from both the test and validation trials were different based on community structure and membership (Figure S1, S2, S3, and S4).

Measures of the correlation between sampling niches can reflect microbial movement. Pearson correlations between adjacent niches under different healthy statuses were calculated by accounting for both other microbial rank orders and the magnitude of relative abundances. The correlation between the respiratory niches was affected by BRD. In BRD calves, the NPS microbiome was highly correlated with NS ($r=0.67$, $p<0.001$), while slight lower correlation co-efficiency between NS and NPS was observed in healthy control $_{arrival}$ ($r=0.59$), BRD $_{arrival}$ ($r=0.61$) and Control ($r=0.64$) subjects (Figure 1). A similar pattern was also observed in correlations between NPS and BAL (healthy $_{arrival}$: $r=0.48$; BRD $_{arrival}$: $r=0.49$; BRD: $r=0.57$; Control: $r=0.47$; $p<0.001$). In addition, the co-efficiency decreased between NS and BAL, and high correlation was observed in morbid animals (healthy arrival: $r=0.35$; BRD $_{arrival}$: $r=0.40$; BRD: $r=0.40$; Control: $r=0.39$; $p<0.001$). In the validation trial, we found a similar pattern with the highest correlation in BRD animals (Figure 2).

To determine the contribution of the upper airway (nasal and nasopharyngeal) microbiota to the lower airway (lung, BAL) microbiota, Source Tracker was performed (Figure 3). For microbial movement within the upper airway, the NPS microbiome in BRD $_{arrival}$ had 41.26% from its NS that was lower than 64.66% of NPS from NS in BRD ($p=0.081$). Other healthy

statuses were not different ($p>0.05$) due to large individual variation (Figure 3 A). We confirmed more the NS microbiome contributed to the NPS in BRD calves compared to BRD $_{arrival}$ from the validation trial ($p<0.001$) (Figure 3 B). Then, the contribution to the lung microbiome using NS and NPS as sources was determined. The lung microbiome in BRD calves had more microbiome from the NPS (48.00%) compared to Control calves (26.22%). At feedlot arrival, BRD $_{arrival}$ and healthy $_{arrival}$ had 32.17% and 39.03% of the lung microbiome from the NPS. However, the pattern of lung microbiome from NPS is opposite with the pattern of lung microbiome from NS. NS microbiome contribution to the lungs during BRD status was 1.35%, significantly lower than 8.43% of BRD $_{arrival}$ and 14.22% of Control ($p<0.05$). In the validation trial, BRD calves' lung microbiome from the NPS community had a higher proportion (68.75%) than BRD $_{arrival}$ cattle (33.75%) and healthy $_{arrival}$ (18.76%) ($p<0.05$). Moreover, BRD calves with a lower proportion of the lung microbiome from NS than BRD $_{arrival}$ was validated (Figure 3 D, E, F).

The prediction of biogeography of BRD pathogens associated with BRD status

To understand potential bacterial pathogens' movement within the bovine respiratory tract, we used logistic regression to model the extent to which abundance of a feature at one site was predictive in its abundance at an adjacent site. However, there were no predictive patterns of major signature bacteria including *Jeotgalicoccus psychrophilus* (F29), *Bifidobacterium pseudolongum* (F31*), Corynebacterium* (F34) and *Streptococcus luteciae* (F37) that demonstrated high abundance in control $_{arrival}$, BRD $_{arrival}$ and Control compared to BRD. (Figure S5). The BRD pathogens that were more abundant in the lung microbiome of BRD calves, which include three *Mycoplasma* (F1, F3, and F7), *Histophilus somni* (F11), *Mannheimia* (F4), and *Pasteurella* (F13), were selected (Figure 4). Figure 4 summarizes the relationships of β-values (regression slopes) between adjacent sites in the bovine respiratory tract. The prediction pattern

(pathogens abundance at one niche can be used to predict its abundance at another) was affected by BRD status. For healthy control $_{arrival}$ status, all six pathogens at one site could not be predicted by another site, while their abundance at one site could be partially or fully predicted by another site for calves under the other three healthy statuses. For F1 with high abundance in the NPS microbiome of BRD $_{arrival}$, BAL is highly predictive of its abundances in both NS and NPS. While, for F1 in BRD and Control calves, not only BAL is predictive by NS and NPS, but also, F1 abundance in NPS is predictive of its abundance in NS. *Mycoplasma* (F3) in BRD $_{arrival}$, BRD and Control is predictive by its adjacent niches, while *Mycoplasma* (F7) in BRD and Control status is predictive. The abundances of *Histophilus somni* (F11) in the lung from BRD $_{arrival}$ and Control was not predicted by its abundance in the upper airway, but F1 in the BRD lung is predictive. *Mannheimia* (F4) had the same predictive pattern with F3. *Pasteurella* (F13) had high abundances in BRD $_{arrival}$ and BRD calves, and BRD lung of is highly predicted by both the NS and NPS. In summary, using logistic regression modeling allowed us to investigate if pathogens had predictable ecological patterns across the bovine respiratory and its association with BRD status.

In the validation trial, logistic models were also performed to determine the features related with pathogens (F1, F3, F7, F11, F4, and F13) (Figure 5). The abundance of these features with over representation in morbid animals produced similar results to the test trial. Compared to the test trial, F1, F3 and F4 in BAL were predictive under healthy control $_{arrival}$, BRD $_{arrival}$ and BRD. F7 and F11 showed a similar predictive pattern with the test trial. However, F13 is not predictive of any niches.

Investigation of the relationship between microbial interactions and BRD status

Network analysis using the SparCC algorithm showed that microbial interaction was associated with spatial dynamics and BRD status (Figure 6). The respiratory microbiota in healthy calves had more complex interactions compared with BRD animals, which reflects the microbial dysbiosis caused by disease. All three niches showed more complex microbial associations at feedlot arrival (both healthy control $_{arrival}$ and BRD $_{arrival}$) than the onset of BRD (BRD and Control). The potential probiotics including (F29, F31, F34 and F37) associated with other microbiota were observed in healthy cattle.

In healthy control $_{arrival}$, *Mycoplasma* (F3) in NS and BAL was negatively correlated with *Microbacteriaceae* (F6). For BRD arrival calves, F4 and F7 positively correlated with each other in the NPS and lung microbiome, and F7 positively associated with F11 in BAL. When cattle were diagnosed with BRD, F1 positively correlated with F7 in NS, and positively associated with F3 in BAL samples. Regarding Control animals, F7 and F11 had positive correlation in NPS, while F7 was negatively correlated with F4. Furthermore, in the lung community of Control calves, although F4, F1, F3 and F7 showed positive correlation with F1 and F3 as nodes, *Enterobacteriaceae* (F9) was negatively correlated with F7 and F3. Collectively, in animals under BRD $_{arrival}$, Control and BRD, more positive interactions between pathogens (F1, F3, F4, F7 and F11) were observed, while simpler correlation among pathogens were even observed in BRD calves.

In the validation trial, we found F4 positively correlated with F11 having negative correlation with F29 in the NPS community at healthy control $_{arrival}$ status, while F4 and F11 had negative correlation with *Succinivibrionaceae* (F20) having high abundance in BAL. In BRD $_{arrival}$, while F3 and F11 had positive correlation in the NS niche, *Enterobacteriaceae* (F9) had

negative correlation with F11 and F3 in lung, and F20 showed a negative association with F4. When calves were clinically diagnosed with BRD, greater associations between pathogens were also observed in all three niches. In NS, F1, F3 and F11 had positive correlation, while F4 showed negative correlation with F3 and F11. Moreover, in the lung microbiome of BRD calves, positive correlation between F1 and F11 was again observed.

Discussion

This is the first study to investigate whether the spatial correlation of the bovine respiratory microbiome is affected by BRD status. The spatial correlation of the microbiome between the adjacent niches was high in BRD calves compared to healthy calves. A larger proportion of microbiota in BRD lung were derived from their upper airway. The BRD pathogens in BRD lung were predictive by using their abundance in communities of the corresponding upper airway tracts. The synergies between pathogens (species associated with *Mycoplasma*, *Mannheimia*, *Pasteurella* and *Histophilus somni*) were found in respiratory niches of diseased calves, while more complex interactions among commensal microbiota were found in healthy calves. These findings indicate that the microbial immigration from the upper airway to the lung is associated with BRD status.

The different physiological and biochemical difference among niches leads niche-specific community structures, while the microbial immigration within the airway is influenced by mucus communication, ventilation, microbial aspiration and coughing (Dickson et al., 2016; Lanaspa et al., 2017). Therefore, the spatial dynamics of the respiratory microbiota demonstrated signature patterns in healthy calves compared to diseased animals. In healthy cattle, the respiratory tract could prevent pathogens' colonization by trapping them in mucous and cilia and subsequently removing them physically (Love et al., 2014). When cattle are diagnosed with BRD, the

physiology of the respiratory tracts, including breathing rhythm, ventilation, immunity and lung lesions, are changed (Buczinski et al., 2018; Thompson et al., 2006), which may lead to changes in the spatial dynamic from the upper airway to the lung. Our results confirmed that the shifts among NS, NPS and BAL based on beta diversity were affected by BRD status, which provides evidence that airway disease could influence the microbial immigration within the bovine respiratory tracts. High correlation between adjacent niches was found, while this correlation was affected by disease in our study as BRD had stronger associations between the upper and lower respiratory tract. In healthy calves, the dynamic mucociliary transport transfers mucus and its contents (infectious agents and bacteria etc.) toward the nasopharynx or oropharynx to be swallowed (Erickson et al., 2015), which could generate weaker correlation between the nostril and lung. However, for BRD calves, the increased amount of mucus secretion, and damaged mucociliary escalator and cilia increase the chances that pathogens and other bacteria enter the lung (Dickson et al., 2014b; Rossi et al., 2019). Together, this community structure shifts of microbiome from the upper airway to the lower respiratory tract and is influenced by disease, and additionally, the nasal microbiome in BRD calves is more prone to move towards the lung compared to healthy cattle.

To quantify the microbiome in the upper airway that contributed to the lung, Source Tracker was utilized. Our data showed that lung microbiota had approximately 9% and 38% derivation from the nasal and nasopharyngeal microbiome, respectively. A study found that lung microbiome in patients with chronic obstructive pulmonary disease had 21% oral source and 8% nasal source (Pragman et al., 2018). This human study did not measure the source of the nasopharyngeal microbiota, while our study confirmed that less lung microbiota were directly from the nostrils compared from the nasopharynx. Another study confirmed that the

nasopharyngeal microbiota is most similar to the lung bacteria in healthy calves, and may be the primary source of the lung microbiota (McMullen et al., 2020). Overall, the main source for lung microbiome is from nasopharynx, while nasal microbiome is the major source for nasopharyngeal community. Furthermore, our analysis found that the BRD lungs had more microbiota were from their nasopharyngeal community, and the nasal microbiome contributed to the NPS more in BRD calves than compared to the contribution in healthy calves. This may be explained by the changes in the respiratory environments by BRD. Source Tracker uses a Bayesian approach to assess the proportion of the microbiome in 'sink' from the possible source environments, and assigns an 'unknown' source environment. In our data, the BRD lung had approximately 49% (in the test trial) and 79% (in the validation trial) microbiota from the upper airway (nostrils and nasopharynx). Other proportions may come from the oral cavity or the selective growth of bacteria. In future BRD studies, oral and the upper aerodigestive microbiomes should be considered as sources for the lung.

To measure the movements of BRD pathogens from the nostrils to lung, a logistic model was performed. This model has been successfully used to predict the relationship between stool and the intestinal microbiome (Yasuda et al., 2015). Our study found that BRD pathogens in BRD lung could be predicted by their upper airway, which provide insight that pathogens' immigration in BRD calves with dysbiosis is predictive. However, pathogens in healthy lungs could not be predictive, which might be due to complex microbiota-host or microbiota-microbiota interactions. New algorithms are necessary to explore the spatial dynamics of the respiratory microbiome in healthy subjects. In addition, the associations of pathogens between nasal and lung microbiome are highly correlated with BRD status, suggesting nasal swabs as an easy and convenient sampling approach for BRD researches.

Microbial interactions during BRD development were reported using the signature microbiota for BRD (Zeineldin et al., 2020). In this study, we found microbial interactions were related with BRD using the top bacterial features. Complex interactions among commensal bacteria were found in healthy respiratory tracts especially in healthy control $_{arrival}$. The commensal signature-microbiota for healthy calves including *Jeotgalicoccus psychrophilus*, *Bifidobacterium pseudolongum*, *Corynebacterium* and *Streptococcus luteciae* were classified as nodes to interact with other bacteria. A property of commensal microbiota is anti-pathogens (Kadiu, 2020). A study found *Streptococcus* could inhibit the biofilm formation capacity of pathogens of the upper respiratory tract (Bidossi et al., 2018). The pathogens, including *Mycoplasma* (e.g. *M. bovis*), and *Mannheimia*, can form biofilms and cause bovine pneumonia (Boukahil et al., 2016; Chen et al., 2018). In our results, the indirectly negative association between *Mycoplasma* and *Streptococcus* was observed in healthy $_{arrival}$ calves, suggesting the ability of commensal microbiota to defend against pathogens. This inhibition can be achieved through the competition of nutrients, modification of local environments, and regulation of mucosal inflammation (Zeineldin et al., 2019). For BRD $_{arrival}$ and clinically healthy control calves, the microbial interactions within each niche were simple and high frequencies of BRD pathogens were observed, such as positive correlation among *Mannheimia* (F4), *Mycoplasma* (F1, F3, and F7), *Histophilus somni* (F11) and *Pasteurella* (F13). The increases of pathogens' interaction and decreases of commensal microbiota could be the reason leading to BRD infections, although the association between BRD development synergy of these common pathogens remains unclear. Metagenomics studies found the main species in BRD nasopharynx included *M. bovis* and *Mannheimia haemolytica* (Gaeta et al., 2017), and the pathogens, such as *Histophilus somni*, *Mannheimia haemolytica*, and *M. bovis*, in the lower respiratory tract (Klima

et al., 2019). Their cooperation is a possible way to increase incidences of BRD. From the latent stage (BRD $_{arrival}$) to BRD appearance, reduction in the interaction of commensal bacteria and increases in the positive correlation between BRD pathogens (e.g. *Mycoplasma* species, *Histophilus somni*) were found in the BRD airway, suggesting that microbial community structure in BRD calves were under a state of dysbiosis by increasing the abundances and frequencies of pathogens. In addition, poor interactions in BRD calves were found. Increases of BRD pathogens in diseased cattle have been observed in previous studies (Holman et al., 2015; Johnston et al., 2017; Lima et al., 2016; Timsit et al., 2018). Therefore, in healthy calves, spatial dynamics of the microbiota are complex and associated with other bacteria in local environments, while pathogens abundant in BRD calves could cooperate with each other, cause disequilibrium and enter the lung to cause infection. It provides further evidences that spatial movements of airway microbiota is related with BRD development, and BRD pathogens in BRD lung could be predicted by their abundances in the BRD upper airway.

Conclusion

In this study, we confirmed that BRD status could affect the spatial dynamics of the respiratory microbiome. High correlations between the nasal and nasopharyngeal microbiome and between the nasopharynx and lung were found, and the correlation co-efficiency increased with BRD development. Additionally, the proportion of BRD lung microbiome were higher from its upper airway compared healthy lung from its upper respiratory tract using the Source Tracker algorithm. BRD pathogens could enter the lung in BRD cattle more easily than healthy cattle. Microbial interactions within a healthy airway were more complex, while dysbiosis of BRD microbiome were abundant with pathogens incidence. Future studies need to be conducted to

determine the spatial shifts of the microbiome with selective growth of pathogens in local environments and BRD development.

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Tables and Figures

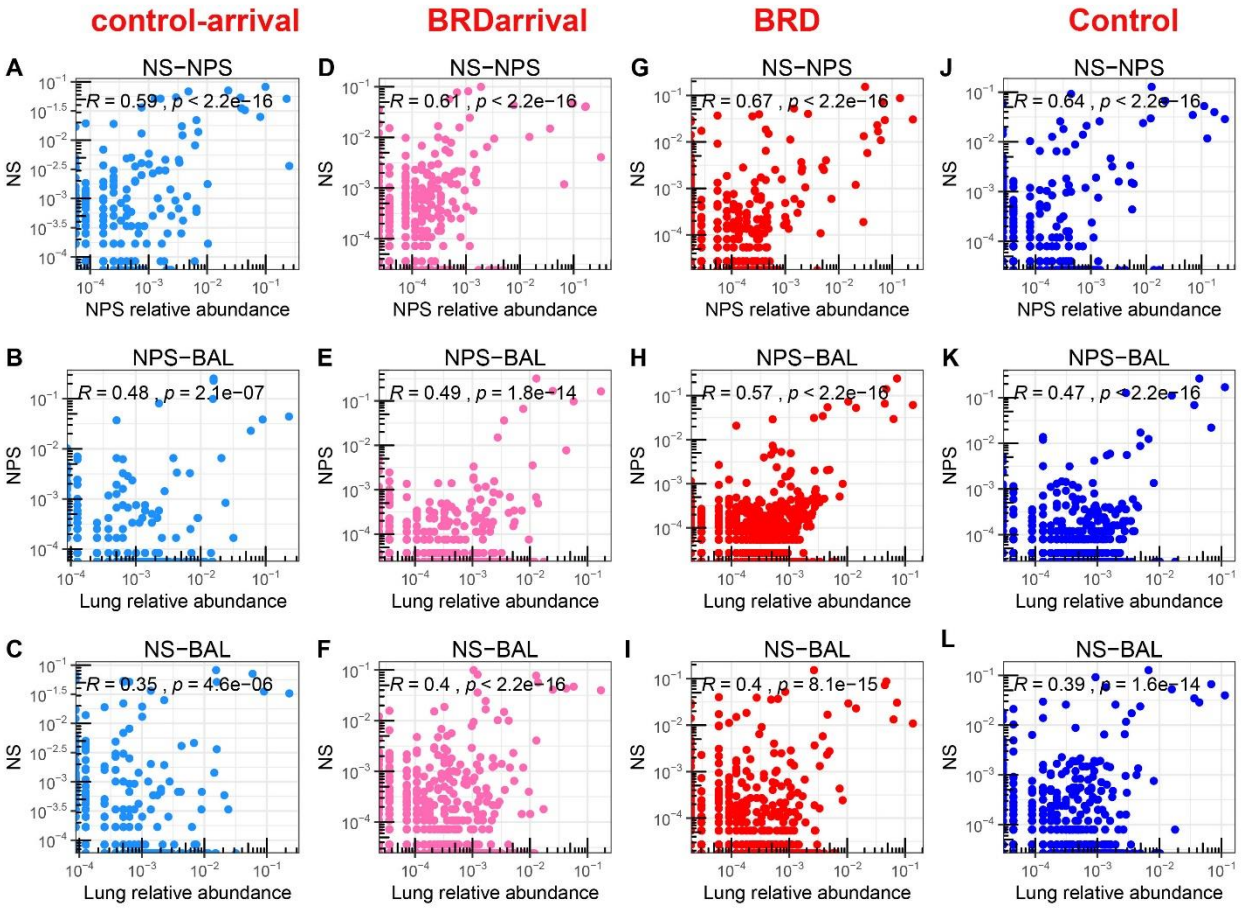

Figure 1 The correlation of the respiratory microbiome associated with BRD status in the test trial

Each dot corresponds to the average relative abundance of a feature across all animals for each of respiratory sampling niches (NS, NPS, and BAL). To measure correlation, Pearson's r was calculated based on features abundance of two niches. Marks on the y axis (horizontal lines) or x axis (vertical lines) margins represent bacterial features with zero measured abundance at one site but non-zero abundance at the other.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: health cattle samples were collected upon feedlot arrival; BRD $_{arrival}$: cattle developed BRD later and samples were collected upon feedlot arrival; Control: clinically healthy cattle samples were collected as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

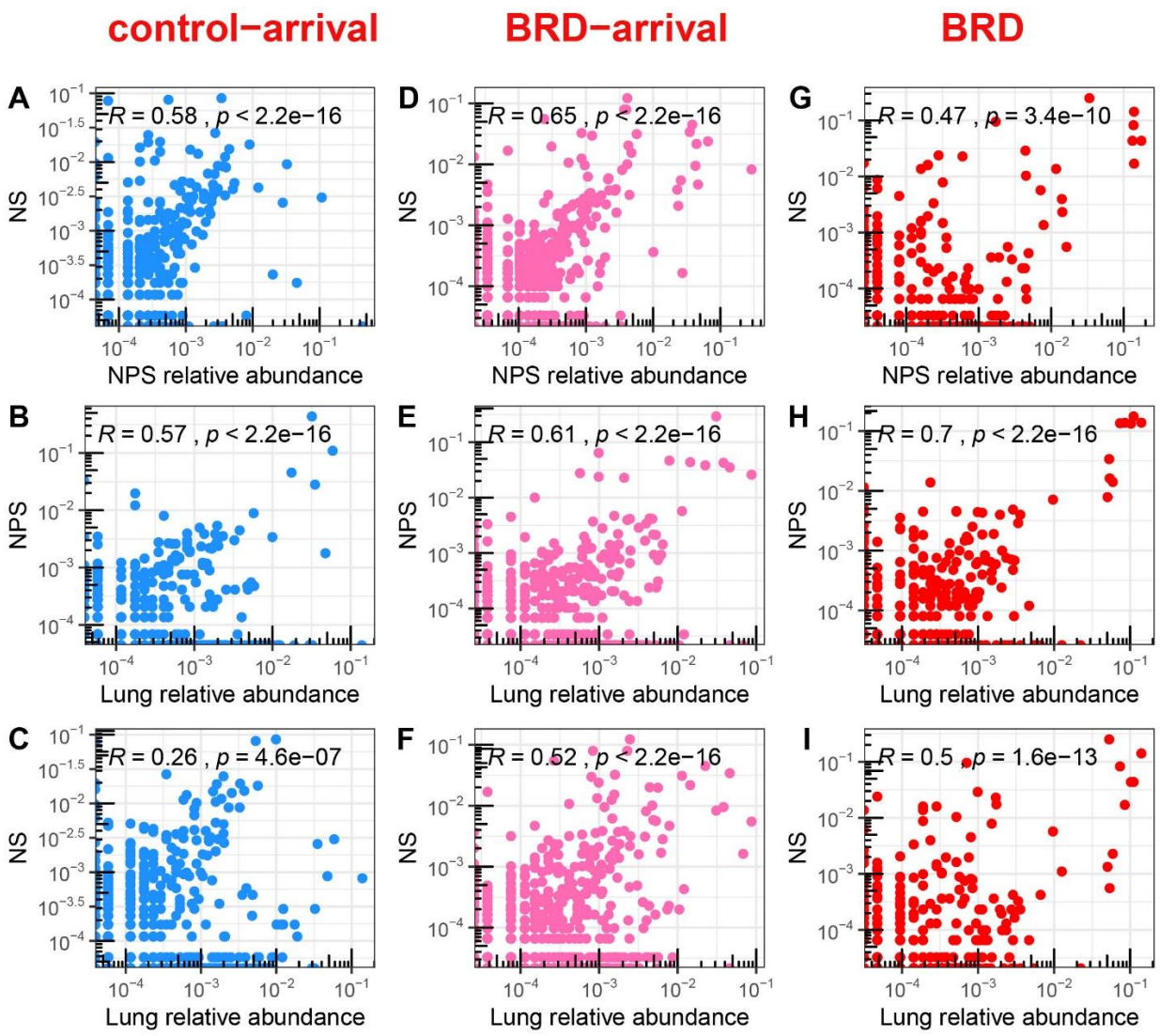

Figure 1 The correlation of the respiratory microbiome associated with BRD status in the validation trial

Each dot corresponds to the average relative abundance of a feature across all animals for each of the respiratory sampling niches (NS, NPS, and BAL). To measure correlation, Pearson's r was calculated based on features abundance of two niches. Marks on the y axis (horizontal lines) or x axis (vertical lines) margins represent bacterial features with zero measured abundance at one site but non-zero abundance at the other.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: health cattle samples were collected upon feedlot arrival; BRD $_{arrival}$: cattle developed BRD later and samples were collected upon feedlot arrival; Control: clinically healthy cattle samples were collected as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

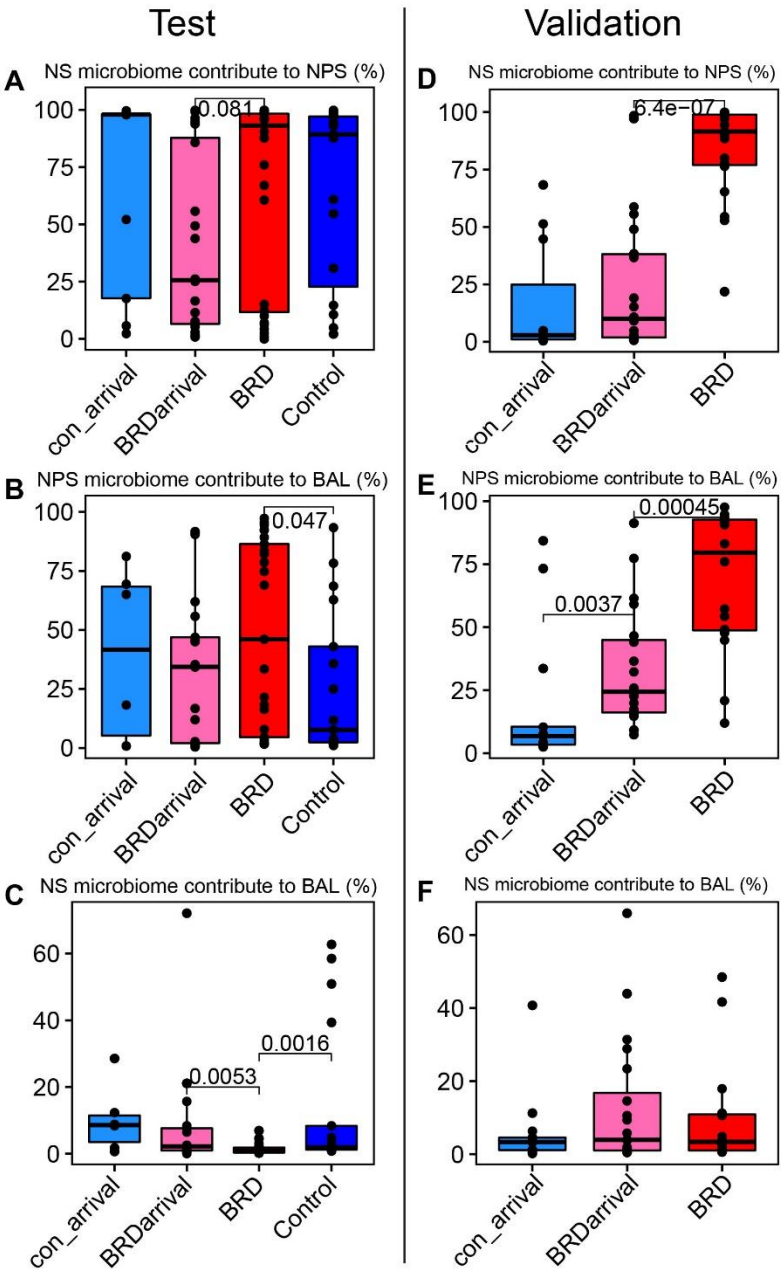

Figure 3 Source Tracker analysis of the contributions of the upper airway to lung

The left panel (A, B, and C) is for the test trial, while the right panel (D, E, and F) is for the validation trial.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: health cattle samples were collected upon feedlot arrival; BRD $_{arrival}$: cattle developed BRD later and samples were collected upon feedlot arrival; Control: clinically healthy cattle samples were collected as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

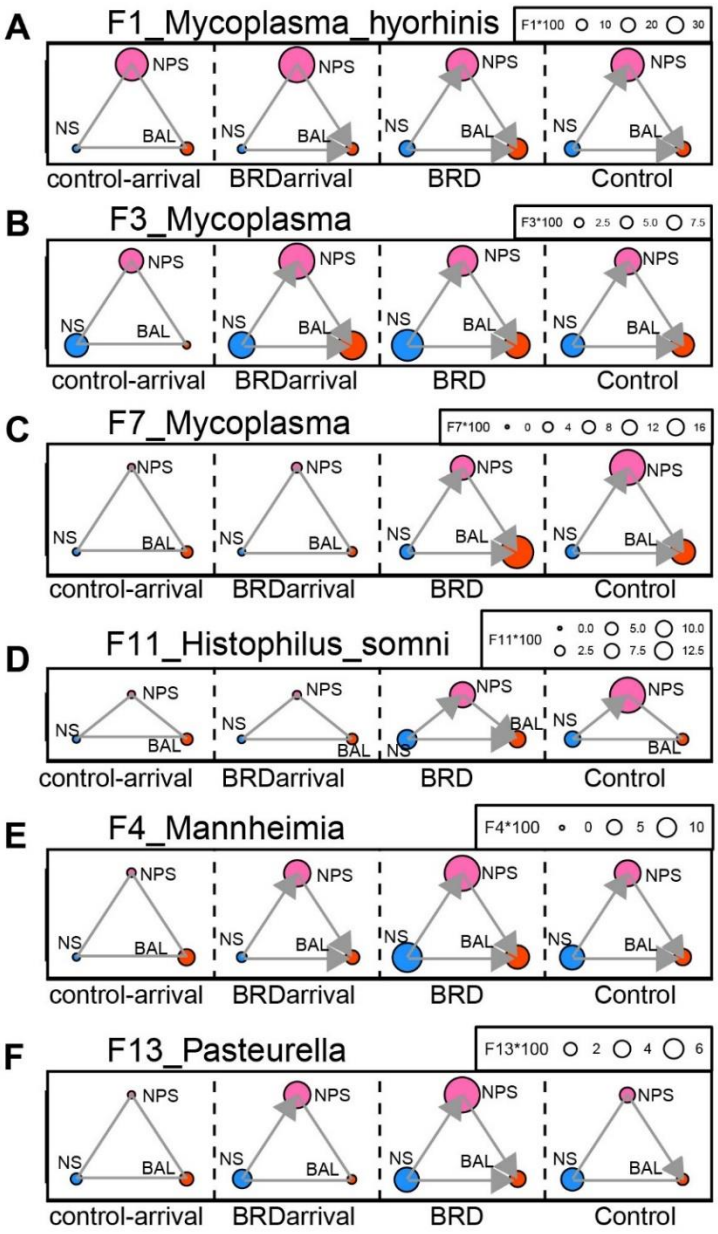

Figure 4 A logistic regression model of bacterial pathogens site enrichment and immigration through the bovine respiratory tract in the test trial

Points on each triangle point represent biogeographic sites in the airway, and point size corresponds to mean relative abundance at each niche across all animals for each feature. Adjacent sites with significant non-zero β (indicating that relative abundance of this feature at one site can predict that of the other) are connected with an arrow. Solid lines without an arrow indicate non-significant β values.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: health cattle samples were collected upon feedlot arrival; BRD $_{arrival}$: cattle developed BRD later and samples were collected upon feedlot arrival; Control: clinically healthy cattle samples were collected as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

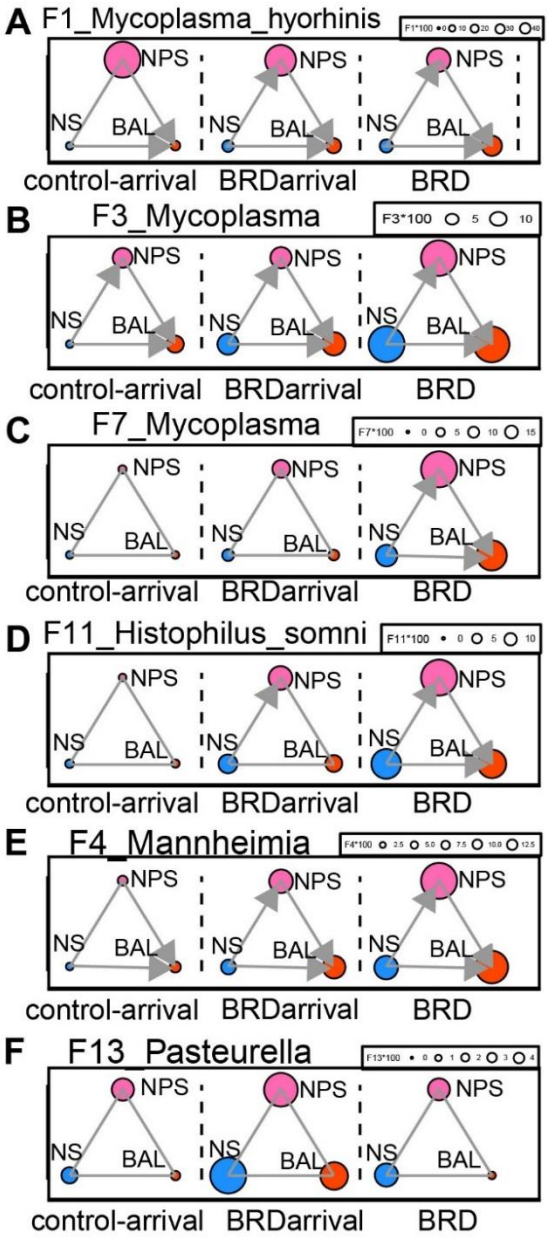

Figure 5 A logistic regression model of bacterial pathogens site enrichment and flow through the bovine respiratory tract in the validation trial

Points on each triangle point represent biogeographic sites in airway, and point size corresponds to mean relative abundance at each niche across all animals for each feature. Adjacent sites with significant non-zero β (indicating that relative abundance of this feature at one site can predict that of the other) are connected with an arrow. Solid lines without an arrow indicate non-significant β values.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: health cattle samples were collected upon feedlot arrival; BRD $_{arrival}$: cattle developed BRD later and samples were collected upon feedlot arrival; Control: clinically healthy cattle samples were collected as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

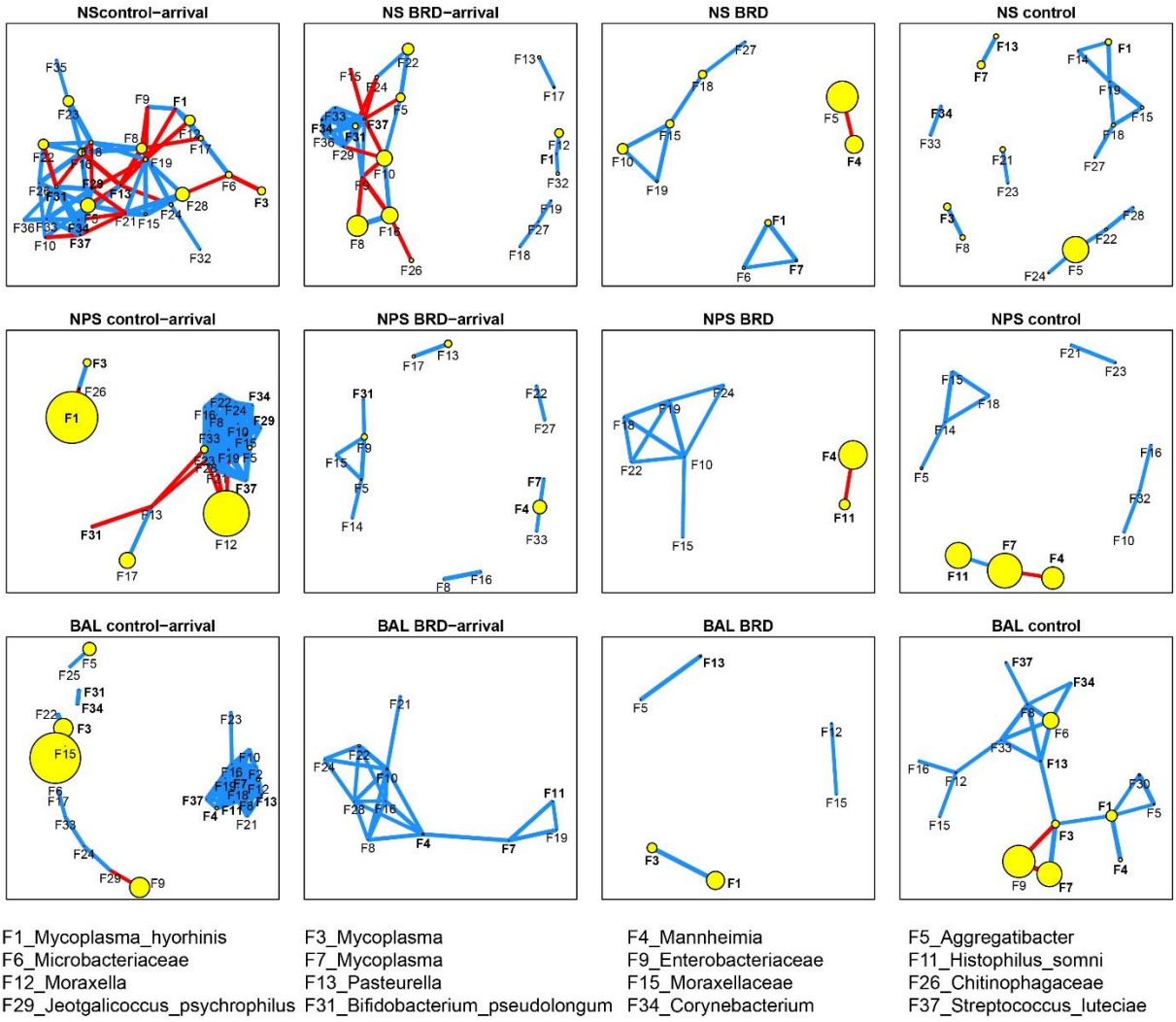

Figure 6 Network analysis of microbial interaction in different sampling niches of calves under different healthy statuses in the test trial

The yellow circles represent relative abundance of each bacterial feature. The blue lines mean positive correlation between bacterial features, while red lines represent negative correlation. The line width represents the coefficients.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: health cattle samples were collected upon feedlot arrival; BRD $_{arrival}$: cattle developed BRD later and samples were collected upon feedlot arrival; Control: clinically healthy cattle samples were collected as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

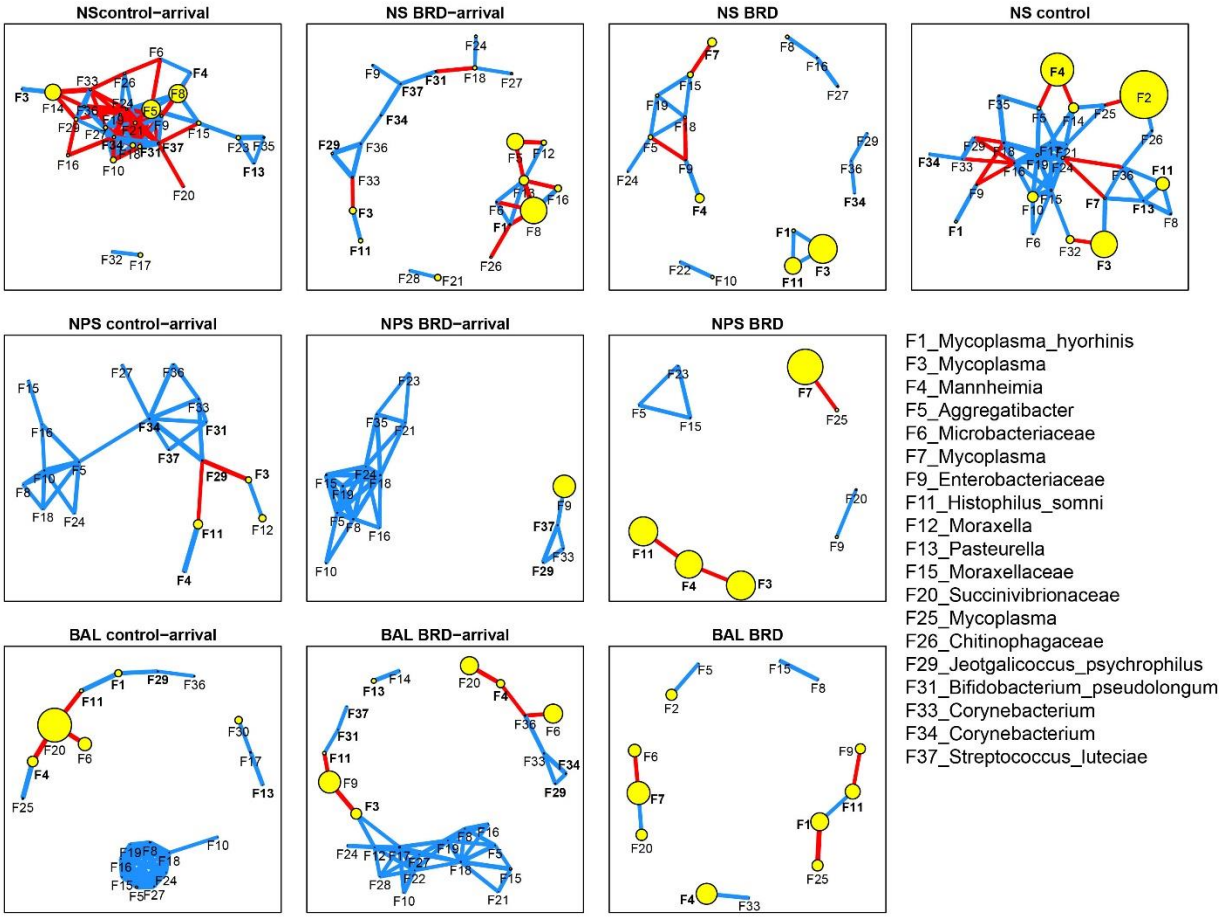

Figure 6 Network analysis of microbial interaction in different sampling niches of calves under different healthy statuses in the validation trial

The yellow circles represent relative abundance of each bacterial feature. The blue lines mean positive correlation between bacterial features, while red lines represent negative correlation. The line width represents the coefficients.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: health cattle samples were collected upon feedlot arrival; BRD $_{arrival}$: cattle developed BRD later and samples were collected upon feedlot arrival; Control: clinically healthy cattle samples were collected as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

Supplemental Materials

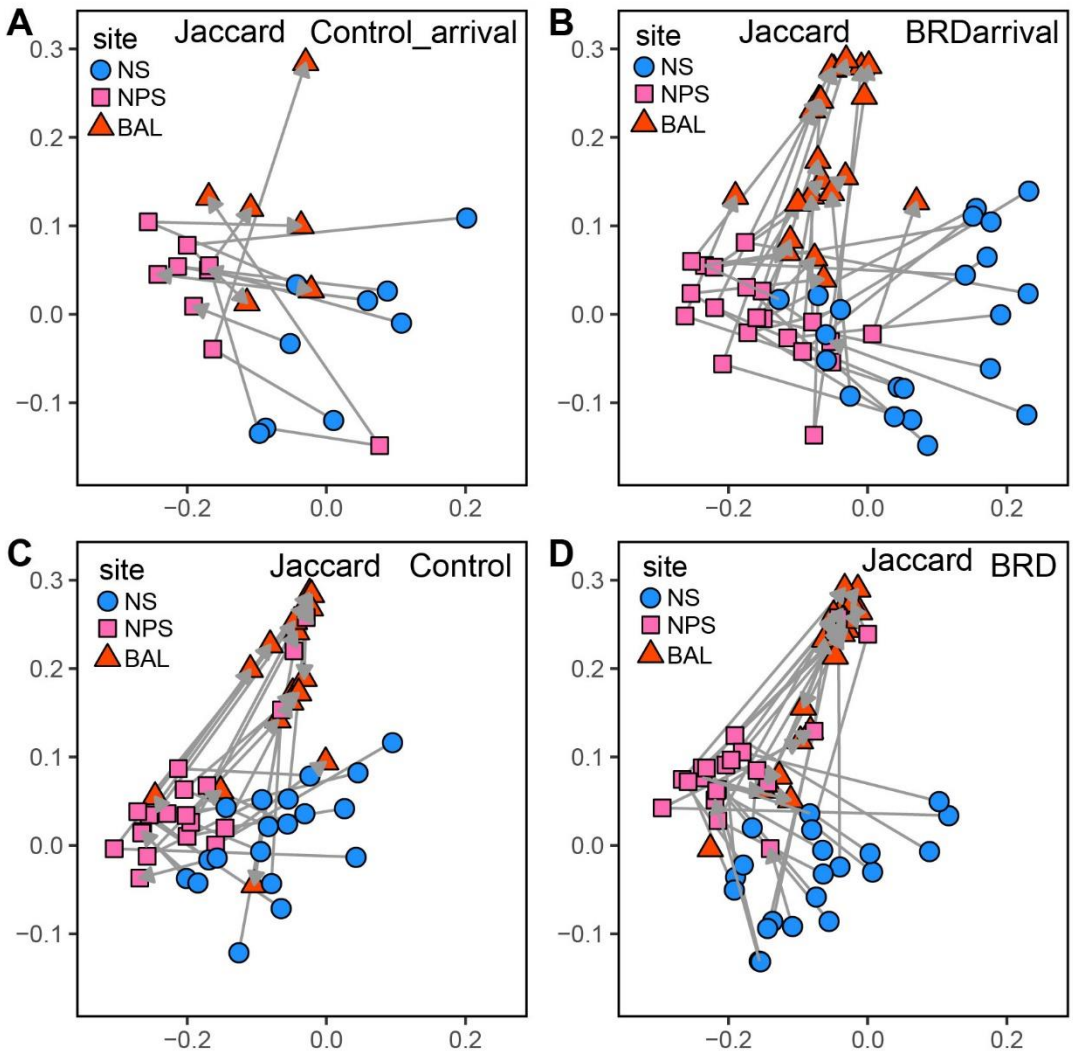

Figure S1 PCoA plot (Jaccard distance) of biogeography microbiome for individual calves in the test trial.

Same animals were connected with gray lines. Sampling niches were distinguished by shape (circle, square, diamond respectively) and color (dodgerblue, hot pink and red, respectively).

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: health cattle samples were collected upon feedlot arrival; BRD $_{arrival}$: cattle developed BRD later and samples were collected upon feedlot arrival; Control: clinically healthy cattle samples were collected as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

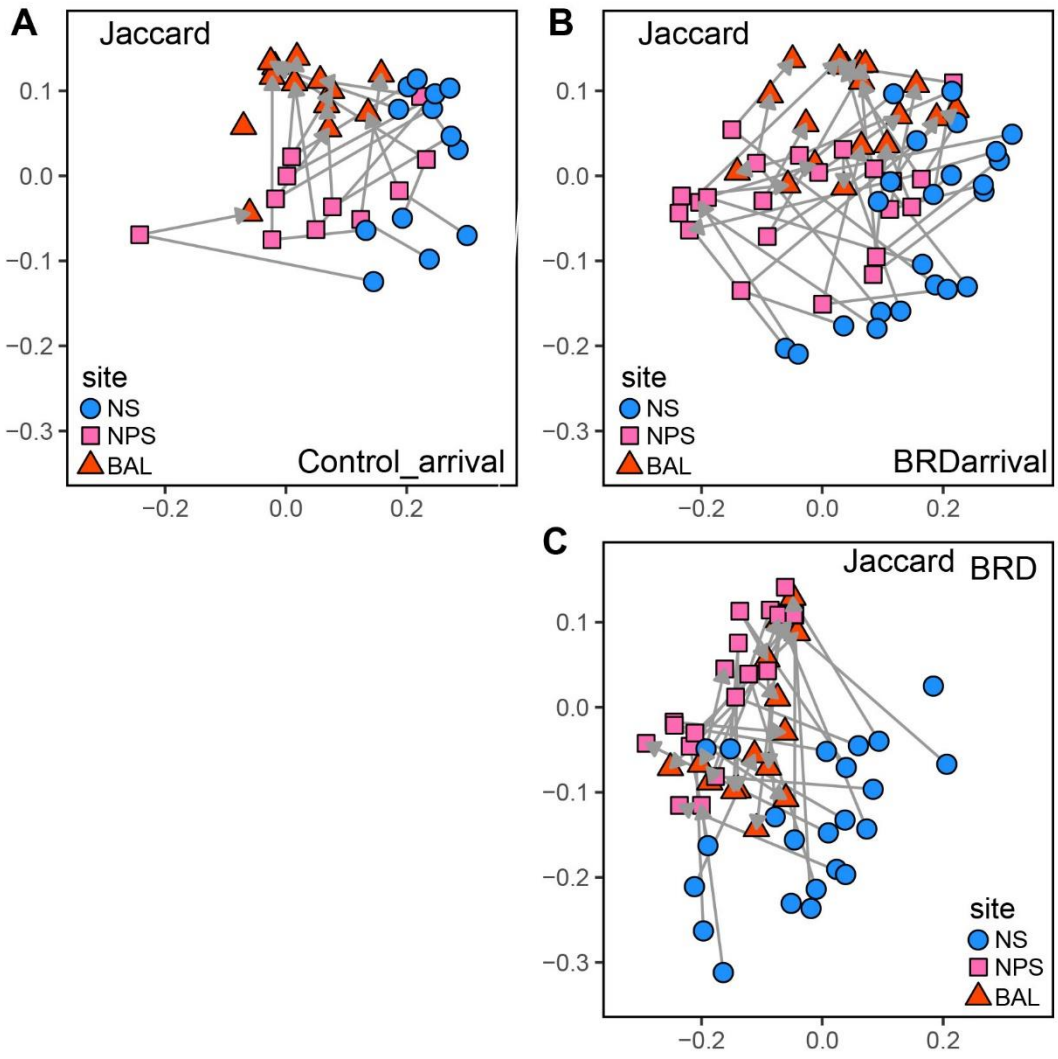

Figure S2 PCoA plot (Jaccard distance) of biogeography microbiome for individual calves in the validation trial.

Same animals were connected with gray lines. Sampling niches were distinguished by shape (circle, square, diamond respectively) and color (dodgerblue, hot pink and red, respectively).

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: health cattle samples were collected upon feedlot arrival; BRD $_{arrival}$: cattle developed BRD later and samples were collected upon feedlot arrival; Control: clinically healthy cattle samples were collected as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

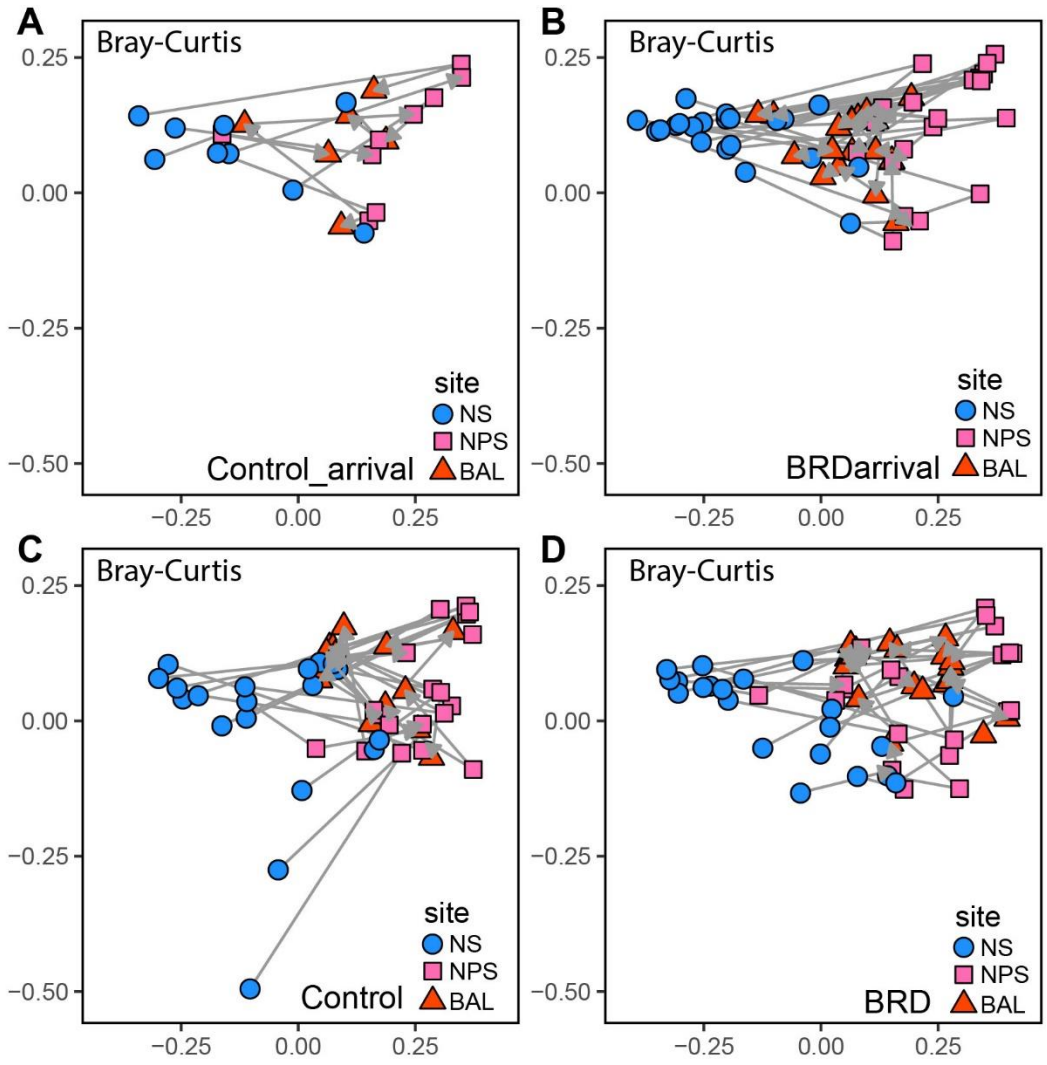

Figure S3 PCoA plot (Bray-Curtis distance) of biogeography microbiome for individual calves in the test trial.

Same animals were connected with gray lines. Sampling niches were distinguished by shape (circle, square, diamond respectively) and color (dodgerblue, hot pink and red, respectively).

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: health cattle samples were collected upon feedlot arrival; BRD $_{arrival}$: cattle developed BRD later and samples were collected upon feedlot arrival; Control: clinically healthy cattle samples were collected as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

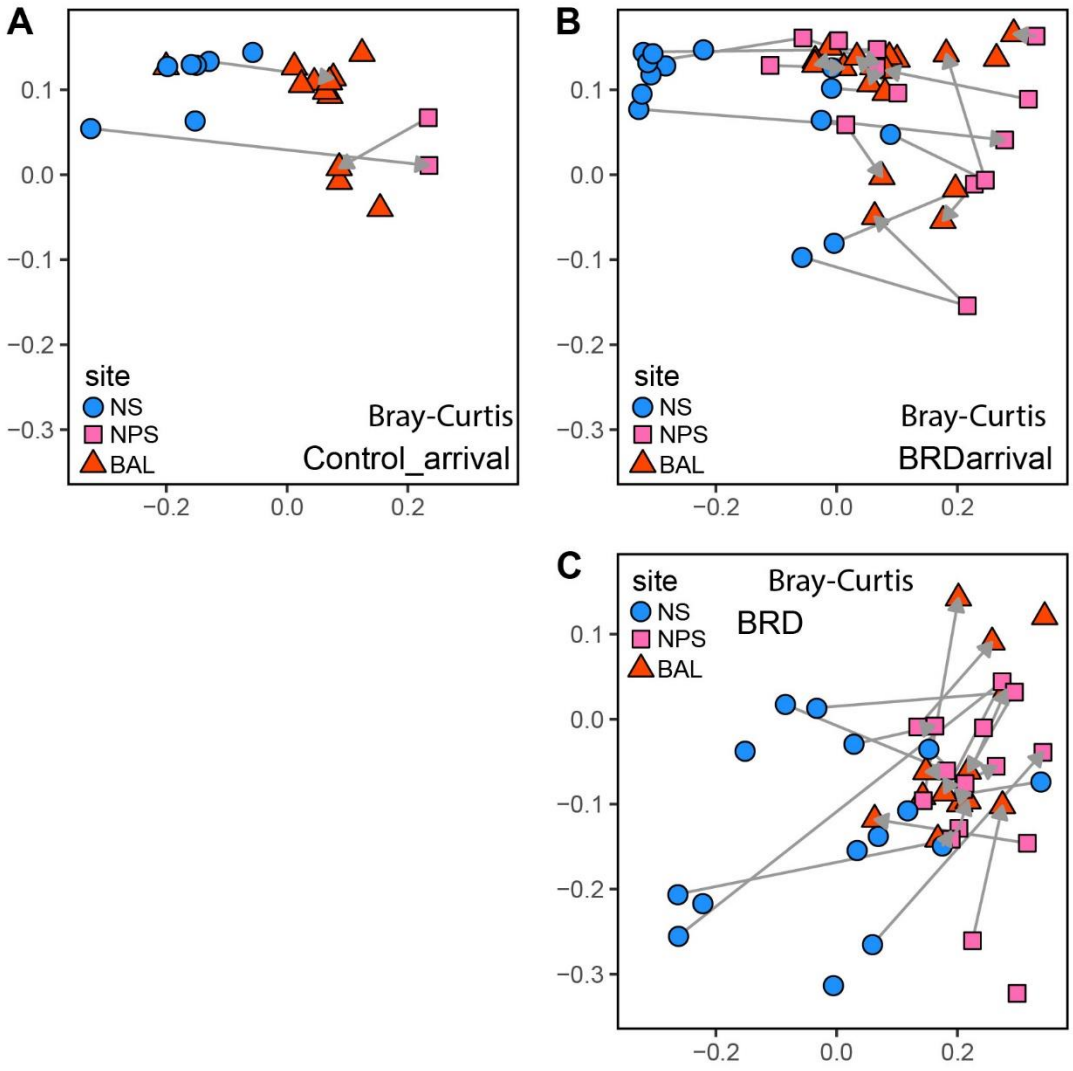

Figure S4 PCoA plot (Bray-Curtis distance) of biogeography microbiome for individual calves in the validation trial.

Same animals were connected with gray lines. Sampling niches were distinguished by shape (circle, square, diamond respectively) and color (dodgerblue, hot pink and red, respectively).

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: health cattle samples were collected upon feedlot arrival; BRD $_{arrival}$: cattle developed BRD later and samples were collected upon feedlot arrival; Control: clinically healthy cattle samples were collected as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

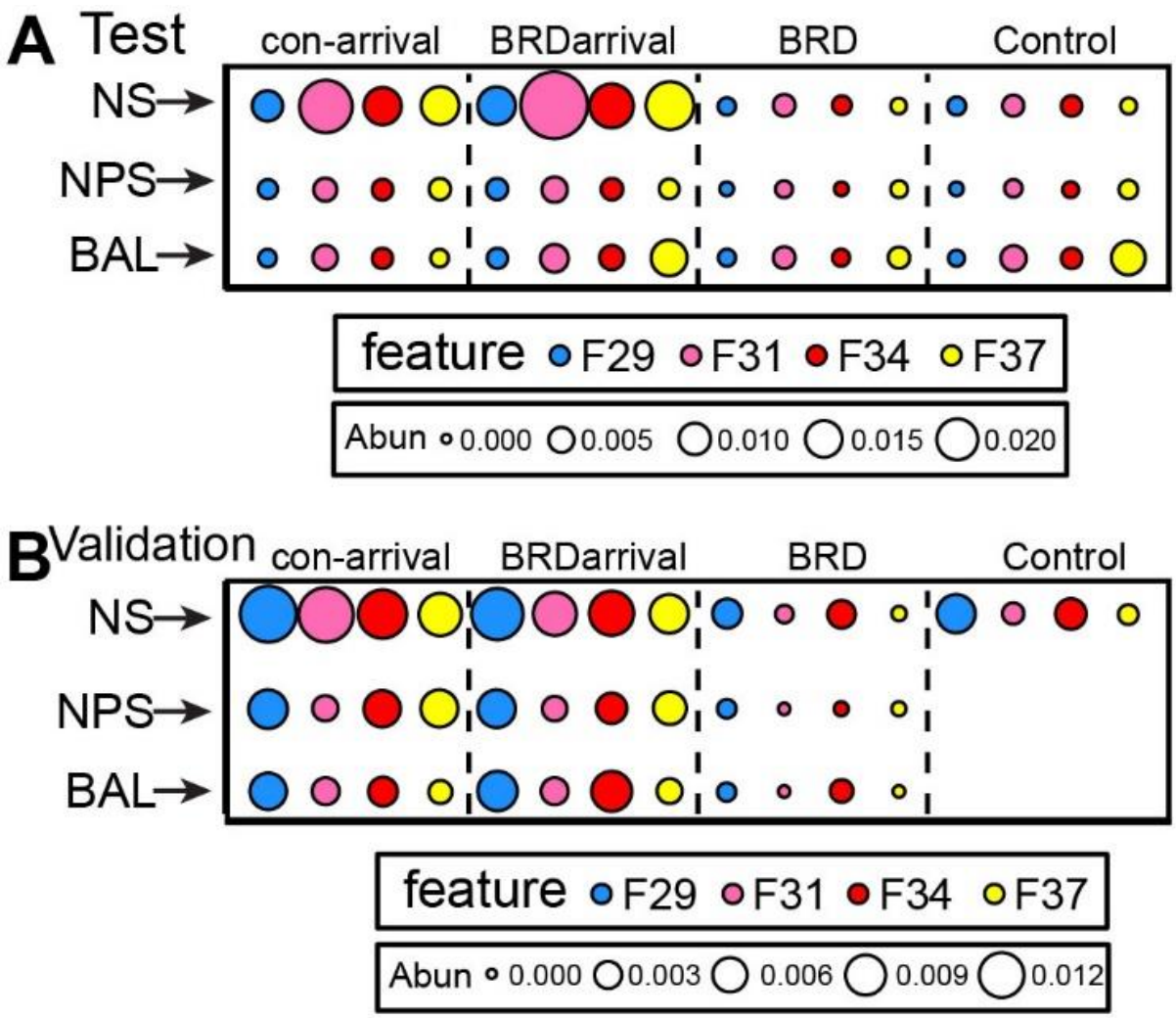

Figure S5 The spatial distribution of the major commensal microbiota of *Jeotgalicoccus psychrophilus* (F29), *Bifidobacterium pseudolongum* (F31*), Corynebacterium* (F34), and *Streptococcus luteciae* (F37)

These microbial distributions in test (A) and validation (B) trials are plotted under different healthy statuses. Color was used to differentiate feature ID. Points point size corresponds to mean relative abundance (Abun in figure legend) at each niche across all animals for each feature.

Abbreviations: Nasal swabs (NS), nasopharyngeal swab (NPS) and bronchoalveolar lavage (BAL). control $_{arrival}$: health cattle samples were collected upon feedlot arrival; BRD $_{arrival}$: cattle developed BRD later and samples were collected upon feedlot arrival; Control: clinically healthy cattle samples were collected as controls for BRD cattle; BRD: cattle were diagnosed with BRD.

Chapter VI. Conclusion

Investigation of the respiratory microbiome benefits to understand the pathogenesis of bovine respiratory disease (BRD). In this dissertation, we firstly characterized the biogeography of the bovine airway microbiome in an animal test trial and a second animal (validation) trial. Significant community structure and composition among nasal, nasopharyngeal, and lung microbiomes were found. The signature microbiota for each niche were identified by random forest algorithm, and common biomarkers for sampling niches from both the test and validation trials were found. Bacterial microbiota associations could be found at both the upper and lower respiratory tracts, and strong correlations between adjacent niches within the airway were confirmed. Our study indicates that nasal swabbing could be a potentially effective approach to investigate the association between the lung microbiome and bovine respiratory disease.

We next sought to determine the relationships between the respiratory microbiota and BRD. By characterizing the microbiota of healthy control $_{\text{arrival}}$ and BRD $_{\text{arrival}}$ calves using a machine-learning based approach, we found signature microbiota could predict the onset of BRD with high accuracy across nasal swabs, nasopharyngeal swabs, and lung samples. Significant shifts in microbial structure from arrival to the onset of BRD were found across the three niches in BRD and healthy control calves with increasing time in the feedlot. The commensal microbiota including *Corynebacterium* (F34) and *Bifidobacterium pseudolongum* (F31) were consistently decreased in both the upper and lower airway, while consistently temporal increases in the relative abundance of several taxa associated with BRD pathogens (e.g. *Mycoplasma* (F7), and *Histophilus somni* (F11)) were also observed. Moreover, the signature microbiota identified by random forest could differentiate BRD and healthy control calves and can be used as biomarkers to diagnose BRD.

We found that the spatial movement of the microbiome within the bovine respiratory tract is associated with BRD status. The correlation between the upper and lower airway microbiota increased in BRD calves compared to other healthy statuses, and a high proportion of BRD lung microbiota were derived from the upper airway compared to healthy calves was also observed. The BRD pathogens in BRD lungs were predicted by using their abundance in BRD subjects' nostrils and nasopharynx, but similar strategies could not be used to predict pathogens in lungs of healthy calves. Complex interactions among the commensal microbiota were found in healthy respiratory tracts, while communities under a state of dysbiosis as well as the interaction of abundant BRD pathogens, were observed as complex in BRD calves.

Appendix

Appendix 1. IACUC approval letter for the first animal trial

UNIVERSITY OF ARKANSAS

Office of Research Compliance

MEMORANDUM

TO: Jiangchao Zhao

FROM: Craig N. Coon, Chairman

DATE: 9/22/15

SUBJECT: IACUC Approval

Expiration Date: Sep 21, 2018

The IACUC has approved your protocol # 16024 "Characterization of the bovine respiratory and gastrointestinal tract microbiome". You may begin work immediately.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond the indicated expiration date, you must submit a newly drafted protocol o prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem
cc: Animal Welfare Veterinarian

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199

Appendix 2. IACUC approval letter for the second animal trial

UNIVERSITY OF ARKANSAS

Office of Research Compliance

To: Jiangchao Zhao
Fr: Craig Coon
Date: April 5th, 2019
Subject: IACUC Approval
Expiration Date: April 4th, 2022

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # **19071:** *Investigation Of The Respiratory Microbiome and Metatranscriptome of Beef Cattle And Their Influences On Bovine Respiratory Disease.*

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond April 4th, 2022, you must submit a newly drafted protocol prior to that date to avoid any interruption. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/tmp

19071