Understanding the Links between the Respiratory Microbiota and Pneumonia in Dairy Calves

By

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DEDICATION

This dissertation is dedicated to my partner Tyson, who has supported me and the unit through late nights of endless writing and on-call shifts, my parents, and to all my Madisonian running friends that have become my second family.

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DISSERTATIONAL ABSTRACT

UNDERSTANDING THE LINKS BETWEEN THE RESPIRATORY MICROBIOTA AND PNEUMONIA IN DAIRY CALVES

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Thesis objectives were to evaluate the association between changes in the respiratory microbiota and lung consolidation (LC) in dairy calves, investigate the effect of *Pasteurella multocida* infection and ampicillin therapy on the upper and lower respiratory tract (LRT) microbiota, and to investigate the host response to Bovine Respiratory Disease (BRD) by identifying differentially expressed (DE) microRNAs (miRNA) in lung tissue.

In a field study of dairy calves, relative abundance (RA) of *Pasteurella* spp. within the nasopharyngeal (NP) microbiota was associated with LC. Decreased NP diversity was associated with clinical BRD. Increased NP diversity prior to challenge with *P. multocida* was associated with decreased clinical BRD severity post challenge. Ampicillin was associated with a higher NP RA of *Pasteurella* spp. and a decrease in the NP RA of *Prevotella* spp. following the challenge; ampicillin had no effect on the NP diversity nor on the LRT microbiota. Dominant taxa within the NP and LRT of calves challenged with *P. multocida* and healthy calves included

gastrointestinal tract (GIT)-associated bacteria (e.g. *Prevotella* spp., *Ruminococcaceae*). Increased NP RA of *Prevotella* spp. was associated with a delay in LC following the challenge.

Host lung miRNAs from a subset of challenged calves and a group of healthy calves were sequenced; 33 bovine DE miRNAs were identified. Upregulation of bta-miR-21-5p and bta-miR-146b were identified in challenged lung, which have previously been linked to virus-induced LC in sheep and *Mycobacterium bovis* infection in cattle, respectively. The majority of predicted target gene functional categories included zinc and metal binding proteins, which may indicate an effect on immune gene expression.

Overall, NP microbial diversity in dairy calves appears to be an important defense strategy against the development of clinical BRD. The GIT microbiota may also impact the respiratory microbiota and LC development. Host miRNA DE was identified in the lung tissue of pneumonic calves; the impact on target genes may influence the host response to changes in the respiratory microbiota. How the immune system is affected by miRNA DE in the context of BRD requires further investigation.

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CHAPTER 1. LITERATURE REVIEW

1.1 INTRODUCTION

Bovine Respiratory Disease (BRD) is a complex, ongoing obstacle to managing animal health in the dairy industry. Specifically, BRD is a predominant cause of morbidity and mortality in pre-weaned dairy calves (Urie et al., 2018). The clinical phenotype of BRD includes upper and/or lower respiratory tract disease; etiopathogenesis involves synergy between commensal bacteria, viruses, environmental stressors, and suppression of host immune capacity (Yates, 1982). Viruses associated with BRD pathogenesis include type 1 bovine herpesvirus, parainfluenza virus type 3, bovine respiratory syncytial virus, and bovine viral diarrheal virus; bovine adenovirus, bovine coronavirus, and bovine influenza may also be pathogenic (Dawson et al., 1965; Jericho and Langford, 1978; Baker et al., 1986; Hartel et al., 2004; Ng et al., 2015). Bacteria primarily associated with BRD pathogenesis include *Pasteurella multocida*, *Mannheimia hemolytica*, *Histophilus somni*, *Mycoplasma bovis*, and *Trueperella pyogenes* (Jericho and Langford, 1978; Ames et al., 1985; Angen et al., 2009; Dagleish et al., 2010). These bacteria also normally colonize the nasopharynx and tonsils and can be isolated from the lower respiratory tract of clinically healthy animals (Allen et al., 1991; Ackermann et al., 2010). The upper respiratory tract commensal bacterial population and BRD pathogenesis are inextricably linked (Ackermann et al., 2010; Caswell, 2014). Characterization of the respiratory microbiota and how fluctuations may increase the risk of or protect against BRD are not well understood (McMullen et al., 2019).

Pathology due to BRD manifests as rhinitis, tonsillitis, laryngitis, tracheitis, and/or suppurative to fibrinosuppurative bronchopneumonia (Jericho and Langford, 1978; Confer et al., 1996; Dagleish et al., 2010). A common sequela of BRD in dairy calves is otitis media and/or otitis interna (Lima et al., 2015); upper respiratory tract disease has been linked to inflammation of cranial nerves VII and VIII and peripheral vestibular structures (Jensen et al., 1983; Van Biervliet et al., 2004). External clinical signs associated with BRD and otitis include depressed appetite, dull attitude, pyrexia, spontaneous and induced coughing, nasal and ocular discharge, and dropped ear position (McGuirk et al, 2008). Calves with subclinical BRD harbor lung lesions consistent with bronchopneumonia; however, they do not display corresponding external clinical signs associated with BRD (Leruste et al., 2012; Ollivett et al., 2015). Lung lesions are diagnosed antemortem using diagnostic imaging techniques, such as ultrasonography; the amount of lung consolidation detected correlates accurately with the severity of non-aerated lung found at necropsy due to BRD pathogenesis (Rabeling et al., 1998; Ollivett et al., 2015).

Clinical and subclinical BRD in dairy calves have both been associated with reduced average daily gain during the preweaning period (Cramer and Ollivett, 2019). Clinical BRD diagnosed 4 or more times prior to a heifer's first lactation has been associated with a doubling of odds that a heifer would be culled prior to completion of her first lactation (Bach, 2011). Lung consolidation specifically, diagnosed at least once prior to 8 weeks of age, was associated with decreased first lactation milk production (Dunn et al., 2018). Additionally, lung consolidation diagnosed at weaning, was associated with a decreased likelihood of pregnancy and an increased risk of culling prior to initiation of first lactation (Teixiera et al., 2017b). The economic impact of BRD is far-reaching due to both short- and long- term consequences. Immediate treatment of cost per calf has been estimated at $10 - $16 (Gorden and Plummer,

2010); however, this cost is likely compounded by the aforementioned negative impact on average daily gain and future heifer productivity (Virtala et al., 1996; Dunn et al., 2018; Cramer and Ollivett, 2019).

The national producer-reported incidence rate of clinical BRD has been estimated at 12% of dairy calves prior to weaning; approximately 14% of overall pre-weaned calf mortality (5% of calves from 104 US operations surveyed) was due to clinical BRD (Urie et al., 2018). Results of a recent epidemiological study, which included 11,470 pre-weaned Holstein calves from 6 herds in California, showed an average pre-weaning clinical BRD prevalence of 8% (range: 3% - 13%) over a one year time period (Dubrovsky et al., 2019). Incidence of BRD may vary widely by region, as results of one study, which included 1,141 calves in New York and Georgia, showed a 61% (range: 21% - 90%) incidence rate of pre-weaning clinical BRD (Heins et al., 2014). Results from another recent study in California, which evaluated 4,636 calves from 100 herds in 3 regions of the state, showed that prevalence of clinical BRD was different by region, by breed in some regions of the state, and changes in prevalence were associated with differences in calving, colostrum, and feeding management practices (Karle et al., 2019). The impact of BRD is likely larger than has been reported, as aforementioned studies did not account for subclinical BRD in case definitions. Less than half of dairy calves with lung consolidation had been previously identified and treated by producers from 13 Montreal dairies enrolled in a cohort study (Buczinski et al., 2014). Results of one study of a Wisconsin herd showed a 67% prevalence of subclinical BRD in pre-weaned dairy calves (Ollivett and Buczinski et al., 2016). Additionally, a recent study performed in Ohio showed that although 26% of calves were diagnosed with clinical BRD, 60% of calves had subclinical BRD based on ultrasonography findings (Cramer and Ollivett, 2019). Therefore, inclusion of subclinical BRD in case

definitions illustrates the substantial impact of BRD as an obstacle to maintaining calf health on dairy farms.

The diversity of pathogens involved as well as variability in host immune capacity complicate BRD prevention and therapeutic strategies (Caswell, 2014; Neibergs et al., 2014). Specific challenges to BRD management in dairy calves include adequate colostrum management (Windeyer et al. 2014; Karle et al., 2019), increased utilization of group housing in the dairy industry (Kung et al., 1997; Jorgensen et al., 2017), inconsistent vaccine efficacy (Aubry et al., 2001; Windeyer et al., 2012; Ollivett et al., 2018), imperfect accuracy of BRD detection methods (Amrine et al., 2013; Buczinski et al., 2015; Ollivett et al, 2015), and balancing antibiotic stewardship with effective therapeutic and preventative antibiotic administration protocols (Forbes et al., 2011; Lhermie et al., 2016, 2017).

Colostrum source and passive transfer are important determinants of host immunity and thus, BRD susceptibility. A serum total protein less than 5.7 g/dl was associated with a higher risk of BRD in the first 5 weeks of life (Windeyer et al. 2014). Additionally, calves fed pooled colostrum or colostrum replacer, compared with their dam's colostrum, were at a higher risk of BRD (Karle et al., 2019). Although group housing, compared with individual housing, has been associated with increased social interaction, improved solid feed transition and improved postweaning weight gain (Chua et al., 2002; Costa et al., 2015), it is also a risk factor for BRD (Svensson et al., 2003; Buczinski et al., 2018). Positive ventilation and poor microbiological air quality in calf housing are also risk factors for BRD (Lago et al., 2006; Urie et al., 2018). While it has been documented that intranasal and parenteral administration of multivalent, modified-live viral (MLV) vaccination induces protective immune responses in calves with circulating maternally-derived antibiotics (Zimmerman et al., 2006; Hill et al., 2012), results of studies

evaluating the impact of vaccination on morbidity and mortality due to BRD in dairy calves have been inconsistent. An early study found no effect of a modified-live *P. multocida* and *M. hemolytica* vaccine on calf morbidity or average daily gain despite a significant increase in serum titers (Aubry et al., 2001). Windeyer et al. (2012) found no effect of parenteral administration of a multivalent, MLV vaccine on the incidence of clinical BRD or mortality in pre-weaned dairy calves. Ollivett et al. (2018) found that intranasal administration of a multivalent, MLV vaccine decreased the risk of calves developing lung consolidation; however, no effect was found on the risk of calves developing external clinical signs of BRD.

Along with the difficulty of inconsistent protection by vaccination programs, diagnosis of BRD in complicated by a lack of an antemortem gold standard test (White and Renter, 2009; Buczinski et al., 2015). Physical examination, specifically thoracic auscultation, is minimally sensitive for detecting lung consolidation (Buczinski et al., 2014). Clinical scoring systems offer an objective systematic approach to screening for BRD cases (McGuirk and Peek, 2014; Love et al., 2014), which increases the accuracy of clinical BRD detection on farms (Buczinski et al., 2014). A Bayesian latent class analysis found that clinical scoring, utilizing methods of McGuirk and Peek (2014), had a sensitivity of 62% (95% credible interval: 48-76%) and specificity of 74% (credible interval: 65-83%) for BRD diagnosis (Buczinski et al., 2015). Importantly, however, cases of subclinical BRD are missed by clinical scoring systems (Ollivett et al., 2015; Buczinski and Ollivett, 2016). Lung ultrasonography has been shown to have high sensitivity and specificity for detecting lung consolidation: 94% (95% CI: 69-100%) and 100% (95% CI: 64-100%), respectively (Ollivett et al., 2015). Another study that performed a Bayesian latent class analysis showed that ultrasonography had a 79% (95% credible interval: 66.4-90.9%) sensitivity and 94% (95% credible interval: 65-83%) specificity for diagnosing

BRD (Buczinski et al., 2015). However, the ultrasound technique utilized in Buczinski et al., 2015 omitted scanning the most cranial aspect of the right lung lobe; this lobe is a common location for lung consolidation in dairy calves (Ollivett and Buczinski, 2016) and was included in the ultrasonography methods of Ollivett et al., 2015. Ultrasonography requires specialty veterinary training, and therefore, subclinical cases will likely go undetected by producers without veterinary oversight. Additionally, unless clinical scoring is implemented consistently, late detection of cases may confound BRD management efforts by producers.

In addition to the challenge of BRD detection, determination of a primary bacterial pathogen, and subsequently, making antibiotic treatment decisions is convoluted (Van Driessche et al., 2017). Sixty-nine percent of deep nasopharyngeal swabs (DNPS) and 21% of bronchoalveolar lavage (BAL) samples from pre-weaned calves with clinical BRD and confirmed lung consolidation resulted in polymicrobial cultures (Van Driessche et al., 2017). Additionally, 82% of DNPS and 39% of BAL samples from healthy calves with no evidence of lung consolidation resulted in polymicrobial cultures (Van Driessche et al., 2017). Bacteria isolated from cases and controls in the study by Van Driessche et al., (2017) exist as commensals within the respiratory tract during mucosal homeostasis (Zeineldin et al., 2019). Given the difficulties of primary pathogen detection and concern of late disease detection, effective treatment interventions continue to be an obstacle for managing BRD on dairies.

Metaphylaxis, treating an entire group of animals at high risk of exposure and infection with an antibiotic prior to individual animals being diagnosed with disease, is commonly used in feedlots to decrease the bacterial load within the respiratory tract within an entire group of animals (Schunicht et al., 2002; Nickell and White, 2010). The goals of metaphylaxis are to improve group health and performance by treating both subclinical and clinical BRD cases

(Nickell and White, 2010). It has been shown that metaphylaxis with a long acting macrolide decreased the risk of clinical BRD and/or otitis, but not clinical BRD alone, in 795 dairy calves in New York (Teixeira et al., 2017a). Although metaphylaxis has positive benefits in commingled calf raising facilities, there is also a risk of exposing a potentially large group of animals to antibiotics even when not required (Lhermie et al., 2016). An alternative therapeutic approach is treating individual animals when clinical signs of BRD are detected (Heins et al., 2014). However, this method is considered a "late approach" treatment strategy, as pulmonary damage occurs rapidly following experimental challenge with *Pasteurella multocida*; clinical signs of respiratory disease at the point of pulmonary insult may be minimal except for an elevation in rectal temperature (Dagleigh et al., 2010; Holschbach et al., 2019). Results from a recent study showed that calves treated with a lower dose of a fluoroquinolone antibiotic 2-4 hours following pyrexia, after an experimental *Mannheimia hemolytica* challenge, had a more rapid elimination of bacteria from BAL samples than calves treated 35-39 hours after developing pyrexia (Lhermie et al., 2016). Ampicillin therapy for 3 days, administered within 24 hours of an experimental *Pasteurella multocida* challenge, had short term beneficial effects on lung consolidation in dairy calves; lung consolidation improved and then re-occurred within 14 days following the challenge (Holschbach et al., 2019). In a randomized field study, calves treated with tulathromycin and calves in a placebo group were equally likely to have lung consolidation at weaning, despite antibiotic-treated calves having a decreased risk of worsening lung consolidation following treatment at their first BRD event (Binversie et al., 2017). Results from another recent study showed that calves treated early for BRD with a fluoroquinolone antibiotic, based on persistent ruminal hyperthermia or increased rectal temperature, had a higher proportion of first line and relapse treatments compared to calves treated later for BRD (i.e.

pyrexia and external clinical signs of BRD were present) (Lhermie et al., 2017). Early intervention with antibiotics may not completely eliminate pulmonary infection, and thus may lead to a higher amount of antibiotic usage on farms unnecessarily.

Utilization of antibiotics as a control strategy for BRD also has the potential to facilitate antimicrobial resistance by exposing bacteria to selective pressure (Portis et al., 2012). According to a national survey of producers, ninety-five percent of calves with BRD are treated with antibiotics; primary antibiotics utilized include macrolides and florfenicol (USDA, 2018). In 10 Belgian beef herds, in which 4 nasal swabs were taken every 6 months for 2 years, there was a significant association between the resistance profile of isolated *Pasteurellaceae* and herd treatment incidences (Catry et al., 2015). Additionally, results from a retrospective study evaluating the antimicrobial susceptibility patterns of *Pasteurella multocida*, *Mannheimia hemolytica*, and *Histophilus somni* from US and Canadian beef cattle isolates from 2000 - 2009 showed an increase in resistance to macrolides tilmicosin and tulathromycin (Portis et al., 2012). A high prevalence of tulathromycin resistance has also been shown in *Mannheimia hemolytica* and *Pasteurella multocida* isolates in samples from the lower airway of feedlot steers and heifers (Timsit et al., 2017a). Oxytetracycline treatment also increased the proportion of the tetracycline resistance gene within the NP microbiota of feedlot calves (Holman et al., 2018). Along with potentially impacting treatment efficacy in dairy calves, antibiotic therapy for BRD management may have an impact on public health, as macrolides are listed as critically important antibiotics in human medicine (WHO, 2019). The contribution of agricultural usage of antibiotics in the development of human antimicrobial resistance; however, is not well-defined (Prescott, 2014). Increased patterns of resistance were identified in fecal *Escherichia coli* isolates from pre-weaned dairy calves exposed to systemic antibiotics; however, resistant populations were

transient and were only observed if calves were treated within a short time interval of fecal sample collection (Berge et al., 2005; Foutz et al., 2018). Although no direct link has been established between antibiotic resistance in dairy calves and human antimicrobial resistance, the possibility exists for environmental contamination of farms with resistant isolates to medically important antibiotics, such as macrolides.

The population health challenge of BRD management, selective pressure exerted on the respiratory microbiota by metaphylaxis, and recent antimicrobial resistance genes identified in *Pasteurellaceae* have encouraged research into further understanding of interactions between the host and respiratory microbiota to facilitate alternative preventative and therapeutic strategies, such as implementing respiratory probiotics. Advances in next-generation sequencing (NGS) have allowed for investigations into the role of the respiratory microbiome and/or microbiota in BRD pathogenesis as well as the host response, specifically transcriptional regulation, to changes in the microbiota. In contrast to microbiome analysis, which infers the whole collection of genomes within the environment, microbiota studies identify bacterial taxa by sequencing a target gene within the environment. The 16S rRNA gene is often selected as the identifier because of its ubiquity within the eubacteria kingdom, which facilitates primer design, and the variable regions of the gene differ between organisms with distinct genomes, with differences equivalent to the evolutionary distance between them (Morgan and Huttenhower, 2012). This literature review and subsequent research chapters will focus on elucidating attributes of the respiratory microbiota associated with environmental factors, development of BRD and antibiotic therapy, as well as how the host may regulate transcription in response to shifts in the microbiota.

1.2 DEFINING THE RESPIRATORY MICROBIOTA

It has been well established from previous microbiological studies that colonization of the NP with a diverse bacterial population is crucial for homeostasis and innate immune function (Ackermann et al., 2010; Caswell, 2014). The upper respiratory tract bacterial community dynamics may involve priming the immune system as well as significant microbe-microbe interactions, which lead to synergistic or exclusion effects that influence the pathogenesis of respiratory disease (Esposito and Principi, 2018). Early culture-dependent studies revealed nasal mucus from healthy and pneumonic dairy calves cultured *P. multocida*, *M. hemolytica*, *Neisseria* spp., beta-hemolytic *Streptococcus* spp., and *Micrococci* spp. (Magwood et al., 1969). Well-known bacterial species within the NP that consist of serotypes commonly identified as commensal bacteria and can function as pathogens in cattle include *M. hemolytica*, *P. multocida*, and *Histophilus somni* (Ackermann et al., 2010).

As recently as the early 2000's, it was thought that the lungs were sterile in healthy animals; bacteria in the lower respiratory tract were postulated to exist only in diseased states, secondary to commensal bacteria residing in the upper respiratory tract proliferating from stress, viral infection, cold temperatures or genetic predisposition (Ackermann et al., 2010). However, in an early study, samples from the trachea, bronchial lymph nodes and lung of healthy feedlot cattle had previously been shown to culture *Bacillus* spp., *Streptococcus* spp., *Streptomyces* spp., *Micrococcus* spp., and *Pseudomonas* spp. (Collier and Rossow, 1964). Corstvet et al. (1973) also isolated *H. somni*, *P. multocida*, *M. hemolytica*, and *Mycoplasma* spp. from the trachea of healthy cattle. Additionally, in feedlot calves, bacteriologic culture revealed similar populations of *Mycoplasma* spp., *H. somni*, *Streptomyces* spp., *Neisseria* spp. and *Bacillus* spp. in both the

NP and bronchoalveolar lavage fluid (BAL) (i.e. fluid aspirated from the lung lobe via access by the distal bronchus) of calves with BRD and healthy calves (Allen et al., 1991) (Figures 1.1,1.2). Culture-dependent studies provided evidence for further investigations using molecular techniques to describe the resident bacterial populations in the lower airways.

In a more recent study that combined culture-dependent methods and PCR, 59% of BAL samples from 56 healthy dairy calves cultured potentially pathogenic bacteria (e.g. *P. multocida*, *H. somni*, *M. hemolytica*, and *Trueperella pyogenes*), with 32% of those calves isolating high numbers of the organisms in pure culture or as dominating isolates in mixed culture (Angen et al., 2009). Earlier culture-dependent studies provided evidence that although NP commensal bacteria are involved in the pathogenesis of BRD, the lower respiratory tract contains populations of resident bacteria, derived from the upper respiratory tract, with a likely role in the development of bronchopneumonia. The increasing accessibility of NGS in the last decade has made it possible to illustrate the complexities within the microbiota of the respiratory system and describe previously unidentified or under-appreciated taxa that may have implications for risk of or protection from BRD.

It is now understood that the entire respiratory tract, including the healthy lung, hosts a diverse bacterial population that includes species with dual commensal-pathogen behavior; these species have been termed ‘pathobionts’ (Hauptmann and Schaible, 2016; Unger and Bogaert, 2017; Thomas et al., 2019). The external and internal factors that drive commensal and pathogenic states of pathobionts are not well-understood (Thomas et al., 2019). These pathobionts exist within specific niches of the respiratory tract with significant overlap (de Steenhuijsen Piters et al., 2015). The niches within the respiratory tract likely differ due to environmental abiotic factors, such as temperature, pH, oxygen levels, and epithelial cell type

(de Steenhuijsen Piters et al., 2015). Specifically, the upper respiratory tract has constant exposure from the external environment, which continuously shapes the habitat of species within the respiratory and gastrointestinal (GI) tracts (de Steenhuijsen Piters et al., 2015).

Interactions between the microbiota and environmental variables can be understood by utilizing ecological principles, such as species dispersal (i.e. movement of bacterial species across space), species diversification (i.e. creation of new species), environmental selection (i.e. differences in fitness among species) and ecological drift (i.e. random shifts in bacterial species abundance) (Vellend, 2010; Costello et al., 2012). How these phenomena affect biodiversity of the respiratory microbiota impacts ecosystem stability and function (de Steenhuijsen Piters et al., 2015), which therefore affects host immunity to pathogen invasion (Dillon et al., 2005). The respiratory microbiota is analogous to a contiguous ecosystem, which allows implementation of community ecological principles to analyze and further understand the diversity of the upper and lower respiratory microbiota and how external factors impact the stability of resident pathobionts.

Based on studies in human literature, colonization of the lung niche is thought to depend primarily on immigration and elimination from the upper respiratory tract in healthy individuals; the relative growth rates of resident bacteria are more likely to affect the lower respiratory microbiota in disease states (Bassis et al., 2015; Dickson et al., 2015). Results from studies in human medicine have shown that the bacterial community within the lung is more closely related to the oral cavity than the nares in healthy individuals; dispersal from the oral cavity to the lungs likely occurs via microaspiration and inhalation (Morris et al., 2013; Bassis et al., 2015). A recent model introduced to explain the interrelationships between the upper and lower respiratory tract microbiota is the adapted island model (Dickson et al., 2015). The model proposes that the

source community for the respiratory tract is the upper airway and in healthy individuals, the distal lung microbiota has decreased community richness and reduced similarity from the upper respiratory tract due to spatial distance (Dickson et al., 2015). This implies the upper respiratory tract has an advantage of pathogen colonization resistance due to increased resident bacterial diversity. Emphasizing therapies or prevention strategies that preserve upper respiratory tract diversity may provide new strategies for BRD management. Development of such strategies requires further understanding of the linked composition of the respiratory microbiota and the effect of environmental variables on pathobiont diversity within both the upper and lower respiratory tract.

1.3 ASSOCIATION BETWEEN ENVIRONMENTAL FACTORS AND THE BOVINE RESPIRATORY MICROBIOTA

1.3.1 ANATOMIC SITE: Anatomic site is an environmental variable that has been shown to impact the composition of the respiratory microbiota in human studies, as was described in section **1.2**. Understanding the variation in the microbiota within the upper respiratory tract itself is crucial for determining sampling techniques for diagnostics and for implementing therapeutics. The bacterial community of the paranasal sinuses (Figure 1.1) was recently evaluated in 99 clinically healthy beef cattle and 34 cattle submitted for post-mortem exam using culture and PCR (Murray et al., 2017). Results of this study showed that 83.5 % of all samples (94.9% of clinically normal and 50% of cattle submitted for necropsy) had no detectable microbes within the paranasal sinuses. The authors surmised that the respiratory epithelium in the paranasal sinuses may have little impact on the development of BRD given the

lack of microbiota detected; however, the study was limited by the lack of more sensitive sequencing technology to detect taxa in low abundance.

McDaneld et al. (2018) further evaluated the upper respiratory tract of feedlot cattle by comparing the microbiota of the nasal cavity to the nasopharynx (Figure 1.1) using NGS. The authors found there was no significant difference in Euclidean distance, a metric used to describe the ecological distance or difference in species composition, between sampling sites within the upper respiratory tract (McDaneld et al., 2018). A significant limitation of this study is the utilization of hypervariable regions V1-V3 as the target of their sequencing, as V2 has been found to have a large phylogenetic distance from the entire 16S rRNA gene (Yang et al., 2016), and the combination of V1-V3 may lead to higher sequencing error due to the increase in amplicon length (Kozich et al., 2013). The hypervariable regions used in the analysis may have affected the accuracy of the measured Euclidean distance between the nasal cavity and NP; however, the results of this study indicate the nasal cavity and NP are continuous in terms of species diversity within the microbiota.

Investigating the relationship between the upper and lower respiratory tract bacterial communities is vital for further understanding the role of the respiratory microbiota in BRD pathogenesis and for potential development of probiotic therapies. In a recent culture-dependent study, there was only fair to moderate agreement between isolation rates of *P. multocida*, *M. hemolytica*, *M. bovis* and *H. somni* on deep NP swabs (see Figure 1.1) and in BAL samples of beef and veal calves with clinical BRD (Van Driessche et al., 2017). Although a recent NGS study showed similarities in some of the genera identified in the upper and lower respiratory tract, the alpha diversity (i.e. a measure of diversity of taxa within each sample) and beta diversity (i.e. a measure of diversity of taxa between samples) of nasal swab and

transtracheal aspirate samples (i.e. fluid aspirate from distal trachea) were significantly different from one another in 28 weaned beef cattle, of which 8 had clinical BRD and 11 were healthy (Nicola et al., 2017). In a study with a larger sample size (n=60 mixed-breed beef steers with BRD and n=60 age- and breed- matched healthy controls), distinct metacommunities were observed in the NP and from the transtracheal aspirates of both steers with BRD and control steers (Timsit et al., 2018). Bacterial species diversity in the nasal swab and NP was significantly higher than the transtracheal aspirates in both studies, which is consistent with the adapted island model by Dickson et al. (2015).

The distinction between microbial community structure of the upper and lower respiratory tract microbiota was also affirmed when comparing the NP and BAL communities (Figure 1.1,1.2). Results from a small study (n = 8), which sampled clinically healthy Charolais feedlot calves, showed significantly different beta diversity between NP and BAL samples (Zeineldin et al., 2017a). Although alpha diversity was numerically higher in the NP samples compared to BAL samples, which is consistent with the adapted island model, it was not significantly different; this is in contrast to findings from Nicola et al., 2017 and Timsit et al., 2018. Interestingly, results of this study showed that the relative abundance (RA) of abundant genera in the NP were significantly correlated with the RA of bacteria in BAL samples (Zeineldin et al., 2017a). Additionally, the most prevalent phyla from the nasal cavity, the NP, and from the distal trachea include Proteobacteria, Actinobacteria, Bacteroidetes, Tenericutes, and Firmicutes (Holman et al., 2017; Nicola et al., 2017; Zeineldin et al. 2017a; Zeineldin et al., 2017b; Stroebel et al., 2018; Timsit et al., 2018; McMullen et al., 2019; Amat et al., 2019). These phyla were also prevalent in dairy calves (Gaeta et al., 2017; Lima et al., 2016; Maynou et al., 2019). These results further solidify that while the upper and lower respiratory tract

bacterial communities are distinct, there is likely a symbiotic relationship in healthy animals that confers resistance to the pathogen-like behavior of resident pathobionts in both feedlot cattle and pre-weaned dairy calves.

1.3.2 TIME: In addition to further understanding the effect of anatomic location on the composition of the respiratory microbiota, knowledge of the temporal stability of bacterial populations is essential for determining the feasibility of manipulating the microbiota for health purposes. Results from a recent study evaluating the rumen and small intestinal microbiota in dairy calves demonstrated significant changes in the RA of taxa from one week of age to 63 days of age (i.e. approximate age of weaning) (Dias et al., 2018). Age has also been shown to significantly affect the composition of the respiratory microbiota in mice (Krone et al., 2014). Longitudinal studies including both dairy calves and feedlot cattle have evaluated the upper respiratory microbiota within the pre-weaning and post-weaning timeframe, respectively.

Temporal variability was observed in the NP microbiota of 70 clinically healthy steers between entry into feedlot and 60 days after feedlot placement (Holman et al., 2015a). In that study, beta diversity was significantly different between time points and dominant genera at entry (*Pseudomonas* spp., *Shewanella* spp., *Acinetobacter* spp. and *Carnobacterium* spp.) were replaced by different genera (*Staphylococcus* spp., *Mycoplasma* spp., *Mannheimia* spp. and *Moraxella* spp.) at day 60. The results of Timsit et al. (2016a) showed a definitive shift in the NP community profile of 30 beef steers, which included changes in beta diversity and the RA of 24 genera, between weaning, feedlot entry and 40 days following entry. A significant shift in the NP beta diversity was also observed in 40 beef heifers during a ten day period of observation following feedlot entry (Timsit et al., 2017b). Significant variation in the NP microbiota composition of feedlot cattle over time after weaning was affirmed by McMullen et

al. (2018) and McDaneld et al. (2018). The most abundant genera in the NP of healthy dairy calves were *Mannheimia* spp., *Mycoplasma* spp., *Moraxella* spp., *Psychrobacter* spp., and *Pseudomonas* spp. at 3 days of age; significant increases in the RA of *Mannheimnia* spp., *Mycoplasma* spp., and *Pseudomonas* spp. occurred within the first 2 weeks of life (Lima et al., 2016). Temporal instability of the NP microbiota indicates that potential manipulation of the microbiota through therapeutics will likely require frequent dosing. Additionally, interpretation of the respiratory microbiota in calves depends on accurately characterizing age group and production class.

1.3.3 TRANSPORT: The variable of time often includes stressful events such as weaning, transport and diet changes that may contribute to the temporal instability of the respiratory microbiota. Stress due to weaning and transport decreases innate host defenses (Caswell, 2014), which affects the ability of the NP microbiota to restrict bacterial overgrowth and infection (Timsit et al., 2016a). Secreted catecholamines can influence the composition of the respiratory microbiota directly by affecting the growth and expression of virulence factors of resident pathobionts (Lyte, 2014). Timsit et al. (2016a) found a rapid significant change in NP beta diversity in 48 hours following weaning and transport of 30 steers. An accelerated shift in NP microbial composition, specifically species richness, was also observed in 14 beef heifers 2 days following transport (Holman et al., 2017). Results from a more recent paper also showed the NP microbiota became more diverse with an increase in species richness 14 days following transport to an auction market and feedlot in 13 clinically healthy steers (Amat et al., 2019). In contrast, results from a larger study that evaluated the NP and transtracheal aspirates of 60 beef heifers before and after transportation and commingling at auction demonstrated no significant effect on the microbiota composition (Stroebel et al., 2018). All heifers in that study were

administered a long-acting macrolide (tildipirosin) parenterally at feedlot arrival, which may have negated the effect of transportation on the NP and lower respiratory tract microbiota (Stroebel et al., 2018). Overall, changes to the respiratory microbiota due to external stressors appear to happen peracutely and mostly increase the diversity of the NP microbiota. Further longitudinal studies are needed to determine the long-term effects of stress on microbial diversity and associated risk of BRD.

1.3.4 DIET: Diet changes in the beef production system (from backgrounding to feedlot arrival) and within the dairy industry (starter consumption or feeding replacer versus waste milk in calves) have the potential to affect the respiratory microbiota. The microbiota within the oral cavity is closely associated with the NP (Figure 1.1) and approximates the lower respiratory microbiota in humans (Morris et al., 2013; Bassis et al., 2015). Changes in the GI microbiome have been linked to chronic inflammatory respiratory disorders in humans via the gut-lung axis of the immune system (Frati et al., 2019). Results from a recent study investigating the effects of dietary selenium on the NP microbiota showed significantly different beta diversity between beef calves not supplemented and calves supplemented with a high oral dose of selenium (Hall et al., 2017). A recent study by Maynou et al. (2019) examined the effect of milk diet (pasteurized waste milk vs. milk replacer) on the nasal microbiota and fecal microbiota of 40 dairy calves. The results of the study showed no difference in the diversity metrics in the fecal or nasal microbiota and a higher RA of *Histophilus somni* and *Streptococcaceae* in the nasal microbiota of calves fed milk replacer (Maynou et al., 2019). The authors of that study hypothesized that subtherapeutic antibiotic concentrations within pasteurized waste milk may have contributed to the decreased RA of taxa in the nasal microbiota; however, the effect was not evident in the fecal microbiota. The GI microbiota may have been affected by antibiotic

residues in the pasteurized waste milk more proximally, but these changes were not likely to be captured by fecal samples.

1.3.5 ANTIBIOTICS/THERAPEUTICS: Antibiotic administration poses a risk of introducing selective pressure that may have lasting effects on commensal populations (Looft et al., 2012), which could disrupt the host's innate immune defense and increase the risk of infection. The majority of research investigating this potential risk has focused on the effect of antibiotics on the GI commensal bacterial population. Oral antibiotics have been shown to decrease the diversity of the fecal microbiota composition in humans (Willmann et al., 2019) and to be associated with significant changes to the fecal bacterial community membership in swine (Allen et al., 2011; Looft et al., 2012). Studies investigating the effects of antibiotics on the fecal microbiota in calves have shown conflicting results. Oral oxytetracycline was not associated with shifts in veal calf (n = 42) fecal community structure or species diversity (Keijser et al., 2019). However, results from a smaller study (n = 7) showed parenteral oxytetracycline was associated with an increase in fecal species richness and differences in the RA of abundant genera (Oultram et al., 2015).

Few studies have evaluated the association between antibiotic administration and the respiratory microbiota. The composition of the NP microbiota underwent significant antibiotic-dependent microbial shifts in piglets up to 14 days following parenteral administration (Zeineldin et al., 2018). Results of a recent study showed that the NP microbiota of Angus heifers (n = 20) treated with tilmicosin at feedlot arrival had reduced species abundance at 6 and 11 days following treatment (Timsit et al., 2017b). This study lacked a control group, so it is possible the changes identified were due to variability over time. Injectable oxytetracycline was shown to be associated with a decrease in NP alpha diversity in beef cattle 60 days after

administration (Holman et al., 2018). A limitation in interpretation of this study is that both the treatment group and control groups received in-feed chlortetracycline at feedlot entry. Therefore, it is difficult to discern whether the decrease in alpha diversity at the 60-day timepoint was due to antibiotic administration or variability over time, as a true control group was not included. In a recent, longitudinal study in healthy feedlot cattle (n = 36), the NP microbiota was more affected by parenteral oxytetracycline and tulathromycin treatment than the fecal microbiota (Holman et al., 2019). Treatment with either oxytetracycline or tulathromycin at feedlot entry had a significant effect on the beta diversity of the NP microbiota up to 34 days after treatment; however, while day 2 and day 5 were the most dissimilar for the NP of antibiotic-treated cattle, oxytetracycline-treated animals remained significantly dissimilar from day 12 to 34. Importantly, oxytetracycline and tulathromycin also increased the proportion of many antibiotic resistance genes in both the fecal and NP microbiome (Holman et al., 2019).

1.4 ASSOCIATION BETWEEN BRD AND RESPIRATORY MICROBIOTA

The link between the respiratory microbiota and clinical disease has been investigated extensively in humans, swine and beef cattle; however, there is minimal data available in pre-weaned dairy calves. Early interactions between pathogens and the respiratory microbiota were shown to alter the immune response in children, which affects disease severity and prognosis (Esposito and Principi, 2018); stable NP microbiota profiles have been associated with reduced consecutive respiratory infections (Biesbroek et al., 2014). Clinically healthy children were also found to have increased NP microbial diversity compared to children diagnosed with pneumonia (Sakwinska et al., 2014). This association between reduced NP bacterial diversity and respiratory disease was also found in piglets (Correa-Fiz et al., 2016). Results of studies in

beef cattle have also found a similar association between reduced NP microbial diversity and clinical BRD.

In one study that evaluated the NP microbiota of beef cattle at feedlot entry and at 60 days following arrival, animals (n = 10) were assigned to two groups: a BRD-group that included cattle previously diagnosed and treated with antibiotics prior to feedlot entry and a control-group that consisted of cattle not previously treated (Holman et al., 2015b). The results of the study showed the BRD-group had reduced alpha diversity and decreased species abundance compared to the control group at both time points. This indicates that changes within the NP microbiota associated with clinical BRD are persistent over time, despite the temporal alterations that occur in healthy animals (Holman et al., 2015b). Results from a recent study that included a larger sample size (n = 120) also showed that feedlot cattle with clinical BRD had reduced alpha diversity in both the NP and in transtracheal aspirate samples (Timsit et al., 2018). This pattern was also observed in the results from a large study (n = 164) that compared the NP microbiota between healthy and BRD- diagnosed feedlot cattle, all of which had never received metaphylaxis upon feedlot entry (McMullen et al., 2019). There was also a study in beef cattle that found no significant difference in the NP microbial diversity between healthy beef cattle and cattle diagnosed with BRD (Zeineldin et al., 2017b). Interestingly, the study design of Zeineldin et al. (2017b) included lung ultrasound (US) as a diagnostic to exclude animals with lung consolidation from the healthy comparison group. All other studies with a central focus of comparing the respiratory microbiota between healthy feedlot cattle and cattle diagnosed with BRD have only utilized clinical signs in their case definitions (Holman et al., 2015b; Timsit et al., 2017b; McDaneld et al., 2018; McMullen et al., 2019; Timsit et al., 2018). There is potential for inaccuracies in comparisons between healthy cattle and cattle

diagnosed with BRD in the aforementioned studies; because lung ultrasonography was not utilized in the case definitions, cattle identified as "healthy" could have harbored subclinical lung consolidation. The association between lung consolidation and changes in the respiratory microbiota requires further investigation.

Along with changes in microbial diversity metrics found to be associated with BRD, studies also identified taxa correlated with health status in feedlot cattle. Zeineldin et al. (2017b) found an increased RA of *Rathayibacter* spp. in healthy cattle and a higher RA of *Solibacillus* spp., *Pasteurella* spp., and *Acinetobacter* spp. in cattle diagnosed with BRD. The RA of *Lactobacillaceae* and *Bacillaceae* families were higher in the NP of cattle that had arrived at the feedlot clinically healthy compared to cattle that arrived with a previous treatment history for BRD (Holman et al., 2015b). Timsit et al. (2018) also reported a higher RA of *Lactobacillus lactis* and *Lactobacillus casei* in the transtracheal samples of clinically healthy cattle compared to cattle diagnosed with BRD. The findings of Holman et al. (2015b) and Timsit et al. (2018) are interesting, because *Lactobacillus* spp. have been shown to function as probiotics in the gastrointestinal tract of swine (Valeriano et al., 2017). Additionally, results from a recent study showed *Lactobacillus* spp. strains were able to adhere to bovine bronchial epithelial cells and complete with and inhibit *Mannheimia hemolytica* growth (Amat et al., 2017).

Timsit et al. (2018) also found a higher RA of *Pasteurella multocida* and *Mannheimia hemolytica* in the NP and transtracheal samples of cattle diagnosed with BRD. Overall, the RA of *Mycoplasma* spp. was high in the NP of all cattle; however, *M. bovis* was higher in the BRD-diagnosed cattle and *M. dispar* was higher in healthy cattle. Speciation of *Mycoplasma* spp. was performed in Timsit et al. (2018) using a newly developed bioinformatic "denoising"

package (e.g. DADA2), which corrects for errors in 16S rRNA sequencing by identifying biological sequences at single nucleotide resolution with amplicon sequencing variants (Nearing et al., 2018). There are challenges with denoising packages: different bioinformatics packages return variable results using the same raw sequence data, possible false positive identification of sequence variants, and potentially inaccurate diversity metrics (Nearing et al., 2018). Resolving taxa to species or strain with a high degree of accuracy likely requires whole genome sequencing.

Despite the ongoing challenges of BRD in pre-weaned dairy calves, there is only a paucity of data that investigates potential links between the respiratory microbiota and disease in dairy calves. Results from a recent longitudinal study with a large sample size (n = 174) compared the NP microbiota of healthy Holstein calves to calves that developed clinical BRD; a higher bacterial load (i.e. increased number of bacterial species present) at 3 days of age was associated with calves that developed clinical BRD in the first month of life (Lima et al., 2016a). This result is in contrast to the majority of studies in feedlot cattle that have shown a potential relationship between increased NP microbial diversity and reduced prevalence of BRD (Holman et al., 2015b; Timsit et al., 2018; McMullen et al., 2019). The study design of Lima et al. (2016) included sampling calves at a much earlier age (3 days) than the aforementioned beef cattle studies. Given the known variability of the NP microbiota over time, the association between increased NP microbial diversity and reduced risk of BRD may solidify at a later age in dairy calves. Results of Lima et al. (2016) also showed an increased RA of *Mannheimia* spp. and *Mycoplasma* spp. in calves that developed BRD and an increased RA of *Pseudomonas* spp. in calves that remained healthy during the study period. A smaller study (n = 16) that used whole genome sequencing to evaluate the NP microbiome in healthy dairy calves and calves

diagnosed with clinical BRD found an increased RA of *Pseudomonas fluorescens* in calves diagnosed with BRD (Gaeta et al., 2017). The results of Gaeta et al. (2017) and Lima et al. (2016) were contradictory in the association of *Pseudomonas* spp. and BRD or healthy status, respectively. Both studies used a case definition for BRD that relied on clinical signs alone. Given the high prevalence of subclinical BRD in dairy calves (Buczinski et al., 2014; Ollivett and Buczinski, 2016; Binversie et al., 2017) and importance of lung consolidation in future productivity (Dunn et al., 2018), further investigations of the link between the respiratory microbiota and BRD in pre-weaned dairy calves should include lung US findings in the case definitions.

The association between the lower respiratory microbiota and BRD in pre-weaned dairy calves has been evaluated by one observational study performed in Ireland (Johnston et al., 2017). The authors used 16S rRNA sequencing to evaluate the microbiota of post-mortem lung and lymph node samples in calves diagnosed with BRD at necropsy (32 beef calves and 6 dairy calves) and calves without clinical BRD at slaughter (20 dairy calves); however, only 8 of those calves had no gross evidence of BRD on post-mortem evaluation. Lung tissue from calves with BRD was sampled from lesions of consolidation or abscessation within the right and left cranial lobes; dairy calves diagnosed with BRD had a higher RA of *Leptotrichiaceae*, *Mycoplasma* spp., *Fusobacterium* spp., *Trueperella* spp. and *Bacteroides* spp. compared to healthy calves with no lesions. Mediastinal lymph node samples from dairy calves BRD lesions had a higher RA of *Leptotrichiaceae*, *Mycoplasma* spp., and *Pasteurellaceae* compared to calves without lesions. The results of Johnston et al. (2017) re-emphasize the importance of including subclinical BRD in case definitions, as only 40% of the "healthy" dairy calves had no evidence of lung consolidation at necropsy. The association of specific taxa with BRD diagnosis in this

study may be specific to management environment or may be biased due to the severity of BRD (i.e. calves with BRD that were enrolled in this study had died and had been submitted for necropsy by producers). Further research is needed to understand the link between the upper and lower respiratory tract microbiota and how the composition of the bacterial community impacts risk of BRD in dairy calves.

1.5 HOST REGULATION OF TRANSCRIPTION AND LINK TO MICROBIOTA

Commensal bacteria play a vital role in the innate immune system by competing with pathogenic bacteria for nutrients and adhesion sites as well as directly antagonizing pathogenic bacteria (Timsit et al., 2016b). The host immune system must, therefore, balance the resident bacterial population by operating as an environmental filter to limit the spatial distribution of the microbiota's accessible niches and by buffering the mucosal epithelium from contact that would induce systemic inflammation (Costello et al., 2012; Hooper et al., 2012). The molecular mechanisms underlying the protection of the host by the microbiota and the feedback control of the host immune system are not well understood (Edwards et al., 2019). Host immunomodulation has been described in the cervicovaginal microbiota of women; high-diversity microbial communities are associated with pro-inflammatory cytokine concentrations (Anahtar et al., 2015). Differences in the microbial composition in this study were strongly linked to increased inflammation and disease susceptibility (Anahtar et al., 2015). Results of studies in mice have shown that the composition of the respiratory microbiota affects the capacity of the innate and adaptive immune responses following experimental *Streptococcus pneumoniae*, *Klebsiella pneumoniae,* and influenza A infection (Ichinohe et al., 2011; Brown et al., 2017). Studies evaluating the mechanistic relationship between the respiratory microbiota, host immune response and susceptibility to BRD have not been performed in cattle (Timsit et

al., 2016b). In addition to furthering understanding of host susceptibility to disease in the context of resident pathobionts, studying the relationship between changes in the microbiota and perturbations to the host immune system may improve early detection of disease by developing sensitive biomarkers.

A promising pathway of communication between the microbiota and host immune system is microRNA (miRNA) (Edwards et al., 2019). MicroRNAs are small (approximately 22 nucleotides in length) non-coding RNAs that post-transcriptionally regulate gene expression and function as important regulators of the innate immune response (Zhou et al., 2011, 2012; Momen-heravi and Bala, 2018). MicroRNAs can modulate epithelial immune responses within the innate immune signaling network, including in the production and secretion of cytokines, expression of adhesion receptors and feedback regulation of immune homeostasis (Zhou et al., 2011). Following epithelial recognition of bacterial antigens by pattern recognition receptors, miRNA genes are transcribed, or co-transcribed in groups, by RNA polymerase II into primary transcripts (reviewed in Zhou et al., 2011, Momen and Haravi, 2018; Figure 1.3). These primary transcripts are modified into 70- to 100-nucleotide long hairpin precursors within the nuclease by the RNAse III Drosha. Precursor miRNAs are actively transported into the cytoplasm by exportin-5 and cleaved by Dicer to form mature miRNAs (a mature miRNA guide strand and miRNA passenger strand). The mature miRNA strand is loaded into the RNA-induced silencing complex, which functions as an RNA interfering molecule by identifying target messenger RNA (mRNA) with complementary base pairing; mRNA is then either degraded or translation is suppressed. Each miRNA can regulate hundreds of genes, which indicates many protein coding genes are under their control (Friedman et al., 2009). Although miRNAs are processed within the nucleus and cytoplasm, they perform functions extracellularly

and circulate ubiquitously throughout body fluids (Weber et al., 2010; Liu et al., 2016). It is also known that miRNAs are exported in extracellular vesicles, which exert autocrine effects and paracrine effects on neighboring cells (Valadi et al., 2007).

Results of many recent studies demonstrate the significance of miRNA expression in the immune response in a variety of host tissues; these expression profiles have been studied as biomarkers for diagnosis as well as indicators of severity and prognosis (Taka et al., 2020). Differential expression profiles of miRNAs have been noted in serum, sputum, skin and bone marrow from humans infected with *Mycobacterium* spp.; many of the miRNAs identified were found to impact the host immune response during exposure and infection (Singh et al., 2013). Results from a recent study showed differential expression of oar-miR-21 within sheep lungs, which correlated with Visna Maedi Virus exposure and infection; oar-miR-21 functions as a regulator of inflammation and cell proliferation (Bilbao-Arribas et al., 2019). Experimental infection of bovine mammary epithelial cells with *Streptococcus uberis* induced a specific miRNA response that was unique to Gram-positive bacteria and could be discerned from lipopolysaccharide induced expression; the targeted genes were crucial to the innate immune response (Lawless et al., 2013). Differential expression of miRNAs in serum was also able to distinguish between healthy cattle and cattle naturally infected with *Mycobacterium avium* subsp. *paratuberculosis* (Gupta et al., 2018). Interestingly, Edwards et al. (2019) found that the RA of *Lactobacillus* spp. within the vaginal mucosa of women correlated positively with miR-193b expression, which targets and silences cell cycle genes; inhibiting vaginal epithelial cell proliferation is a host defense mechanism against *Chlamydia trachomatis* infection. Researchers also found that changes in the gastrointestinal microbiota affected miRNA expression, which suppressed inhibition of TNF-α and led to increased susceptibility of allergic

airway disease in mice (Shi et al., 2018). These studies provide compelling evidence of interdependence between epigenetic modifications, commensal bacterial populations, and host immunity.

1.6 ASSOCIATION BETWEEN BRD AND MICRORNA

There has been very little data evaluating the link between miRNA expression and BRD. One recent study identified serum miRNAs associated with antibody response, ascertained by direct ELISA testing, to natural *M. bovis* exposure in 16 beef steers (Casas et al., 2016). The identified miRNAs: bta-let-7b, bta-miR-24-3p, bta-miR-92a, and bta-miR-423-5p, have been previously recognized as playing a significant role in regulation of the immune response. Casas et al. (2016) did not include clinical data in their analysis; therefore, the relationship between the differentially expressed miRNAs and clinical or subclinical BRD was not interpretable. Another study, built on the findings of a relationship between *M. bovis* exposure and miRNA expression by Casas et al. (2016), evaluated serum miRNA differential expression in 9 dairy calves following experimental Bovine Viral Diarrhea Virus infection delivered nasally (Taxis et al., 2017). Results of this study showed that serum bta-miR-423-5p and bta-miR-151-3p were significantly different between challenged and control calves over time; peak expression of bta-miR-423-5p occurred at 4 days post challenge and peak expression of bta-miR-151-3p occurred at 9 days post-challenge. The results indicated differential expression of miRNAs may facilitate in distinguishing between acute and chronic respiratory infections in calves.

Altered miRNA expression has been associated with the pathogenesis of respiratory disease in humans, with much of the focus on the role of miRNA in the host immune response and lung development (Rupani et al., 2013; Booton and Lindsay, 2014; Maltby et al., 2016). Although it is known that the lung microbiota has a significant impact on lung immune

homeostasis and miRNA is an important regulator of the immune response (Fujita et al., 2018), the association between miRNAs and alterations in the respiratory microbiota have not been evaluated specifically. Aforementioned results of Edwards et al. (2019) and Shi et al. (2018) (section 1.6) emphasize the linkage between the microbiota, miRNA expression and disease susceptibility. Further understanding of how host-microbiota interactions are associated with BRD pathogenesis will require studies that evaluate differential miRNA expression within the respiratory tract.

1.7 CONCLUSIONS AND THESIS OBJECTIVES

Bovine respiratory disease is economically significant in the dairy industry and has a multifactorial etiopathogenesis that is not fully understood. Next generation sequencing has illuminated complexities of the respiratory microbiota and how changes in the commensal bacterial population may affect host susceptibility to BRD. The respiratory microbiota is significantly impacted by external variables, including anatomic site, stress (e.g. transportation), and antibiotic administration; however, the majority of studies have evaluated these variables is in beef production systems. There is a deficit in data that describe the effects of environmental variables on the respiratory microbiota in pre-weaned dairy calves. Also, studies up to this point have only evaluated the respiratory microbiota in the context of naturally occurring BRD; effects of specific bacterial challenge have not been investigated. Additionally, there has been a noted association between decreased NP microbial diversity and clinical signs of BRD. This result has been found in multiple studies involving feedlot cattle with clinical BRD following weaning. Further studies are needed to assess the validity of this finding in the context of pre-weaned dairy calves. Given the significance of lung consolidation in the future of productivity and prevalence of subclinical pneumonia in dairy calves, it is critical to investigate the

association between changes in the respiratory microbiota and BRD, with inclusion of subclinical disease in the case definition.

Knowledge of how the composition of the respiratory microbiota increases the risk of or protects against BRD creates opportunities for the development of alternative treatment strategies. Probiotics applied to the upper respiratory tract have shown promise in preventing lower respiratory tract infection in mice (Kanmani et al., 2017) and children (Leyer et al., 2009). Amat et al. (2016) also described the ability of *Lactobacillus* spp. to adhere to bovine bronchial epithelial cells and compete against *M. hemolytica in vitro*. Research that promotes understanding and development of alternative strategies to antimicrobials is in compliance with the global One Health effort of preserving efficacy of medically important antibiotics and reducing antimicrobial resistance in veterinary and human medicine (McEwen and Collignon, 2017). This effort has an important impact on the dairy industry, as producers and veterinarians will have to balance protection of the food supply and human health with progressive objectives to improve animal health and welfare.

The composition of the respiratory microbiota and BRD pathogenesis are further complicated by intercommunication with the host. Interactions between the microbial community and the host play a crucial role in mucosal homeostasis and host health (Kogut et al., 2019). Evaluating differential miRNA expression associated with BRD in pre-weaned dairy calves will provide preliminary data that describe the host immune response to changes in the respiratory microbiota. This information could potentially inform sensitive diagnostic biomarkers and improve our understanding of variation in host susceptibility to BRD.

Specifically, the objectives of this thesis are:

1) Describe the association between changes in the composition of the NP microbiota and lung consolidation in pre-weaned dairy calves.
2) Evaluate the effect of experimental *Pasteurella multocida* infection and antibiotic therapy on the upper and lower respiratory tract microbiota of pre-weaned dairy calves

    2 a) Evaluate the effect of anatomic site on the respiratory microbiota of a subset of pre-weaned dairy calves excluded from experimental bacterial challenge.
3) Investigate the association between changes in the respiratory microbiota and differential miRNA expression in lung tissue of healthy pre-weaned dairy calves and of calves inoculated with *Pasteurella multocida*.

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1.9 FIGURES

Figure 1.1

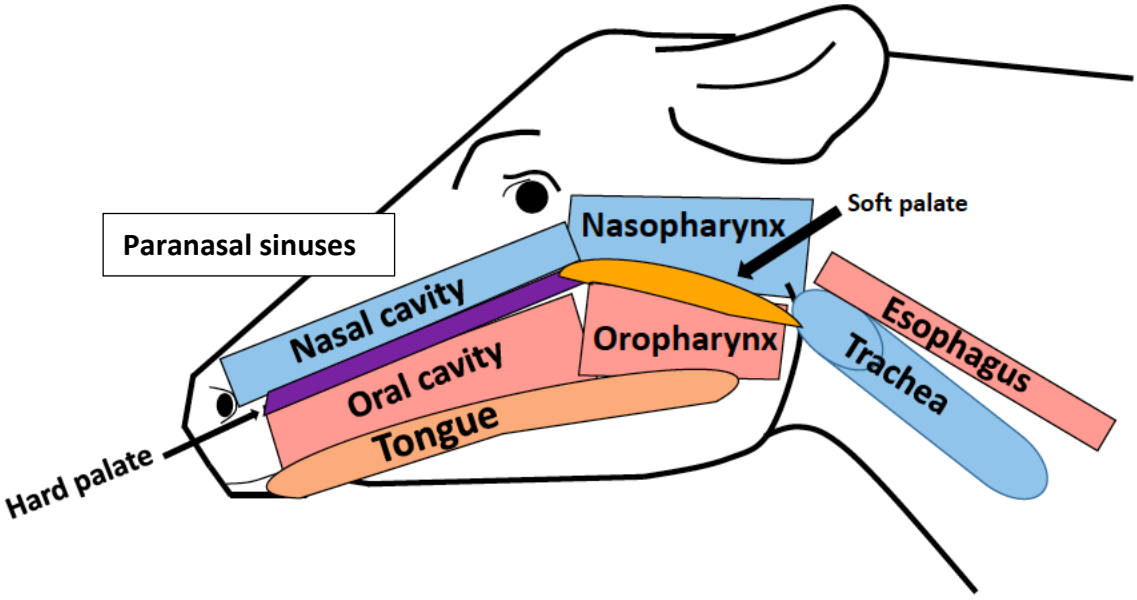

Figure 1.1 Anatomy of the bovine upper respiratory tract
Modified from Stenfeldt and Arzt (2019)

Figure 1.2

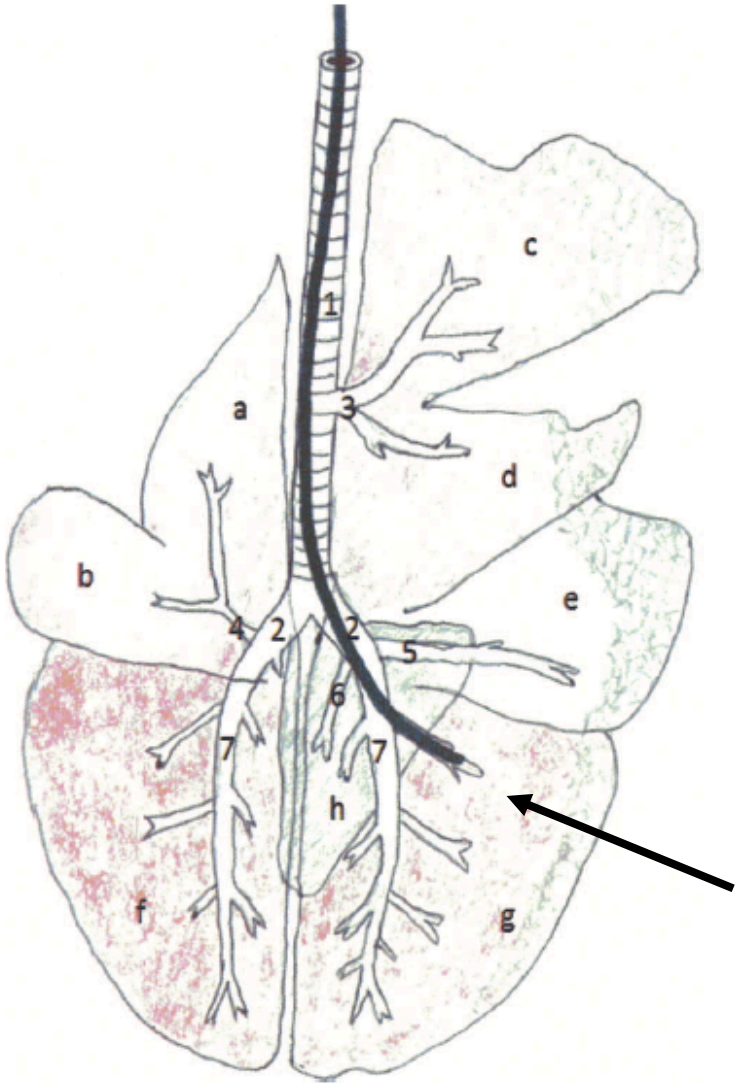

Figure 1.2 Lower Respiratory Tract in cattle includes the distal trachea (1), mainstem bronchi (2) and bronchioles (3-7). Lung anatomy: Left cranial (a) and caudal (b) aspects of the cranial lung lobe, left caudal lung lobe (f), Right cranial (c) and caudal (d) aspects of the cranial lung lobe, Middle lung lobe (e), Right caudal lung lobe (g), and Accessory lung lobe (h). Black arrow is pointing to the typical sample collection location of a bronchoalveolar lavage (BAL). Modified from Van Driessche et al. (2016)

Figure 1.3

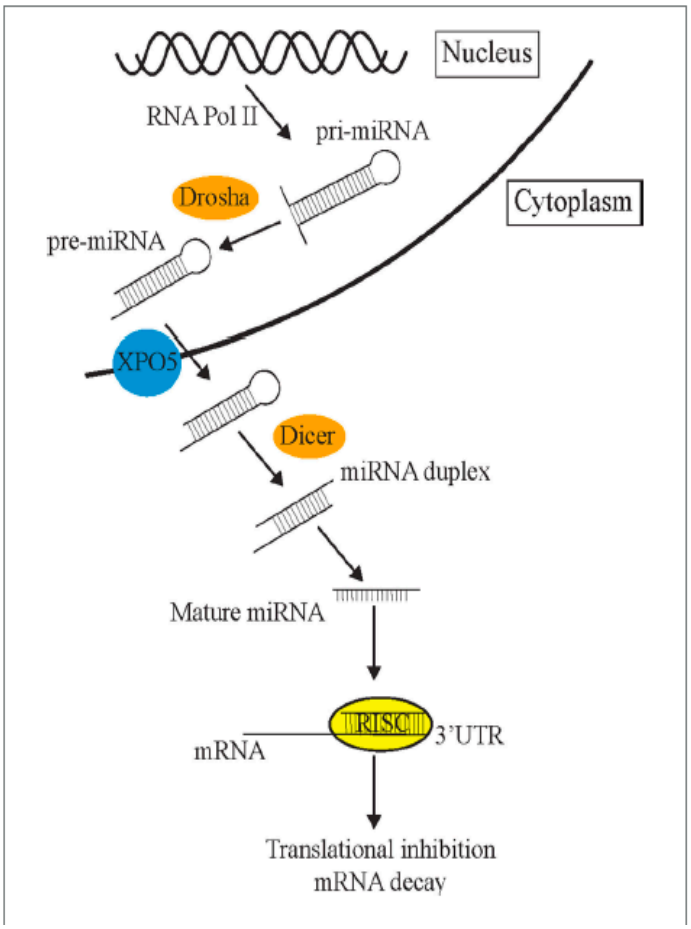

Figure 1.3 The formation of microRNA: Primary miRNAs (pri-miRNAs) are transcribed from the genome within the nucleus. Precursor miRNA (pre-miRNA) are cleaved by Drosha in the nucleus. The precursor is then exported to the cytoplasm where it is further cleaved by Dicer to form a mature miRNA of 21-23 nucleotides. One strand joins the RISC complex and scans the transcriptome (messenger RNA) for complementary target transcripts. This leads to translational inhibition or mRNA degradation. (Davis et al., 2015)

CHAPTER 2. THE NASOPHARYNGEAL MICROBIOTA OF PRE-WEANED DAIRY CALVES WITH AND WITHOUT ULTRASONOGRAPHIC LUNG LESIONS

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The nasopharyngeal microbiota of pre-weaned dairy calves with and without ultrasonographic lung lesions. Raabis. This cross-sectional study compares the microbial composition of the nasopharynx between calves diagnosed with and without **Bovine Respiratory Disease (BRD)** using ultrasonography and clinical scoring. Severity of pneumonia was associated with an increased relative abundance of *Pasteurella* spp. in the nasopharynx. Increased microbial diversity of the nasopharynx may be associated with reduced prevalence of clinical BRD. Further understanding of the respiratory microbiota will require longitudinal studies with larger sample sizes to determine shifts in the microbiota that may impact the risk of BRD.

The nasopharyngeal microbiota of pre-weaned dairy calves with and without ultrasonographic lung lesions

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2.1 ABSTRACT

The primary objective of this cross-sectional study was to identify the prevalence of changes in the **NP (nasopharyngeal microbiota)** associated with pneumonia in pre-weaned dairy calves. Characteristics of the NP microbiota were compared between calves with and without pneumonia, as diagnosed by **ultrasonography (US)**. Secondary objectives were to compare the composition of the NP microbiota between calves by age, **clinical respiratory score (CRS)**, and previous antibiotic therapy. Holstein heifer calves (n = 50) from a southern WI dairy were enrolled at either 3 or 6 wk of age; 4 calves were sampled at both time points. Antibiotic treatment history was also collected for the 30 d prior to enrollment. For the purpose of this study, pneumonia was defined as having lobar pneumonia, as diagnosed by US, in at least 1 lung lobe. Following examination by CRS and US, a deep nasopharyngeal swab was obtained for 16S rRNA amplicon sequencing. Alpha diversity was reduced in calves that were CRS positive and beta diversity tended to be different in calves previously treated with antibiotics and in calves that were CRS positive. Diversity metrics were not different between calves with and without pneumonia. The most dominant genera identified was *Mycoplasma* spp.; however, there was no association between **relative abundance (RA)** and pneumonia status. The RA of *Mycoplasma* spp. was higher in calves at 3 wk of age compared to 6 wk of age. Relative abundance of *Pasteurella* spp. was increased in calves with pneumonia and the RA was highest in calves with severe pneumonia. Calves that were not treated previously with antibiotics had a higher RA of *Chryseobacterium* spp*., Psychrobacter* spp., and *Stenotrophomonas* spp. These genera, except for *Stenotrophomonas* spp., significantly affected the observed difference in beta diversity between calves treated and not previously treated with antibiotics. Further research evaluating the respiratory microbiota of dairy calves should

include utilization of US to diagnose pneumonia, as shifts in the microbiota may not be consistent among calves with clinical signs of pneumonia and calves with lung lesions. In addition, longitudinal studies with larger sample sizes will be required to determine associations between shifts in the NP microbiota and antibiotic therapy.

Key words: bovine respiratory disease, nasopharyngeal microbiota, lung ultrasound

2.2 INTRODUCTION

Bovine respiratory disease (BRD) causes morbidity and mortality in the dairy industry with significant economic consequences. The prevalence of producer reported BRD in pre-weaned dairy calves varies by farm; however, national surveys documented estimates of 12% morbidity (USDA, 2018) and 14% of pre-weaning mortality, which is estimated at 5% overall from all causes (Urie et al., 2018). A recent study in California documented an average BRD prevalence, as diagnosed by clinical scoring system, of 8% (range: 3% - 13%) in pre-weaned dairy calves across six herds (Dubrovsky et al., 2019). The economic impact extends beyond morbidity and mortality. Clinical and subclinical BRD have been shown to reduce average daily gain (Donovan et al., 1998; Cramer and Ollivett, 2019). Results from Bach (2011) also showed that 4 or more clinical BRD events, prior to weaning, doubled the odds a calf will leave the herd before completing her first lactation. Additionally, lung consolidation increases the risk of pre-weaning mortality, and decreases the likelihood of pregnancy and first lactation milk production (Buczinski et al., 2014; Teixeira et al., 2017; Dunn et al., 2018). Management of BRD in calves is complicated by diverse pathogens, adequacy of passive transfer of immunity, heritability, nutrition, and environmental exposure (Stanton et al., 2012; Caswell, 2014; Neibergs et al., 2014; Quick et al., 2020). Moreover, the upper respiratory commensal bacterial

population is known to play a crucial role in respiratory health (Caswell, 2014); however, the characterization of its role in the pathogenesis of BRD, due to perturbations of the microbiota, is not well understood (McMullen et al., 2019).

Early diagnosis is essential for control of BRD, as late identification may lead to greater risk of treatment failure, chronic disease, and a higher number of calves weaning with "dirty lungs" (i.e. lung consolidation) (Ollivett, 2020). Clinical scoring systems facilitate recognition of calves with clinical BRD; however, these systems are insensitive to detecting calves with subclinical disease and do not reliably correlate with lung lesions identified at necropsy (Leruste et al., 2012; Ollivett et al., 2015, Holschbach et al., 2019). In individual herds with enzootic BRD, subclinical lung lesions may be detected in more than 50% of pre-weaned calves (Buczinski et al., 2014; Ollivett and Buczinski, 2016; Binversie et al., 2017). **Lung ultrasonography (US)** is a non-invasive diagnostic tool that is highly accurate in identifying lung consolidation (Rabeling et al., 1998; Buczinski et al., 2015; Ollivett et al., 2015). Integrating **clinical respiratory scoring (CRS)** and US can improve detection of BRD by capturing subclinical cases that may be missed by physical examination alone (Ollivett et al., 2015; Cramer and Ollivett, 2019).

Next-generation sequencing, technology that generates large numbers of unique reads of DNA fragments in parallel, has allowed for investigations into the role of the respiratory microbiota in BRD pathogenesis. Correlations between the composition and diversity of the **nasopharyngeal (NP)** microbiota and a clinical diagnosis of BRD have been identified in beef cattle (Holman et al., 2015b; Timsit et al., 2018; McMullen et al., 2019). Additionally, in feedlot cattle, shifts in the nasopharyngeal microbiota composition have been associated with transport, time and antibiotic therapy (Holman et al., 2015a; Holman et al., 2015b; Timsit et al.,

2016; Holman et al., 2017). However, only a few studies have evaluated the relationship between the upper respiratory microbiota and BRD in pre-weaned dairy calves (Gaeta et al., 2017; Lima et al., 2016), and none have classified BRD using US to include calves with lung lesions. Therefore, the primary objective of this cross-sectional study was to identify the prevalence of changes in the NP microbiota associated with pneumonia, as diagnosed by US, in pre-weaned dairy calves. Secondary objectives were to compare the NP microbiota of calves by age, CRS, and by whether antibiotics had been administered previously.

2.3 MATERIALS AND METHODS

Animals and facilities

The present cross-sectional study was conducted at a commercial dairy in southern Wisconsin from July to August 2017. We selected 50 Holstein heifer calves from a larger cohort (n = 257) enrolled in a **Genome-Wide Association Study (GWAS)** (Quick et al., 2020). Throughout the study period, calves were fed 9 L/d pasteurized waste milk with added balancer and housed in individual calf pens in a naturally ventilated barn equipped with supplemental positive pressure tubes.

Enrollment and calf scoring criteria, records analysis

As part of the large GWAS study designs (Quick et al., 2020), all calves were evaluated at 3 wk and 6 wk of age by trained evaluators using the University of Wisconsin School of Veterinary Medicine Calf Health Scorer iPad application[a] (McGuirk and Peek, 2014). At each examination, calves were assigned a **lung US score (USS)** and CRS, as previously described (Cramer and Ollivett, 2019; Holschbach et al., 2019). In brief, CRS assigned 0 to 3 points (i.e. 0 = normal, 3 = severely abnormal) for each of the following categories: rectal temperature, nasal discharge, ocular discharge, cough and ear position. Lung US was performed using a portable ultrasound unit (Ibex

Pro, E. I. Medical, Loveland, CO) with a linear rectal transducer (frequency of 6.2 megahertz) at the following settings: depth of 9 cm, gain of 18 **decibel (dB)**, near of 25 dB and far of 36 dB. The USS assigned was based on a 0 to 5 point scale (i.e. 0 = normal, aerated lung with no consolidation, 5 = 3 or more lung lobes consolidated), as previously described (Ollivett and Buczinski, 2016).

For the purposes of the current study, a convenience sample of calves with pneumonia (i.e. at least 1 lung lobe consolidated or USS ≥ 3) and age-matched calves without pneumonia (i.e. USS = 0) were selected for enrollment. The calves were enrolled in 6 separate farm visits. At each visit, calves were enrolled in order of examination under the GWAS study design; the first calf identified to have pneumonia on US was enrolled and the next age-matched calf identified without pneumonia was included. Calves from both groups with a CRS ≥ 2 in at least 2 categories were additionally grouped as **CRS positive (CRS+);** otherwise, calves were considered **CRS negative (CRS-)**. Using farm management software, antibiotic treatment histories, including the 30 d prior to enrollment, were also obtained for each calf.

Deep nasopharyngeal swab collection

Immediately following examination, a deep **nasopharyngeal (NP)** swab was collected from each calf using an 84-cm, sterile, double-guarded culture swab (Jorgensen Labs Inc., Loveland, CO). The external nares were wiped clean with dry gauze. The swab was inserted ventromedially, into the ventral nasal meatus, approximately 15 cm; once in place, the swab was pushed through two guards, rotated clockwise 360° for 30 seconds, retracted back into the second guard, and then removed from the nares. The swabs were then broken at the pre-scored edge and placed in 15 mL Cornig^{TM} centrifuge tubes (Thermo Fisher Scientific, Waltham, MA),

containing 3 mL of sterile PBS (Thermo Fisher Scientific, Waltham, MA). The tubes were then placed on ice for transport and stored in a -80°C freezer until further processing.

DNA Extraction and 16S rRNA Amplicon Sequencing

Deep nasopharyngeal swabs were thawed at room temperature and vortexed within extraction buffer. Total genomic DNA was then extracted by lysing and mechanically disrupting bacterial cells and purifying DNA via phenol and phenol:chloroform:isoamyl alcohol extraction, as has been previously described (Stevenson and Weimer, 2007; Dias et al., 2018). Extracted DNA was quantified using a Qubit fluorometer and a broad range dsDNA assay kit (Thermo Fisher Scientific, Waltham, MA). The V4 hypervariable region of the 16S rRNA gene was amplified with dual-indexed primers (F – GTGCCAGCMGCCGCGGTAA ; R - GGACTACHVGGGTWTCTAAT), which included adapters for sequencing using Illumina technology (F - AATGATACGGCGACCACCGAGATCTACAC ; R-CAAGCAGAAGACGGCATACGAGAT) (Kozich et al., 2013). PCR consisted of 25 ng template DNA, 0.5 μL of forward and reverse primers, 0.125 μL of Ex Taq DNA polymerase (Takara Bio USA, Inc. Mountain View, CA), 2.5 μL of 10X Ex Taq Buffer (Takara Bio USA, Inc. Mountain View, CA), 2.0 μL dNTP Mixture (Takara Bio USA, Inc. Mountain View, CA) and sterilized distilled water to 25 μL. PCR was performed using the following conditions: 30 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. Negative and positive controls were also performed.

The PCR products were recovered by gel electrophoresis in a 1% (wt/vol) low-melt agarose gel (Sigma-Aldrich, St. Louis, MO) using a Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, CA). Purified PCR products were quantified using a Qubit high sensitivity kit (Thermo Fisher Scientific, Waltham, MA) and a Synergy 2 Multi-Mode plate reader (BioTek,

Winooski, VT). Samples were then equimolar pooled to a 4 nM final library for sequencing. The final library was sequenced with an Illumina MiSeq using a v2 2 x 250 reagent kit at 10pmol/L with a 10% PhiX control, following the manufacturer's recommend protocol (Illumina, Inc., San Diego, CA). Raw sequence data generated from this study were deposited into NCBI's Short Read Archive (SRA) and are publicly available under BioProject PRJNA588247.

Bioinformatics and Statistical Analysis

The generated sequences were processed in mothur (v.1.40.0) (Schloss et al., 2009) with modified procedures (Kozich et al., 2013), as has been previously described (Dias et al., 2018, 2017). Briefly, paired-end reads were joined using the default parameters of the *make.contigs* command. Quality of the data was controlled by removing ambiguous bp, excessively short (< 200 bp) and long (> 500 bp) sequences, as well as removing homopolymers greater than 8 bp using the *screen.seqs* command. Sequences were then aligned to the SILVA 16S rRNA database (v 132) (Pruesse et al., 2007) and chimeric sequences were detected using the Uchime algorithm (Edgar et al., 2011) and removed. The resulting set of high-quality sequences were then classified, using the GreenGenes database (DeSantis et al., 2006), into **operational taxonomic units (OTU)** by the OptiClust algorithm (Westcott and Schloss, 2017) using a similarity cutoff of 97%. Depth of sequencing for each sample was evaluated by Good's coverage. Samples were normalized in mothur to the sample with the smallest number of sequences using the *normalize.shared* command (2000 sequences).

Normalized OTU counts were used to determine diversity metrics and the **relative abundance (RA)** of taxa in each sample. Relative abundance for each OTU, assigned a specific taxon, was defined as the OTU count / total OTU in each sample. Alpha diversity metrics,

including **Shannon's diversity index (SDI)** and **Chao1 index (Chao1)**, were calculated for all samples using mothur. Beta diversity was determined using the Bray-Curtis dissimilarity index, as calculated using the vegan package in RStudio (v 1.0.44) (RStudio Inc., Boston, MA). Differences in the Bray-Curtis index between groups were visualized using non-metric multidimensional scaling.

Statistical analyses of taxonomy and diversity data were performed in RStudio. The calf was considered the experimental unit. Outcomes of interest included diversity metrics and the RA of dominant taxa. For the diversity data, the primary predictor analyzed was the presence of pneumonia as diagnosed by US. Secondary predictors included age, CRS, and previous parenteral **antibiotic (ABC)** administration for BRD: calves that **were previously treated with ABCs (ABC+)** and calves that **were not previously treated with ABCs (ABC-)**. Alpha diversity metrics were compared between calves with pneumonia (calves with USS $\geq$ 3) and without pneumonia (calves with USS = 0), 3 and 6 wk old calves, CRS+ and CRS- calves, and between ABC+ and ABC- calves using a student's t test or a Wilcoxon rank sum test, depending on whether the data distribution was parametric or non-parametric, respectively. Alpha diversity metrics were also compared among calves with pneumonia that had different USS (e.g. USS = 3 or USS = 4) using an ANOVA or a Kruskal-Wallis test, depending on whether the data was normally distributed.

Dominant taxa were determined by identifying mean percent RA > 1 % using the phyloseq package (McMurdie and Holmes, 2013). Relative abundance values of dominant taxa were compared between groups as described for the alpha diversity metrics. Percent RA of genera, which were significantly different between comparison groups, were converted to categorical variables (greater than the median percent RA or less than or equal to the median

percent RA). Multiple logistic regression was used to evaluate the effect of primary and secondary predictors (i.e. pneumonia, age, CRS and ABC) on the odds of the percent RA of genera in a sample being greater than the median of all samples. A model was fit for each genus that was identified as significantly different between levels of a predictor variable (i.e. pneumonia diagnosis, age group, CRS+ or CRS-, and ABC+ or ABC-) in the univariable analysis (Tables 2.3 -2. 6). All predictors and the interaction of age and pneumonia diagnosis were forced into the models and model selection was performed with backwards elimination using the Bayesian Information Criterion.

Equality of beta dispersion between groups, for the Bray-Curtis dissimilarity metric, was assessed using the betadisper function of the vegan package. Bray-Curtis was compared between groups using a **permutational multivariate analysis of variance (PERMANOVA)** if the assumption of equal beta dispersion was met. An analysis of similarities was utilized if the assumption of equal beta dispersion was not met. If the RA of genera was significantly different between comparison groups, it was added as a vector using the envfit function in the vegan package. Alpha was set at $\alpha < 0.05$. A tendency was defined as $0.05 \leq P \leq 0.07$.

2.4 RESULTS

Descriptive data

Fifty calves were enrolled (n = 25 calves with USS ≥ 3 and n = 25 calves with USS = 0). Two samples were lost during transport from the farm. Therefore, 48 samples were sequenced (n = 23 calves with USS ≥ 3 and n = 25 calves with USS = 0). The median age (**interquartile range (IQR)**) at enrollment was 44 d (27, 45). The median age at enrollment was not different between calves with an USS ≥ 3 and calves with USS = 0 ($P = 0.19$). A summary of the clinical

data collected from enrolled calves is provided in Table 2.1. Parenteral antibiotics, administered by farm personnel to treat BRD in the 30 d prior to enrollment, included oxytetracycline (1 calf) and florfenicol (6 calves).

Sequencing

After sequence trimming, quality filtering, and normalization, a total of 2,281,405 sequences were obtained. The number of sequences obtained per sample was (mean ± **standard deviation (SD)**) 47,530 ± 39,320. There was no difference in the number of sequences between calves that had an USS ≥ 3 and calves with an USS = 0 ($P = 0.84$). Good's coverage was 0.98 for one sample; all other samples were greater than or equal to 0.99, which indicates adequate sequence coverage. A total of 7,398 unique OTU were identified following normalization of data.

Alpha diversity

Alpha diversity metrics (i.e. SDI and Chao1) are reported for each predictor variable in Table 2.2. Lung USS was not associated with a difference in SDI ($P = 0.86$) or Chao1 ($P = 0.78$). In addition, no differences were found in SDI nor Chao1 among age categories ($P \geq 0.50$), nor between antibiotic treatment groups ($P \geq 0.20$). While we found no difference in SDI between CRS+ and CRS- calves ($P = 0.60$), there was an association between increased Chao1 in CRS- calves compared to Chao1 in CRS+ calves ($P = 0.04$) (Table 2.2).

Beta diversity

There was no difference in beta diversity between calves with an USS ≥ 3 and calves with an USS = 0 ($P = 0.74$), between lung USS categories ($P = 0.73$), nor between age categories ($P = 0.63$) (Figure 2.1). Additionally, beta diversity tended to be different between ABC+ and ABC- calves ($P = 0.05$) and between CRS+ and CRS- calves ($P = 0.06$) (Figure 2.2). Vectors

depicting significant differences in the RA of genera between ABC+ and ABC- calves as well as CRS+ and CRS- calves were added to adjacent plots (Figure 2.2).

Phyla

Five phyla were identified with a mean percent RA > 1%. These include Tenericutes, Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria (Figure 2.3). Additional phyla identified with a mean percent RA > 0.1% but < 1% included Fusobacteria, Cyanobacteria, Fibrobacteres, Spirochaetes, and other unclassified bacteria (included as Others category in Figure 2.3). No differences in the RA of phyla were identified between calves with an USS 3 and calves with an USS = 0, nor between CRS+ and CRS- calves. The median (IQR) percent RA of Tenericutes was higher in the NP microbiota of 3 wk old calves (49 (47, 69)) compared to 6 wk old calves (24 (9, 56)) ($P = 0.03$). The median (IQR) percent RA of Bacteroidetes tended to be higher in the NP microbiota of 6 wk old calves (9 (3, 17)) compared to 3 wk old calves (4 (1, 8)) ($P = 0.06$). The median (IQR) percent RA of Actinobacteria was higher in ABC- (4 (2, 8)) compared to ABC+ (2 (1, 2)) ($P = 0.02$).

Families

A total of fifteen families were identified with a mean RA >1%. These include *Alcaligenaceae, Brucellaceae, Clostridiaceae, Enterobacteriaceae, Lachnospiraceae, Microbacteriaceae, Moraxellaceae, Mycoplasmataceae, Pasteurellaceae, Prevotellaceae, Rhizobiaceae, Sphingobacteriaceae, Staphylococcaceae, Veillonellaceae*, *Canthomonadaceae*, *[Weeksellaceae],* and an unclassified phylum of Bacteroidetes.

The median (IQR) percent RA of *Pasteurellaceae* was higher in the NP microbiota of calves with an USS ≥ 3 (2 (0.5, 6)) compared to calves with an USS = 0 (0.20 (0, 1.50)) ($P = 0.02$). Calves with an USS = 4 had the highest median (IQR) percent RA of *Pasteurellaceae* (6

(2, 26)) compared to calves with an USS = 0 (0.21 (0, 1.5)) ($P = 0.04$). There were no other differences in the RA of families between calves with an USS ≥ 3 and calves with an USS = 0.

The median (IQR) percent RA of *Mycoplasmataceae* was higher in the NP microbiota of calves 3 wk of age (49 (47, 69)) compared to calves 6 wk of age (24 (9, 56)) ($P = 0.03$). There were no other differences in the RA of families between calves 3 wk of age and calves 6 wk of age.

The median (IQR) percent RA of *Pasteurellaceae* tended to be higher in the NP microbiota of CRS+ (3.5 (2, 13)) compared to CRS- (0.50 (0, 3)) ($P = 0.05$). The median (IQR) percent RA of *Brucellaceae* was higher in the NP microbiota of CRS- (0.40 (0.1, 4)) compared to CRS+ (0 (0, 0.1)) ($P = 0.01$). No other differences in the NP microbiota were detected among calves CRS+ and CRS-.

The median (IQR) percent RA of *Brucellaceae* also tended to be higher in the NP microbiota of ABC- (0.2 (0, 4)) compared to ABC+ (0.1 (0, 0.1)) ($P = 0.06$). The median (IQR) percent RA of *Moraxellaceae* was higher in the NP microbiota of ABC- (13 (4, 24)) compared to ABC+ (2 (2, 5)) ($P = 0.01$). The median (IQR) percent of *Xanthomonadaceae* was higher in the NP microbiota of ABC- (0.1 0, 2)) compared to ABC+ (0 (0, 0.1)) ($P = 0.02$).

Genera

Sixteen genera with a mean percent RA > 1% were identified in the NP microbiota; this included 14 resolved genera, 1 unclassified family and 1 unclassified phylum (Tables 2.3 – 2.6; Figures 2.4 - 2.7). Overall, *Mycoplasma* spp. was the most abundant genus; however, there was no difference in the median (IQR) percent RA between the NP microbiota of calves with an USS ≥ 3 and calves with an USS = 0 ($P = 0.75$). There was a higher median (IQR) percent RA of *Pasteurella* spp. in the NP microbiota of calves with an USS ≥ 3 (2 (0.2, 6)) compared with

calves with an USS = 0 (0.1 (0, 1)) (*P* = 0.01) (Table 2.3). Specifically, calves with an USS = 4 had the highest median (IQR) percent RA of *Pasteurella* spp. at 6 (2, 26) compared to calves with an USS = 0, which had a median (IQR) percent RA of 0.05 (0, 0.1)) (P = 0.01). The odds of a calf having a percent RA of *Pasteurella* spp. within the NP microbiota higher than the median percent RA of all calves was 5 times higher for calves with an USS ≥ 3 than for calves with an USS = 0 (95% CI 1.4 – 17; *P* = 0.01).

Calves that were sampled at 3 wk of age had a higher median (IQR) percent RA of *Mycoplasma* spp. (49 (46, 69)) in their NP microbiota compared with calves sampled at 6 wk of age (24 (9, 56)) (P = 0.02) (Table 2.4). The odds of 3 wk-old calves having a percent RA of *Mycoplasma* spp. in their NP microbiota higher than the median percent RA of all calves were 8.3 times higher than calves sampled at 6 wk of age (95% CI: 1.6 – 43; *P* = 0.01). Calves sampled at 6 wk of age had an increased median (IQR) percent RA of *Tetrathiobacter* spp. (0.1 (0, 0.1)) in their NP microbiota compared with calves sampled at 3 wk of age (0 (0, 0)) (*P* = 0.03) (Table 2.4). Calves at 6 wk of age had 6 times higher odds of having a percent RA of *Tetrathiobacter* spp. within the NP microbiota higher than the median percent RA of all calves, (95% CI: 1.1 – 30.2; *P* = 0.04).

The median (IQR) percent RA of *Pasteurella* spp. in the NP microbiota of CRS+ (3 (2, 13)) was higher than the median (IQR) percent RA *Pasteurella* spp. in CRS- calves (0.1 (0, 2)) (*P* = 0.04) (Table 2.5). Although CRS+ calves had 9.5 higher odds (95% CI 1.1 – 84.4) of having a percent RA of *Pasteurella* spp. in the NP microbiota greater than the median of all calves, the CRS predictor was eliminated from stepwise model selection. The median (IQR) percent RA of *Stenotrophomonas* spp. tended to be higher in the NP microbiota of CRS- (0 (0, 2)) compared to CRS+ (0 (0, 0)) (*P* = 0.05). The odds of CRS- calves having a percent RA of

Stenotrophomonas spp. in the NP microbiota higher than the median RA of all calves were 6.3 (95% CI: 0.7, 56.3) times higher than CRS+ calves; however, the CRS predictor was eliminated from the stepwise model selection.

Calves that were not treated previously with antibiotics had a higher median (IQR) percent RA of *Chryseobacterium* spp. (0.3 (0.05, 3)) ($P = 0.01$), *Psychrobacter* spp. (4 (1, 16)) ($P = 0.01$), and *Stenotrophomonas* spp. (0 0, 2)) (P = 0.02) within the NP microbiota. The median (IQR) percent RA in ABC+ calves for *Chryseobacterium* spp., *Psychrobacter* spp., and *Stenotrophomonas* spp. was 0.05 (0.03, 0.05), 0.7 (0.4, 1) and 0 (0, 0), respectively. The median (IQR) percent RA of *Sphingobacterium* spp. in ABC- calves (0.4 (0.05, 1)) tended to be higher than ABC+ calves (0.05 (0, 0.1)) ($P = 0.06$). The median (IQR) percent RA of an unclassified Bacteroidetes phylum in the NP microbiota of ABC+ calves was higher (3 (1, 9)) compared to ABC- (0.3 (0, 2)) ($P = 0.02$). Stepwise model selection maintained only the predictor ABC for *Chryseobacterium* spp., *Psychrobacter* spp., *Stenotrophomonas* spp. and *Sphingobacterium* spp. The odds of ABC- calves having a higher percent RA of *Chryseobacterium* spp. in the NP microbiota than the median percent RA of all calves were 2.0×10^8 (95% CI 0, ∞) ($P = 0.99$). The odds of ABC- calves having a higher percent RA of *Psychrobacter* spp. in the NP microbiota than the median percent RA of all calves were 7.7 (95% CI 0.9, 70) ($P = 0.07$). The odds of ABC- calves having a higher percent RA of *Sphingobacterium* spp. in the NP microbiota than the median percent RA of all calves were also 7.7 (95% CI 0.9, 70) ($P = 0.07$). The odds of ABC- calves having a higher percent RA of *Stenotrophomonas* spp. in the NP microbiota than the median percent RA of all calves were 4.0×10^7 (95% CI 0, ∞) (P = 0.99).

Antibiotic treatment history was also the only predictor remaining in the model following backwards elimination when evaluating the odds that the percent RA of unclassified

Bacteriodetes was higher in the NP microbiota of ABC+ calves than the median of all other calves. The odds of an ABC+ calf having an increased percent RA of unclassified Bacteroidetes greater than the median RA were 7.7 (95% CI 0.9, 70) ($P = 0.07$).

2.5 DISCUSSION

To the best of our knowledge, this is the first report to investigate the association between ultrasonographic lung consolidation and the NP microbiota of pre-weaned dairy calves. Given that lung lesions can be found in ≥ 50% of pre-weaned dairy calves without apparent clinical signs (Ollivett and Buczinski, 2016; Binversie et al., 2017), it is important to illustrate changes in the NP microbiota associated with increased risk of or protection against lung consolidation, which could impact long-term productivity (Adams and Buczinski, 2016; Dunn et al., 2018).

In a recent longitudinal study evaluating the NP microbiota of pre-weaned dairy calves, a higher number of 16S rRNA genes quantified from the NP at 3 d of age was associated with a higher prevalence of clinical BRD in the first 35 d of life compared with calves that had a lower number of 16S rRNA genes at 3 d of age; however, diversity indices Chao1 and SDI were not different between calves that did and did not develop clinical BRD at 35 d of age (Lima et al., 2016). In the current study, SDI and Chao1 were not different between calves by pneumonia diagnosis (i.e. USS≥ 3 or USS = 0), age category or by previous antibiotic treatment history.

Chao1 index was, however, significantly lower in CRS+ calves compared with CRS- calves. This indicates that calves with clinical BRD had reduced NP alpha diversity as measured by Chao1, a metric that estimates species richness by counting the minimal number of unique I in each sample (Chao, 1984). Although SDI, a diversity metric that accounts for abundance and evenness (Ludwig, 1988), was numerically lower in CRS+ calves, it was not statistically significant. Shannon diversity index is not sensitive to low abundance taxa (Dejong, 1975); this

may have affected our ability to observe a significant difference in SDI between CRS+ and CRS- calves.

Our finding of reduced Chao1 in the NP of CRS+ calves is consistent with the findings of studies evaluating the NP microbiota of weaned beef calves and beef cattle (Holman et al., 2015b; Timsit et al., 2018; McMullen et al., 2019). Decreased bacterial richness and diversity has also been noted in the NP microbiota of children diagnosed with pneumonia compared with healthy controls (Sakwinska et al., 2014). Results of our study are inconsistent with the results of Lima et al. (2016), as authors of that study found an increase in the number of different 16S rRNA genes associated with clinical BRD. The definition of clinical BRD in our study was mainly different from the definition of BRD in Lima et al. (2016) in that our definition was based on scoring criteria that included nasal discharge. The definition of clinical BRD in Lima et al. (2016) included cough, fever, tachypnea and crackles or wheezes on thoracic auscultation (Lima et al., 2016). Nasal discharge has been included in scoring systems that reliably predict carriage of BRD associated bacterial and viral pathogens (Love et al., 2014). This difference in case definitions may have led to the inconsistency in results between our study and Lima et al. (2016). Additionally, the current study design was cross-sectional, while Lima et al. (2016) collected longitudinal data; the difference in study designs may also have affected our ability to interpret differences in the data.

The lack of a significant reduction in alpha diversity in calves with pneumonia (i.e. USS ≥ 3) in our study (this group included 7 CRS+ calves and 16 CRS- calves) may be due to small sample size, chronicity of US lesions or due to a lack of a direct link between NP diversity and lung consolidation. This study design evaluated USS at one point in time, which did not allow for determination of US lesion duration. In weaned beef cattle, the NP microbial diversity and

richness have been shown to increase over time (Holman et al., 2017). Therefore, the NP microbial diversity could have stabilized if the US lesion was chronic in duration. To further determine whether NP microbial diversity is associated with US lesions irrespective of CRS, a larger sample size comparing exclusive groups (i.e. CRS+ with USS ≥ 3, CRS+ with USS = 0, CRS- with USS = 0, and CRS- with USS ≥ 3) is indicated.

Results of previous studies in beef calves have shown significant differences in NP beta diversity between sampling timepoints before and after transport, days on feed and between calves diagnosed with BRD and healthy calves (Holman et al., 2017; McMullen et al., 2018; McMullen et al., 2019). Our study showed that NP beta diversity tended to be different between antibiotic treatment groups and between CRS+ and CRS- calves. The genera that significantly influenced the tendency for differences in beta diversity among CRS+ and CRS- calves were *Pasteurella* spp. and *Stenotrophomonas* spp. The genera that significantly influenced the tendency for differences in beta diversity among antibiotic treatment groups were *Chryseobacterium* spp., *Psychrobacter* spp., *Sphingobacterium* spp., and *Stenotrophomonas* spp.

In our study, the lack of significant differences in beta diversity may be due to a small number of CRS+ and ABC+ calves that were enrolled. Additionally, calves treated with antibiotics received short-term treatment in the previous month before enrollment; any effects on the NP microbial alpha and beta diversity may have been transient. We note that a recent investigation into the effects of metaphylactic oxytetracycline on the NP microbial diversity in feedlot cattle showed significantly reduced alpha diversity over 60 d after treatment (Holman et al., 2018).

Overall, the identified phyla in calves with and without pneumonia (Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, Tenericutes) were consistent with previous

descriptions of the most prevalent phyla within the NP microbiota of dairy calves (Lima et al., 2016; Gaeta et al., 2017; Maynou et al., 2019). Visually, the distribution of mean percent RA of phyla among calves with an USS ≥ 3 and calves with an USS = 0 was similar (Figure 2.3). This is consistent with what has previously been observed from comparisons of the NP commensal bacteria among dairy and beef calves with clinical BRD and healthy calves (Holman et al., 2015b; Lima et al., 2016; McMullen et al., 2019).

When comparing abundant genera in the current study, calves with an USS ≥ 3 in this study had a higher median percent RA of *Pasteurella* spp. compared with calves with an USS = 0 and calves with an USS=4 had the highest median RA of *Pasteurella* spp. In addition, CRS+ calves tended to have a higher median percent RA of *Pasteurella* spp. within the NP compared to CRS- calves. Calves with an USS ≥ 3 also had higher odds of having a RA of *Pasteurella* spp. within the NP microbiota that was higher than the median RA of all calves. Our results indicate a potentially significant association between increased NP *Pasteurella* spp. and lung consolidation.

Lima et al. (2016) found that *Pasteurella* spp. was higher in healthy dairy calves compared to calves with otitis; however, authors found no difference in the genus RA between calves with clinical BRD and healthy calves. In Gaeta et al. (2017), approximately half of the healthy calves (n=10) and half of the calves with BRD (n=6) had *P. multocida* identified within their NP; however, no difference in the genus RA was found between healthy and BRD-affected calves. *Pasteurella multocida* was detected in a majority of BRD-affected feedlot cattle, but not in healthy cattle at feedlot arrival; however, it was not detected in BRD-affected cattle 60 d after entry (Holman et al., 2015b). In another longitudinal study, McMullen et al. (2019) identified *Pasteurella* spp. in both healthy and BRD-affected feedlot cattle; however, there was no

significant difference in RA between groups of cattle. Our study results suggest that NP *Pasteurella* spp. may be specifically important in the development of lung consolidation in dairy calves. Given the high levels of antibiotic resistance recently reported for *P. multocida* against oxytetracycline and tulathromycin (Timsit et al., 2017), it is crucial to understand the mechanisms that increase the risk of *P. multocida* overgrowth and development of lung consolidation and clinical BRD.

The RA of *Mycoplasma* spp. was not different between calves with and without pneumonia. Lima et al. (2016) found a significantly higher RA of *Mycoplasma* spp. in the NP of dairy calves with clinical BRD compared to healthy calves; the mean RA of *Mycoplasma* spp. was 33.3% in calves with BRD (specifically pneumonia and otitis) at 28 d of age compared to 11% in healthy calves. Gaeta et al. (2017) identified a low RA of *Mycoplasma bovis* in both BRD-affected and healthy dairy calves and no association with BRD; mean RA for both groups was < 2%. Results of studies evaluating feedlot cattle NP microbiota have been conflicting in showing associations between *Mycoplasma* spp. carriage and BRD (Holman et al., 2015b; McMullen et al., 2019). Results of studies have also shown *Mycoplasma* spp. in healthy beef cattle, with a significant increase in RA noted over time after arrival at a feedlot (Holman et al., 2017; Holman et al., 2015a; Timsit et al., 2016).

In our study, calves sampled at 3 wk of age had a higher median RA of *Mycoplasma* spp. in the NP microbiota compared to calves at 6 wk of age; younger calves also had increased odds of the RA of *Mycoplasma* spp. being greater than the median in the NP microbiota of all calves. This finding was unexpected. Given the high prevalence of *Mycoplasma* spp. in this study, we hypothesize this farm had high levels of *Mycoplasma* spp. in the environment, which likely influenced the density of *Mycoplasma* spp. NP carriage in younger calves. A higher density of

NP carriage of *P. multocida* in healthy beef cattle was recently shown to be associated with slower clearance of the organism over time (Thomas et al., 2019). The significance of early *Mycoplasma* spp. NP carriage in dairy calves, in terms of lung consolidation, clinical BRD and future productivity, requires further investigation.

Calves that were sampled at 6 wk of age had a higher median RA of *Tetrathiobacter* spp.; 6 wk-old calves also had higher odds of having a percent RA in the NP microbiota higher than the median of all calves. To the best of our knowledge, this genus has not previously been identified in studies of the bovine respiratory microbiota. This chemolithoautotroph belongs to the Betaproteobacteria and is classified to the family *Alcaligenaceae*; it was recently classified to the genus *Advenella* (Gibello et al., 2009). This genus is phylogenetically related to pathogens in the genera *Taylorella* spp. and *Bordetella* spp. (Ghosh et al., 2011). The clinical significance of identifying *Tetrathiobacter* spp. in the NP of pre-weaned dairy calves is unknown and requires further investigation.

Calves that were ABC- had a higher RA of *Chryseobacterium* spp., *Psychrobacter* spp., *Stenotrophomonas* spp. and tended to have an increased RA of *Sphingobacterium* spp. The genus *Stenotrophomonas* spp. also tended to be higher in CRS- calves. Calves that were ABC+ had an increased RA of an unclassified Bacteroidetes phylum compared to ABC- calves. Calves that were ABC- only tended to have higher odds of the percent RA of *Psychrobacter* spp. and *Spingobacterium* spp. being greater than the median of all calves; coefficients for the other genera showed no tendencies or significance. *Chryseobacterium* spp. and *Psychrobacter* spp. have been identified previously in the NP microbiota in healthy and BRD-affected feedlot cattle (Holman et al., 2015a; McMullen et al., 2019). *Psychrobacter* spp. has also been previously identified in the NP microbiota of healthy and BRD-affected pre-weaned dairy calves (Gaeta et

al., 2017; Lima et al., 2016). *Stenotrophomonas* spp. has been identified in the NP microbiota of healthy feedlot cattle (Holman et al., 2015a); however, the significance of this finding is unknown. *Sphingobacterium* spp. has also been identified in the NP of healthy feedlot cattle with unknown significance (Holman et al., 2015a). The significance of identified changes in the NP microbiota due to previous antimicrobial therapy is unknown, as the sample size in our study was small and the timing of administration was not uniform for each calf. Further investigation into the effects of antibiotic therapy on the NP microbiota and how changes to the microbiota might influence risk of clinical BRD and lung consolidation is required.

We acknowledge that there are limitations to our study design. Selected calves were enrolled as a convenience sample, which introduces potential sampling bias as this sample may not be representative of the dairy calf population on this farm. In addition, 4 calves were sampled at both 3 wk and 6 wk, due to the data collection schedule of the GWAS. This may have impacted the independence of these samples that was assumed in our data analysis. Sample sizes of calves that were CRS+ and ABC+ were small, which likely impacted our statistical power to make inferences about the relationship of these predictor variables to the NP microbiota. Finally, the duration of identified lung lesions was not definitively known, given the cross-sectional design of the study. Of the 35 calves sampled at 6 wk of age, 7 / 35 (20 %) had a pneumonia diagnosis (USS ≥ 3) recorded at both 3 wk of age and 6 wk of age. Therefore, lung lesions identified during this study may have persisted at least 3 wk prior to deep NP swab collection. This may have impacted our ability to discern acute shifts in the NP microbiota, such as changes in the NP alpha diversity metrics that may correlate with the development of lung lesions, as the NP microbiota may have stabilized over the duration of chronic lesion development.

2.6 CONCLUSIONS

In a specific population of pre-weaned dairy calves, significant lung consolidation was not associated with a change in alpha diversity within the NP microbiota; however, clinical respiratory signs were associated with a decrease in alpha diversity, as measured by Chao1. Clinical respiratory signs and previous antibiotic therapy also tended to be associated with a difference in beta diversity. Lung consolidation and clinical BRD were associated with an increased RA of *Pasteurella* spp., which was also highest for calves with the most severe lung consolidation. This finding emphasizes the importance of this genus in the potential long-term sequelae of BRD, as lung consolidation may impact future productivity. *Mycoplasma* spp. was the most abundant genus in all calves; however, only younger calves had a higher RA of *Mycoplasma* spp. compared to older calves. The impact of early NP *Mycoplasma* spp. carriage on the risk of BRD requires further study.

Future research of the respiratory microbiota in dairy calves should include longitudinal studies that utilize US to diagnose BRD, to include subclinical cases, and to determine associations between shifts in the microbiota and the duration of lung lesions. Further understanding of the link between the upper respiratory tract microbiota and pulmonary pathology may influence sampling protocols and data interpretation in future studies of the bovine respiratory microbiome.

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APPENDIX

[a] https://www.vetmed.wisc.edu/fapm/svm-dairy-apps/calf-health-scorer-chs/

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2.8 TABLES AND FIGURES

Table 2.1 Summary of clinical and historical data collected from enrolled calves (n = 48)

Predictor	n (% of total)
USS score[1]	
USS = 4	6 (13%)
USS = 3	17 (35%)
USS = 0	25 (52%)
Age category:	
3 wk	13 (27%)
6 wk	35 (73%)
3 wk and 6 wk[2]	4 (8%)
Clinical respiratory score[3]	
CRS+	8 (17%)
USS ≥ 3	7/8
USS = 0	1/8
CRS-	40 (83%)
USS ≥ 3	16/40
USS = 0	24/40
Antibiotic history[4]	
ABC+	7 (15%)
USS ≥ 3	3/7
USS = 0	4/7
ABC-	41 (85%)
USS ≥ 3	20/41
USS = 0	21/41

[1]Lung Ultrasound score

[2]Calves enrolled at both 3 wk and 6 wk of age

[3]Clinical respiratory score positive (CRS+) includes calves with a CRS ≥ 2 for at least 2 parameters scored using the University of Wisconsin School Calf Health scoring system. Clinical respiratory score negative (CRS-) included calves with a CRS < 2 for parameters scored.

[4]Antibiotic history (ABC+, ABC-) includes administration of antimicrobials by farm staff for treatment of respiratory disease in the 30 d prior to enrollment.

Table 2.2 Diversity metrics among levels of predictor variables; results are reported as mean ± standard deviation and median (interquartile range) (n = 48)

Predictor	SDI [1]	Chao1[2]
Pneumonia diagnosis[3]		
USS ≥ 3	2.51 ± 0.72[a]	512 (343, 700)[a]
USS = 0	2.54 ± 0.85[a]	544 (375, 712)[a]
Age category		
3 wk	2.36 ± 1.03[a]	538 (358, 733)[a]
6 wk	2.54 ± 0.68[a]	519 (371, 709)[a]
Clinical respiratory score[4]		
CRS+	2.36 ± 0.96[a]	338 (290, 498)[a]
CRS-	2.52 ± 0.76[a]	539 (384, 736)[b]
Antibiotic history[5]		
ABC+	2.30 ± 0.58[a]	461 (373, 461)[a]
ABC-	2.53 ± 0.81[a]	529 (357, 756)[a]

[a-b] Means or medians between levels of a predictor with different superscripts differ ($P < 0.05$).
[1]Shannon diversity index.
[2]Chao1 index.
[3]Pneumonia diagnosis includes 2 levels: calves with an USS = 0 and calves with an USS ≥ 3.
[4]Clinical respiratory score positive (CRS+) includes calves with a CRS ≥ 2 for at least 2 parameters scored using the University of Wisconsin School Calf Health scoring system. Clinical respiratory score negative (CRS-) included calves with a CRS < 2 for parameters scored.
[5]Antibiotic history (ABC+, ABC-) indicates whether administration of antimicrobials by farm staff for treatment of respiratory disease in the 30 d prior to enrollment occurred or did not occur, respectively.

Table 2.3. Relative abundance of genera/resolved taxa by pneumonia diagnosis; results are reported as median (interquartile range) (n = 48)

Genera/resolved taxa	USS ≥ 3[1]	USS = 0[1]	*P* value
Acinetobacter spp.	3 (1, 5)	3 (1, 4)	0.60
Agrobacterium spp.	1 (0.1, 2)	0.4 (0.2, 2)	0.75
Chryseobacterium spp.	0.2 (0.1, 2)	0.2 (0.1, 2)	0.98
Clostridium spp.	1 (0.3, 1.5)	0.4 (0.1, 6)	0.81
Escherichia spp.	1.40 (0.5, 2)	1 (0.3, 9)	0.93
Mycoplasma spp.	45 (13, 64)	35 (13, 52)	0.75
Pasteurella spp.	2 (0.2, 6)	0.1 (0,1)	0.01**
Prevotella spp.	1 (0.3, 3)	1 (0.4, 2)	0.73
Pseudochrobactrum spp.	0 (0, 1)	0 (0, 2.5)	0.86
Psychrobacter spp.	2.5 (1, 13)	4 (1, 13)	0.85
Sphingobacterium spp.	0.2 (0, 1)	0.3 (0.1, 1)	0.73
Staphylococcus spp.	1 (1, 2)	1 (0.3, 1)	0.16
Stenotrophomonas spp.	0 (0, 1)	0 (0, 0.5)	0.67
Tetrathiobacter spp.	0 (0, 0.1)	0 (0, 0.1)	0.95
Microbacteriaceae[2]	0.50 (0.1, 1)	0.4 (0, 1)	0.87
Bacteroidetes[3]	0.4 (0.1, 2)	0.4 (0, 7)	0.71

[1]Pneumonia diagnosis includes 2 levels: calves with and lung US score of 0 and calves with a lung US score ≥ 3. *P* value represents the result of Kruskal-Wallis tests between groups.
[2] *Microbacteriaceae* is a family that could not be classified into a resolved genus
[3]Bacteroides is a phylum that could not be classified into a resolved genus
† $P \leq 0.07$, *$P \leq 0.05$, **$P \leq 0.01$, *** $P \leq 0.001$; reported significance is the result of Kruskal-Wallis tests between the predictor levels.

Table 2.4. Relative abundance of genera/resolved taxa by age category; results are reported as median (interquartile range) (n = 48)

Genera/resolved taxa	3 wk	6 wk	*P* value
Acinetobacter spp.	3 (0.4, 5)	3 (1, 4)	0.47
Agrobacterium spp.	1 (0.2, 2.5)	0.5 (0, 1)	0.54
Chryseobacterium spp.	0.4 (0.1, 2)	0.2 (0.1, 2)	0.68
Clostridium spp.	0.4 (0.1, 1)	1 (0.2, 4)	0.23
Escherichia spp.	1 (0.2, 2)	1 (0.3, 8)	0.34
Megamonas spp.	0.3 (0.1, 0.5)	0.2 (0.1, 1)	0.83
Mycoplasma spp.	49 (46, 69)	24 (9, 56)	0.02*
Pasteurella spp.	0.1 (0, 1)	1.4 (0, 4)	0.12
Prevotella spp.	1 (0.3, 3)	1 (0.3, 2.5)	0.98
Pseudochrobactrum spp.	0 (0, 6)	0 (0, 0.05)	0.23
Psychrobacter spp.	4.2 (2, 9)	2.5 (1, 17)	0.97
Sphingobacterium spp.	0.2 (0, 1)	0.3 (0.03, 1)	0.65
Staphylococcus spp.	1 (1, 2)	1 (0.4, 1)	0.49
Stenotrophomonas spp.	0 (0, 1.3)	0 (0, 0.1)	0.57
Tetrathiobacter spp.	0 (0, 0)	0.1 (0, 0.1)	0.03*
Microbacteriaceae[1]	0.1 (0, 1)	0.5 (0.1, 2)	0.20
Bacteroidetes[2]	0.3 (0.1, 0.5)	1 (0.03, 6)	0.18

[1]*Microbacteriaceae* is a family that could not be classified into a resolved genus
[2]Bacteroides is a phylum that could not be classified into a resolved genus
† $P \leq 0.07$, *$P \leq 0.05$, **$P \leq 0.01$, *** $P \leq 0.001$; reported significance is the result of Kruskal-Wallis tests between the predictor levels.

Table 2.5. Relative abundance of genera/resolved taxa by clinical respiratory score; results are reported as median (interquartile range) (n = 48)

Genera/resolved taxa	CRS+[1]	CRS-[2]	*P* value
Acinetobacter spp.	1 (0.5, 2)	3.3 (1, 4.5)	0.09
Agrobacterium spp.	0.5 (0.04, 2)	0.5 (0.2, 2)	0.39
Chryseobacterium spp.	0.1 (0.1, 0.3)	0.2 (0.1, 3)	0.38
Clostridium spp.	1.3 (0.4, 4)	0.5 (0.2, 3)	0.56
Escherichia spp.	2 (1, 5)	1 (0.3, 4.5)	0.70
Megamonas spp.	0.2 (0.1, 0.6)	0.2 (0.1, 0.6)	0.82
Mycoplasma spp.	43 (23, 61)	36 (12, 55)	0.51
Pasteurella spp.	3 (2, 13)	0.1 (0, 2)	0.04*
Prevotella spp.	0.7 (0.1, 2)	1 (0.3, 3)	0.49
Pseudochrobactrum spp.	0 (0, 0)	0 (0, 3)	0.10
Psychrobacter spp.	2 (1, 3)	4 (1, 14.5)	0.26
Sphingobacterium spp.	0.1 (0, 0.4)	0.3 (0.1, 1)	0.15
Staphylococcus spp.	1 (0.4, 2)	0.6 (0.4. 1)	0.99
Stenotrophomonas spp.	0 (0)	0 (0, 2)	0.05*
Tetrathiobacter spp.	0.1 (0, 0.1)	0 (0, 0.05)	0.35
Microbacteriaceae[3]	0.6 (0.1, 3)	0.4 (0.05, 1)	0.70
Bacteroidetes[4]	1 (0.2, 2)	0.3 (0.04, 3)	0.66

[1]Clinical respiratory score positive (CRS+) includes calves with a CRS ≥ 2 for at least 2 parameters scored using the University of Wisconsin School Calf Health scoring system.
[2] CRS- includes calves with a CRS < 2 for parameters scored.
[3]*Microbacteriaceae* is a family that could not be classified into a resolved genus
[4]Bacteroides is a phylum that could not be classified into a resolved genus
† $P \leq 0.07$, *$P \leq 0.05$, **$P \leq 0.01$, *** $P \leq 0.001$; reported significance is the result of Kruskal-Wallis tests between the predictor levels.

Table 2.6. Relative abundance of genera/resolved taxa by antibiotic treatment history; results are reported as median (interquartile range) (n = 48)

Genera/resolved taxa	ABC +[1]	ABC-[2]	*P* value
Acinetobacter spp.	1 (0.6, 1.5)	3 (1, 5)	0.08
Agrobacterium spp.	0.1 (0.03, 1)	0.5 (0.2, 2)	0.34
Chryseobacterium spp.	0.05 (0.03, 0.05)	0.3 (0.05, 3)	0.01**
Clostridium spp.	2 (0.5, 7)	0.5 (0.2, 3.5)	0.18
Corynebacterium spp.	0.3 (0.3, 0.4)	0.4 (0.2, 1)	0.34
Escherichia spp.	2 (1, 12)	1 (0.3, 5)	0.20
Megamonas spp.	0.2 (0.2, 1)	0.2 (0.05, 0.6)	0.32
Mycoplasma spp.	63 (34, 74)	35 (10, 52)	0.10
Pasteurella spp.	2 (0.6, 7)	0.3 (0, 3)	0.25
Prevotella spp.	1 (0.7, 2)	1 (0.3, 3)	0.53
Pseudochrobactrum spp.	0 (0, 0)	0 (0, 3)	0.11
Psychrobacter spp.	0.7 (0.4, 1)	4 (1, 16)	0.01**
Sphingobacterium spp.	0.05 (0, 0.1)	0.4 (0.05, 1)	0.06†
Staphylococcus spp.	0.4 (0.4, 0.6)	0.6 (0.5, 2)	0.14
Stenotrophomonas spp.	0 (0, 0)	0 (0, 2)	0.02*
Tetrathiobacter spp.	0 (0, 0.05)	0 (0, 0.05)	0.76
Microbacteriaceae[3]	0.4 (0.2, 1)	0.4 (0.05, 1)	0.58
Bacteroidetes[4]	3 (1, 9)	0.3 (0, 2)	0.02*

[1]Antibiotic history positive (ABC+) includes calves administered antimicrobials by farm staff for treatment of respiratory disease in the 30 d prior to enrollment.
[2]ABC- includes calves not previously administered antimicrobials.
[3]*Microbacteriaceae* is a family that could not be classified into a resolved genus.
[4]Bacteroides is a phylum that could not be classified into a resolved genus.
† $P \leq 0.07$, *$P \leq 0.05$, **$P \leq 0.01$, *** $P \leq 0.001$; reported significance is the result of Kruskal-Wallis tests between the predictor levels.

Figure 2.1. Raabis

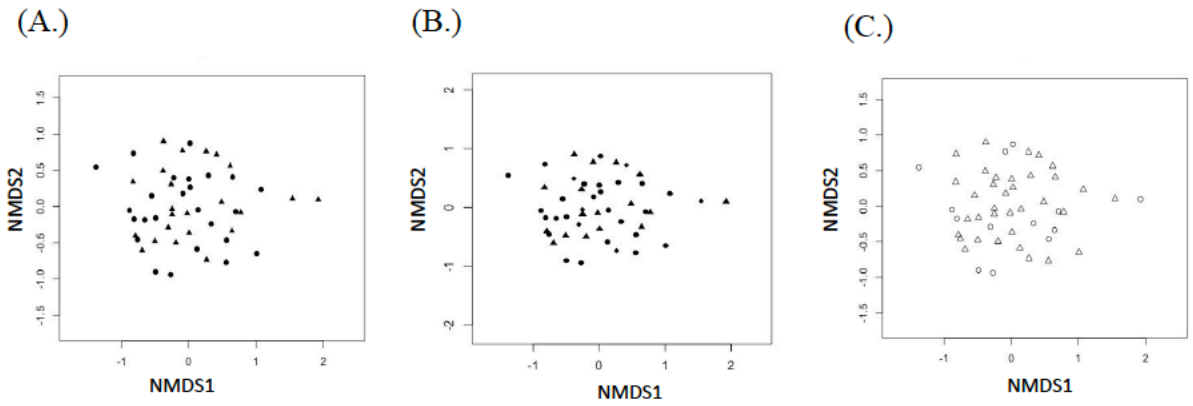

Figure 2.2. Raabis

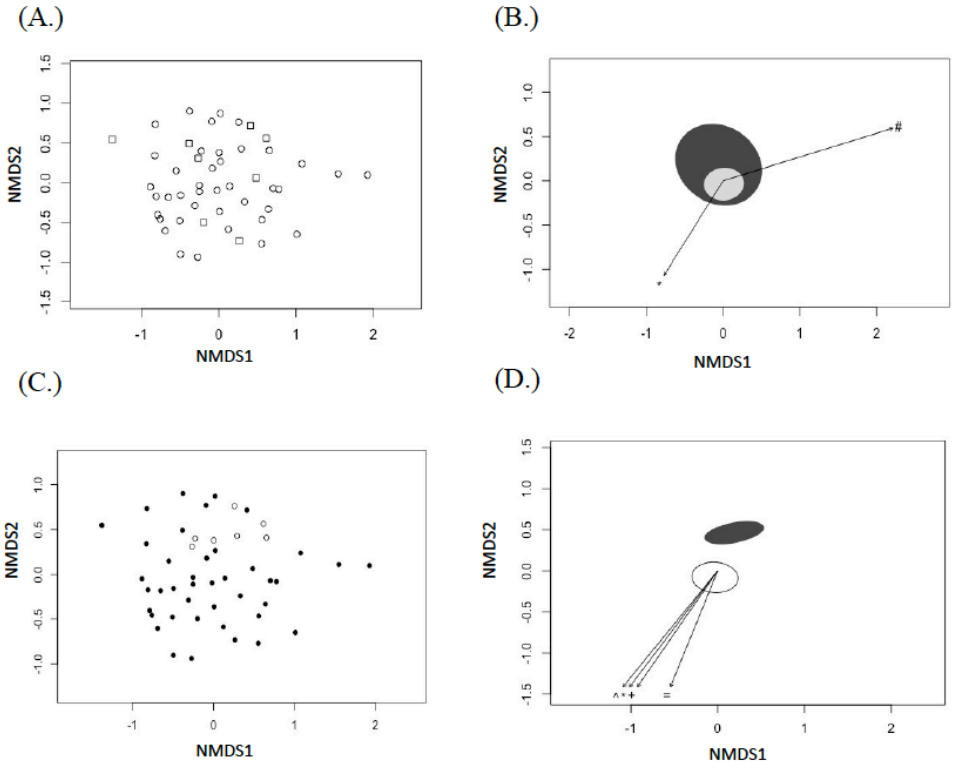

Figure 2.3. Raabis

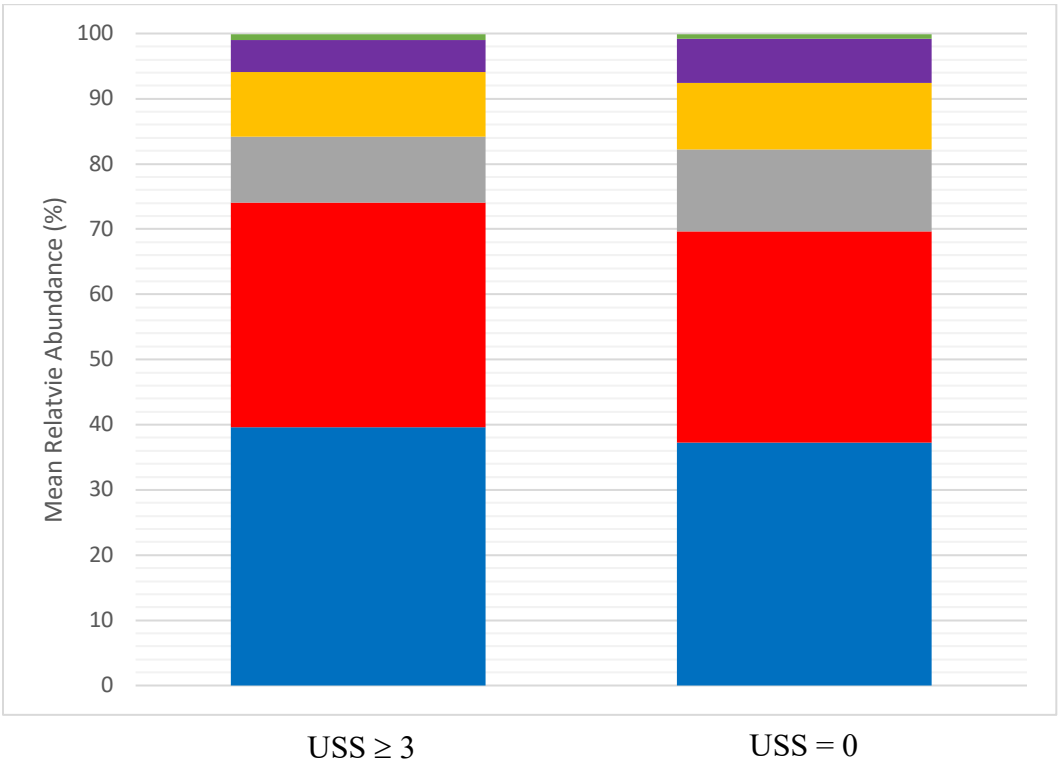

Figure 2.4. Raabis

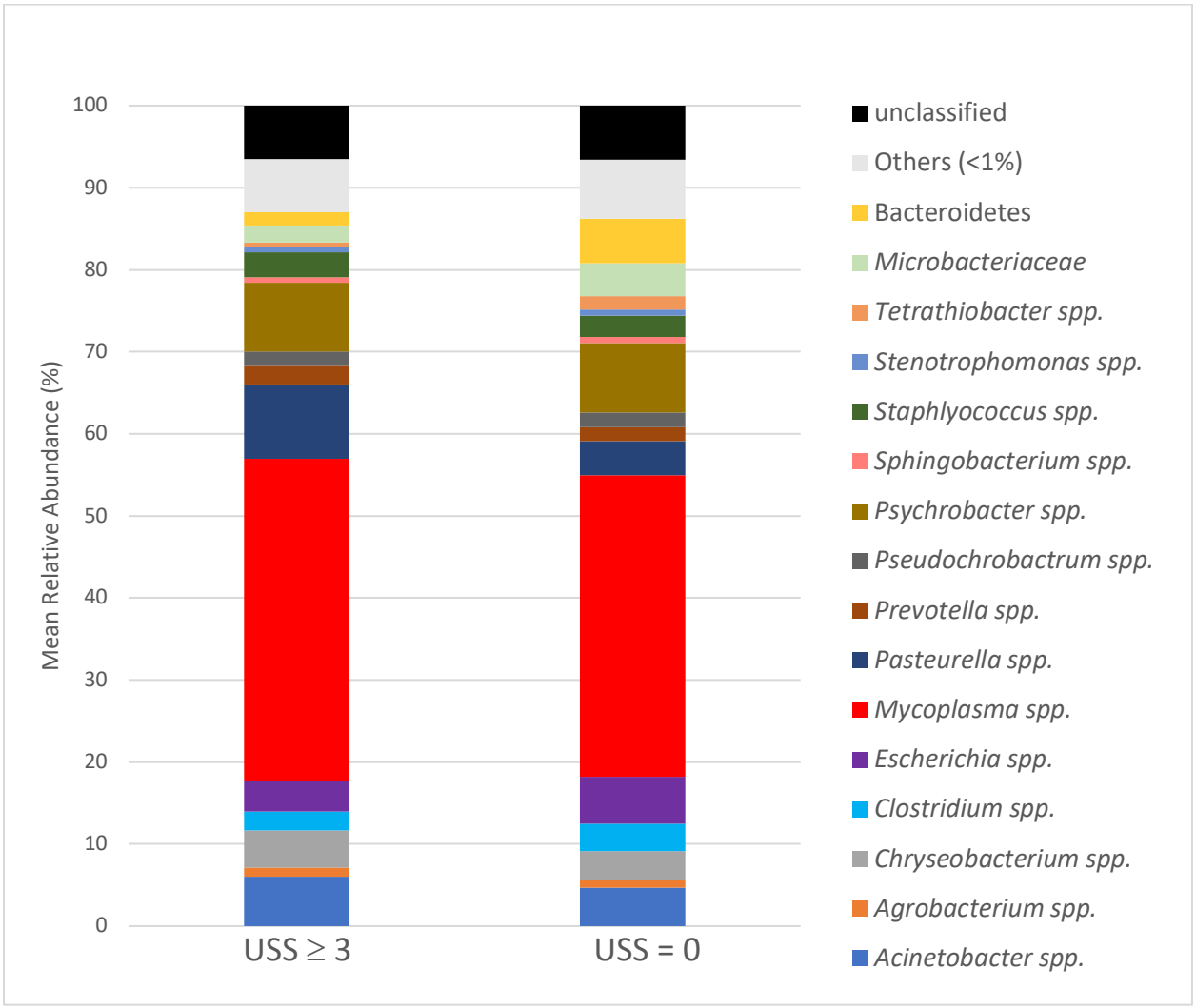

Figure 2.5. Raabis

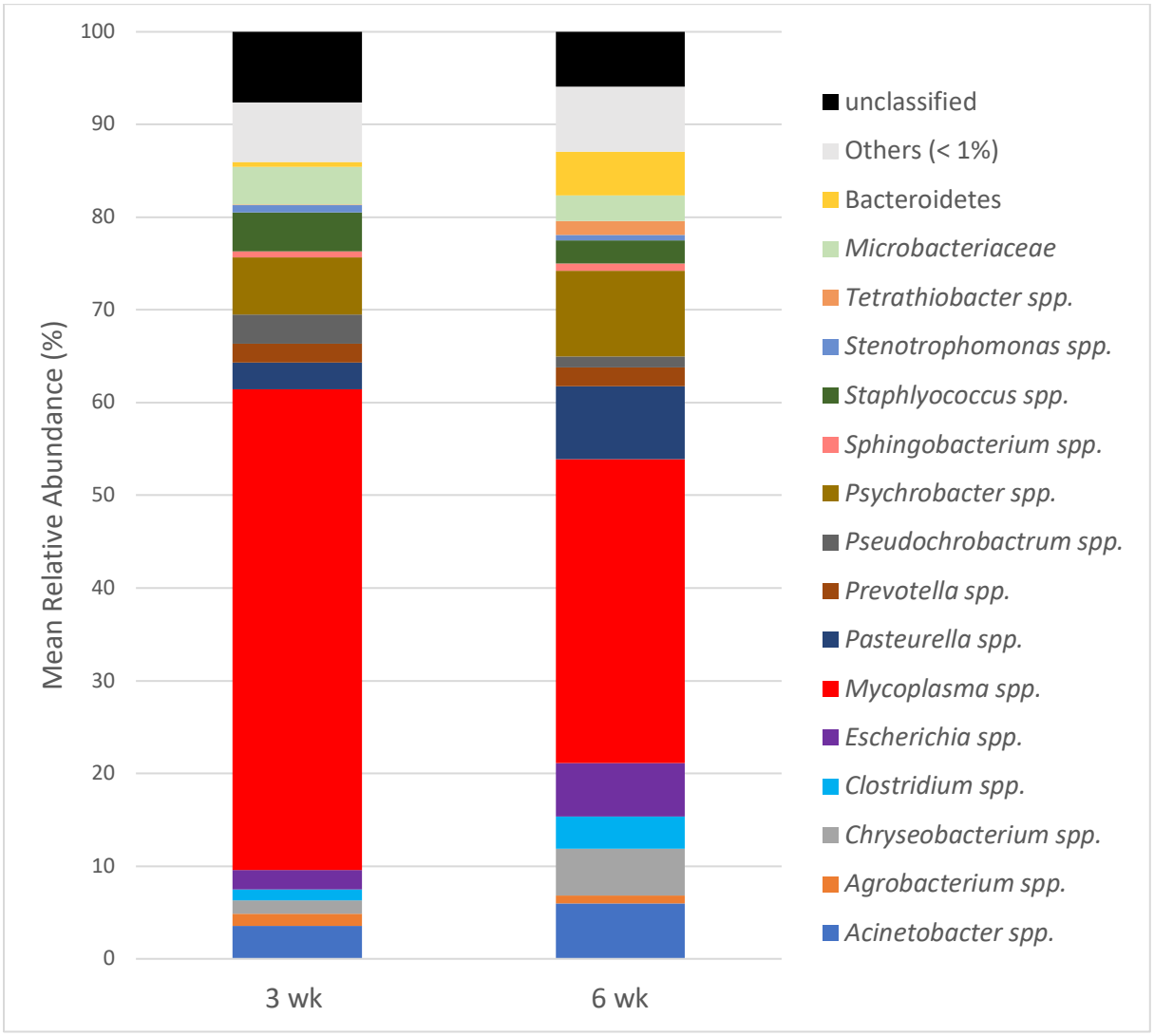

Figure 2.6. Raabis

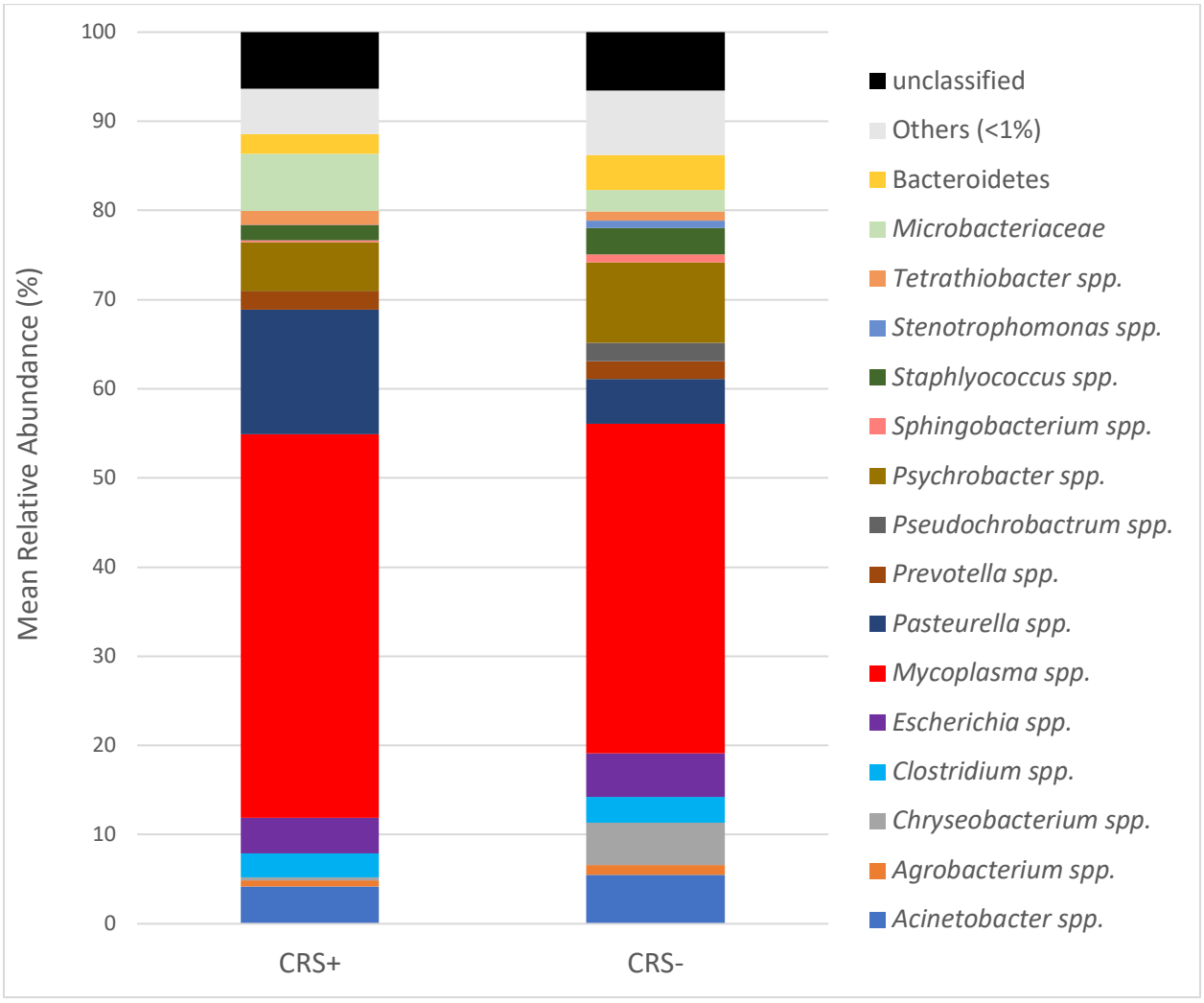

Figure 2.7. Raabis

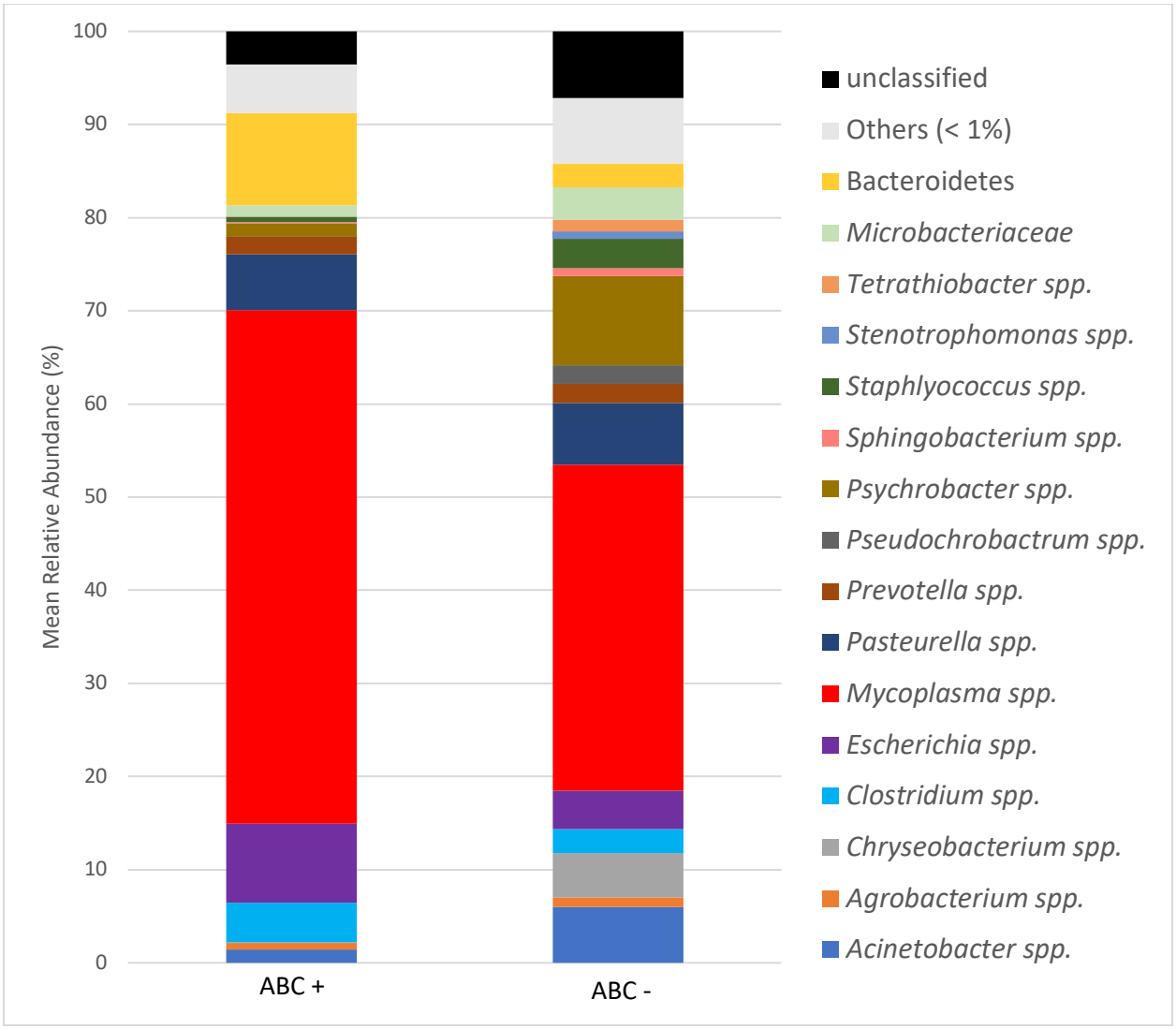

Figure 2.1: **Non-metric multidimensional scaling (NMDS)** plots depicting Bray-Curtis dissimilarity between calves with USS ≥ 3 and calves with USS = 0 (A.), calves with USS = 0, USS = 3, and USS = 4 (B.) and age categories (C.). In (A.), black triangles represent calves with USS ≥ 3 and black circles represent calves with USS = 0. In (B.), black circles represent USS = 0, black triangles represent USS = 3, and black diamonds represent USS = 4. In (C.), open circles represent calves at 3 wk of age and open triangles represent calves at 6 wk of age. Analysis of beta diversity between groups was tested using a permutational multivariate analysis of variance or an analysis of similarities. There were no significant differences between calves with USS ≥ 3 and calves with USS = 0 (P = 0.74), calves with USS = 0, USS = 3, and USS = 4 (P = 0.73), nor between calves at 3 wk and 6 wk of age (P = 0.63).
USS = Lung ultrasound score; n = 48

Figure 2.2: **Non-metric multidimensional scaling (NMDS)** plots depicting Bray-Curtis dissimilarity between calves between calves that are **clinical respiratory score positive (CRS+)** and calves that are **clinical respiratory score negative (CRS-)** (A.), calves **treated previously with antibiotics (ABC+)** and calves **not previously treated with antibiotics (ABC-)** (C.) Ellipse plots (B., D.) demonstrate the 95% CI and vectors for RA of genera that were significantly different between CRS+ vs. CRS- and ABC+ vs. ABC-, respectively. In (A.), open squares represent CRS+ and open circles represent ABC-. In (B.), the black ellipse represents the 95% CI of the Bray-Curtis dissimilarity metric among CRS+. The open circle ellipse represents the 95% CI of the Bray-Curtis dissimilarity metric among CRS-. Vector labels: * = *Stenotrophomonas* spp., # = *Pasteurella* spp. In (C.), open circles represent ABC+ and black

circles represent ABC-. In (D.), the black ellipse represents the 95% CI of the Bray-Curtis dissimilarity metric among ABC+. The open circle ellipse represents the 95% CI of the Bray-Curtis dissimilarity metric among ABC-. Vectors labels: + = *Sphingobacterium* spp., * = *Stenotrophomonas* spp., ^ = *Chryseobacterium* spp., "=" = *Psychrobacter* app. Analysis of beta diversity between groups was tested using a permutational multivariate analysis of variance or an analysis of similarities. There was a tendency for a difference in beta diversity between CRS+ and CRS- calves ($P = 0.06$) and between ABC+ and ABC- calves ($P = 0.05$); n = 48

Figure 2.3: Mean percent **relative abundance (RA)** of 10 abundant phyla identified in the NP microbiota of calves with an USS ≥ 3 and calves with an USS = 0. Others category includes phyla with <1% mean RA (Fusobacteria, Cyanobacteria, Fibrobacteres, Spirochaetes, and a kingdom of unclassified bacteria). Blue = Tenericutes, red=Proteobacteria, gray = Bacteroidetes, yellow = Firmicutes, purple = Actinobacteria, green= others; n = 48

Figure 2.4: Mean percent **relative abundance (RA)** of 16 abundant genera identified in the NP microbiota of calves with an USS ≥ 3 and calves with an USS = 0. Others category includes genera with <1% mean RA.
USS = Lung ultrasound score; n = 48

Figure 2.5: Mean percent **relative abundance (RA)** of 16 abundant genera identified in the NP microbiota of calves of 3 wk and 6 wk of age. Others category includes genera with <1% mean RA; n = 48

Figure 2.6: Mean percent **relative abundance (RA)** of 16 abundant genera identified in the NP microbiota of CRS+ and CRS- calves. Others category includes genera with <1% mean RA. CRS= clinical respiratory score. CRS+ includes calves with a CRS ≥ 2 for at least 2 parameters scored using the University of Wisconsin School Calf Health scoring system. CRS- includes calves with a CRS < 2 for parameters scored; n= 48

Figure 2.7: Mean percent **relative abundance (RA)** of 16 abundant genera identified in the NP microbiota of ABC+ and ABC- calves. Others category includes genera with <1% mean RA. ABC= antibiotics administered in 30 d prior to enrollment; ABC+ includes calves administered antimicrobials by farm staff for treatment of respiratory disease in the 30 d prior to enrollment. ABC- includes calves not previously administered antimicrobials in the 30 d prior to enrollment; n = 48

CHAPTER 3. THE EFFECTS OF EXPERIMENTAL BACTERIAL CHALLENGE WITH *PASTEURELLA MULTOCIDA* AND PARENTERAL AMPICILLIN ON THE RESPIRATORY MICROTIOTA OF PRE-WEANED HOLSTEIN CALVES

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The effects of experimental bacterial challenge with *Pasteurella multocida* and antibiotic treatment on the respiratory microbiota of pre-weaned Holstein calves

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Keywords:
Respiratory microbiota
Dairy calves
Pasteurella multocida
Ampicillin

Highlights:

- Respiratory microbiota of dairy calves included gastrointestinal tract bacteria.
- Nasopharyngeal microbial diversity associated with decreased disease severity.
- Ampicillin therapy had no effect on respiratory microbiota diversity.
- Ampicillin associated with increased *Pasteurella* spp. in the nasopharynx.

3.1 Abstract

The association between changes in the respiratory microbiota and risk of Bovine Respiratory Disease (BRD) in dairy calves is not well understood. In this study, we investigated characteristics of the nasopharyngeal (NP) microbiota associated with disease severity following inoculation with *Pasteurella multocida*. We also evaluated the effects of anatomic site and ampicillin on the respiratory microbiota following inoculation. Calves (n = 30) were inoculated with *P. multocida* and randomly allocated into an ampicillin treatment group (AMP; n = 17) or placebo group (PLAC; n = 11) when lung lesions developed. Deep NP swabs (DNPS) were collected before and after inoculation. Monitoring was performed daily until euthanasia at day 14. Swabs and tissue samples were collected for analysis. The V4 hypervariable region of the 16S rRNA gene was amplified and sequenced using an Illumina MiSeq. Increased species abundance in the pre-challenge DNPS was associated with a decrease in severity of clinical respiratory disease. While NP beta diversity was affected by bacterial challenge, ampicillin had no effect on the alpha diversity nor the relative abundance (RA) of genera in the NP tonsil, lymph node and lung microbiota. Ampicillin therapy actually increased the RA of NP *Pasteurella* spp. and decreased the RA of NP *Prevotella* spp. Anatomic site did not affect microbial diversity; however, the RA of *Microbacteriaceae* and *Prevotella* spp. were higher in DNPS compared to tissue samples. Common taxa among all sample types included gastrointestinal (GIT)-associated bacteria, which indicates a possible link between GIT microbiota and BRD in dairy calves.

3.2 1. Introduction

Bovine respiratory disease (BRD) is a persistent obstacle to managing calf health in dairy herds. Overall BRD prevalence is variable by farm; producer reported BRD in pre-weaned dairy calves was recently estimated at 12% nationally (USDA, 2018), while a longitudinal study reported an average prevalence of 8% (range: 3 - 13%) from 6 dairy herds in California (Dubrovsky et al., 2019). The respiratory tract microbiota is increasingly being recognized as a complex, dynamic and interconnected community that significantly influences the risk of and development of BRD. The mechanisms by which resident pathobionts facilitate colonization resistance, while also retaining the capacity to incite pathology, are not well understood (Thomas et al., 2019).

Evidence of an association between decreased nasopharyngeal (NP) microbial diversity and clinical respiratory disease has been documented in children, piglets and beef cattle (Sakwinska et al., 2014; Holman et al., 2015; Correa-Fiz et al., 2016). An increase in the number of different NP bacterial species at 3 d of age was associated with an increased risk of clinical BRD in dairy calves; however, specific diversity metrics were not associated with disease (Lima et al., 2016). The variability of previous study results and a relative paucity of data describing the microbiota of dairy calves indicates further studies are needed to understand the relationship between shifts in microbiota diversity and BRD pathogenesis.

Additionally, the diagnoses of BRD in recent microbiota studies were based solely on clinical signs (McDaneld et al., 2018; Timsit et al., 2018; McMullen et al., 2019) that may develop late in the disease process; therefore, results may not accurately depict the respiratory microbiota of calves with acute BRD or subclinical lung lesions. Further understanding of the respiratory microbiota in dairy calves also requires investigation of the lower respiratory

microbiota associated with BRD, as the effects of anatomic site on microbiota composition are not well described in cattle (Zeineldin et al., 2019). Results from human studies suggest that in healthy individuals, the upper respiratory tract microbiota retains the highest diversity and community richness decreases in correlation with spatial distance from the upper airway (Dickson et al., 2015).

Our primary objective was to investigate whether characteristics of the NP microbiota were associated with protection against or increased respiratory disease severity following experimental intratracheal challenge with *Pasteurella multocida*, a well-known resident microbe that is frequently isolated from cases of BRD in pre-weaned dairy calves (Caswell, 2007). Secondary objectives were to compare the composition of the NP microbiota before and after experimental challenge, as well as determine the effect of parenteral ampicillin on the NP, NP tonsil, tracheobronchial lymph node and lung microbiota 14 days after challenge. An additional secondary objective was to compare the respiratory microbiota composition among anatomic sites in a subset of calves that had not been experimentally challenged.

Our main hypothesis was that calves with increased diversity in the NP microbiota would have less severe respiratory disease following experimental challenge. Additionally, we hypothesized that *P. multocida* challenge would decrease diversity and increase the relative abundance (RA) of *Pasteurella* spp. in the upper and lower respiratory microbiota. Furthermore, we hypothesized that ampicillin would decrease diversity and the RA of *Pasteurella* spp. in the respiratory microbiota. Lastly, we hypothesized that alpha diversity would be higher in the upper respiratory microbiota compared to the lower respiratory microbiota; however, the composition of taxa would be similar among the respiratory tract samples.

3.3 2. Materials and methods

2.1. Study overview

This report consists of two trials. Trial 1 was a cohort study that was a derivative of a randomized controlled trial (RCT), which was carried out between July 11, 2017 and August 2, 2017, at the University of Wisconsin Livestock Laboratory in Madison, Wisconsin (Holschbach et al., 2019). The RCT protocol was approved by the University of Wisconsin Institutional Animal Care and Use Committee (protocol #A005636-A02) and the Institutional Biosafety Committee (protocol #B00000610). Trial 2 was an observational study in which calves that did not meet the inclusion criteria of the RCT were enrolled by convenience.

2.2. Enrollment, study design and data collection

A total of 39 Holstein bull calves, at the mean ± SD age of 52 ± 6 d, in this study were enrolled into trial 1 (n = 30) and trial 2 (n =9). Trial 1 calves were challenged intratracheally with an ampicillin-sensitive *Pasteurella multocida* A1 strain, randomized into an antibiotic treatment group following development of significant lung lesions, and then monitored for respiratory health for 14 days, as was previously described by Holschbach et. al. (2019). Trial 2 calves consisted of 9 sacrificed calves (SAC) that were excluded from the larger RCT and were not challenged or randomized into an antibiotic treatment group. A visual timeline describing study design, the experimental groups and the data collection sequence is provided in Supplemental Figure 3.1.

Respiratory health was evaluated in calves using standardized clinical respiratory scoring (CRS) and lung US scoring (USS) system, as was described by Holschbach et al. (2019). In summary, CRS assigned 0 to 3 points (i.e. 0 = normal, 3 = severely abnormal) for each of the

following categories: rectal temperature, nasal discharge, ocular discharge, cough, and ear position. Lung USS was performed by scanning both the right and left lung fields and assigning a score from 0 to 5 (i.e. 0 = normal aerated lung, 5 = severe lung consolidation), as previously described (Holschbach et al., 2019). In brief, calves with a score 0 or 1 were considered normal, calves with a score of 2 (i.e. ≥ 1 cm^2 consolidation) had significant lung consolidation and calves with scores of 3, 4 or 5 had 1, 2, or 3 or more lung lobes completely consolidated, respectively. The presence of ≥ 1 cm^2 consolidation (i.e. USS = 2) has previously been associated with decreased average daily gain in pre-weaned dairy calves (Cramer and Ollivett, 2019).

Samples for respiratory microbiota analysis were taken at 3 different time points for trial 1. The first deep nasopharyngeal swab (DNPS #1) was taken pre-challenge using a 33″ long, double guarded polystyrene cotton uterine swabs (JorVet, Jorgensen Labs, Loveland, CO). Lung US scoring and CRS were performed at 2, 6, 12 and 24 h following the *P. multocida* challenge. For the first 24 h monitoring period, if USS = 2 (i.e. ≥ 1 cm^2 lung consolidation was present) and ≥ 6 h had elapsed since the challenge, DNPS #2 was collected as described above, and calves were randomized into 2 treatment groups: an ampicillin treatment group (AMP) and a saline placebo group (PLAC). The AMP group received 250 mg/L ampicillin at 6.6 mg/kg i.m. once daily for 3 d (Polyflex, 250 mg/mL; Boehringer Ingelheim Vetmedica, Duluth, GA). The PLAC group received an equal volume of sterile saline i.m. once daily for 3 d. Calves were evaluated for 14 d following the challenge by once daily USS and twice daily CRS. Following the final evaluation of calves on d 14, DNPS #3 was collected. At the time of collection, all DNPS were placed in sterile PBS and immediately transported on ice to a -80°C refrigeration unit.

After DNPS #3 collection at d 14, challenged calves were sedated with 0.1 mg/kg xylazine i.v. (AnaSed injection, 20 mg/mL; Akorn Animal Health, Lake Forest, IL) and

euthanized with an intravenous overdose (1 mL/10 lbs) of barbiturate (Beuthanasia-D Special, Merck Animal Health, Kenilworth, NJ). Postmortem sample collection was performed immediately following euthanasia. Using aseptic technique, an approximately 3-5 cm^3 sample was dissected from the right NP tonsil, tracheobronchial lymph nodes (LN) (proximal to the carina of the trachea), and from the lung lobe with the most severe lesion(s) upon gross examination. If lung lobe lesions were equivalent in severity, the most cranial lobe was selected for sample collection. Postmortem samples were also stored in sterile PBS and transported on ice to a -80°C refrigeration unit.

Deep nasopharyngeal swabs were collected premortem, as described above, from SAC calves. Calves were then sedated and euthanized as described above. Postmortem sample collection occurred immediately after euthanasia, as was described for trial 1. Lung samples were primarily collected from the right middle lung lobe. If there was evidence of lung consolidation, the sample consisted of lung tissue from the lung lobe that had gross evidence of consolidation. Deep nasopharyngeal swabs and postmortem samples from SAC calves were placed in sterile PBS and immediately transported on ice to a -80°C refrigeration unit.

2.3 DNA extraction and 16S rRNA amplicon sequencing

Deep nasopharyngeal swabs were thawed at room temperature and vortexed within extraction buffer. Following thawing at room temperature, tissue samples were further processed aseptically on individual sterile petri dishes. An approximately 1 cm^3 specimen was dissected from each sample and vortexed within extraction buffer. Total genomic DNA was then extracted by lysing and mechanically disrupting microbial cells and purifying DNA via phenol and phenol:chloroform:isoamyl alcohol extraction, as has been previously described (Dias et al., 2018). A Qubit fluorometer and a broad range dsDNA assay kit was used to quantify total

genomic DNA (Thermo Fisher Scientific, Waltham, MA). The V4 hypervariable region of the 16S rRNA gene was amplified with dual-indexed primers (F – GTGCCAGCMGCCGCGGTAA ; R - GGACTACHVGGGTWTCTAAT) as previously described (Kozich et al., 2013), which included adapters for Illumina sequencing technology (F - AATGATACGGCGACCACCGAGATCTACAC ; R- CAAGCAGAAGACGGCATACGAGAT). PCR included 25 ng template DNA, 0.5 μL of forward and reverse primers, 0.125 μL of Ex Taq DNA polymerase (Takara Bio USA, Inc. Mountain View, CA), 2.5 μL of 10X Ex Taq Buffer (Takara Bio USA, Inc. Mountain View, CA), 2.0 μL dNTP Mixture (Takara Bio USA, Inc. Mountain View, CA) and sterile distilled water to 25 μL. PCR was performed under the following specifications: 30 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. Negative and positive controls were also included.

PCR products were recovered by gel electrophoresis in a 1% (wt/vol) low-melt agarose gel (Sigma-Aldrich, St. Louis, MO) using a Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, CA). Purified products were quantified using a Qubit high sensitivity kit (Thermo Fisher Scientific, Waltham, MA) and a Synergy 2 Multi-Mode plate reader (BioTek, Winooski, VT). Samples were then equimolar pooled to a 4 nM final library for sequencing. The final library was sequenced with an Illumina MiSeq using a v2 2 x 250 reagent kit at 10 pmol/L with a 10% PhiX control, following the manufacturer's protocol (Illumina, Inc., San Diego, CA). Raw sequence data generated from this study were deposited into NCBI's Short Read Archive (SRA) and are publicly available under BioProject PRJNA625907.

2.5 Sequencing and bioinformatics workflow

Sequences were processed in mothur (v.1.40.0) (Schloss et al., 2009) with modified procedures (Kozich et al., 2013), as has been previously described (Dias et al., 2018). Briefly, paired-end reads were joined using the default parameters of the *make.contigs* command. Data quality was controlled by removing ambiguous bp, excessively short (< 200 bp) and long (> 500 bp) sequences, as well as removing homopolymers greater than 8 bp using the *screen.seqs* command. Sequences were then aligned to the SILVA 16S rRNA database (v 132) (Pruesse et al., 2007) and chimeric sequences were detected and removed using the Uchime algorithm (Edgar et al., 2011). The processed high-quality sequences were then classified, using the GreenGenes database (DeSantis et al., 2006), into operational taxonomic units (OTU) by the OptiClust algorithm (Westcott and Schloss, 2017) using a similarity cutoff of 97%. Depth of sequencing for each sample was evaluated by Good's coverage. Samples were normalized in mothur to the sample with the smallest number of sequences (2000 sequences) using the *normalize.shared* command.

Normalized OTU counts were used to determine diversity metrics and the RA of taxa in each sample. Dominant taxa were determined by identifying mean percent RA > 1 % using the phyloseq package (McMurdie and Holmes, 2013). Alpha diversity metrics, including Shannon's diversity index (SDI) and Chao1 index (Chao1), were calculated for all samples using mothur. Beta diversity was determined using the Bray-Curtis dissimilarity index, as calculated using the vegan package in RStudio (v 1.2.5) (RStudio Inc., Boston, MA). Differences in the Bray-Curtis index between groups were used to create a sample-wise distance matrix that was visualized using multidimensional scaling (MDS). Non-metric MDS (NMDS) was used if the base stress of creating the ordination plots was non-linear; metric MDS was utilized if the base stress followed linear regression. Equality of beta dispersion between groups was assessed using the betadisper

function of the vegan package. Bray-Curtis was compared between treatment groups (i.e. AMP, PLAC) and sample types (DNPS, NP tonsil, lung and LN) using a permutational multivariate analysis of variance (PERMANOVA) as implemented in the vegan package. The adonis function was used if the assumption of equal beta dispersion was met. An analysis of similarities was utilized if the assumption of equal beta dispersion was not met. A temporal beta-diversity index, which accounts for repeated measures, was calculated by the adespatial package to compare the beta diversity among DNPS #1, #2 and #3, and among 14 d samples, as previously described (Legendre, 2019).

2.6 Statistical analysis

All statistical analyses were performed in RStudio (v 1.2.5033). The experimental unit was defined as the individual calf in both trials. Sample size calculations were performed for the RCT objectives, as described in Holschbach et al. (2019). For the primary objective of whether characteristics of the NP microbiota were protective against or increased the risk of respiratory disease severity following challenge, outcome variables of interest included severity of CRS and USS as well as time to significant lesion development following challenge (i.e. USS = 2) in AMP and PLAC calves. The severity of CRS and USS were defined as the area under the curve (AUC) of each total score over time (e.g. AUC_{CRS} and AUC_{USS}). The AUC values were determined by graphing both scores individually (y-axis) by time point (x-axis) and calculating the AUC by summing the trapezoidal area between sequential time points. Time to significant lesion development was defined as a categorical variable (i.e. 2 h, 6 h, and 12 h after challenge).

Predictors included alpha diversity metrics of DNPS #1 ($Chao1_{DNPS\ \#1}$, $SDI_{DNPS\ \#1}$, the RA of dominant genera/unclassified taxa within DNPS #1, and treatment category (e.g. AMP, PLAC). The distributions of continuous variables were analyzed by evaluating histograms.

Continuous variables that were normally distributed included AUC_{CRS} and $Chao1_{DNPS\ \#1}$. Continuous variables that were not normally distributed included $SDI_{DNPS\ \#1}$, AUC_{USS}, and RA data of dominant genera/unclassified taxa within DNPS #1, #2 and #3.

The relationship between the predictor variable $Chao1_{DNPS\ \#1}$ and the outcome variable AUC_{CRS} was evaluated with multiple linear regression, controlling for treatment group. The model was evaluated with a Q-Q plot and residuals were evaluated for homoscedasticity. The strength of association between $SDI_{DNPS\ \#1}$ and AUC_{CRS}, AUC_{USS} and $SDI_{DNPS\ \#1}$ and $Chao1_{DNPS\ \#1}$ and AUC_{USS} were evaluated by Spearman's rank correlation coefficients. Relationships between clinical data (i.e. AUC_{CRS} and AUC_{USS}) and dominant taxa were also assessed with Spearman's rank correlation coefficients. The RA of dominant genera/unclassified taxa within DNPS #1 were compared among groups of calves that developed significant lesions 2 h, 6 h and 12 h following the challenge with Kruskal-Wallis tests. Shannon diversity index $_{DNPS\ \#1}$ and $Chao1_{DNPS\ \#1}$ were also compared among these groups by a Kruskal-Wallis test and an ANOVA, respectively. Dunn's multiple comparison test and Tukey's HSD test were used to further analyze specific group differences following a significant result in the Kruskal-Wallis test or ANOVA, respectively.

To address the secondary objective of evaluating the effect of bacterial challenge on the composition of the NP microbiota, composition characteristics were compared among the 3 DNPS in trial 1. Shannon diversity index and Chao1 were compared among DNPS #1, #2 and #3 using a 2-way repeated measures ANOVA, including treatment group as a fixed effect and calf as a random effect. The Bray-Curtis beta diversity metric was compared between DNPS #1 and #2, DNPS #2 and #3, and DNPS #1 and #3, using a temporal beta-diversity index. The effect of treatment group on beta diversity was evaluated for each DNPS. The dominant genera identified

among the 3 DNPS each displayed a Poisson distribution. Therefore, generalized linear mixed models were fit by maximum likelihood with the Laplace Approximation using the lme4 package; DNPS number and treatment group were fixed effects and calf was included as a random effect. If over dispersion was detected, a negative binomial family distribution was used, as previously described (Zhang et al., 2017). Nested models, including interactions, were compared with likelihood ratio tests.

To meet the objective of determining changes in the respiratory microbiota 14 d after experimental challenge (i.e. in DNPS#3, NP Tonsil, LN, lung samples), microbiota composition, alpha diversity metrics, the Bray-Curtis beta diversity metric, and the RA of dominant genera were compared by treatment group and analyzed as described above. Common dominant genera among anatomic sample types that were collected at d 14 (i.e. DNPS #3, NP tonsil, LN and lung) were evaluated for effects of sample site type and treatment group on the RA using the generalized linear mixed models described above, accounting for calf as a random effect.

The trial 2 objective, of describing changes in the composition of the respiratory microbiota by anatomic site, was met by comparing alpha diversity metrics, the Bray-Curtis beta diversity metric and dominant genera among the DNPS, NP tonsil, LN and lung samples of SAC calf samples. The alpha and beta diversity metrics were compared as described above. The RA of dominant genera were compared among sample types using a Friedman's test to account for repeated measures and the non-parametric distribution of RA data. Pairwise comparisons were performed with Nemenyi multiple comparison tests. Alpha was set at 0.05.

3.4 3. Results

3.2 Outcomes of Trial 1

All challenged calves survived the entire study period. A total of 28/30 calves developed pneumonia following the challenge (i.e. developed an USS = 2 within 12 h of *P. multocida* inoculation). There were 2 calves that did not respond to the challenge with significant ultrasonographic evidence of pneumonia; therefore, samples from these calves were excluded from further analyses. With this exclusion, 28 calves were randomized into the 2 treatment groups: 17 AMP calves and 11 PLAC calves. The resultant sample sizes for challenged calves, taking into account samples excluded because of sequencing error, is provided in Table 3.1. The AUC for clinical data by treatment group is presented in Supplemental Table 3.1.

3.2 Outcomes of Trial 2

Two out of the 9 calves had an USS = 2 prior to sample collection. One out of 9 calves were CRS positive prior to sample collection. Overall, SAC samples included DNPS (n = 9), NP tonsils (n = 9), LN (n = 9), and lung (n = 9).

3.3. Sequencing

Following quality filtering and normalization, a total of 6,872,209 sequences were recovered. The number of sequences per sample obtained from challenged calves was (mean ± SD) 31,488 ± 42,856. The number of sequences per sample, specifically from DNPS #1, #2, and #3, was (mean ± SD) 34,866 ± 53,786. The number of sequences per sample in DNPS #1 (23,230 ± 20,912) was lower than the number of sequences per sample in DNPS #2 (39,006 ± 28,252), when taking into account calf as a random effect ($P = 0.03$). There was no difference between the number of sequences in DNPS #3 (41,560 ± 84,801) and DNPS #1 or DNPS #2 ($P > 0.13$). There was no difference in the number of sequences among DNPS #3 (41,560 ± 84,801), NP tonsil (20,476 ± 6,908), LN (25,331± 19415), and lung (38,834 ± 45,215). The number of sequences per sample obtained from SAC calves was (mean ± SD) 50,075 ± 8,5172. Good's

coverage was (mean ± SD) 99.4% (± 0.01%) for samples from challenged calf and 99.8% (± 0.002%) for samples from SAC calves, which indicate adequate coverage in all sample groups. Following normalization of all sequence data, there was a total of 7,398 unique OTUs identified. There were 7 samples in which 16S rRNA DNA could not be amplified from after repeated PCR attempts; therefore, these samples were excluded from the study. The sample types excluded were DNPS #1 (n = 5), DNPS #2 (n = 1), and lung (n = 1) (Table 3.1).

3.4 Trial 1: Association between DNPS #1 composition and severity of respiratory disease following P. multocida challenge

Pre-challenge NP alpha diversity, as measured by $Chao1_{DNPS\ \#1}$, was associated with a -0.03 unit decrease in AUC_{CRS} of challenged calves, regardless of treatment group ($P = 0.01$) (Figure 3.1). A weak to moderate linear negative correlation (rho = -0.38) was observed between $Chao1_{DNPS\ \#1}$ and AUC_{USS} ($P = 0.07$) and a moderate negative linear correlation (r = -0.52) was observed between $Chao1_{DNPS\ \#1}$ and AUC_{CRS} ($P = 0.01$) (Table 3.2). There was no significant correlation between $SDI_{DNPS\ \#1}$ and AUC_{CRS} nor $SDI_{DNPS\#1}$ and AUC_{USS} ($P \geq 0.22$) (Table 3.2; Figure 3.1). When evaluating the relationship between clinical outcome data (i.e. AUC_{CRS} and AUC_{USS}) and dominant taxa (i.e. genera and unclassified families) in DNPS #1, weak to moderate negative linear correlations (r = -0.37) were observed between the RA of *Acinetobacter* spp. and *Helococcus* spp. and AUC_{CRS} (Supplemental Table 3.2; $P = 0.09$).

Calves that developed a significant lesion (i.e. USS = 2) 2h following experimental challenge had a higher RA of *Mycoplasma* spp. in DNPS #1 compared to calves that developed a significant lesion 12 h after challenge ($P = 0.01$) (Figure 3.2). Compared to calves with an USS=2 at 2 h post challenge, calves that took 12 h to develop a significant lung lesion, following *P. multocida* exposure, had higher RA of *Prevotella* spp., [*Prevotella* spp.], *Bacteroides* spp.,

Blautia spp., *Ruminococcaceae*, *Faecalibacterium* spp., and *Lachnospiraceae* in DNPS #1 ($P \leq 0.01$) (Figure 3.2). Time until significant lesion development was also associated with $SDI_{DNPS\#1.}$ Calves that took 12 h to develop a significant lung lesion had a higher median (IQR) $SDI_{DNPS\ \#1}$ (5 (4.9, 5.3)) than calves that developed a significant lung lesion in 2 h (2.7 (2.2, 3.3)) ($P = 0.02$). Although the mean ± SD $Chao1_{DNPS\ \#1}$ was numerically highest in calves that developed an USS = 2 at 12 h post challenge (1012.54 ± 253.15) compared to calves that had an USS = 2 at 2 h post challenge (638.30 ± 150.23), this difference was not statistically significant ($P = 0.20$).

3.5 Trial 1: Effect of bacterial challenge and treatment group on the composition of NP microbiota

The effects of *P. multocida* challenge and treatment group (i.e. AMP, PLAC) did not significantly affect Chao1 among DNPS #1, #2, #3, when controlling for repeated sampling ($P > 0.09$). Similarly, bacterial challenge and treatment group did not significantly affect SDI among the 3 DNPS, when controlling for repeated sampling ($P > 0.07$) (Table 3.3). Beta diversity, as measured by the Bray-Curtis dissimilarity index, was affected by bacterial challenge in the short-term (Supplemental Figure 3.2). The mean temporal beta diversity index, calculated from the difference of OTU loss and gain, was -0.0049 between DNPS #1 and DNPS #2 ($P = 0.04$). This difference was 0.0065 between DNPS #2 and DNPS #3 ($P = 0.01$). However, when comparing DNPS #1 and DNPS #3, the mean difference was 0.0016 ($P = 0.53$). The R^2 values from the PERMANOVA evaluating the effect of treatment group on Bray-Curtis dissimilarity was 0.031 for DNPS #1 ($P = 0.73$), was 0.014 for DNPS #2 ($P = 0.98$) and was 0.055 for DNPS#3 ($P = 0.26$) (Supplemental Figure 3.2).

Dominant taxa, including phyla and resolved genera or unclassified families, identified in DNPS #1, #2, and #3 are displayed in Figure 3.3. Output from the mixed models evaluating the effects of DNPS number (i.e. experimental challenge) and treatment group (i.e. AMP or PLAC) on the expected RA of genera (or resolved taxa) is displayed in Supplemental Table 3.3. Treatment group had no effect on the expected RA of unclassified *Microbacteriaceae*, *Bacteroides* spp., and *Blautia* spp., which were higher in DNPS #1; however, AMP calves had an expected RA (95% CI) of *Prevotella* spp. 0.59 (0.38, 0.91) lower than PLAC calves.

Predictably, the expected RA (95% CI) of *Pasteurella* spp. was 8.44 (2.82, 25.25) higher in DNPS #2 and was 53.10 (18.57, 151.81) higher in DNPS #3 compared to DNPS #1. The effect of treatment group (i.e. AMP calves compared to PLAC calves) actually increased the expected RA (95% CI) of *Pasteurella* spp. by an order of 6.84 (2.43, 19.27). The impacts of taxa, which were significantly affected by DNPS swab number and/or treatment group, on the beta diversity of DNPS #1, #2, and #3, are displayed in Supplemental Figure 3.2(d-f).

3.6 Trial 1: Effect of treatment group on the composition of the respiratory tract microbiota 14 d after experimental challenge

There was no effect of treatment group on SDI nor Chao1 among DNPS #3, NP tonsil, LN, and lung samples in challenged calves ($P = 0.78$, $P = 0.74$, respectively) (Table 3.4). Additionally, there were no differences in Chao1 between d 14 sample types ($P = 0.53$). The mean ± SD of SDI in NP tonsil samples (4.19 ± 0.76) was higher than in DNPS #3 (3.30 ± 1.32; $P = 0.04$) and in lung samples (3.41 ± 1.35) ($P = 0.02$) (Supplemental Table 3.4). Beta diversity, as measured by the Bray-Curtis dissimilarity index, was affected by sample type at 14 d post challenge; however, treatment group had no significant effect on beta diversity ($P > 0.14$) (Supplemental Figure 3.3). The R^2 values from the PERMANOVAs evaluating the effect of

treatment group on Bray-Curtis dissimilarity for each sample type at d 14 were 0.060 ($P = 0.14$) for DNPS #3, 0.02 ($P = 0.84$) for NP tonsil, 0.04 ($P = 0.43$) for LN and 0.04 ($P = 0.49$) for lung samples (Supplemental Figure 3.3). The mean temporal beta diversity index difference between DNPS #3 and NP tonsil was 0.005 ($P = 0.02$) and between DNPS#3 and LN was 0.0047 ($P = 0.06$). Differences in mean temporal beta diversity index between DNPS #3 and lung (0.0019), NP tonsil and LN (-0.0005), NP tonsil and lung (-0.0032), and LN and lung (0.0027) were not significant ($P > 0.12$).

The dominant genera or classified taxa common to all 14 d sample types (DNPS #3, NP tonsil, LN and Lung) included *Bacteroides* spp., *Blautia* spp., *Ruminococcaceae*, *Clostridium* spp., *Prevotella* spp., *Acinetobacter* spp., *Psychrobacter* spp., *Escherichia* spp., and *Lachnospiraceae* (Figure 3.3). Treatment group did not significantly affect the expected RA of common, dominant, genera among 14 d samples (Supplemental Table 3.5). *Pasteurella* spp. was not identified as a dominant genus in NP tonsil samples of challenged calves; however, it was identified in DNPS #3, LN and lung samples. Along with *Pasteurella* spp., *Mycoplasma* spp. and *Microbacteriaceae* were identified as dominant taxa in DNPS #3 and lung samples exclusively. Deep nasopharyngeal swab #3 and NP tonsil shared only [*Prevotella*] spp. exclusively and NP tonsil, LN and DNPS #3 shared *Faecalibacterium* spp. and *Corynebacterium* spp. exclusively (Figure 3.3; Supplemental Table 3.6). The effects of common dominant taxa on the beta diversity of 14 d samples, which were significantly affected by sample type, are displayed as vectors in Supplemental Figure 3.3e-h.

3.7 Trial 2: The effect of anatomic sample type on the respiratory microbiota in SAC calves

The alpha diversity metric Chao1 was numerically highest in DNPS and NP tonsil compared to LN and lung (Table 3.5). In contrast, SDI was numerically lower in DNPS samples

compared to lung samples; however, SDI in NP tonsil was higher than DNPS, LN and lung samples. Both Chao1 and SDI were not significantly different by anatomic sample type, when including calf as a random effect ($P = 0.24$; $P = 0.06$, respectively). Beta diversity, as measured by the Bray-Curtis dissimilarity metric, for SAC samples is displayed in Supplemental Figure 3.4. There was no visual separation of the samples by sample type on the NMDS plots (Supplemental Figure 3.4a). The mean temporal beta diversity index difference was 0.0038 between DNPS and NP tonsil ($P = 0.21$), 0.0031 between DNPS and LN ($P = 0.34$), and 0.0050 between DNPS and lung ($P = 0.05$). The trend between DNPS and lung is displayed in Supplemental Figure 3.4b.

The dominant taxa identified in SAC samples were similar to those identified in challenged calves (Supplemental Figure 3.5). The dominant genera or unclassified families common to all sample types included some of the same taxa identified in challenged calves: *Ruminococcaceae*, *Blautia* spp., *Bacteroides* spp., and *Prevotella* spp. Additionally, *Faecalibacterium* spp. and [*Prevotella*] spp. were also identified as common among anatomic sample types (Supplemental Figure 3.5). The genera or unclassified families identified solely in DNPS samples included *Pasteurella* spp., *Moraxellaceae*, *Escherichia* spp., *Chlamydia* spp., and *Mannheimia* spp. Identified taxa that were exclusive to DNPS and lung samples included *Microbacteriaceae* and *Mycoplasma* spp. There were no taxa identified that were exclusive to DNPS and LN samples. Only *Acinetobacter* spp. was identified as an exclusively common genus in DNPS and NP tonsil. The median (IQR) percent RA of common dominant taxa are displayed in Supplemental Table 3.7. The RA of *Microbacteriaceae* was highest in DNPS and was significantly different from NP tonsil samples ($P = 0.03$). The RA of *Prevotella* spp. was highest in LN and lung samples, both of which were significantly different from DNPS ($P =$

0.0002; $P = 0.003$, respectively). The impacts of the RA of *Microbacteriaceae* and *Prevotella* spp. on the beta diversity of SAC samples are displayed in Supplemental Figure 3.4.

3.5 4. Discussion

The respiratory microbiota is a complex community that functions as a host defense strategy and a facilitator of pathogenesis under certain conditions. Dysbiosis, manifested as reduced NP microbial diversity, has been associated with clinical BRD in feedlot cattle (Holman et al., 2015; Timsit et al., 2018; McMullen et al., 2019). The association between decreased NP microbial species abundance and increased severity of clinical respiratory disease following bacterial challenge is consistent with results of aforementioned feedlot cattle studies. Our results contrast the findings of Lima et al. (2016), which showed that higher NP species abundance at 3 d of age was associated with a higher risk of pneumonia during the first month of life in pre-weaned dairy calves. Given the evidence of temporal variability in the NP microbiota (Holman et al., 2017), the relationship between diversity and BRD may diverge between studies because of the difference in age of enrollment (i.e. 3 d versus 52 $\pm$ 6 d of age).

Interestingly, alpha diversity metrics (i.e. Chao1 and SDI) were not significantly affected by bacterial challenge in our study. There is debate as to whether alpha diversity metrics extrapolated from macroecology are sensitive enough to describe low-abundance OTUs (Johnson and Burnet, 2016). The respiratory tract microbiota is considered to have low biomass overall (Zeineldin et al., 2019); therefore, alpha diversity metrics may not precisely illustrate the stability of the NP microbial community. The acute shift in beta diversity between DNPS #1 and DNPS #2 was consistent with results from previous studies in feedlot cattle that have noted significant shifts in NP beta diversity within 48 h of stressful events, such as transportation and weaning (Timsit et al., 2016; Holman et al., 2017).

Additionally, there was no effect of ampicillin on the diversity metrics of all samples following bacterial challenge. These findings are in contrast to results from a recent study in healthy feedlot cattle that showed treatment with either oxytetracycline or tulathromycin affected the NP microbiota beta diversity up to 34 d after treatment (Holman et al., 2019). Surprisingly, AMP treatment was associated with an increased expected RA of *Pasteurella* spp. within the NP compared to PLAC calves. Ampicillin was administered at a mid-range dose for a conservative duration (6.6 mg/kg i.m. for 3 d; label range: 4.4 -11 mg/kg i.m. for 3 - 7 d) and DNPS #3 was collected 11 d following treatment. The RA of *Pasteurella* spp. may have decreased initially but could have been allowed to replicate following the decrease of other ampicillin-sensitive bacteria. This pattern is consistent with the immediate improvement in USS following treatment of AMP calves and subsequent reoccurrence of lung lesions during the final 4 days of the trial (Holschbach et al., 2019). Further longitudinal studies that include different doses and durations are needed. Additionally, the impact of antibiotics primarily used to treat BRD in dairy calves (e.g. macrolides, florfenicol), as reported by the results of a national producer survey (USDA, 2018), should be investigated.

The RA of oral and GIT-associated bacteria, such as *Prevotella* spp., *Bacteroides* spp., *Blautia* spp., *Ruminococcaceae*, *Lachnospiraceae*, and *Faecalibacterium* spp., were dominant in samples from the NP, NP tonsil, LN and lung of challenged and SAC calves. Although these bacteria have been previously identified within the NP of dairy calves and the NP and trachea of feedlot cattle, they have not been linked to BRD pathogenesis (Lima et al., 2016; Maynou et al., 2019; Timsit et al., 2018). Interestingly, increased RA of each of these aforementioned taxa, within the NP, was associated with a delay in lung lesion development following experimental challenge. *Ruminococcaceae* and *Lachnospiraceae* have been associated with anti-inflammatory

properties in the GIT; abundance of both families has been negatively associated with immune-mediated inflammation in humans (Frank et al., 2007; Fujimoto et al., 2013). Enrichment of *Prevotella* spp. specifically in the bronchoalveolar lavage samples of humans has been associated with increased lower airway inflammation (Segal et al., 2013). Interestingly, the mean % RA of *Prevotella* spp. was increased in the lung samples of both challenged and SAC calves. Although NP enrichment of *Prevotella* spp. within the NP was associated with slower lung lesion development, the genus was not correlated respiratory disease severity. Aspiration-derived bacteria, have been shown to facilitate regulation of constitutive inflammation within the mucosal surface of the human respiratory tract (Segal et al., 2016). Pre-weaned dairy calves are developing ruminants and therefore, regurgitate ruminal bacteria into the oral cavity; these bacteria may have effects on the respiratory microbiota via microaspiration, which may impact BRD pathogenesis.

Lung lesion development was more rapid in calves with NP enrichment of *Mycoplasma* spp. in DNPS#1. Increased RA of NP *Mycoplasma* spp. has been previously associated with an increased risk of pneumonia in dairy calves (Lima et al., 2016). Deep nasopharyngeal swab #1 also had an increased expected RA of *Psychrobacter* spp. and *Microbacteriaceae*. Nasopharyngeal *Psychrobacter* spp. and *Rathayibacter*, a genus of *Microbacteriaceae*, have previously been linked to health in dairy calves and feedlot cattle, respectively (Lima et al., 2016; Zeineldin et al., 2017a). These genera may facilitate homeostasis of the NP microbiota. The expected RA of *Acinetobacter* spp. was highest in DNPS #2. Nasopharyngeal enrichment of *Acinetobacter* spp. has been correlated with BRD in feedlot cattle (Zeineldin et al., 2017a). Enrichment of NP *Acinetobacter* spp. may be an early indicator of a shift in microbial homeostasis.

Common dominant taxa identified among 14 d samples were similar to those identified in the first two swabs. Although taxa were similar, *Pasteurella* spp. was not a dominant genus in the NP tonsil, which may indicate a difference in the surface microbiota collected by DNPS and the microbiota attached to mucosal tissue. To the authors' knowledge, the tonsil microbiota has not been previously described in cattle. Only one previous study evaluated the tissue microbiota of lung and mediastinal lymph nodes in dairy calves (n = 26) and beef calves (n = 32); results indicated a higher RA of *Mycoplasma* spp. and *Leptotrichiaceae* in both lung and LN of calves with BRD (Johnston et al., 2017). Bacteria and viruses involved in pneumonia pathogenesis circulate to LN from the lung and impact the host immune response (Bashiruddin et al., 2005; Johnston et al., 2019). Therefore, further understanding of the lower respiratory microbiota may depend upon both the LN and lung resident bacteria.

Anatomic location in SAC calves did not significantly affect alpha or beta diversity metrics. This was in contrast to studies in feedlot cattle and humans that showed a decrease in alpha diversity in the lower respiratory tract compared to the NP (Dickson et al., 2015; Zeineldin et al., 2017b; Timsit et al., 2018). The common taxa identified within samples from trial 2 were dominated by GIT and oral cavity-associated bacteria, which is comparable to the results of samples from trial 1. The sample size of this group was small; thus, a lack of power may have limited our ability to appreciate differences in diversity metrics between anatomic sites.

There are limitations to this study that require consideration. There was a 14-d lag period between DNPS#2 and DNPS#3 and tissue samples, which hindered our ability to detect acute changes in the respiratory microbiota following *P. multocida* challenge and ampicillin treatment. Additionally, the lower respiratory microbiota was not sampled prior to the challenge or antibiotic therapy, which impedes interpretation of those variables. Post-mortem lung tissue

sample collection may have also introduced variability, as lesion severity and exact location differed among challenged calves. Also, 2 / 9 SAC calves had evidence of BRD, which may have affected the composition of the respiratory microbiota and obscured our ability to detect composition changes due to anatomic site alone.

3.6 5. Conclusions

Our primary hypothesis was supported in that challenged calves with a higher species abundance within the NP microbiota had less severe clinical respiratory disease, as measured by cumulative respiratory score, in the 14 days following experimental inoculation. Experimental challenge significantly altered the beta diversity of the NP microbiota acutely; however, parenteral ampicillin had no significant effect on alpha or beta diversity following the challenge. As hypothesized, inoculation did increase the RA of *Pasteurella* spp. in the NP; however, ampicillin actually increased the RA of *Pasteurella* spp. in the NP. Parenteral ampicillin did not have a significant effect on the diversity or RA of taxa within tissue samples. Lastly, our results did not support previous trends in results from feedlot cattle and human studies that the upper respiratory tract has greater alpha diversity than the lower respiratory tract in health; however, given numerical trends were identified, we suspect lack of power in our data contributed to this contrast. Common dominant genera and unclassified families identified within the NP and tissue samples of challenged calves and SAC calves included bacteria associated with the oral cavity and GIT, such as *Ruminococcaceae*, *Lachnospiraceae, Blautia* spp., *Bacteroides* spp. and *Prevotella* spp. These taxa were associated with slower lung lesion development, which may suggest protective effects. How interactions between the GIT microbiota and pathobionts associated with the respiratory tract contribute to BRD risk requires further investigation.

Conflict of Interest

Financial support for this study was provided by the National Institutes of Health Training Grant (T32OD010423) and the University of Wisconsin Agricultural Research Stations.

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3.8 TABLES AND FIGURES

Table 3.1. Summary of group sample sizes in challenged calves

Sample type	Sample size (N)	AMP (n)	PLAC (n)
DNPS #1	23	14	9
DNPS #2	27	17	10
DNPS #3	28	17	11
tonsil	28	17	11
LN	28	17	11
lung	27	16	11

Table 3.1. A summary of group sample sizes (N) with the number of calves (n) in AMP and PLAC treatment groups for each sample type. The excluded calves were due to inadequate response to the experimental challenge (n = 2) and sequencing errors (n = 7). DNPS = deep nasopharyngeal swab, LN = lymph node, AMP = ampicillin treatment group, PLAC = placebo group

Table 3.2. Spearman's correlation coefficient between alpha diversity metrics of DNPS #1 and severity of respiratory disease

Dependent variable	Independent variable	Spearman's rho	95% CI	*P* value
AUC_{USS}	$SDI_{DNPS\ \#1}$	0.02	-0.40 - 0.41	0.91
AUC_{CRS}	$SDI_{DNPS\ \#1}$	-0.27	-0.61 - 0.13	0.22
AUC_{USS}	$Chao1_{DNPS\ \#1}$	-0.38	-0.73 - 0.003	0.07
AUC_{CRS}	$Chao1_{DNPS\ \#1}$	-0.52	-0.79 - -0.10	0.01

Table 3.2. Spearman's rank correlation coefficients, including the 95% confidence interval, between outcome variables describing severity of respiratory disease (AUC_{CRS} and AUC_{USS}) and predictors ($SDI_{DNPS\ \#1}$, $Chao1_{DNPS\ \#1}$); n = 28

Table 3.3. Mean ± SD of Chao1 and SD1 by DNPS for each treatment group

DNPS	Chao1		SDI	
	AMP	PLAC	AMP	PLAC
DNPS #1	733.30 ± 360.40	693.31± 318.30	3.20 ± 1.60	4.10 ± 0.90
DNPS #2	787.64 ± 254.10	733.10 ± 287.60	3.24 ± 1.23	3.44 ± 1.30
DNPS #3	559.20 ± 248.53	643.50 ± 234.20	3.11 ± 1.34	3.72 ± 1.30

Table 3.3. Mean ± SD of alpha diversity metrics (Chao1 index (Chao1) and Shannon Diversity Index (SDI)) for Deep nasopharyngeal swabs (DNPS) before challenge (DNPS #1), at significant lesion development (DNPS #2), and at 14 d following the challenge (DNPS #3). Samples balanced to include the same number of calves (n = 23) in each sample type group. The Mean ± SD of Chao1 was not different by swab number ($P = 0.08$) or treatment group ($P = 0.97$). The Mean ± SD of SDI was not different by swab number ($P = 0.67$) or treatment group ($P = 0.07$). AMP = ampicillin treated group; PLAC = placebo group

Table 3.4. Alpha diversity metrics (mean ± SD) for 14 d samples by treatment group

Respiratory microbiota at 14 d post challenge	Chao1		SDI	
	AMP	PLAC	AMP	PLAC
DNPS #3	556.66 ± 256.46	643.45 ± 234.20	3.01 ± 1.32	3.72 ± 1.27
NP Tonsil	501.18 ± 175.43	520.16 ± 311.13	4.18 ± 0.82	4.21 ± 0.69
LN	477.25 ± 255.65	579.93 ± 213.77	3.87 ± 0.91	4.21 ± 1.04
Lung	597.43 ± 234.71	417.77 ± 246.34	3.66 ± 1.13	3.06 ± 1.61

Table 3.4. Mean ± SD Chao1 index (Chao1) and Shannon Diversity Index (SDI) in 14 d samples. Samples balanced to include the same number of calves (n = 27) in each sample type group.

Table 3.5. Mean ± SD of Chao1 and Median (IQR) of SDI by sample type in SAC calves

Sample type	Chao1	SDI
DNPS	598.12 ± 225.91	2.5 (1.8, 3.2)
NP tonsil	526.48 ± 80.63	4.8 (4.1, 5.0)
LN	506.28 ± 197.70	4.1 (3.5, 4.7)
Lung	476.98 ± 195.94	3.5 (2.8, 4.8)

Table 3.5. Mean ± SD of Chao1 and Median (IQR) of SDI by sample type in SAC (n =9)

Figure 3.1. Scatter plots of relationship between alpha diversity metrics in DNPS #1 and severity of pneumonia following experimental challenge

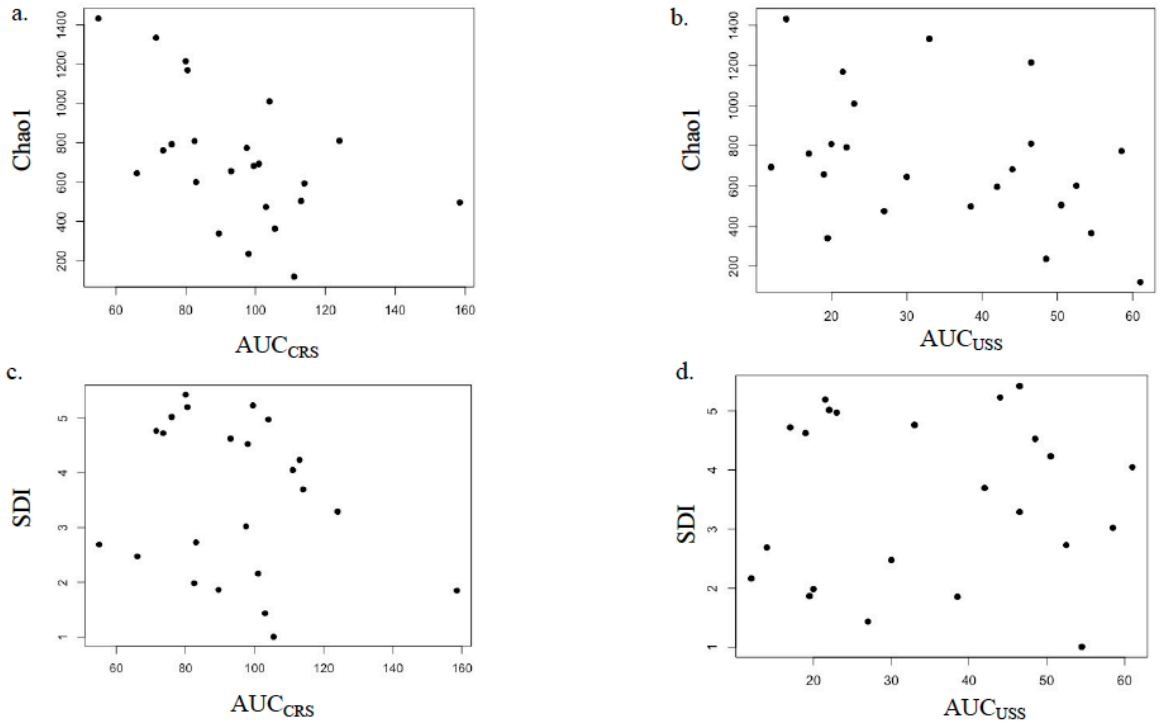


Figure 3.1. a) Area under the curve for clinical respiratory score over 14 d post challenge (AUC_{CRS}) by Chao1 index (Chao1) (n =23) (b) Area under the curve for ultrasound score (AUC_{USS}) by Chao1. (c) AUC_{CRS} by Shannon diversity index (SDI). (d) AUC_{USS} by SDI.

Figure 3.2. Percent relative abundance (RA) of dominant taxa in deep nasopharyngeal swab (DNPS) #1 by time of significant lesion development following experimental challenge.

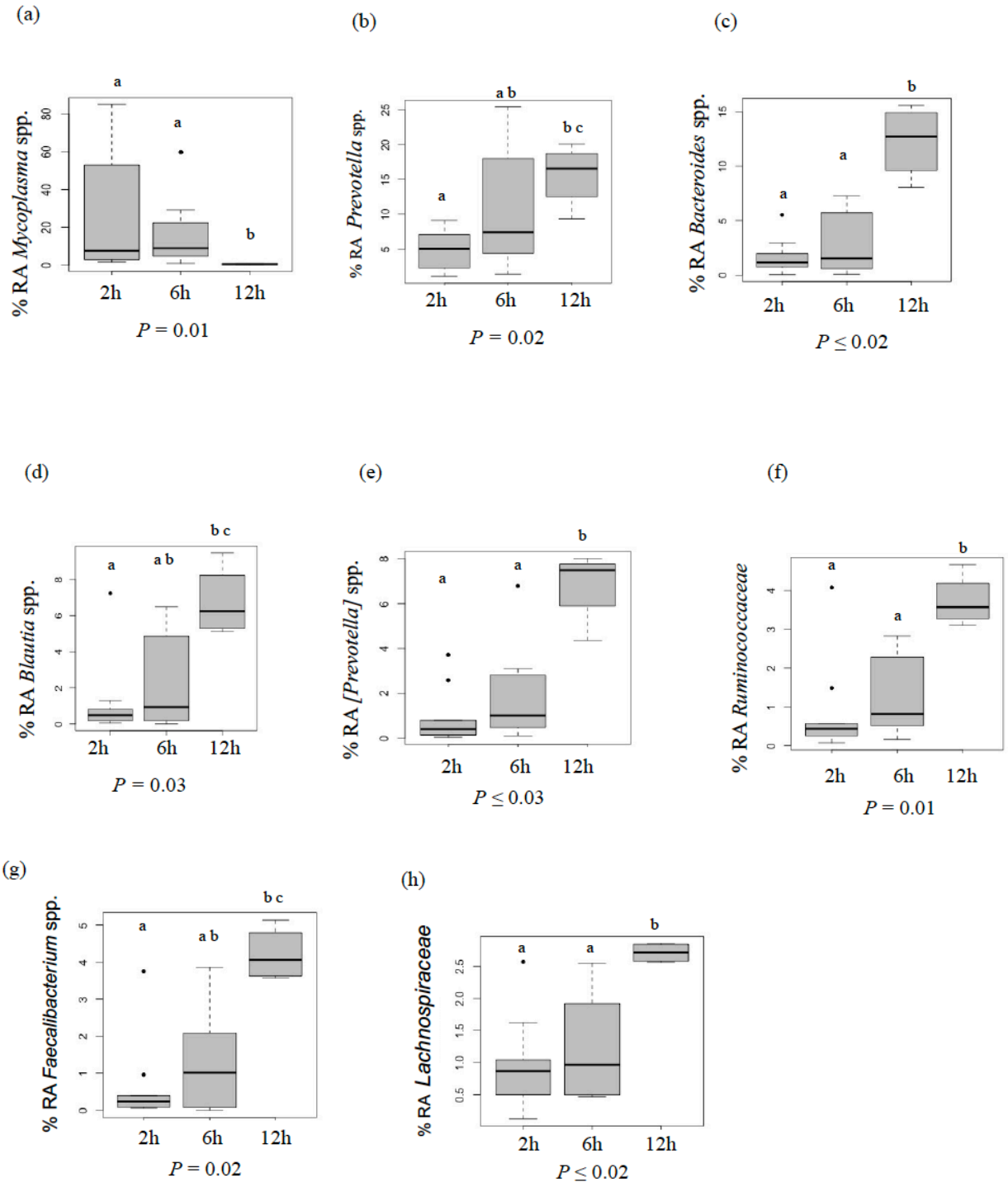
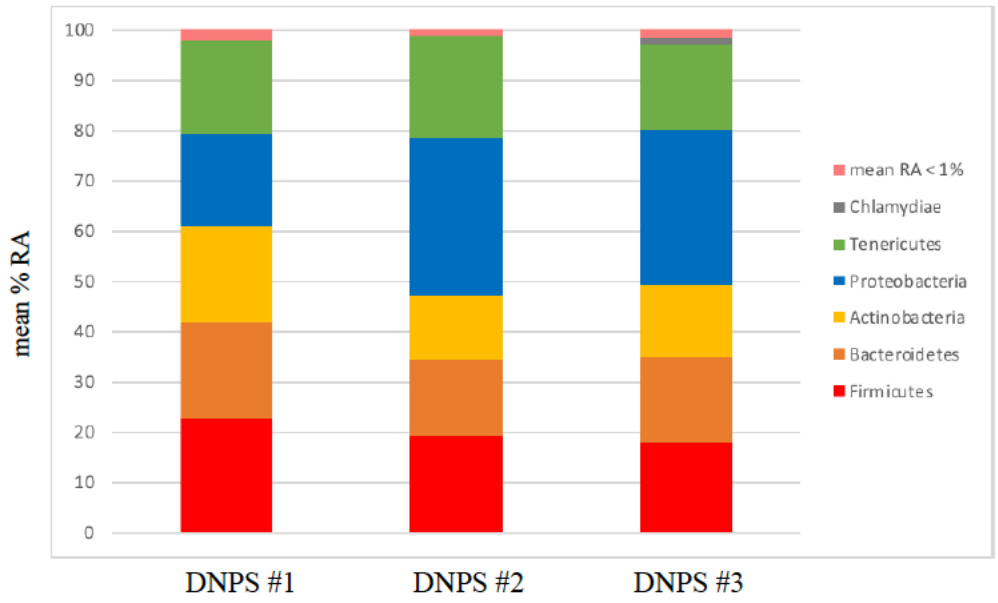


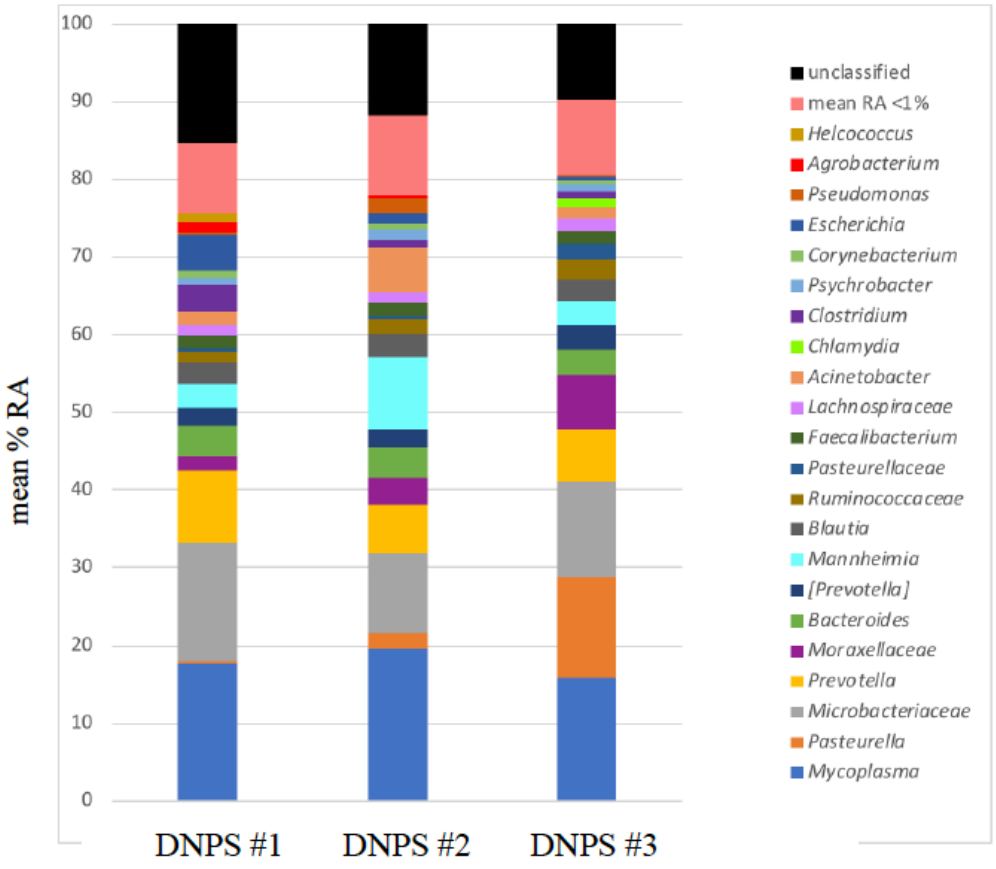
Figure 3.2. Boxplots include the minimum, first quartile, median, third quartile, maximum, and outlier values (filled in circles) of % RA for dominant taxa identified as significantly different among points at which significant ultrasonographic evidence of pneumonia (USS = 2) was recorded following experimental challenge (n=23). Taxa displayed include: (a) *Mycoplasma* spp., (b) *Prevotella s*pp., (c) *Bacteroides* spp., (d) *Blautia* spp., (e) [*Prevotella* spp.], (f) *Ruminococcaceae*, (g) *Faecalibacterium* spp., (h) *Lachnospiraceae*. Time points post-challenge included 2h (n = 9), 6h (n =10), and 12 h (n = 4). Kruskal-Wallis tests were performed for each taxon. Superscript letters that differ are statistically significant ($P < 0.05$).

Figure 3.3. Dominant taxa in DNPS #1, #2, #3, NP tonsil, LN and lung of challenged calves

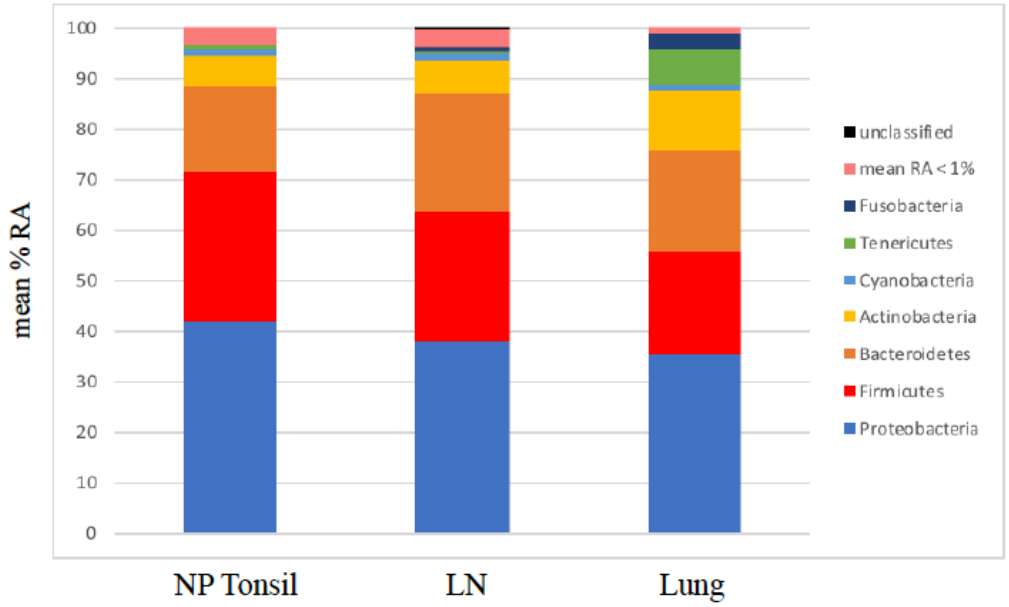
(a)



(b.)


mean % RA
100
90
80
70
60
50
40
30
20
10
0
DNPS #1
DNPS #2
DNPS #3
unclassified
mean RA <1%
Helcococcus
Agrobacterium
Pseudomonas
Escherichia
Corynebacterium
Psychrobacter
Clostridium
Chlamydia
Acinetobacter
Lachnospiraceae
Faecalibacterium
Pasteurellaceae
Ruminococcaceae
Blautia
Mannheimia
[Prevotella]
Bacteroides
Moraxellaceae
Prevotella
Microbacteriaceae
Pasteurella
Mycoplasma

(c)


mean % RA
100
90
80
70
60
50
40
30
20
10
0
NP Tonsil
LN
Lung
unclassified
mean RA < 1%
Fusobacteria
Tenericutes
Cyanobacteria
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria

(d)

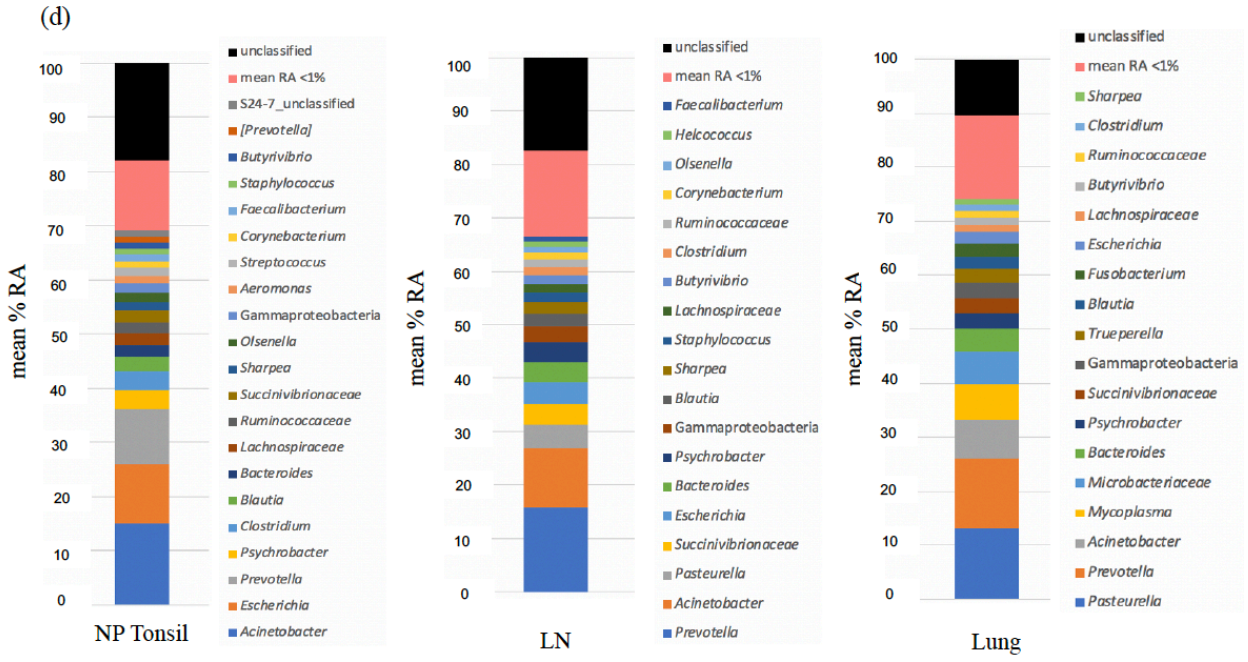

mean % RA
NP Tonsil
unclassified
mean RA <1%
S24-7_unclassified
[Prevotella]
Butyrivibrio
Staphylococcus
Faecalibacterium
Corynebacterium
Streptococcus
Aeromonas
Gammaproteobacteria
Olsenella
Sharpea
Succinivibrionaceae
Ruminococcaceae
Lachnospiraceae
Bacteroides
Blautia
Clostridium
Psychrobacter
Prevotella
Escherichia
Acinetobacter
LN
Faecalibacterium
Helcococcus
Olsenella
Corynebacterium
Ruminococcaceae
Clostridium
Butyrivibrio
Lachnospiraceae
Staphylococcus
Sharpea
Blautia
Gammaproteobacteria
Psychrobacter
Bacteroides
Escherichia
Succinivibrionaceae
Pasteurella
Acinetobacter
Prevotella
Lung
Sharpea
Clostridium
Ruminococcaceae
Butyrivibrio
Lachnospiraceae
Escherichia
Fusobacterium
Blautia
Trueperella
Gammaproteobacteria
Succinivibrionaceae
Psychrobacter
Bacteroides
Microbacteriaceae
Mycoplasma
Acinetobacter
Prevotella
Pasteurella

Figure 3.3 (a) Mean percent relative abundance (RA) of dominant phyla in deep nasopharyngeal swab (DNPS) #1, DNPS #2 and DNPS #3 of challenged calves. (n=23) (b) Mean percent RA of dominant genera in DNPS #1, DNPS #2, and DNPS #3 of challenged calves (n=23) (c) Mean percent RA of dominant phyla in Nasopharyngeal (NP) tonsil, lymph node (LN) and lung of challenged calves (n=27) (d) Mean percent RA of dominant genera in NP tonsil, LN and lung of challenged calves (n=27). Dominant taxa were defined by identifying a mean RA > 1% in at least one of the sample type groups. Unclassified refers to operational taxonomic units (OTU) that were unable to be resolved. Mean RA <1% includes the sum of resolved phyla and genera that had a mean RA of less than 1%.

3.9 SUPPLEMENTARY TABLES AND FIGURES

Supplement Table 3.1. Area under the curve (AUC) for clinical data; presented as median (interquartile range) or mean ± standard deviation (SD), by experimental treatment group in challenged calves

Clinical data	AMP	PLAC	*P* value
AUC_{CRS}	92.9 ± 28.5	95.8 ± 17	0.74
AUC_{USS}	23 (20, 38.5)	46 (38, 51.5)	0.03

Supplemental Table 3.1. Area under the curve (AUC) for clinical respiratory score (CRS) and ultrasound score (USS) presented as median (interquartile range) and mean ± SD (n = 28)

Supplemental Table 3.2. Spearman's rank correlation coefficients between RA of dominant taxa (genera and unclassified families) in DNPS #1 and severity of respiratory disease

Dominant taxa	Dependent variable	Spearman's rho	95% CI	*P* value
Prevotella spp.	AUC_{CRS}	-0.007	-0.42 - 0.42	0.97
	AUC_{USS}	0.05	-0.37 - 0.44	0.82
Mycoplasma spp.	AUC_{CRS}	-0.13	-0.51 - 0.26	0.55
	AUC_{USS}	-0.09	-0.54 - 0.40	0.70
Microbacteriaceae	AUC_{CRS}	0.005	-0.51 - 0.46	0.98
	AUC_{USS}	-0.25	-0.60 - 0.13	0.25
Escherichia spp.	AUC_{CRS}	-0.16	-0.62 - 0.28	0.46
	AUC_{USS}	0.04	-0.42 - 0.50	0.86
Bacteroides spp.	AUC_{CRS}	-0.18	-0.56 - 0.27	0.42
	AUC_{USS}	-0.12	-0.61 - 0.37	0.60
Clostridium spp.	AUC_{CRS}	-0.16	-0.58 - 0.30	0.47
	AUC_{USS}	0.20	-0.29 - 0.63	0.40
Mannheimia spp.	AUC_{CRS}	-0.06	-0.44 - 0.35	0.77
	AUC_{USS}	-0.08	-0.46 - 0.34	0.71
Blautia spp.	AUC_{CRS}	-0.20	-0.56 - 0.25	0.46
	AUC_{USS}	-0.20	-0.65- 0.30	0.20
[Prevotella] spp.	AUC_{CRS}	-0.02	-0.45 - 0.36	0.93
	AUC_{USS}	0.05	-0.44 - 0.45	0.84
Moraxellaceae	AUC_{CRS}	-0.12	-0.56 - 0.32	0.57
	AUC_{USS}	-0.21	-0.66 - 0.33	0.35
Acinetobacter spp.	AUC_{CRS}	-0.37	-0.66 - 0.004	0.08
	AUC_{USS}	0.20	-0.26 - 0.58	0.36
Ruminococcaceae	AUC_{CRS}	-0.15	-0.51 - 0.23	0.50
	AUC_{USS}	-0.14	-0.58 - 0.36	0.52
Faecalibacterium spp.	AUC_{CRS}	-0.26	-0.60 - 0.17	0.23
	AUC_{USS}	-0.30	-0.70 - 0.19	0.22
Agrobacterium spp.	AUC_{CRS}	-0.23	-0.60 - 0.17	0.27
	AUC_{USS}	0.12	-0.33 - 0.54	0.60
Lachnospiraceae	AUC_{CRS}	-0.31	-0.60 - 0.16	0.22
	AUC_{USS}	-0.13	-0.53 - 0.28	0.54
Helococcus spp.	AUC_{CRS}	-0.37	-0.73 - 0.07	0.09
	AUC_{USS}	-0.15	-0.57 - 0.28	0.50
Corynebacterium spp.	AUC_{CRS}	0.02	-0.40 - 0.41	0.92
	AUC_{USS}	0.20	-0.41 - 0.60	0.50

Supplemental Table 3.2. Spearman's rank correlation coefficients, including the 95% confidence interval, between outcome variables describing severity of respiratory disease (AUC_{CRS} and AUC_{USS}) and dominant genera, including unclassified families, in DNPS #1; n = 23

Supplemental Table 3.3 Expected coefficients for RA of dominant genera in DNPS #1, #2, and #3

Resolved taxa	Expected RA by DNPS (95% CI)	*P*	Expected RA by treatment group (95% CI)	*P*	Calf effect (SD)
Mycoplasma spp.	DNPS #1: 6.38 (2.44, 16.68) DNPS #2: 1.05 (0.68, 1.60) DNPS #3: 0.95 (0.62, 1.46)	0.0002 0.84 0.83	AMP: 1.65 (0.51, 5.32) PLAC: REF	0.40	1.32
Pasteurella spp.	DNPS #1: 0.03 (0.01, 0.13) DNPS #2: 8.44 (2.82, 25.25) DNPS #3: 53.10 (18.57, 151.81)	<0.0001 0.0001 <0.0001	AMP: 6.84 (2.43, 19.27) PLAC: REF	0.0003	0.91
Microbacteriaceae	DNPS #1: 4.89 (1.62, 14.76) DNPS #2: 0.66 (0.43, 1.03) DNPS #3: 1.12 (0.29, 4.35)	0.005 0.07 0.25	AMP: 1.12 (0.29, 4.35) PLAC: REF	0.87	2.94
Prevotella spp.	DNPS #1: 10.60 (7.47, 15.04) DNPS #2: 0.67 (0.54, 0.84) DNPS #3 0.72 (0.59, 0.89)	<0.0001 0.0003 0.003	AMP: 0.59 (0.38, 0.91) PLAC: REF	0.02	0.47
Moraxellaceae	DNPS #1: 0.24 (0.03, 1.73) DNPS #2: 0.63 (0.25, 1.57) DNPS #3: 2.84 (1.30, 6.17)	0.16 0.33 0.01	AMP: 1.90 (0.19, 18.92) PLAC: REF	0.58	2.38
Bacteroides spp.	DNPS #1: 3.38 (1.97, 5.79) DNPS #2: 0.96 (0.71, 1.31) DNPS #3: 0.84 (0.61, 1.15)	<0.0001 0.81 0.28	AMP: 0.73 (0.37, 1.41) PLAC: REF	0.35	0.71
[*Prevotella*] spp.	DNPS #1: 1.84 (0.95, 0.36) DNPS #2: 0.99 (0.51, 1.92) DNPS #3: 1.43 (0.74, 2.75)	0.07 0.97 0.29	AMP: 0.83 (0.41, 1.65) PLAC: REF	0.59	0.48
Mannheimia spp.	DNPS #1: 0.14 (0.02, 1.18) DNPS #2: 4.92 (2.21, 10.95) DNPS #3: 2.10 (0.85, 5.05)	0.07 <0.0001 0.11	AMP: 2.0 (0.19, 9.04) PLAC: REF	0.60	2.35
Blautia spp.	DNPS #1: 2.2 (1.23, 3.98) DNPS #2: 1.22 (0.85, 1.75) DNPS #3:1.10 (0.76, 1.59)	0.008 0.29 0.62	AMP: 0.67 (0.33, 1.35) PLAC: REF	0.26	0.74

Ruminococcaceae	DNPS #1: 1.15 (0.60, 2.19)	0.67	AMP: 0.91 (0.50, 1.66)	0.76	0
	DNPS #2: 1.13 (0.53, 2.40)	0.76	PLAC: REF		
	DNPS #3: 1.90 (0.92, 3.84)	0.10			
Pasteurellaceae	DNPS #1: 0.01 (0.003, 0.07)	<0.0001	AMP: 6.35 (0.66, 60.98)	0.11	1.93
	DNPS #2: 2.5 (0.49, 12.88)	0.27	PLAC: REF		
	DNPS #3: 21.50 (5.21, 88.73)	<0.0001			
Faecalibacterium	DNPS #1: 1.29 (0.67, 2.47)	0.44	AMP: 0.86 (0.46, 1.59)	0.62	0
	DNPS #2: 1.08 (0.51, 2.27)	0.85	PLAC: REF		
	DNPS #3: 1.03 (0.49, 2.18)	0.94			
Lachnospiraceae	DNPS #1: 0.90 (0.43, 1.90)	0.78	AMP: 0.87 (0.43, 1.76)	0.70	0
	DNPS #2: 1.00 (0.42, 2.36)	0.99	PLAC: REF		
	DNPS #3: 1.20 (0.52, 2.79)	0.67			
Acinetobacter spp.	DNPS #1: 0.86 (0.32, 2.33)	0.77	AMP: 0.97 (0.31, 3.01)	0.95	1.11
	DNPS #2: 2.69 (1.36, 5.33)	0.005	PLAC: REF		
	DNPS #3: 0.45 (0.19, 1.07)	0.07			
Clostridium spp.	DNPS #1: 2.38 (0.97, 5.82)	0.06	AMP: 0.40 (0.12, 1.29)	0.12	1.20
	DNPS #2: 0.19 (0.10, 0.34)	<0.0001	PLAC: REF		
	DNPS #3: 0.20 (0.11, 0.36)	<0.0001			
Psychrobacter spp.	DNPS #1: 0.34 (0.13, 0.87)	0.03	AMP: 1.32 (0.50, 3.50)	0.57	0.82
	DNPS #2: 1.77 (0.90, 3.49)	0.10	PLAC: REF		
	DNPS #3: 0.92 (0.42, 2.02)	0.84			
Corynebacterium spp.	DNPS #1: 0.35 (0.09, 1.46)	0.15	AMP: 0.70 (0.13, 3.70)	0.67	1.48
	DNPS #2: 0.59 (0.27, 1.28)	0.18	PLAC: REF		
	DNPS #3: 0.06 (0.01, 0.44)	0.01			
Escherichia spp.	DNPS #1: 2.26 (0.77, 6.66)	0.14	AMP: 0.50 (0.12, 2.04)	0.32	1.48
	DNPS #2: 0.19 (0.12, 0.32)	<0.0001	PLAC: REF		
	DNPS #3: 0.04 (0.02, 0.12)	<0.0001			

Supplemental Table 3.3 Expected count coefficients of dominant taxa (i.e. genera or unclassified families with a MRA > 1%) are the result of a generalized mixed model using the Poisson or negative binomial distributions. Calf was included in models as a random effect. If treatment group was significant, a likelihood ratio test was performed to ensure both fixed effects should be included in the model. *Pseudomonas* spp., *Agrobacterium* spp., *Helcoccus* spp., *Faecalibacterium* spp., and unclassified *Lachnospiraceae* models failed to converge; (n = 23)

Supplement Table 3.4. Alpha diversity metrics (mean ± SD) for 14 d samples

Respiratory microbiota at 14 d post challenge	Chao1	SDI
DNPS #3	592.02 ± 246.84	3.30 ± 1.32[b]
NP Tonsil	508.91 ± 234.68	4.19 ± 0.76[a]
LN	519.09 ± 240.67	4.01 ± 0.96[a,b]
Lung	524.24 ± 251.43	3.41 ± 1.35[b]

Supplement Table 3.4 Mean ± SD Chao1 index (Chao1) and Shannon Diversity Index (SDI) in 14 d samples. Samples are balanced to include the same number of calves (n = 27) in each sample type group. Superscripts with different letters indicate $P < 0.05$ between sample types.

Supplemental Table 3.5. Expected count coefficients for RA of dominant genera common among 14 d samples

Genera/resolved taxa	Expected RA by sample type (95% CI)	*P*	Expected RA by treatment group (95% CI)	*P*	Calf effect (SD)
Bacteroides spp.	DNPS #3: 1.60 (1.14, 2.15) NP tonsil: 0.62 (0.44, 0.90) LN: 2.71 (1.69, 4.36) Lung: 1.12 (0.82, 1.55)	<0.01 0.01 <0.0001 0.48	AMP: 0.82 (0.44, 1.53) PLAC: REF	0.54	0.64
Blautia spp.	DNPS #3: 1.23 (1.00, 1.50) NP tonsil: 1.01 (0.82, 1.26) LN: 2.12 (1.48, 3.05) Lung: 0.90 (0.72, 1.12)	0.05 0.91 <0.0001 0.34	AMP: 0.87 (0.55, 1.40) PLAC: REF	0.58	0.50
Ruminococcaceae	DNPS #3: 1.68 (1.31, 2.16) NP tonsil: 1.23 (0.94, 1.61) LN: 1.37 (0.99, 1.89) Lung: 0.63 (0.45, 0.88)	<0.0001 0.14 0.06 0.01	AMP: 0.82 (0.54, 1.25) PLAC: REF	0.36	0.34
Clostridium spp.	DNPS #3: 0.59 (0.40, 0.87) NP tonsil: 2.67 (2.08, 3.43) LN: 0.96 (0.62, 1.48) Lung: 0.59 (0.40, 0.87)	0.007 <0.0001 0.84 0.007	AMP: 0.94 (0.55, 1.63) PLAC: REF	0.83	0.55
Prevotella spp.	DNPS #3: 0.72 (0.56, 0.93) NP tonsil: 0.87 (0.67, 1.13) LN: 7.43 (5.00, 11.07) Lung: 1.20 (0.93, 1.54)	0.01 0.30 <0.0001 0.16	AMP: 1.34 (0.81, 2.25) PLAC: REF	0.30	0.54
Acinetobacter spp.	DNPS #3: 0.08 (0.05, 0.14) NP tonsil: 3.16 (2.33, 4.29) LN: 3.10 (1.67, 5.76) Lung: 1.82 (1.31, 2.53)	<0.0001 <0.0001 0.0003 0.003	AMP: 0.97 (0.44, 2.21) PLAC: REF	0.93	0.90

Psychrobacter spp.	DNPS #3: 0.19 (0.12, 0.31)	<0.0001	AMP: 1.08 (0.62, 1.87)	0.78	0.62
	NP tonsil: 1.82 (1.44, 2.30)	<0.0001	PLAC: REF		
	LN: 1.42 (0.90, 2.24)	0.13			
	Lung: 1.50 (1.18, 1.92)	0.001			
Escherichia spp.	DNPS #3: 0.08 (0.04, 0.19)	<0.0001	AMP: 1.28 (0.47, 3.43)	0.63	1.13
	NP tonsil: 6.39 (4.23, 9.65)	<0.0001	PLAC: REF		
	LN: 0.85 (0.38, 1.92)	0.70			
	Lung: 1.03 (0.66, 1.63)	0.90			
Lachnospiraceae	DNPS #3: 0.92 (0.66, 1.26)	0.59	AMP: 0.98 (0.63, 1.53)	0.94	0.33
	NP tonsil: 1.50 (1.13, 1.97)	0.005	PLAC: REF		
	LN: 1.05 (0.74, 1.48)	0.80			
	Lung: 0.75 (0.53, 1.05)	0.10			

Supplement Table 3.5Expected count coefficients of dominant taxa in 14 d samples (i.e. genera or unclassified families with a MRA > 1%) are the result of a generalized mixed model using the Poisson or negative binomial distributions. (n = 27) Calf was included in models as a random effect. If treatment group was significant, a likelihood ratio test was performed to ensure both fixed effects should be included in the model.

Supplemental Table 3.6 Expected count coefficients for RA of dominant genera common between DNPS #3 and at least one another 14 d sample type

Genera/resolved taxa	Expected RA by sample type (95% CI)	*P*	Expected RA by treatment group (95% CI)	*P*	Calf effect (SD)
Mycoplasma spp.	DNPS #3: 11.70 (7.68, 17.80) NP tonsil: 0.25 (0.14. 0.45) LN: 0.98 (0.43, 2.19) Lung: 0.25 (0.14, 0.45)	<0.0001 <0.0001 0.95 0.0001	AMP: 0.90 (0.34, 2.43) PLAC: REF	0.83	1.14
Microbacteria-ceae	DNPS #3: 21.81 (10.55, 45.10) NP tonsil: 0.06 (0.01, 0.27) LN: 0.18 (0.05, 0.71) Lung: 5.28 (2.64, 10.55)	<0.0001 0.0003 0.01 <0.0001	AMP: 0.83 (0.17, 3.98) PLAC: REF	0.81	1.83
[*Prevotella*] spp.	DNPS #3: 4.78 (3.38, 6.76) NP tonsil: 1.16 (0.75, 1.81) LN: 0.65 (0.37, 1.16) Lung: 0.73 (0.44, 1.22)	<0.0001 0.5 0.14 0.23	AMP: 0.55 (0.28, 1.10) PLAC: REF	0.09	0.71
Faecali-bacterium spp.	DNPS #3: 1.83 (1.1.33, 2.52) NP tonsil: 0.94 (0.64, 1.39) LN: 0.67 (0.41, 1.09) Lung: 0.70 (0.45, 1.07)	0.0002 0.75 0.11 0.10	AMP: 0.95 (0.51, 1.74) PLAC: REF	0.86	0.54

Corynebacterium spp.	DNPS #3: 0.13 (0.03, 0.56) NP tonsil: 2.52 (1.35, 4.69) LN: 0.37 (0.19, 0.71) Lung: 1.13 (0.56, 2.31)	0.006 0.04 0.003 0.73	AMP: 0.54 (0.28, 1.04) PLAC: REF	0.07	0.45
Pasteurella spp.			AMP:1.31 (0.60, 3.10) PLAC: REF	0.53	2.10

Supplement Table 3.6. Expected count coefficients of dominant taxa in 14 d samples (i.e. genera or unclassified families with a MRA > 1%) are the result of a generalized mixed model using the Poisson or negative binomial distributions (n = 27). Calf was included in models as a random effect. If treatment group was significant, a likelihood ratio test was performed to ensure both fixed effects should be included in the model. The *Pasteurella* spp. model would not converge under any modified conditions; therefore, sample type was omitted from the model.

Supplemental Table 3.7. Median (IQR) RA of common dominant genera for SAC calves by sample type

Genera/classified taxa	DNPS	NP tonsil	LN	Lung	*P* value
Ruminococcaceae	0.6 (0.2, 1.20)	2.4 (1.4, 3.8)	1.5 (0.7, 2.5)	1.0 (0.7, 3.0)	0.17
Faecalibacterium spp.	1.1 (0.2, 2)	3.5 (2.8, 5.1)	1.2 (1.0, 2.5)	1.0 (0.4, 3.2)	0.07
Blautia spp.	1.8 (0.2, 2.4)	3.4 (2.0, 7.0)	2.0 (1.2, 2.1)	1.2 (0.6, 2.4)	0.07
[Prevotella] spp.	2.0 (0.4, 2.6)	2.7 (1.7, 4.3)	1.6 (0.5, 2.5)	0.8 (0.6, 2.8)	0.77
Bacteroides spp.	2.4 (0.8, 4.0)	3.7 (3.2, 8.0)	1.6 (0.62, 2.5)	1.1 (0.7, 3.3)	0.32
Acinetobacter spp.	0.8 (0.3, 1.7)	1.9 (0.7, 2.6)	0.9 (0.5, 1.4)	0.6 (0.5, 1.0)	0.53
Microbacteriaceae	8.5 (0.1, 40.0)[a]	0.03 (0.01, 0.2)[b]	0.1 (0.02, 0.2)[ab]	0.7 (0.1, 20)[ab]	0.03
Mycoplasma spp.	0.8 (0.3, 1.7)	1.9 (0.7, 2.6)	0.9 (0.5, 1.4)	0.6 (0.5, 1.0)	0.53
Prevotella spp.	2.8 (1.5, 4.2)[a]	15.8 (9.9, 23.2)[ab]	28.1 (20.2, 32.0)[b]	17.6 (16.5, 26.0)[b]	0.0001

Supplemental Table 3.7. Different superscript letters indicate significant difference between sample types ($P < 0.05$). value refers to Friedman test. Pairwise comparisons performed with Nemenyi multiple comparison tests; (n = 27)

Supplemental Figure 3.1. Challenge study timeline

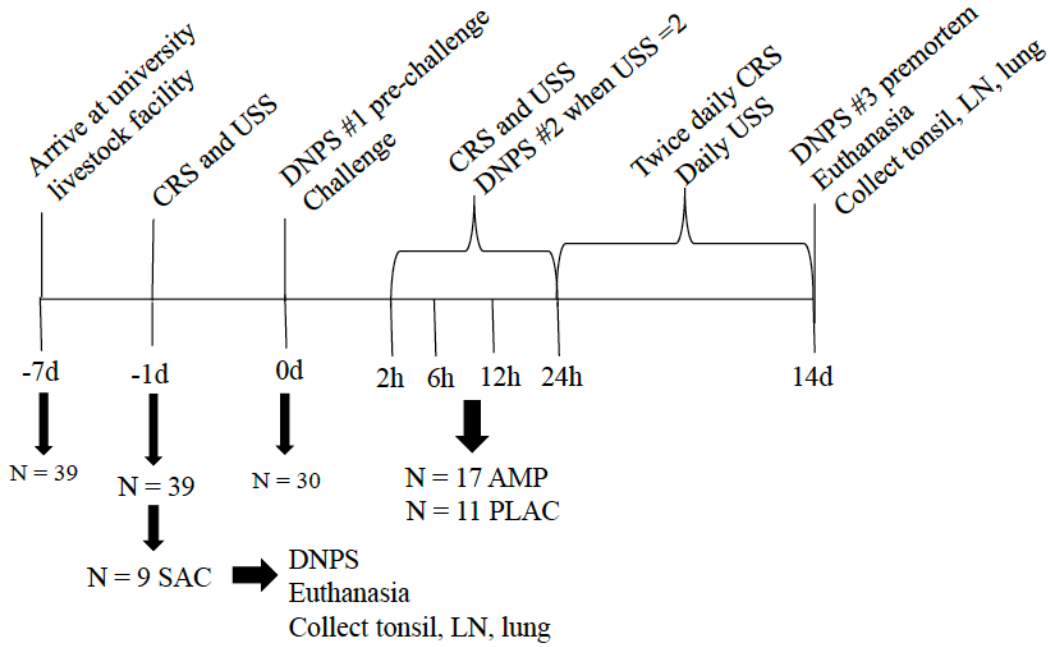


Figure 3.1. Timeline describing overall study design, identification of experimental groups, and data collection. Calves (N = 39) were transported to the livestock facility on -7 d. Following clinical respiratory scoring (CRS) and lung US scoring (USS), a total of 9 calves were selected for sacrifice (SAC) on -1 d. A deep nasopharyngeal swab (DNPS) was taken from SAC calves premortem and tonsil, lymph node (LN) and lung samples were collected following euthanasia. The remaining calves (N = 30) were challenged with *P. multocida* on 0 d following collection of DNPS #1. Challenged calves were monitored at 2 h, 6 h, 12 h, and 24 h by CRS and USS. When USS = 2 (i.e. ≥ 1 cm^2 consolidation was present) and ≥ 6 h had elapsed since the challenge, DNPS #2 was collected, and calves were randomized into an ampicillin treatment group (AMP; N = 17) and a saline placebo group (PLAC; N = 11). Calves were then evaluated twice daily by CRS and daily by USS until 14 d. Prior to euthanasia, DNPS #3 was collected and tonsil, LN and lung samples were compiled postmortem.

Supplemental Figure 3.2: Non-metric multidimensional scaling (NMDS) plots displaying Bray-Curtis dissimilarity metric for deep nasopharyngeal swabs (DNPS) #1, #2, #3

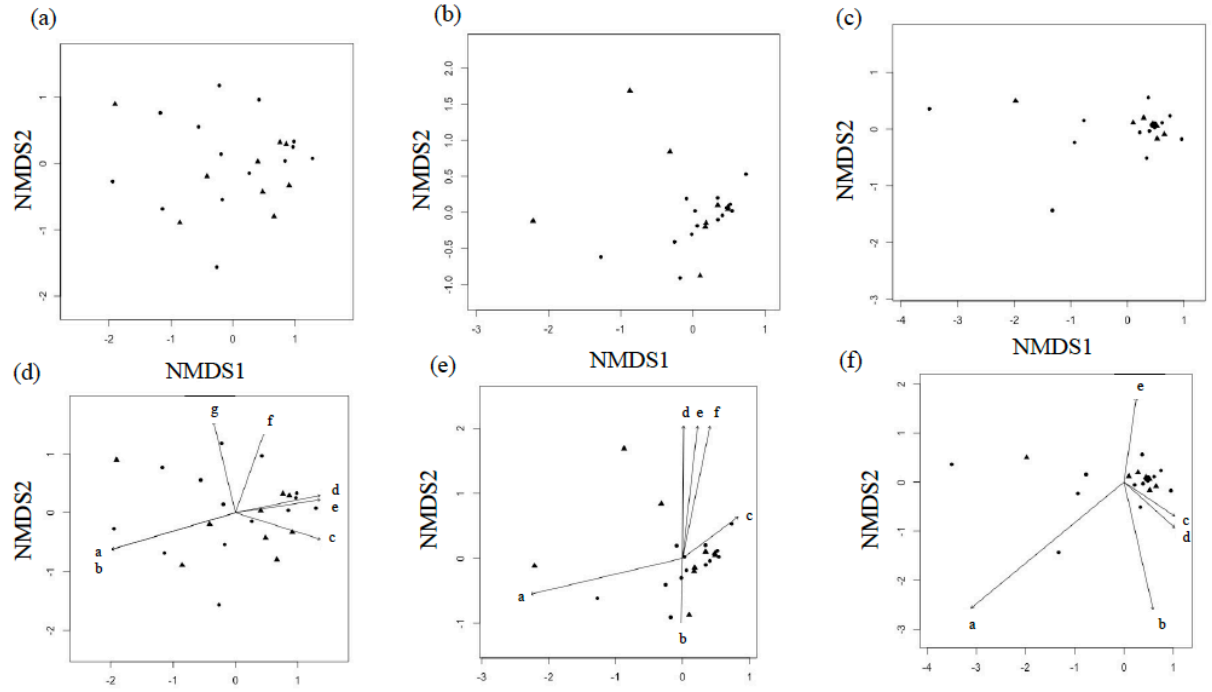
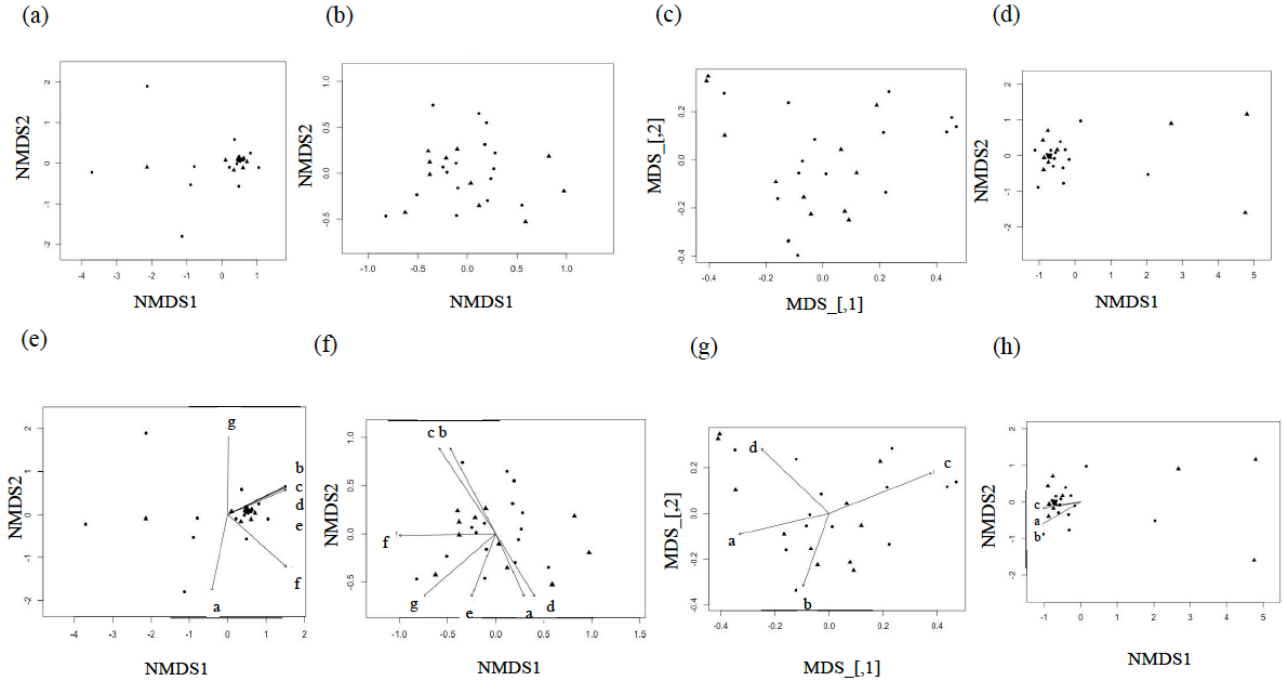


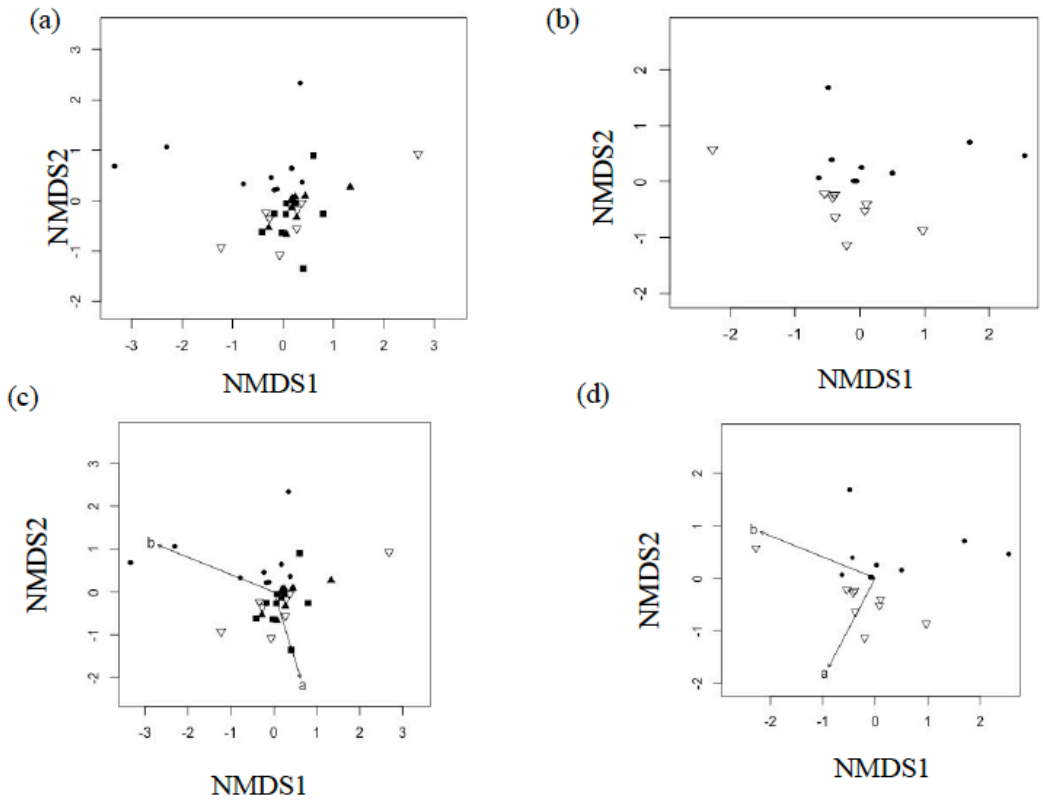
Figure 3.2. Data points in NMDS plots include AMP calves (filled in circles) and PLAC calves (filled in triangles). Plots are balanced (n = 23) (a) DNPS #1. (b) DNPS #2. (c) DNPS #3. (d) DNPS #1 plot with vectors fit for relative abundance (RA) of identified taxa that were significantly different from other swabs in generalized mixed models; a: *Pasteurella* spp., b: *Mycoplasma* spp., c: *Preveotella* spp., d: *Bacteroides* spp., e: *Blautia* spp., f: *Microbacteriaceae*, g: *Psychrobacter* spp. (e) DNPS #2 plot with vectors fit for RA of identified taxa that were significantly different from other swabs in generalized mixed models; a: *Acinetobacter* spp., b: *Mannheimia* spp., c: *Pasteurella* spp., d: *Clostridium* spp., e: *Escherichia* spp., f: *Prevotella* spp. (f) DNPS #3 plot with vectors fit for RA of identified taxa that were significantly different from other swabs in generalized mixed models: a: *Pasteurella* spp., b: *Escherichia* spp.; c: *Corynebacterium* spp., d: *Clostridium* spp., e: *Moraxellaceae.*

Supplemental Figure 3.3 Non-metric multidimensional scaling (NMDS) and multidimensional scaling (MDS) plots of the Bray-Curtis dissimilarity metric in 14 d samples



Supplemental Figure 3.3. Data points in NMDS and MDS plots include AMP calves (filled in circles) and PLAC calves (filled in triangles). Plots are balanced (n = 27). (a) DNPS #3 (b) NP tonsil (c) lymph node (LN) (d) Lung (n = 27) (e) DNPS #3 plot with vectors fit for relative abundance (RA) of identified taxa that were significantly higher than other 14 d sample types in generalized mixed models; a: *Mycoplasma* spp., b: *Blautia* spp., c: *Ruminococcaceae*, d: *[Prevotella]* spp., e: *Faecalibacterium* spp. (f) NP tonsil with vectors fit for relative abundance (RA) of identified taxa that were significantly higher than other 14 d sample types in generalized mixed models; a: *Clostridium* spp., b: *Acinetobacter* spp., c: *Psychrobacter* spp., d: *Escherichia* spp., e: *Lachnospiraceae*, f: *[Prevotella]* spp., g: *Corynebacterium* spp. (g) LN plot with vectors fit for relative abundance (RA) of identified taxa that were significantly higher than other 14 d sample types in generalized mixed models; a: *Blautia* spp., b: *Bacteroides* spp., c: *Prevotella* spp., d: *Acinetobacter* spp. (h) Lung plot with vectors fit for relative abundance (RA) of identified taxa that were significantly higher than other 14 d sample types in generalized mixed models; a: *Acinetobacter* spp., b: *Psychrobacter* spp., c: *Microbacteriaceae*

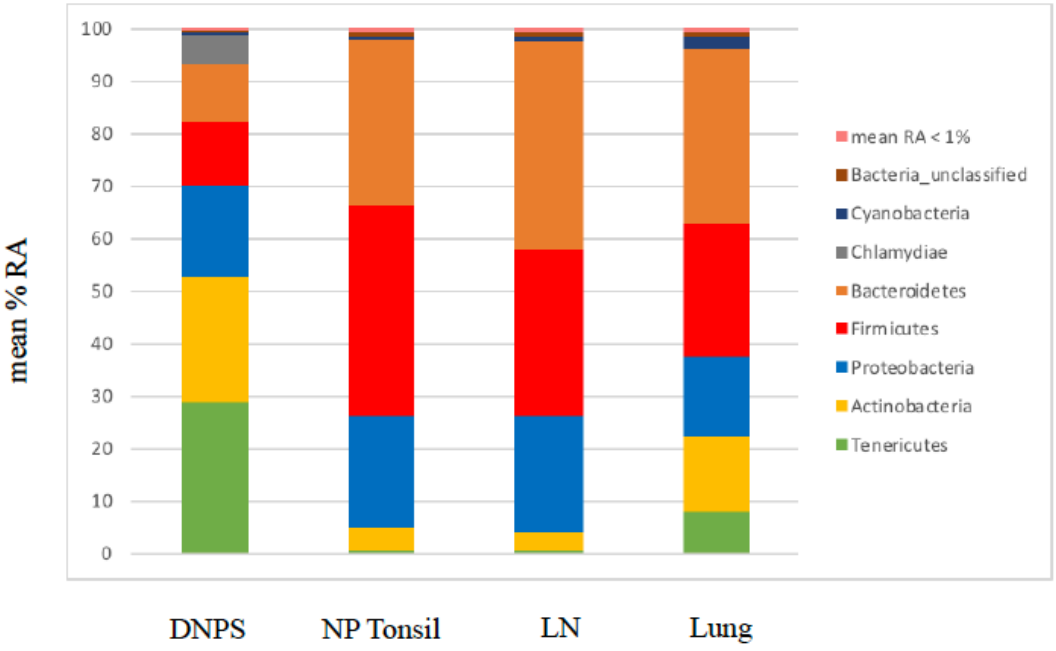
Supplemental Figure 3.4. Non-metric multidimensional scaling (NMDS) plots displaying the Bray-Curtis dissimilarity metric for calves excluded from the challenge



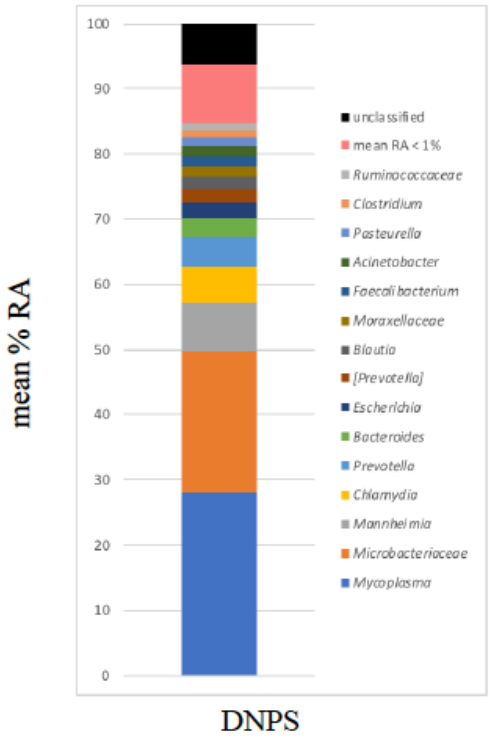
Supplemental Figure 3.4 NMDS plots displaying beta diversity of calves excluded from the challenge (SAC; n = 9). (a) Deep nasopharyngeal swab (DNPS) = open triangle; NP tonsil = filled in square; lymph node (LN) = filled in triangle; Lung = filled in circle. (b) Beta diversity of DNPS (open triangles) and Lung (filled in circles) samples that tended to be different based on the temporal beta diversity index difference ($P = 0.06$). (c) Plot in (a) with vectors included that represent common dominant taxa among 14 d samples with a significant difference between sample types; a: *Prevotella* spp., b: *Microbacteriaceae*. (d) Plot in (b) with vectors included that represent common dominant taxa among 14 d samples with a significant difference between sample types; a: *Prevotella* spp., b: *Microbacteriaceae*. (d) Plot in (b) with vectors included that represent common dominant taxa among 14 d samples with a significant difference between sample types; a: *Prevotella* spp., b: *Microbacteriaceae*.

Supplemental Figure 3.5 Dominant taxa in DNPS, NP tonsil, LN and lung of calves excluded from the challenge (Trial 2)

(a)

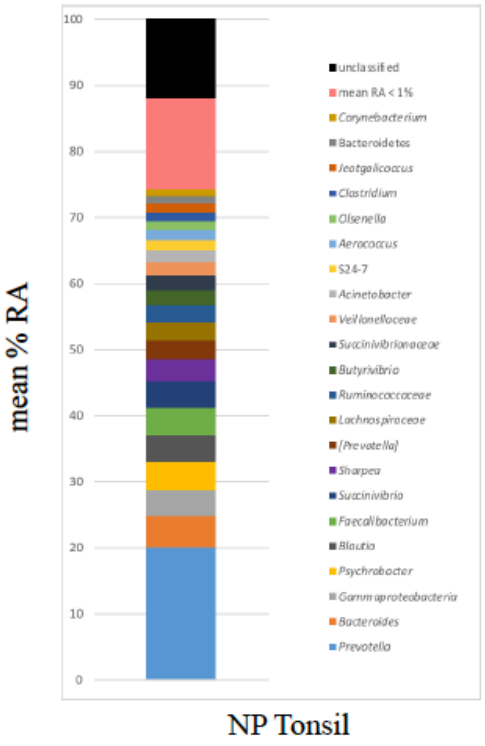


(b)

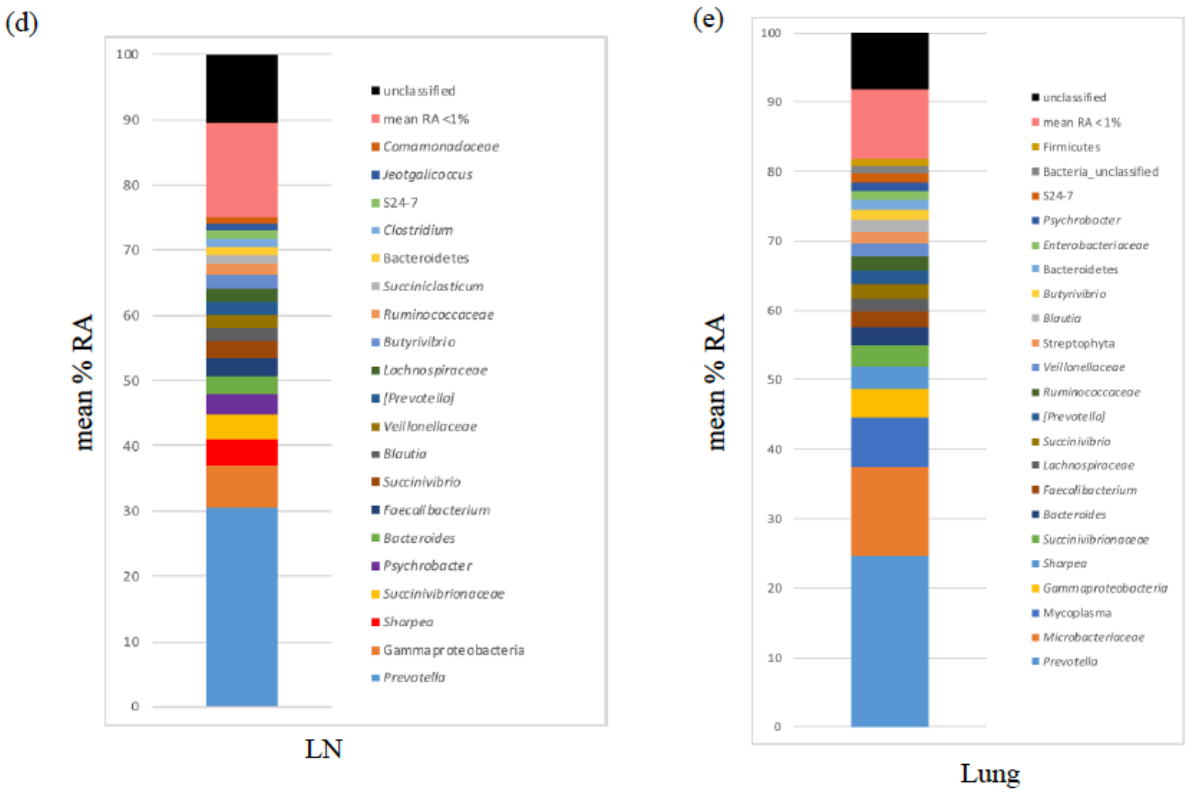


DNPS

(c)



NP Tonsil



Supplemental Figure 3.5 (a) Mean percent relative abundance (RA) of dominant phyla in the deep nasopharyngeal swab (DNPS), NP tonsil, lymph node (LN) and lung of excluded calves (SAC; n = 9). (b) Mean percent RA of dominant genera in the NP tonsil. (c) Mean percent RA of dominant phyla in the LN. (d) Mean percent RA of dominant genera in the lung. Dominant taxa were defined by identifying a mean RA > 1% in at least one of the anatomic sample type groups. Unclassified refers to operational taxonomic units (OTU) that were unable to be resolved. Mean RA <1% includes the sum of resolved phyla and genera that had a mean RA of less than 1%.

CHAPTER 4. LUNG MICRORNA DIFFERENTIAL EXPRESSION CORRELATES WITH MICROBIOTA CHANGES IN DAIRY CALVES FOLLOWING EXPERIMENTAL PASTEURELLA MULTOCIDA INFECTION

Formatted for Frontiers in Genetics (Livestock Genomics Specialty)

Running Title: Calf lung microRNA and microbiota

Lung microRNA differential expression correlates with microbiota changes in dairy calves following experimental *Pasteurella multocida* infection

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4.1 ABSTRACT

Bovine respiratory disease is a complex management problem in dairy herds and host susceptibility has been linked to stability of the respiratory microbiota and variability of host immunity. Previous studies have identified microRNA (miRNA) as a potential link between the host immune system and commensal bacterial populations. The purpose of this observational study was to identify differentially expressed (DE) miRNAs in postmortem lung tissue of pre-

weaned Holstein bull calves experimentally challenged with 10^{10} cfu of ampicillin-sensitive *Pasteurella multocida* (CHALL; n = 4) compared to a control group that was not challenged (CON; n = 5). Secondary objectives included analyzing predicted target genes with subsequent functional categories for identified miRNAs, evaluating changes observed in the lung microbiota secondary to *P. multocida* infection and determining whether miRNA DE was correlated with changes in the composition of the microbiota. Significant DE was identified in 33 known bovine miRNAs; 9 were highly DE (≥ 2 log2FC) and 6 had average normalized read counts ≥ 1000. The majority of predicted functional annotation categories of miRNA target genes included proteins involved in zinc binding, indicating a possible link to the host immune system. Additionally, bta-miR-21-5p and bt-miR-146b were DE in CHALL calves. These miRNAs have been previously linked to virus-induced lung lesions in sheep and *Mycobacterium bovis* infection in cattle, respectively.

There was a higher relative abundance (RA) of *Pasteurella* spp. and *Escherichia* spp. and a lower RA of *Butyrivibrio* spp. in the lung microbiota of CHALL calves compared to CON calves. Although alpha diversity metrics were not different between CHALL and CON calves, beta diversity tended to be different between groups. The RA of *Pasteurella* spp. was positively correlated with bta-miR-212 expression and negatively correlated with bta-miR-10b expression. The expression of bta-miR-10b was also negatively correlated with the RA of *Escherichia* spp. and positively correlated with the RA of *Butyrivibrio* spp. Alpha diversity, as measured by Shannon Diversity index, was negatively correlated with the expression of bta-miR-21-5p. The links between miRNA expression, zinc, and host susceptibility to BRD require further investigation with larger sample sizes and additional samples from the entire respiratory tract.

Keywords: pneumonia, lung microbiota, BRD, bta-miR-21, bta-miR-146b, bta-miR-212, bta-miR-149-5p

4.2 INTRODUCTION

Bovine respiratory disease (BRD) is a multifactorial cause of morbidity and mortality in dairy calves with significant economic impact. Lung lesions have been associated with reduced average daily gain, increased risk of pre-weaning mortality and decreased first lactation milk production (Buczinski et al., 2014; Dunn et al., 2018; Cramer and Ollivett, 2019). Results of a national producer survey showed a 12% incidence rate of clinical BRD in pre-weaned dairy calves (Urie et al., 2018). Results of a recent epidemiological study, which included 11,470 pre-weaned Holstein calves from 6 dairy herds in California that were diagnosed using a clinical scoring system, showed an average pre-weaning clinical BRD prevalence of 8% (range: 3% - 13%) over a one year time period (Dubrovsky et al., 2019). Subclinical disease can increase prevalence estimates by more than double (Ollivett and Buczinski, 2016; Binversie et al., 2017). Management of BRD is complicated by delayed identification leading to possible treatment failure (Binversie et al., 2017), as well as primary pathogens of BRD residing within the commensal bacterial population of the respiratory tract in healthy calves (i.e. pathobionts) (Thomas et al., 2019).

The complex ecological factors that affect the homeostasis of the respiratory microbiota as well as facilitate pathology by pathobionts within the respiratory tract are not well understood in cattle (Timsit et al., 2016; Zeineldin et al., 2019). It is known that host immunity and the integrity of the respiratory microbiota both contribute to BRD susceptibility, as resident microbes are important for mucosal barrier function and colonization resistance (Zeineldin et al., 2019). Evidence from studies in feedlot cattle indicate that preserving diversity of the

nasopharyngeal microbiota may be important for maintaining respiratory health, as decreased nasopharyngeal microbial diversity was associated with clinical BRD, as diagnosed by external clinical signs (Holman et al., 2015; McMullen et al., 2019). In a human study, host immunomodulation was described in the cervicovaginal microbiota; a high-diversity microbiota was associated with pro-inflammatory cytokine concentrations within the genital tract (Anahtar et al., 2015). Additionally, results of a study in mice showed that the composition of the respiratory microbiota affected both the innate and adaptive immune responses to an experimental respiratory challenge (Ichinohe et al., 2011). Overall, the mechanisms by which the host is protected by the microbiota and how the microbiota interacts with the host immune system have not been elucidated (Edwards et al., 2019). Recently, microRNA (miRNA) was proposed as a potential link between the host microbiota and the immune system (Edwards et al., 2019).

MicroRNAs are endogenous, short non-coding RNAs with a typical length of 21-23 nucleotides. Once transcribed and modified into mature strands, miRNA pairs with messenger RNA (mRNA) to direct post-transcriptional repression of protein-coding genes, either by destabilizing the mRNA transcript, which is the predominant function in mammals, or by inhibiting translation (Friedman et al., 2009; Guo et al., 2010). Importantly, miRNAs have been shown to function as regulators of the innate immune response (Zhou et al., 2011; Zhou et al., 2012; Momen-heravi and Bala, 2018). Edwards et al. (2019) reported a significant positive correlation between the relative abundance (RA) of *Lactobacillus* spp. within the vaginal microbiota and hsa-miR-193b expression, which targets and inhibits cell cycle genes; impeding vaginal epithelial cell proliferation is a defense mechanism that prevents *Chlamydia trachomatis* infection. Disruption of the gastrointestinal tract microbiota in mice led to decreased expression

of miR-130a, which disinhibited production of TNF-alpha and increased susceptibility to allergic airway disease (Shi et al., 2018). These studies provide evidence of a close relationship between commensal bacterial populations, miRNA expression and disease susceptibility.

There is a dearth of information regarding differential expression (DE) of miRNA in the context of pneumonia and disruption of the respiratory microbiota. Results from studies in humans have demonstrated that severity of pneumonia is associated with miRNA DE and subsequent immune dysregulation (Moran et al., 2015; Hoffmann et al., 2016). Human host cells, such as alveolar macrophages, have also been shown to release extracellular vesicles, such as exosomes and microvesicles, which contain miRNAs; these transcripts regulate lung inflammation, and therefore, the airway microenvironment (Ismail et al., 2013; Fujita et al., 2018). Natural *Mycoplasma bovis* exposure in steers, quantified by direct ELISA testing of antibody levels, was shown to correlate with DE of circulating miRNAs involved in immune response regulation (Casas et al., 2016). Serum DE of miRNAs was also found to correlate with time point following experimentally delivered Bovine Viral Diarrhea Virus infection in calves (Taxis et al., 2017). Further understanding of how the stability of the respiratory microbiota affects host susceptibility to BRD requires investigating communication between the respiratory epithelium, the microbiota and the immune system. Given the evidence of miRNA-driven regulation of host immunity and potential relationship to the resident microbiota, which plays a role in maintaining barrier integrity of the respiratory mucosa, evaluating miRNA DE in dairy calves diagnosed with BRD is indicated.

The primary objective of this study was to identify DE miRNAs in lung tissue associated with experimental *Pasteurella multocida* infection in pre-weaned dairy calves. A secondary objective was to perform target gene prediction and functional annotation analysis to provide

preliminary data regarding lung specific miRNA DE and possible impact on host proteins. Additional secondary objectives of this study were to evaluate changes observed in the lung microbiota secondary to *P. multocida* infection and to evaluate whether DE of specific miRNA were correlated with changes in the composition of the microbiota.

4.3 MATERIALS AND METHODS

Animal use approval

This observational study was performed as a derivative of a larger randomized controlled study evaluating the effect of parenteral ampicillin on experimentally induced BRD and the respiratory microbiota. The study was carried out between July 11, 2017 and August 2, 2017 at the University of Wisconsin Livestock Laboratory in Madison, Wisconsin and approved by the University of Wisconsin Institutional Animal Care and Use committee (protocol #A005636-A02) and the Institutional Biosafety Committee (protocol #B00000610) (Holschbach et al., 2019).

Animals and facilities

Forty-five Holstein bull calves were raised on a commercial dairy in southern Wisconsin, as described by Holschbach et al. (2019). Calf respiratory health was monitored for 3 wk at the dairy by performing lung ultrasound scores (USS) and clinical respiratory scores (CRS), as was previously described (Holschbach et al., 2019). Lung US scores were assigned using a 5-point scale, as previously described (Cramer and Ollivett, 2019; Holschbach et al., 2019). Clinical respiratory scores, performed using the University of Wisconsin Calf Scoring Application, were assigned on a 0 – 3 scale for each evaluated parameter (rectal temperature, cough, nasal discharge, ocular discharge and ear position) that was summed into a total respiratory score (McGuirk and Peek, 2014). Calves were considered to be CRS positive if they had a score ≥ 2 for at least 2 parameters.

Thirty-nine calves remained healthy during the monitoring period and were transported to the University of Wisconsin-Madison Livestock Laboratory at a mean ± standard deviation (SD) age of 52 ± 6 d. The day prior to experimental challenge, CRS and USS were collected; 9 calves were excluded from the challenge because they either did not meet the challenge inclusion criteria (i.e. USS = 0, CRS negative, and a rectal temperature ≤103°F) or they were randomly selected for exclusion to maintain an equal number of calves per pen in the larger study (Holschbach et al., 2019). Excluded calves were sedated with 0.1 mg/kg xylazine i.v. (AnaSed injection, 20 mg/mL; Akorn Animal Health, Lake Forest, IL) and euthanized with an intravenous overdose (1 mL/10 lbs) of barbiturate (Beuthanasia-D Special, Merck Animal Health, Kenilworth, NJ). Postmortem sample collection was performed immediately following euthanasia. A total of 5 calves (CON) were conveniently selected from the excluded calves to be included in this separate observational study.

Using aseptic technique, an approximately 5 cm^3 sample was dissected from the right middle lung lobe of each CON calf. If there was evidence of lung consolidation, the sample consisted of lung tissue from the region of consolidation. Collected samples were divided equally into one half for microbiota analysis, which was placed in sterile PBS and immediately transported on ice to a -80°C refrigeration unit, and the other half for miRNA analysis, which was aseptically dissected into 4-5 mm^2 fragments; these portions were sealed in Eppendorf safe-lock tubes (Eppendorf North America, Hauppauge, NY), flash frozen in liquid nitrogen, and stored within a separate -80°C refrigeration unit.

A total of 30 calves were challenged with *Pasteurella multocida* via intratracheal delivery of a 25-ml suspension containing 10^{10} cfu of ampicillin-sensitive *P. multocida* A1, as previously described (Holschbach et al., 2019). When calves developed significant lung consolidation,

which was defined as USS = 2 (i.e. ≥ 1 cm^2 lung consolidation), within the first 24 h, they were randomized into an ampicillin treatment group and a placebo group. Calves were monitored with USS and CRS over 14 d following the challenge; they were then sedated and euthanized as was described for the excluded calves. Postmortem sample collection was performed immediately following euthanasia. A total of 4 challenge calves were conveniently selected to be included in this observational study (CHALL).

Using aseptic technique, an approximately 5 cm^3 sample was dissected from the lung lobe with the most severe lesion(s) upon gross examination for each CHALL calf. If there was evidence of lung consolidation, the sample consisted of lung tissue from the region of consolidation. Collected samples were divided equally into one half for microbiota analysis, which was placed in sterile PBS and immediately transported on ice to a -80°C refrigeration unit, and the other half for miRNA analysis, which was aseptically dissected into 4-5 mm^2 fragments; these portions were sealed in Eppendorf safe-lock tubes (Eppendorf North America, Hauppauge, NY), flash frozen in liquid nitrogen, and stored within a separate -80°C refrigeration unit.

Therefore, the current study enrolled 5 CON and 4 CHALL postmortem lung samples for both microbiota and miRNA analyses.

RNA extraction, quantification and small RNA sequencing

Frozen lung tissue samples intended for miRNA analysis were thawed at room temperature and homogenized into fine powders using a mortar and pestle. RNAs were extracted following the miRNeasy mini protocol (Qiagen, Germany) using a QIAcube instrument (Qiagen, Germany) according to the manufacturer's instructions. The quality of extracted RNAs was evaluated using a Bioanalyzer RNA 6000 nano kit (Agilent Technologies, Santa Clara, CA). Purified RNAs were stored at -80°C until sequencing. Small RNA sequencing library

preparation was performed using the Illumina TruSeq small RNA library preparation kit following the manufacturer's instructions. For each sample, 1μg of total RNA was used for library preparation. Quantification of prepared libraries was performed using a KAPA library quantification kit (KapaBiosystems, Boston, MA) with an ABI7300 RT-qPCR instrument (Thermo Fisher Scientific, Waltham, MA). Libraries were assessed for quality using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) to ensure miRNA-derived cDNA was present (i.e. 140-150 bp length). Libraries were further normalized to ensure equivalent quantity before sequencing. Single-end, 50 bp reads were acquired using an Illumina NextSeq 500 instrument with a 75-cycle kit.

DNA extraction and 16S rRNA amplicon sequencing

Bacterial DNA from CON and CHALL calves in this study was extracted and sequenced in one batch that included samples from calves enrolled in the larger randomized control study. Following thawing at room temperature, tissue samples were further processed aseptically on individual sterile petri dishes. An approximately 1 cm^3 specimen was dissected from each sample and vortexed within extraction buffer. Total genomic DNA was then extracted by lysing and mechanically disrupting microbial cells and purifying DNA via phenol and phenol:chloroform:isoamyl alcohol extraction, as has been previously described (Dias et al., 2018). A Qubit fluorometer and a broad range dsDNA assay kit was used to quantify total genomic DNA (Thermo Fisher Scientific, Waltham, MA). The V4 hypervariable region of the 16S rRNA gene was amplified with dual-indexed primers (F – GTGCCAGCMGCCGCGGTAA ; R - GGACTACHVGGGTWTCTAAT) as previously described (Kozich et al., 2013), which included adapters for Illumina sequencing technology (F - AATGATACGGCGACCACCGAGATCTACAC ; R-

CAAGCAGAAGACGGCATACGAGAT). PCR included 25 ng template DNA, 0.5 μL of forward and reverse primers, 0.125 μL of Ex Taq DNA polymerase (Takara Bio USA, Inc. Mountain View, CA), 2.5 μL of 10X Ex Taq Buffer (Takara Bio USA, Inc. Mountain View, CA), 2.0 μL dNTP Mixture (Takara Bio USA, Inc. Mountain View, CA) and sterile distilled water to 25 μL. PCR was performed under the following specifications: 30 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. Negative and positive controls were also included in the extraction and PCR procedures.

PCR products were recovered by gel electrophoresis in a 1% (wt/vol) low-melt agarose gel (Sigma-Aldrich, St. Louis, MO) using a Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, CA). Purified products were quantified using a Qubit high sensitivity kit (Thermo Fisher Scientific, Waltham, MA) and a Synergy 2 Multi-Mode plate reader (BioTek, Winooski, VT). Samples were then equimolar pooled to a 4 nM final library for sequencing. The final library was sequenced with an Illumina MiSeq using a v2 2 x 250 reagent kit at 10 pmol/L with a 10% PhiX control, following the manufacturer's protocol (Illumina, Inc., San Diego, CA). Raw sequence data generated from this study were deposited into NCBI's Short Read Archive (SRA) and are publicly available under BioProject PRJNA625907.

Bioinformatics and Statistical analysis

For isolated miRNA from CON and CHALL calves, FASTA files containing sequenced cDNA were analyzed using miRDeep2 for miRNA identification and expression analysis (Friedlander et al., 2012). The *miRDeep2.pl*, *mapper.pl,* and *quantifier.pl* scripts were used to identify known miRNAs in the sequencing data, map sequences to the *Bos taurus* reference genome (NCBI UMD 3.1, GenBank accession: GCA_000003055.5) and calculate a raw read count matrix. Differential expression (DE) was then calculated in DEseq2 using RStudio (v

1.2.5033) using normalized raw read counts by a per million scaling factor. Filtering criteria used to determine significant DE included miRNAs with mean raw read counts > 20 and log2 Fold Change (log2FC) ≥ 1.0 or -1.0 (Love et al., 2014). Alpha was set at < 0.05.

For miRNAs determined to have significant DE, the DIANA mirPub database was searched for previously published literature relating those particular miRNAs with diseases and tissue-specific expression in cattle (Vergoulis et al., 2015). Prediction of effective miRNA target genes was also performed using TargetScan 7.1 with bovine specific filters (Agarwal et al., 2015). A functional annotation analysis was then performed to identify gene function categories of the identified target genes using DAVID 6.7 (Huang et al., 2007). Functional categories of target proteins were identified using UniProtKB Keywords. From the functional category that included the highest percentage of target genes, three enriched functional groups with the highest number of target genes, were recorded using UniProtKB Keywords. The threshold of EASE score, a modified Fisher Exact test, was utilized to determine significant enrichment ($P < 0.05$ indicates a strongly enriched functional annotation category) (Huang et al., 2009).

The data obtained from 16S rRNA sequencing were processed using mothur (v.1.40.0) (Schloss et al., 2009) with modified procedures (Kozich et al., 2013), as has been previously described (Dias et al., 2017, 2018). In brief, paired-end reads were joined using the *make.contigs* command with default parameters. Quality was controlled by removing ambiguous bp, excessively short (< 200 bp) and long (> 500 bp) sequences, as well as removing homopolymers greater than 8 bp using the *screen.seqs* command. Sequences were then aligned to the SILVA 16S rRNA database (v 132) (Pruesse et al., 2007) and chimeric sequences were detected and removed using the Uchime algorithm (Edgar et al., 2011). The high-quality sequences were then classified, using the GreenGenes database (DeSantis et al., 2006), into operational taxonomic

units (OTU) by the OptiClust algorithm (Westcott and Schloss, 2017) using a similarity cutoff of 97%. Depth of sequencing for each sample was evaluated by Good's coverage. Samples were normalized in mothur to the sample with the smallest number of sequences using the *normalize.shared* command.

Normalized OTU counts were used to determine diversity metrics and the relative abundance (RA) of taxa in each sample. The RA of each resolved taxa was defined as the number of OTUs assigned a specific taxa divided by all OTUs within samples. Dominant taxa were determined by identifying a mean RA > 1% using the phyloseq package (McMurdie and Holmes, 2013). Dominant genera or unclassified families were compared between CHALL and CON calves using Kruskal Wallis tests. Alpha diversity metrics, including Shannon's diversity index (SDI) and Chao1 index (Chao1), were calculated using mothur. Alpha diversity metrics were compared between CHALL and CON calves with a Wilcoxon rank sum test or a student's t-test, depending on whether the distribution was non-parametric or parametric, respectively.

Beta diversity was determined using the Bray-Curtis dissimilarity index, as calculated using the vegan package in RStudio (v 1.2.5) (RStudio Inc., Boston, MA). Differences in the Bray-Curtis index between CHALL and CON calves were used to create a sample-wise distance matrix that was visualized using multidimensional scaling (MDS). Non-metric MDS (NMDS) was used if the base stress of creating the ordination plots was non-linear; metric MDS was utilized if the base stress followed linear regression. Equality of beta dispersion between groups was assessed using the betadisper function of the vegan package. Bray-Curtis was compared between groups (i.e. CON, CHALL) using a permutational multivariate analysis of variance (PERMANOVA) as implemented in the vegan package. The adonis function was used if the

assumption of equal beta dispersion was met. An analysis of similarities was utilized if the assumption of equal beta dispersion was not met.

To evaluate potential relationships between microbiota composition and DE of miRNAs, Spearman's rank correlation coefficient was evaluated between normalized counts of significantly DE miRNAs and the RA of dominant genera or unclassified families. Correlations between DE miRNA and alpha diversity metrics (e.g. SDI and Chao1) were also performed. Alpha was set at $P < 0.05$.

4.4 RESULTS

Descriptive results for CHALL and CON calves

A total of 4 / 9 calves (CHALL (n = 2) and CON (n = 2)) enrolled in the current study had excellent passive transfer of immunity, 4 / 9 (CHALL (n = 2) and CON (n = 2)) had good passive transfer of immunity, and 1 / 9 (CON (n = 1)) had fair passive transfer or immunity, according to published standards based on measured serum total protein levels (Godden et al., 2019). The CON group included 1 / 5 calves with an USS = 2 (i.e. ≥ 1 cm^2 lung consolidation) and 1 / 5 calves that was respiratory score positive premortem. Upon postmortem examination, it was confirmed that 4 / 5 calves had no gross evidence of lung consolidation. Additionally, the calf that had been assigned an USS = 2, had a 1 cm^2 area of lobular consolidation in the cranial aspect of the right cranial lung lobe; this lesion is consistent with bronchopneumonia.

One out of four calves in the CHALL group received ampicillin (6.6 mg/kg i.m) for 3 days, as per the larger study protocol objectives. A total of 3 / 4 calves developed acute lobular pneumonia following *P. multocida* inoculation (i.e. developed an USS = 2 within 12 h of exposure). Overall, 2 / 4 calves developed severe lobar pneumonia (i.e. USS = 5) and 1 / 4 calves only developed lobular pneumonia (i.e. USS = 2) following the challenge. One calf out of

the four CHALL calves did not develop pneumonia following the challenge. Upon postmortem examination at 14 d post challenge, the 3 calves that developed acute lobular pneumonia following the *P. multocida* challenge had lung consolidation consistent with bronchopneumonia. The gross lesions for these CHALL calves were 2 cm^2 of lobular consolidation in one lung lobe (n = 1), lobar consolidation in 4 lung lobes (n = 1), and lobar consolidation in 1 lung lobe (n =1). The calf that did not develop lung consolidation following the *P. multocida* challenge had no gross evidence of lung consolidation.

Sequencing results

The mean ± SD RIN for extracted RNA was 5.8 ± 2.0. A total of 120,380,702 reads were obtained from miRNA sequencing and 32,347,870 reads were mapped to known bovine miRNAs. A total of 793 unique miRNAs were identified with at least one read among all lung samples. The mean ± SD of reads mapped to the identified miRNAs was 3,594,208 ± 1,634,589. The mean ± SD of reads in CHALL calves was 2,336,910 ± 619,372 and in CON calves was 4,600,046 ± 1,486,811 ($P = 0.02$). A total of 277,439 16S rRNA sequences were obtained with Good's coverage ≥ 99.3 % for all samples. The mean ± SD number of 16S rRNA sequences was 30,827 ± 12,542. In CHALL calves, the mean ± SD number of sequences was 21,509 ± 4,766 and in CON calves was 38,191 ± 11,998 ($P = 0.05$).

Lung miRNA DE in CHALL calves vs. CON calves

A total of 33 miRNAs were identified in lung samples as significantly DE in CHALL compared to CON calves (Table 4.1). Figure 4.1 (a-d) displays the normalized read counts by comparison group for each miRNA. A total of 9 miRNAs were upregulated ≥ 2 log2FC in CHALL compared to CON calves: bta-miR-146b, bta-miR-205, bta-miR-223, bta-miR-2285t, bta-miR-212, bta-miR-2419-3p, bta-miR-1246, bta-miR-183, and bta-miR-376a. The highly DE

miRNAs that were isolated at a BaseMean count ≥ 1000 and were upregulated included bta-miR-146b and bta-miR-223 in CHALL calves. There were 4 miRNAs that were downregulated in CHALL compared to CON calves: bta-miR-10b, bta-miR-190a, bta-miR-129-3p, and bta-miR-383. Only bta-miR-10b was isolated at a normalized read count ≥ 1000. Additional miRNAs with a BaseMean count ≥ 1000 and log2FC ≥ 1.0 included bta-miR-182, bta-miR-21-5p and bta-miR-375.

Results of the mirPub search determined literature evidence of associations between all the significantly DE miRNAs identified in this study and tissue specific expression or disease status in cattle (Table 4.2). A total of 13 / 33 (39 %) of miRNAs have been previously described as DE in mammary epithelium, serum or milk during periods of physiologic stress on the mammary gland (e.g. lactation, heat stress or mastitis): bta-miR-146b, bta-miR-223, bta-miR-2285t, bta-miR-2419-3p, bta-miR-376a, bta-miR-182, bta-miR-2484, bta-miR-210, bta-miR-155, bta-miR-375, bta-miR-6518, bta-miR-363, bta-miR-21-5p. Six identified miRNAs (18 %) were described as DE in serum or macrophages during bacterial infection (e.g. *Mycobacterium avium* subsp. *paratuberculosis*, *Mycobacterium bovis*) or viral infection (e.g. Foot and Mouth Disease): bta-miR-132, bta-miR-677, bta-miR-21-3p, bta-miR-149-5p, bta-miR-10b, bta-miR-129-3p. A total of 7 / 33 (21 %) miRNAs were described as DE within oocytes or plasma during the estrous cycle or pregnancy: bta-miR-383, bta-miR-190a, bta-miR-665, bta-miR-582, bta-miR-96, bta-miR-183, bta-miR-212. The remaining 7 miRNAs identified were previously described as generally serum-, plasma- or tissue-specific; one miRNA (bta-miR-2440) was found to be DE in plasma following hypoxia stress in Jerseys.

The targeted genes of 25 / 33 (76%) DE miRNAs were predicted to function in the general category of coiled coil regions within proteins (Table 4.3). Identified target genes of 22 /

33 (67%) DE miRNAs were predicted to include proteins with metal-binding or zinc ion binding properties. The majority of functional annotation categories predicted included protein domains, cellular components and molecular function.

Microbiota composition in CHALL and CON calves

The dominant phyla and genera identified within lung samples of CHALL and CON calves are displayed in Figure 4.2 (a, b). Visually, the RA of phylum Proteobacteria and genus *Pasteurella* spp. were increased in the CHALL calves compared to CON calves. *Pasteurella* spp. was not identified as a dominant genus in CON calves. Identified genera that were significantly different between CHALL and CON calves are displayed in Figure 4.3 (a-d). The median (IQR) percent RA of *Pasteurella* spp. was 32 % (6.2, 58) in CHALL calves and 0 % (0, 0) in CON calves ($P = 0.01$). The median (IQR) percent RA of *Escherichia* spp. was 2 % (1.8, 2.3) in CHALL calves and was 0.20 % (0.10, 0.24) in CON calves ($P = 0.01$). The median (IQR) percent RA of *Pseudomonas* spp. was 1.6 % (0.40, 2.70) in CHALL calves and was 0.02 % (0.01, 0.04) in CON calves ($P = 0.03$). Finally, the median (IQR) percent RA of *Butyrivibrio* spp. was 0.40 % (0.30, 0.62) in CHALL calves and 1.4 % (1.30, 1.70) in CON calves ($P = 0.03$).

Results of the comparisons of alpha diversity metrics between CHALL and CON calves are displayed in Figure 4.4 (a, b). The mean $\pm$ SD SDI in CHALL calves was 3.29 $\pm$ 0.80 and was 4.04 $\pm$ 1.59 in CON calves ($P = 0.40$). The median (IQR) of Chao1 was 677 (515, 790) in CHALL calves and was 481 (310, 593) in CON calves ($P = 0.73$). Metric multidimensional scaling plots of the Bray-Curtis dissimilarity metric in CHALL and CON calves are displayed in Figure 4.5 (a, b). The PERMANOVA results indicated an R^2 of 0.198 between CHALL and CON lung samples ($P = 0.06$). Vectors depicting the effects of the RA of *Pasteurella* spp.,

Escherichia spp., *Pseudomonas* spp. and *Butyrivibrio* spp. on the beta diversity of CHALL and CON calves are shown in Figure 4.5b.

Correlations between miRNAs and microbiota composition

The Spearman rank correlation coefficients describing the association between the RA of *Pasteurella* spp., *Escherichia* spp., *Pseudomonas* spp., *Butyrivibrio* spp. and DE miRNAs identified in this study are listed in Tables 4.4, 4.5, 4.6 and 4.7. The RA of *Pasteurella* spp. was strongly, positively correlated with bta-miR-212 (Spearman's rho 0.71 (95% CI: 0.15, 1.00), P = 0.03), bta-miR-132 (0.80 (0.30, 0.96), P = 0.01), bta-miR-582 (0.82 (0.57, 1.0), P = 0.01), and was moderately, positively correlated with bta-miR-6518 (0.70 (0.08, 1.00), P = 0.04). The RA of *Pasteurella* spp. was strongly, negatively correlated with bta-miR-10b (-0.85 (-0.99, -0.40), P = 0.003), bta-miR-129-3p (-0.89 (-0.99, -0.60), P = 0.003), and was moderately negatively correlated with bta-miR-383 (-0.70 (-0.90, -0.42), P = 0.03). The RA of *Escherichia* spp. was strongly, positively correlated with bta-miR-132 (0.78 (0.20, 1.00), P = 0.01) and was strongly, negatively correlated with bta-miR-10b (-0.78 (-1.00, -0.11), P = 0.02), bta-miR-129-3p (-0.73 (-0.90, -0.29), P = 0.03), and bta-miR-383 (-0.81 (-0.92, -0.55), P = 0.01). MicroRNA bta-miR-1246 was strongly positively correlated (0.82 (0.25, 0.99)) with the RA of *Pseudomonas* spp. in all calves (P = 0.01). This was also the case for bta-miR-132 (0.85 (0.45, 1.00), P = 0.003), bta-miR-677 (0.90 (0.53, 1.00), P = 0.002), bta-miR-2404 (0.90 (0.27, 1.00), P = 0.001), bta-miR-2484 (0.90 (0.43, 1.00), P = 0.001), bta-miR-2440 (0.91 (0.57, 0.99), P = 0.001), and bta-miR-6518 (0.73(0.01, 0.97), P = 0.03). The RA of *Pseudomonas* spp. was weak to moderately, negatively correlated with bta-miR-190a (-0.67 (-0.94, 0.12), P = 0.04). MicroRNA bta-miR-3957 (-0.70 (-0.98, -0.14), P = 0.04) was moderately, negatively correlated with the RA of

Butyrivibrio spp. In contrast, the miRNA bta-miR-10b was strongly, positively correlated with the RA of *Butyrivibrio* spp.

The Spearman rank correlation coefficients describing the association between DE miRNAs and alpha diversity metrics are displayed in Tables 4.8 and 4.9. When evaluating the correlation between alpha diversity metrics and DE miRNAs, bta-miR-21-5p was moderately, negatively correlated with SDI (-0.70 (-0.95, -0.08), $P = 0.04$). Chao1 index was moderately, positively correlated with bta-miR-1246 (0.70 (0.14, 1.00), $P = 0.03$).

4.5 DISCUSSION

To the authors' knowledge, this is the first report of DE miRNA in the lung tissue of dairy calves experimentally challenged with *P. multocida* compared to calves that were not challenged. Lung tissue has been analyzed for DE of miRNAs in ruminants previously; a recent study in sheep evaluated the DE of miRNA in postmortem lung tissue at different timepoints during Visna Maedi Virus exposure and infection (Bilbao-Arribas et al., 2019). Results of Bilbao-Arribas et al. (2019) showed DE of oar-miR-21, which functions as a regulator of inflammation and was hypothesized by the authors to be a cause of lung lesions in Visna Maedi Virus infections.

Our study identified DE of bta-miR-21-3p and bta-miR-21-5p in CHALL lung samples versus CON lung samples with a log2FC of 1.50 and 1.08, respectively. MicroRNAs are processed from precursor hairpin miRNAs that are cleaved in the cytoplasm into a guide strand (5') and a passenger strand (3'); the less stable guide strand was thought of as preferentially selected to function as the mature miRNA with the passenger strand subsequently destroyed (Ro et al., 2007). Recently, it has been established that the passenger strand can have as many gene targets as the guide strand; in some tissues both strands co-accumulate and in other tissues strand

selection is maintained (Ro et al., 2007). In our study, lung DE of bta-miR-21 included both strands; functional annotation categories of predicted target genes for the 5' and the 3' strands included the nucleus and DNA-binding and phosphoprotein and acetylation, respectively. Both bta-miR-21 strands were not correlated with a significantly dominant genus within the lung microbiota; however, bta-miR-21-5p was moderately negatively correlated with SDI. Shannon diversity index was numerically higher in CON calf samples, and although this was not statistically significant, it is consistent with the negative correlation with bta-miR-21-5p read counts, as bta-miR-21-5p was DE in CHALL versus CON calves. Previous studies in cattle have identified bta-miR-21-3p DE in serum during experimentally induced Foot and Mouth Disease Viral infection (Stenfeldt et al., 2017). Both bta-miR-21-3p and bta-miR-21-5p were found to be DE in mammary epithelium following experimental challenge with *Escherichia coli* and *Staphylococcus aureus*; bta-miR-21-5p was among the highest miRNA expressed at all timepoints (Jin et al., 2014). This miRNA may have implications for lower respiratory tract inflammation and lung lesion development in dairy calves.

Additionally, identified miRNAs bta-miR-149-5p and bta-miR-146b were previously shown to be DE in alveolar macrophages of cattle infected with *Mycobacterium bovis* up to 48 hours post infection (Vegh et al., 2015). In our study, the predicted functional annotation categories of these miRNA's target genes included zinc-finger associated proteins (bta-miR-146b), and transport and nucleotide-binding (bta-miR-149-5p). The normalized read counts of miRNAs bta-miR-149-5p and bta-miR-146b were not correlated with dominant genera nor alpha diversity metrics. Although miRNAs bta-miR-21-5p, bta-miR-149-5p and bta-miR-46b have been associated with pulmonary infection in ruminants, the other miRNAs identified in this study did not match with miRNAs DE in serum of Holstein calves following respiratory challenge with

Bovine Viral Diarrhea Virus (Taxis et al., 2017) or with miRNAs correlated to ELISA-quantified *Mycoplasma bovis* antibody levels in feedlot cattle following natural exposure (Casas et al., 2016).

The miRNAs identified in this study included 9 miRNAs with high DE (≥ 2 Log2FC). Of these miRNAs, the functional annotation category predictions for target genes of bta-miR-146b, bta-miR-2285t, bta-miR-212, bta-miR-2419-3p, bta-miR-1246, bta-miR-183 and bta-miR-376a included zinc or metal-binding proteins. Additionally, DE miRNAs with a high average read count (BaseMean ≥ 1000) included zinc or metal-binding proteins in the functional annotation categories of their predicted target genes; these miRNAs included bta-miR-146b, bta-miR-223, bta-miR-182, bta-miR-21-5p, bta-miR-10b, and bta-miR-375. Zinc is an essential trace element that is known to play a crucial role in modulating the immune system in response to pathogens and also functions as an antioxidant (Shankar and Prasad, 1998). Specifically, zinc's crucial roles in both the innate and adaptive immunes response include signaling as well as neutrophil and macrophage adhesion; when availability of zinc is reduced by disruption of transport mechanisms, survival and proliferation of immune cells is reduced (Bonaventura et al., 2015). The immune system is highly proliferative, an thus, tight regulation of zinc availability is crucial for immunity (Bonaventura et al., 2015).

In a recent study, dairy calves that received an injectable trace mineral supplement containing zinc showed an enhanced immune response (defined as increased peripheral blood mononuclear cells with increased production of interferon-γ) following a vaccination protocol that included a modified-live viral component for respiratory viruses and an attenuated-live *Mannheimia-Pasteurella* bacterin component (Palomares et al., 2016). Interestingly, bacteria identified within the NP of dairy calves diagnosed with clinical BRD, using whole genome

sequencing, had a higher level of cobalt-zinc-cadmium resistance genes compared with bacteria in the NP of healthy calves (Gaeta et al., 2017). Co-selection of heavy metal- and antibiotic-resistance has been known to occur in some species of bacteria, because the genes are physically linked, especially on plasmids (Baker-Austin et al., 2006). It is plausible that host miRNA expression in CHALL calves may target and inhibit proteins that bind or transport zinc due to signaling from changes within the host microbiota. The association between host immune cells, zinc transport and the respiratory microbiota in the context of BRD pathogenesis requires further investigation.

In our study, the phyla identified within the lung microbiota of CHALL and CON calves were similar to previous studies evaluating the tracheal microbiota of feedlot cattle (Timsit et al., 2018). The alpha diversity was not different among CHALL and CON calves; however, beta diversity, as measured by the Bray-Curtis dissimilarity metric, tended to be different between groups. The RA of *Pasteurella* spp. and *Escherichia* spp., which were higher in CHALL calf samples, had similar effects on the beta diversity of CHALL calves, while the RA of *Pseudomonas* spp. and *Butyrivibrio* spp. had similar effects on the beta diversity of CON calves; however, the RA of *Pseudomonas* spp. was higher in CHALL calves and the RA of *Butyrivibrio* spp. was higher in CON calves (Figure 4.5b). Increased RA of *Escherichia* spp. and *Pseudomonas* spp. have previously been identified in the NP microbiota of healthy dairy calves in one study (Lima et al., 2016); however, another study found that increased RA of *Pseudomonas* spp. within the NP of dairy calves was associated with clinical BRD (Gaeta et al., 2017). Less than 1% of both genera were identified in the tracheal microbiota of feedlot cattle (Timsit et al., 2018). The impact of *Escherichia* spp., *Pseudomonas* spp., and *Butyrivibrio* spp., on lung pathology in dairy calves is not well understood.

There was a strong, positive correlation between the RA of *Pasteurella* spp. and the normalized read count of bta-miR-212, which was in the high DE category of miRNAs identified in this study; bta-miR-212 was also predicted to target host genes associated with metal-binding, consistent with possible interactions with the host immune response. Results from the previous literature have identified bta-miR-212 to be DE in pre-ovulatory dominant follicles (Gebremedhn et al., 2015). Differential expression of miRNAs associated with follicular development have been shown to target genes involved in TGF-β signaling (Salilew-Wondim et al., 2014), which indicates bta-miR-212 may have an anti-inflammatory role associated with *P. multocida* infection.

The RA of *Pasteurella* spp. was strongly, negatively correlated with the normalized read count of bta-miR-10b. Additionally, the normalized read count of bta-miR-10b was strongly, negatively correlated with the RA of *Escherichia* spp. and strongly, positively correlated with the RA of *Butyrivibrio* spp. This is consistent with the downregulation of bta-miR-10b in CHALL calves compared to CON calves. The predicted functional annotation categories of bta-miR-10b's targets included zinc-binding and DNA-binding, which could indicate an interaction with the host immune system and transcription factor regulation. The identification of bta-miR-10b in previous studies included DE in serum during Foot and Mouth Disease infection (Stenfeldt et al., 2017) and within oocytes during maturation of the dominant follicle (Salilew-Wondim et al., 2014). The RA of *Pseudomonas* spp. was positively correlated with the normalized read count of bta-miR-1246. The predicted functional annotation category for target genes of bta-miR-1246 also included zinc and metal binding proteins. Casas et al. (2016) previously reported that DE of bta-miR-1246 in the serum of feedlot cattle was associated with seasonal variability.

There are limitations to the interpretation of data from this study. The number of raw reads was different between CHALL and CON calves; however, data analysis was performed using counts normalized for sequencing depth. The mean RIN for extracted RNA in this study was lower than is ideal for study of messenger RNA (mRNA); lower RIN values (< 8) indicate RNA degradation (Schroeder et al., 2006). However, there is evidence to suggest that RIN values are not accurate for determining miRNA integrity, as miRNAs are more robust than mRNA (Jung et al., 2010). There is still a potential that mRNA within the samples degraded into smaller fragments, which could have contaminated the downstream analyses. This risk was minimized by enriching our library for small RNA during gel excision of the cDNA construct following reverse transcription; additionally, sequenced libraries were mapped specifically to known bovine miRNAs.

Although the experimental challenge successfully increased the RA of *Pasteurella* spp.in the lungs of CHALL calves compared to CON calves that were not challenged, samples were taken 14 d following the experimental challenge. This time may have affected the diversity of the lung microbiota and increased variability among CHALL samples; temporal variability of the microbiota has been reported to be significant in previous studies (McDaneld et al., 2018; McMullen et al., 2018). The variation in microbiota composition over time may have had a more significant effect in this study given the small sample size. The small sample size may also have impacted our ability to detect significant differences in diversity metrics between CHALL and CON calves. Additional limitations with the experimental challenge included one calf that did not develop pneumonia following *P. multocida* inoculation and an additional CHALL calf that was treated with ampicillin for 3 days after inoculation, as per the randomization protocol of

the larger study. Investigators were blinded to CRS and USS results, as well as the treatment groups, and calves were selected for the current study during postmortem examination.

An additional limitation was the target gene analysis performed on the identified DE miRNAs. As was identified in our results, each miRNA is predicted to target hundreds to thousands of genes. The biologic function of miRNA binding sites (i.e. degree of mRNA degradation) is dependent on the dose-sensitivity of the host gene; therefore, it is unlikely that all of the predicted targets are sensitive to regulation by miRNAs (Pinzón et al., 2017). Therefore, a portion of the predicted target genes that were entered into DAVID for functional annotation analyses were likely to have been false positives. Further RNA-seq would be needed to verify whether mRNA levels were repressed while miRNAs were simultaneously DE. Quantification of miRNAs by quantitative RT-PCR would also provide stronger evidence of miRNA interactions and inform target gene dose sensitivity.

4.6 CONCLUSIONS

A total of 33 miRNAs were identified as DE in lung tissue of calves 14 days after experimental *P. multocida* infection; 9 of those were highly DE and 6 had an average normalized read count ≥ 1000. Two identified miRNAs from the same family (bta-miR-21-5p and bta-miR-21-3p) were previously identified in sheep lung tissue during viral exposure and infection; bta-miR-21-5p is a crucial regulator of inflammation and may impact the severity of lung pathology due to infection. Additionally, bta-miR-146b, a miRNA previously found to be DE in alveolar macrophages during *Mycobacterium bovis* infection, was found to be highly DE in lung tissue of CHALL calves and was identified at a high average read count. The majority of highly DE miRNAs identified had predicted target genes within functional categories that included proteins with metal binding or zinc binding regions. Zinc is an important element in the innate and

adaptive immune system; additionally, antibiotic- resistance and metal-resistance are co-selected in some bacteria. The microRNA bta-miR-212, which was highly DE in CHALL calves, was positively correlated with the RA of *Pasteurella* spp. Additionally, the normalized read counts of bta-miR-10b were negative correlated with the RA of *Pasteurella* spp. and *Escherichia* spp. within the microbiota and positively correlated with the RA of *Butyrivibrio* spp. Host miRNAs are DE within the lungs of dairy calves 14 d following infection with *Pasteurella multocida*. The function of these miRNAs may be to target the immune system by way of zinc or metal binding proteins. Larger sample sizes as well as samples from other portions of the respiratory tract may elucidate further evidence of communication between changes in the respiratory microbiota and host miRNA expression.

CONFLICT OF INTEREST

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AUTHOR CONTRIBTUIONS

SMR and WL conceived the project and interpreted the results. CHH, SMR and TLO conceived and carried out the experimental challenge. CR and WL performed the miRNA sequencing. SMR and GS performed the 16S rRNA amplicon sequencing. SMR and WL performed bioinformatics and data analysis. SMR wrote the manuscript. WL, TLO and GS reviewed and edited the manuscript.

CONTRIBUTION TO THE FIELD STATEMENT

Bovine respiratory disease (BRD) is an important obstacle to dairy calf health management. Although stability of the respiratory microbiota is known to be an important host defense against development of disease in cattle, very little information in cattle has been published that investigates potential mechanisms of communication between the host and commensal microbial population. There has been evidence in the literature of a link between the mucosal microbiota and host miRNA expression with impact on host susceptibility to disease. This paper is the first to investigate potential miRNA expression in the lung tissue of dairy calves that have been diagnosed with BRD. This study provides preliminary information that will allow for future investigations into miRNAs as possible biomarkers for respiratory microbiota dysbiosis and subsequent risk of BRD.

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4.8 TABLES AND FIGURES

TABLE 4.1 Differentially expressed miRNAs in CHALL vs. CON calves

miRNA	BaseMean[1]	log2FC[2]	*P* value
bta-miR-146b	7835.3	3.26	<0.001
bta-miR-205	204	2.79	0.000
bta-miR-223	1879.52	2.77	0.003
bta-miR-2285t	71	2.42	0.002
bta-miR-212	4.04	2.38	0.007
bta-miR-2419-3p	3.19	2.20	0.013
bta-miR-1246	321.33	2.10	0.001
bta-miR-183	94.14	2.06	<0.001
bta-miR-376a	2.16	2.05	0.043
bta-miR-182	996.33	1.95	0.000
bta-miR-132	92.27	1.94	0.000
bta-miR-677	60.48	1.76	0.004
bta-miR-96	7.41	1.75	0.024
bta-miR-2404	61.10	1.71	0.011
bta-miR-2484	204.35	1.65	0.002
bta-miR-3957	9.69	1.53	0.008
bta-miR-21-3p	823.13	1.51	0.002
bta-miR-210	237.27	1.44	0.000
bta-miR-2440	12.86	1.39	0.004
bta-miR-155	1503.10	1.36	0.000
bta-miR-582	11.61	1.26	0.017
bta-miR-375	2874.59	1.19	0.002
bta-miR-6518	13.86	1.19	0.006
bta-miR-363	114.02	1.15	0.010
bta-miR-665	38.05	1.12	0.001
bta-miR-21-5p	180975.24	1.08	0.003
bta-miR-149-5p	85.33	1.05	0.006
bta-miR-2474	10.72	1.05	0.035
bta-miR-493	125.82	1.05	0.013
bta-miR-10b	32441.02	-1.08	<0.001
bta-miR-190a	8.84	-1.32	0.03
bta-miR-129-3p	4.10	-1.84	0.02
bta-miR-383	3.37	-1.86	0.04

TABLE 4.1. miRNAs that were differentially expressed (DE) between CHALL and CON lung samples. [1]Basemean = mean of normalized counts of all samples, normalized for sequencing depth. [2]log2FC = Log base 2 of the fold change in miRNA expression in CHALL compared to CON lung samples. The *P* value is calculated from the DESeq2 package, which fits a negative binomial generalized linear model and applies a Wald test to each miRNA gene.
CHALL = calves experimentally challenged with *Pasteurella multocida* (n=4); CON = calves not exposed to bacterial challenge (n=5)

TABLE 4.2 Differentially expressed miRNAs and associations previously published

miRNA	mirPub references
bta-miR-146b	-DE in blood-isolated-monocytes following experimentally induced mastitis with *Streptococcus uberis* (Lawless et al., 2014a) -DE in alveolar macrophages following *Mycobacterium bovis* infection (Vegh et al., 2015)
bta-miR-205	-highly expressed in bovine mammary epithelium (Lawless et al., 2013)
bta-miR-223	-DE in milk- isolated-monocytes following experimentally induced mastitis with *Streptococcus uberis* (Lawless et al., 2014a) -in humans, regulates neutrophil proliferation (Chen et al., 2004)
bta-miR-2285t	-DE in mammary tissue in response to heat stress in dairy cattle (Li et al., 2018)
bta-miR-212	-DE in granulosa cells of pre-ovulatory dominant follicles (Gebremedhn et al., 2015)
bta-miR-2419-3p	-DE in mammary glands during lactation (Billa et al., 2019)
bta-miR-1246	-DE in serum of beef cattle by season (Casas et al., 2016)
bta-miR-183	-DE in granulosa cells of pre-ovulatory follicles (Gebremedhn et al., 2015)
bta-miR-376a	-DE in serum of heat stressed Holstein cows (Zheng et al., 2014)
bta-miR-182	-DE in milk-isolated-monocytes following experimentally induced mastitis with *Streptococcus uberis* (Lawless et al., 2014a)
bta-miR-132	-DE in serum during Foot and Mouth Disease viral infection (Stenfeldt et al., 2017)
bta-miR-677	-DE in macrophages infected with *Mycobacterium avium* subsp. *paratuberculosis* (Wang et al., 2019)
bta-miR-96	-DE in granulosa cells of pre-ovulatory dominant follicles (Gebremedhn et al., 2015)
bta-miR-2404	-DE in skeletal muscle satellite cells (Zhang et al., 2016)
bta-miR-2484	-DE in mammary gland during *Staphylococcus aureus* infection (Li et al., 2015)
bta-miR-3957	-tissue specific expression in the bovine liver (Sun et al., 2019)
bta-miR-21-3p	-DE in serum during Foot and Mouth Disease viral infection (Stenfeldt et al., 2017) -DE in bovine mammary epithelium following challenge with *Escherichia coli* or *Staphylococcus aureus* (Jin et al., 2014)
bta-miR-210	-downregulated in milk-isolated-monocytes with *S. uberis* induced mastitis; upregulated in response to LPS (Lawless et al., 2013, 2014a)
bta-miR-2440	-DE in plasma of high-altitude hypoxia-stressed Jerseys (Kong et al., 2019)
bta-miR-155	-identical homolog to human miRNA -critical for B cell development (Lawless et al., 2014b) -crucial for T and B lymphocyte and dendritic cell function (Rodriguez et al., 2007) -DE in monocytes following exposure to LPS (Dilda et al., 2012)

	-DE in bovine mammary epithelium following challenge with *Streptococcus uberis* (Naeem et al., 2012).
bta-miR-582	-DE in granulosa cells of dominant follicles (Zielak-Steciwko et al., 2014)
bta-miR-375	-DE in blood-isolated-monocytes following experimentally induced mastitis with *Streptococcus uberis* (Lawless et al., 2014a)
bta-miR-6518	-DE in milk during lactation (Do et al., 2017)
bta-miR-363	-DE in milk-isolated-monocytes following experimentally induced mastitis with *Streptococcus uberis* (Lawless et al., 2014a)
bta-miR-665	-DE in plasma between day 0 and day 60 of pregnancy (Ioannidis and Donadeu, 2017)
bta-miR-21-5p	-highly conserved in humans and cattle -induced by LPS, negatively regulates TL4 and enhances IL-10 production in mice (Sheedy et al., 2010) -DE in bovine mammary epithelium following challenge with *Escherichia coli* or *Staphylococcus aureus* (Jin et al., 2014)
bta-miR-493	-DE in bovine exosomes and serum (Zhao et al., 2016)
bta-miR-149-5p	-DE in alveolar macrophages following *Mycobacterium bovis* infection (Vegh et al., 2015)
bta-miR-2474	-DE in adipose tissues of steers (Romao et al., 2014)
bta-miR-10b	-DE in serum during Foot and Mouth Disease viral infection (Stenfeldt et al., 2017) -DE in subordinate and subordinate and dominant follicles (Salilew-Wondim et al., 2014).
bta-miR-190a	-DE in oocyte during maturation (Gilchrist et al., 2016)
bta-miR-129-3p	-DE in serum during Foot and Mouth Disease viral infection (Stenfeldt et al., 2017)
bta-miR-383	-DE in granulosa cells during estrous cycle (Salilew-Wondim et al., 2014)

TABLE 4.3. Differentially expressed miRNAs and predicted target functional categories

miRNA	Number of Target Genes	Functional Annotation Categories	*P* values
bta-miR-146b	248	Transport (e.g. amino acids, bacteriocin export) Zinc-finger	4.3E-2 6.8E-2
bta-miR-205	532	Phosphoprotein Nucleus (e.g. transcription factors) Coiled coil	7.4E-4 1.9E-6 3.1E-2
bta-miR-223	366	Coiled coil Nucleus Transcription	8.9E-4 2.0E-4 1.3E-4
bta-miR-2285t	3064	Coiled coil Nucleus Metal-binding	1.1E-2 5.2E-2 6.9E-2
bta-miR-212	365	Phosphoprotein Nucleus Metal-binding	3.9E-3 5.0E-2 6.0E-2
bta-miR-2419-3p	1346	Coiled coil Nucleus Metal-binding	3.1E-3 1.8E-2 2.3E-2
bta-miR-1246	2379	Coiled coil Metal binding Zinc binding	4.0E-3 6.7E-6 1.0E-6
bta-miR-183	472	Phosphoprotein Coiled coil Metal-binding	5.6E-4 1.6E-2 1.3E-3
bta-miR-376a	234	Coiled coil Metal-binding Transferase	2.7E-2 6.5E-2 6.7E-2
bta-miR-182	1217	Coiled coil Metal-binding Zinc binding	2.2E-5 4.2E-3 2.2E-5
bta-miR-132	474	Coiled coil Nucleus Metal-binding	2.1E-4 8.8E-3 2.4E-2
bta-miR-677	4292	Phosphoprotein Nucleus Metal-binding	3.3E-2 6.8E-2 2.2E-2
bta-miR-96	1093	Coiled coil Phosphoprotein Metal-binding	1.7E-4 2.1E-2 5.5E-2

bta-miR-2404	2795	Coiled coil	4.7E-2
		Nucleus	2.3E-2
		Metal-binding	5.7E-2
bta-miR-2484	2020	Phosphoprotein	4.9E-5
		Coiled coil	9.4E-3
		Nucleus	1.8E-4
bta-miR-3957	689	Coiled coil	2.6E-2
		Metal-binding	2.9E-2
		Zn-binding	6.1E-2
bta-miR-21-3p	4755	Phosphoprotein	6.7E-4
		Cytoplasm	1.8E-4
		Acetylation	1.1E-3
bta-miR-210	2593	Coiled coil	3.7E-3
		Metal-binding	5.7E-2
		Glycoprotein	6.3E-2
bta-miR-2440	3509	Membrane	1.5E-2
		Signaling	6.9E-2
		Disulfide bond (e.g. immunoglobulin)	7.8E-2
bta-miR-155	495	Phosphoprotein	3.9E-2
		Nucleus	1.2E-3
		Metal-binding	6.1E-2
bta-miR-582	596	Coiled coil	4.2E-2
		Nucleus	3.7E-2
		Zinc-binding	4.1E-2
bta-miR-375	5150	Coiled coil	6.5E-4
		Metal-binding	5.7E-5
		Zinc-binding	6.6E-7
bta-miR-6518	1355	Coiled coil	4.2E-2
		Nucleus	3.7E-2
		Zinc-binding	3.7E-2
bta-miR-363	1818	Coiled coil	5.8E-3
		Nucleus	8.5E-2
		Metal-binding	2.2E-3
bta-miR-665	2301	Coiled coil	8.5E-2
		Nucleotide-binding	7.6E-2
		Zinc-finger	1.1E-2
bta-miR-21-5p	355	Coiled coil	5.2E-3
		Nucleus	1.1E-2
		DNA-binding	7.3E-3
bta-miR-493	171	Nucleus	8.5E-3
		Cytoplasm	2.6E-2
		Methylation	6.7E-4
bta-miR-149-5p	454	Cytoplasm	5.0E-2
		Transport	9.1E-3
		Nucleotide-binding	5.8E-2

bta-miR-2474	636	Coiled coil	4.9E-3
		Transferase	1.7E-2
		Zinc-binding	5.3E-2
bta-miR-10b	299	Coiled coil	1.0E-3
		DNA binding	9.3E-4
		Zinc-binding	3.7E-2
bta-miR-190a	208	Phosphoprotein	1.0E-2
		Coiled coil	2.3E-2
		Nucleus	9.3E-3
bta-miR-129-3p	482	Phosphoprotein	1.0E-2
		Coiled coil	1.3E-2
		Nucleus	8.3E-3
bta-miR-383	212	Nucleus	2.4E-4
		Coiled coil	2.1E-2
		Cytoplasm	4.6E-2

TABLE 4.4 Spearman rank correlation coefficients describing the relationship between the relative abundance of *Pasteurella* spp. and the normalized read counts of DE miRNAs among all calves.

miRNA	Spearman's rho (95% CI)	*P* value
bta-miR-146b	0.27 (-0.70, 0.80)	0.36
bta-miR-205	0.50 (-0.36, 0.98)	0.17
bta-miR-223	0.49 (-0.26, 0.92)	0.17
bta-miR-2285t	0.06 (-0.85, 0.69)	0.90
bta-miR-212	0.71 (0.15, 1.00)	0.03*
bta-miR-2419-3p	0.67 (0.28, 0.97)	0.05
bta-miR-1246	0.42(-0.45, 0.93)	0.26
bta-miR-183	0.41 (-0.34, 0.90)	0.27
bta-miR-376a	0.32 (-0.28, 0.92)	0.40
bta-miR-182	0.55 (-0.25, 0.98)	0.12
bta-miR-132	0.80 (0.30, 0.96)	0.01*
bta-miR-677	0.65 (0, 0.98)	0.06
bta-miR-96	0.39 (-0.48, 0.90)	0.30
bta-miR-2404	0.47 (-0.30, 0.90)	0.20
bta-miR-2484	0.44 (-0.37, 0.93)	0.23
bta-miR-3957	0.48 (-0.20, 0.83)	0.20
bta-miR-21-3p	0.58 (-0.03, 0.92)	0.10
bta-miR-210	0.56 (-0.25, 1.00)	0.11
bta-miR-2440	0.41 (-0.34, 0.92)	0.27
bta-miR-155	0.41 (-0.47, 0.92)	0.27
bta-miR-582	0.82 (0.57, 1.0)	0.01*
bta-miR-375	0.41 (-0.55, 0.96)	0.27
bta-miR-6518	0.70 (0.08, 1.00)	0.04*
bta-miR-363	0.27 (-0.67, 0.91)	0.50
bta-miR-665	0.57 (-0.023, 0.88)	0.10
bta-miR-21-5p	0.60 (-0.09, 0.97)	0.09
bta-miR-149-5p	0.54 (-0.25, 0.99)	0.13
bta-miR-2474	0.51 (-0.33, 0.93)	0.16
bta-miR-493	0.30 (-0.63, 0.92)	0.42
bta-miR-10b	-0.85 (-0.99, -0.40)	0.003**
bta-miR-190a	-0.58 (-0.99, 0.18)	0.10
bta-miR-129-3p	-0.89 (-0.99, -0.60)	0.003**
bta-miR-383	-0.70 (-0.90, -0.42)	0.03*

TABLE 4.4 *P* values are the results of a Spearman rank correlation coefficient; **P* < 0.05, ***P* <0.01, ****P* < 0.001 (n=9)

TABLE 4.5 Spearman rank correlation coefficients describing the relationship between the relative abundance of *Escherichia* spp. and the normalized read counts of DE miRNAs among all calves.

miRNA	Spearman's rho (95% CI)	*P* value
bta-miR-146b	0.12 (-0.72, 0.75)	0.78
bta-miR-205	0.49 (-0.41, 1.00)	0.18
bta-miR-223	0.43 (-0.35, 0.98)	0.25
bta-miR-2285t	-0.11 (-0.89, 0.60)	0.78
bta-miR-212	0.66 (0.10, 0.93)	0.05
bta-miR-2419-3p	0.55 (0, 0.92)	0.13
bta-miR-1246	0.44 (-0.37, 0.87)	0.24
bta-miR-183	0.46 (-0.32, 0.95)	0.21
bta-miR-376a	0.32 (-0.28, 0.88)	0.42
bta-miR-182	0.48 (-0.38, 1.00)	0.20
bta-miR-132	0.78 (0.20, 1.00)	0.01*
bta-miR-677	0.65 (-0.11, 0.98)	0.07
bta-miR-96	0.28 (-0.75, 0.92)	0.46
bta-miR-2404	0.55 (-0.31, 0.99)	0.13
bta-miR-2484	0.51 (-0.34, 0.99)	0.16
bta-miR-3957	0.34 (-0.39, 0.70)	0.37
bta-miR-21-3p	0.52 (-0.16, 0.95)	0.16
bta-miR-210	0.60 (-0.14, 1.00)	0.10
bta-miR-2440	0.40 (-0.32, 0.91)	0.28
bta-miR-155	0.33 (-0.48, 0.88)	0.40
bta-miR-582	0.67 (0.23, 0.93)	0.05
bta-miR-375	0.37 (-0.53, 1.00)	0.34
bta-miR-6518	0.66 (-0.08, 0.99)	0.05
bta-miR-363	0.31 (-0.50, 0.94)	0.42
bta-miR-665	0.41 (-0.33, 0.85)	0.27
bta-miR-21-5p	0.43 (-0.30, 0.95)	0.25
bta-miR-149-5p	0.49 (-0.31, 0.95)	0.18
bta-miR-2474	0.36 (-0.49, 0.89)	0.34
bta-miR-493	0.17 (-0.73, 0.72)	0.70
bta-miR-10b	-0.78 (-1.00, -0.11)	0.02*
bta-miR-190a	-0.55 (-0.98, 0.26)	0.12
bta-miR-129-3p	-0.73 (-0.90, -0.29)	0.03*
bta-miR-383	-0.81 (-0.92, -0.55)	0.01*

TABLE 4.5 *P* values are the results of a Spearman rank correlation coefficient; **P* < 0.05, ***P* <0.01, ****P* < 0.001 (n=9)

TABLE 4.6 Spearman rank correlation coefficients describing the relationship between the relative abundance of *Pseudomonas* spp. and the normalized read counts of DE miRNAs among all calves.

miRNA	Spearman's rho (95% CI)	*P* value
bta-miR-146b	-0.33 (-0.79, 0.77)	0.95
bta-miR-205	0.22 (-0.57, 0.90)	0.57
bta-miR-223	-0.10 (-0.81, 0.85)	0.81
bta-miR-2285t	-0.37 (-0.99, 0.57)	0.33
bta-miR-212	0.46 (-0.16, 0.81)	0.22
bta-miR-2419-3p	0.18 (-0.42, 0.74)	0.64
bta-miR-1246	0.82 (0.25, 0.99)	0.01*
bta-miR-183	0.42 (-0.43, 0.97)	0.30
bta-miR-376a	0.62 (0.28, 0.90)	0.07
bta-miR-182	0.40 (-0.37, 0.90)	0.29
bta-miR-132	0.85 (0.45, 1.00)	0.003**
bta-miR-677	0.90 (0.53, 1.00)	0.002**
bta-miR-96	0.32 (-0.42, 0.87)	0.40
bta-miR-2404	0.90 (0.27, 1.00)	0.001**
bta-miR-2484	0.90 (0.43, 1.00)	0.001**
bta-miR-3957	0.50 (-0.32, 0.93)	0.17
bta-miR-21-3p	0 (-0.66, 0.63)	1.00
bta-miR-210	0.17 (-0.55, 0.90)	0.70
bta-miR-2440	0.91 (0.57, 0.99)	0.001**
bta-miR-155	0.17 (-0.64, 0.72)	0.70
bta-miR-582	0.43 (-0.24, 0.85)	0.25
bta-miR-375	0.07 (-0.82, 0.75)	0.90
bta-miR-6518	0.73 (0.01, 0.97)	0.03*
bta-miR-363	0.13 (-0.58, 0.89)	0.73
bta-miR-665	0.54 (-0.20, 0.92)	0.14
bta-miR-21-5p	0.10 (-0.62, 0.75)	0.81
bta-miR-149-5p	0.17 (-0.53, 0.69)	0.66
bta-miR-2474	0.39 (-0.33, 0.88)	0.29
bta-miR-493	0.3 (-0.43, 0.89)	0.44
bta-miR-10b	-0.56 (-0.93, 0.17)	0.12
bta-miR-190a	-0.67 (-0.94, 0.12)	0.04*
bta-miR-129-3p	-0.46 (-0.88, 0.14)	0.22
bta-miR-383	-0.37 (-0.85, 0.35)	0.33

TABLE 4.6 *P* values are the results of a Spearman rank correlation coefficient; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (n=9)

TABLE 4.7 Spearman rank correlation coefficients describing the relationship between the relative abundance of *Butyrivibrio* spp. and the normalized read counts of DE miRNAs among all calves.

miRNA	Spearman's rho (95% CI)	*P* value
bta-miR-146b	-0.20 (-0.81, 0.58)	0.61
bta-miR-205	-0.26 (-0.84, 0.69)	0.50
bta-miR-223	0.20 (-0.66, 0.3)	0.61
bta-miR-2285t	0.11 (-0.85, 0.82)	0.78
bta-miR-212	-0.50 (-0.85, 0.10)	0.18
bta-miR-2419-3p	-0.23 (-0.78, 0.32)	0.55
bta-miR-1246	-0.25 (-0.82, 0.61)	0.51
bta-miR-183	-0.28 (-0.98, 0.53)	0.46
bta-miR-376a	-0.41 (-0.85, 0.29)	0.27
bta-miR-182	-0.23 (-0.79, 0.73)	0.55
bta-miR-132	-0.42 (-0.84, 0.36)	0.25
bta-miR-677	-0.43 (-0.85, 0.32)	0.25
bta-miR-96	-0.50 (-0.93, 0.35)	0.17
bta-miR-2404	-0.39 (-0.91, 0.53)	0.30
bta-miR-2484	-0.33 (-0.94, 0.62)	0.38
bta-miR-3957	-0.70 (-0.98, -0.14)	0.04*
bta-miR-21-3p	-0.30 (-0.84, 0.58)	0.50
bta-miR-210	-0.23 (-0.80, 0.63)	0.55
bta-miR-2440	-0.55 (-0.95, 0.37)	0.13
bta-miR-155	-0.60 (-1.00, 0.26)	0.10
bta-miR-582	-0.39 (-0.81, 0.33)	0.30
bta-miR-375	-0.37 (-0.90, 0.53)	0.33
bta-miR-6518	-0.38 (-0.83, 0.35)	0.32
bta-miR-363	-0.05 (-0.86, 0.71)	0.90
bta-miR-665	-0.61 (-0.92, 0.10)	0.08
bta-miR-21-5p	-0.47 (-0.83, 0.36)	0.21
bta-miR-149-5p	-0.56 (-0.93, 0.11)	0.11
bta-miR-2474	-0.62 (-0.95, 0.10)	0.07
bta-miR-493	-0.23 (-0.85, 0.56)	0.55
bta-miR-10b	0.82 (0.42, 0.95)	0.01*
bta-miR-190a	0.21 (-0.57, 0.78)	0.60
bta-miR-129-3p	0.50 (-0.17, 0.89)	0.22
bta-miR-383	0.33 (-0.32, 0.91)	0.33

TABLE 4.7 *P* values are the results of a Spearman rank correlation coefficient; **P* < 0.05, ***P* <0.01, ****P* < 0.001 (n=9)

TABLE 4.8 Spearman rank correlation coefficients describing the relationship between the Shannon Diversity Index and the normalized read counts of DE miRNAs among all calves.

miRNA	Spearman's rho (95% CI)	*P* value
bta-miR-146b	-0.35 (-0.84, 0.42)	0.36
bta-miR-205	-0.34 (-0.89, 0.44)	0.36
bta-miR-223	-0.33 (-0.89, 0.36)	0.40
bta-miR-2285t	-0.24 (-0.70, 0.60)	0.53
bta-miR-212	-0.26 (-0.86, 0.48)	0.50
bta-miR-2419-3p	-0.34 (-0.85, 0.31)	0.37
bta-miR-1246	0.23 (-0.60, 0.94)	0.56
bta-miR-183	0 (-0.74, 0.83)	1.00
bta-miR-376a	0.10 (-0.53, 0.58)	0.80
bta-miR-182	-0.27 (-0.84, 0.59)	0.49
bta-miR-132	-0.06 (-0.84, 0.67)	0.90
bta-miR-677	0.08 (-0.82, 0.81)	0.84
bta-miR-96	-0.25 (-0.78, 0.54)	0.52
bta-miR-2404	0.23 (-0.66, 0.89)	0.56
bta-miR-2484	0.24 (-0.69, 0.92)	0.53
bta-miR-3957	-0.26 (-0.72, 0.45)	0.51
bta-miR-21-3p	-0.55 (-0.98, 0.24)	0.13
bta-miR-210	-0.27 (-0.98, 0.63)	0.49
bta-miR-2440	0.15 (-0.62, 0.75)	0.71
bta-miR-155	-28 (-0.89, 0.50)	0.50
bta-miR-582	-0.41 (-0.86, 0.23)	0.30
bta-miR-375	-0.42 (-0.98, 0.43)	0.27
bta-miR-6518	-0.08 (-0.84, 0.76)	0.84
bta-miR-363	0.22 (-0.65, 0.83)	0.56
bta-miR-665	-0.38 (-0.88, 0.39)	0.32
bta-miR-21-5p	-0.70 (-0.95, -0.08)	0.04*
bta-miR-149-5p	-0.36 (-0.95, 0.45)	0.35
bta-miR-2474	-0.31 (-0.81, 0.39)	0.41
bta-miR-493	-0.03 (-0.68, 0.67)	0.95
bta-miR-10b	0.50 (-0.23, 0.95)	0.18
bta-miR-190a	-0.08 (-0.85, 0.92)	0.84
bta-miR-129-3p	0.46 (-0.21, 0.89)	0.22
bta-miR-383	0.36 (-0.74, 0.93)	0.34

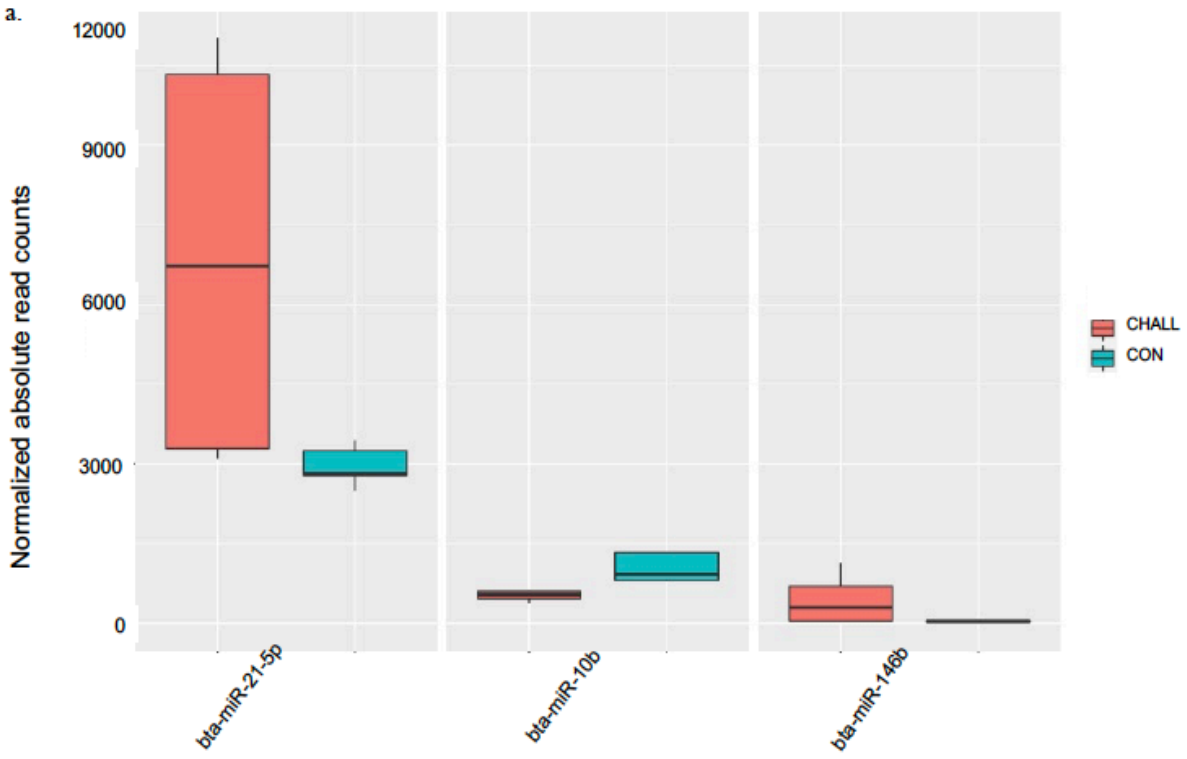
TABLE 4.8 *P* values are the results of a Spearman rank correlation coefficient; **P* < 0.05, ***P* <0.01, ****P* < 0.001 (n=9)

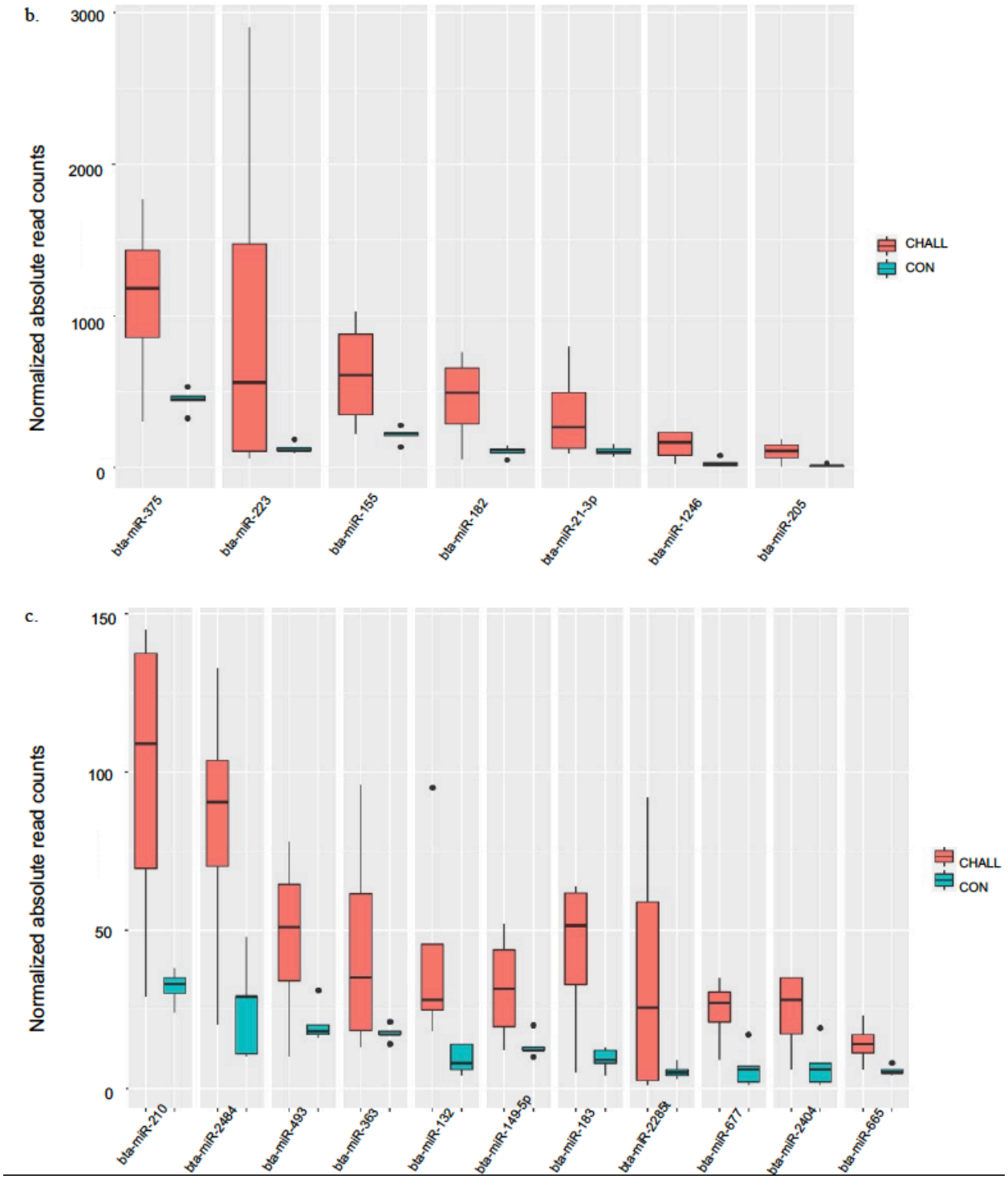
TABLE 4.9 Spearman rank correlation coefficients describing the relationship between the Chao1 Index and the normalized read counts of DE miRNAs among all calves.

miRNA	Spearman's rho (95% CI)	*P* value
bta-miR-146b	0 (-0.85, 0.81)	1.00
bta-miR-205	-0.29 (-0.95, 0.53)	0.46
bta-miR-223	-0.38 (-0.98, 0.46)	0.31
bta-miR-2285t	-0.13 (-0.95, 0.62)	0.73
bta-miR-212	-0.03 (-0.81, 0.85)	0.94
bta-miR-2419-3p	-0.18 (-0.84, 0.74)	0.64
bta-miR-1246	0.70 (0.14, 1.00)	0.03*
bta-miR-183	0.083 (-0.72, 0.64)	0.84
bta-miR-376a	0.52 (0.11, 0.89)	0.15
bta-miR-182	-0.10 (-0.86, 0.62)	0.81
bta-miR-132	0.34 (-0.47, 0.95)	0.37
bta-miR-677	0.60 (-0.08, 0.85)	0.10
bta-miR-96	0.04 (-0.78, 0.80)	0.91
bta-miR-2404	0.66 (0.14, 0.90)	0.06
bta-miR-2484	0.65 (0.11, 0.90)	0.06
bta-miR-3957	0.28 (-0.64, 0.92)	0.46
bta-miR-21-3p	-0.30 (-0.95, 0.59)	0.44
bta-miR-210	-0.35 (-1.00, 0.56)	0.36
bta-miR-2440	0.75 (0.21, 0.91)	0.02
bta-miR-155	0.02 (-0.86, 0.90)	0.98
bta-miR-582	0.04 (-0.76, 0.78)	0.92
bta-miR-375	-0.35 (-0.98, 0.58)	0.36
bta-miR-6518	0.18 (-0.74, 0.93)	0.64
bta-miR-363	0.04 (-0.74, 0.82)	0.91
bta-miR-665	0.26 (-0.59, 0.87)	0.50
bta-miR-21-5p	-0.18 (-0.94, 0.69)	0.64
bta-miR-149-5p	-0.22 (-0.87, 0.86)	0.57
bta-miR-2474	0.12 (-0.65, 0.93)	0.76
bta-miR-493	0.22 (-0.69, 0.81)	0.58
bta-miR-10b	0.10 (-0.84, 0.83)	0.81
bta-miR-190a	-0.32 (-0.96, 0.52)	0.40
bta-miR-129-3p	0 (-0.76, 0.74)	1.00
bta-miR-383	0.28 (-0.55, 0.84)	0.47

TABLE 4.9 *P* values are the results of a Spearman rank correlation coefficient; **P* < 0.05, ***P* <0.01, ****P* < 0.001 (n=9)

FIGURE 4.1. Normalized read counts of differentially expressed miRNAs of lung samples by challenge group




b.
Normalized absolute read counts
3000
2000
1000
0
bta-miR-375
bta-miR-223
bta-miR-155
bta-miR-182
bta-miR-21-3p
bta-miR-1246
bta-miR-205
CHALL
CON
c.
Normalized absolute read counts
150
100
50
0
bta-miR-210
bta-miR-2484
bta-miR-493
bta-miR-363
bta-miR-132
bta-miR-149-5p
bta-miR-183
bta-miR-2285
bta-miR-677
bta-miR-2404
bta-miR-665
CHALL
CON

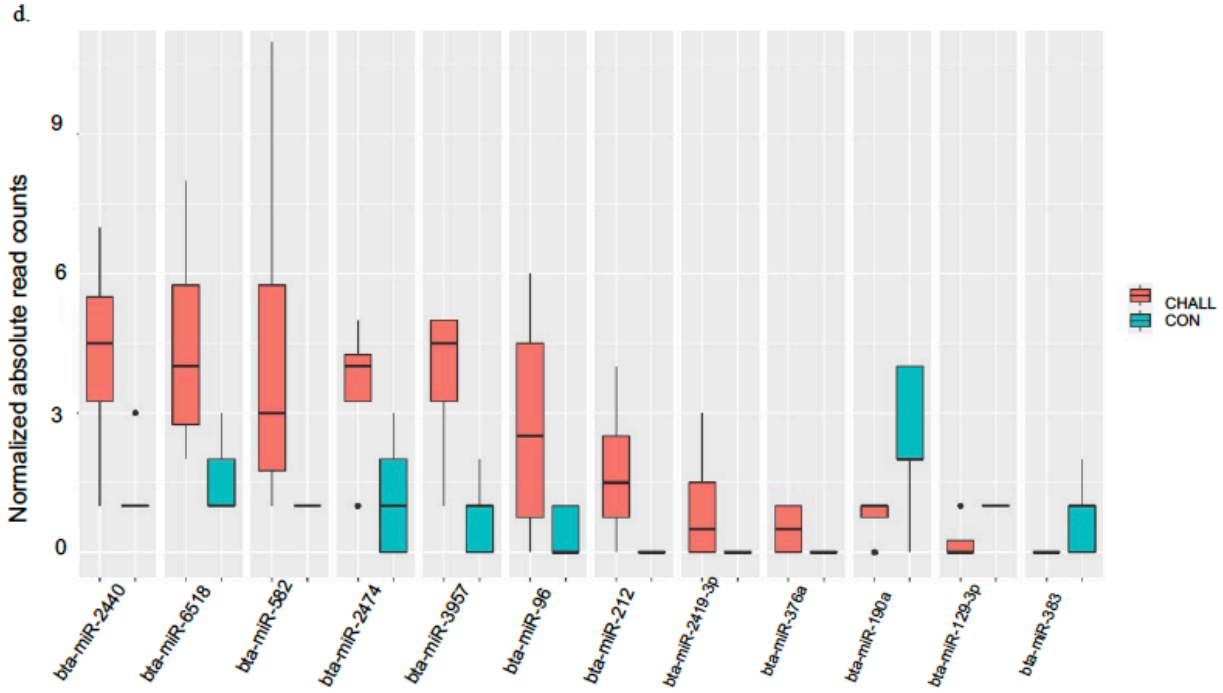
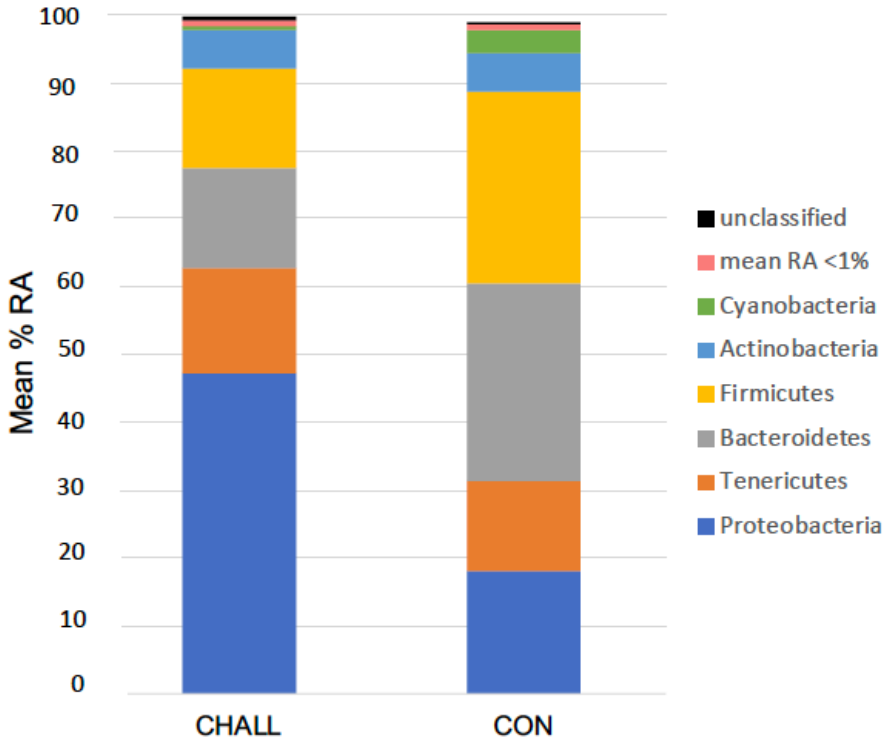


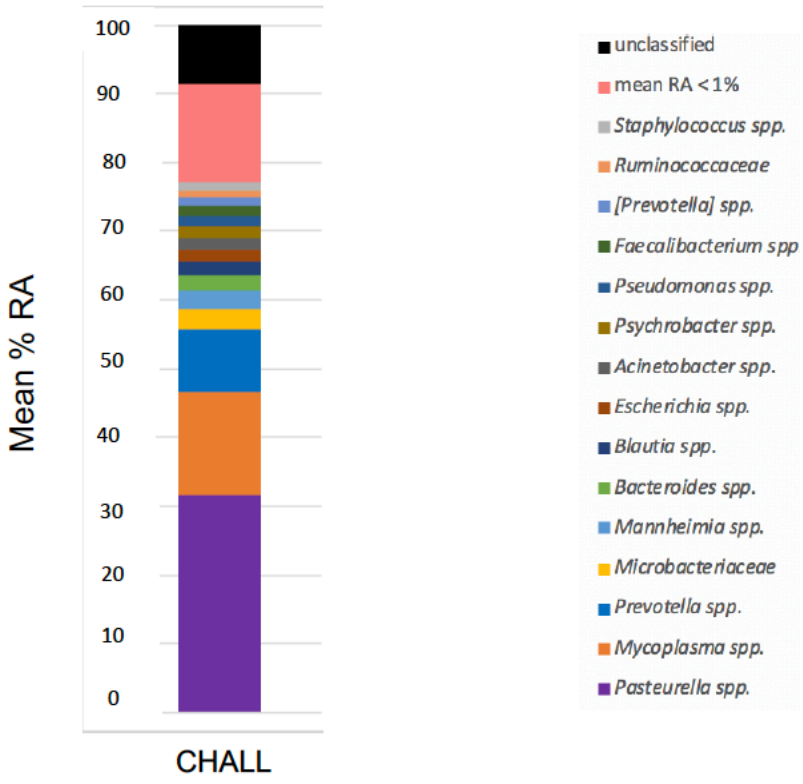
FIGURE 4.1 Boxplots of normalized absolute read counts of differentially expressed miRNAs in CHALL and CON calves. Boxplots include the minimum, Quartile 1, median, Quartile 3, and the maximum, respectively. Outliers are indicated with closed circles. (a.) miRNAs with the interquartile range of normalized absolute read counts equal to 1,500-10,000. (b.) miRNAs with the interquartile range of normalized absolute read counts equal to 200-1,500. (c.) miRNAs with the interquartile range of normalized absolute read counts equal to 25-150. (d.) miRNAs with the interquartile range of normalized absolute read counts < 10.
CHALL = calves experimentally challenged with *Pasteurella multocida* (n = 4); CON = calves not exposed to bacterial challenge (n = 5)

FIGURE 4.2 Percent relative abundance of dominant taxa within the lung microbiota of CHALL and CON calves

(a.)



(b.)


100
90
80
70
60
50
40
30
20
10
0
Mean % RA
CHALL
unclassified
mean RA < 1%
Staphylococcus spp.
Ruminococcaceae
[Prevotella] spp.
Faecalibacterium spp
Pseudomonas spp.
Psychrobacter spp.
Acinetobacter spp.
Escherichia spp.
Blautia spp.
Bacteroides spp.
Mannheimia spp.
Microbacteriaceae
Prevotella spp.
Mycoplasma spp.
Pasteurella spp.

(c.)

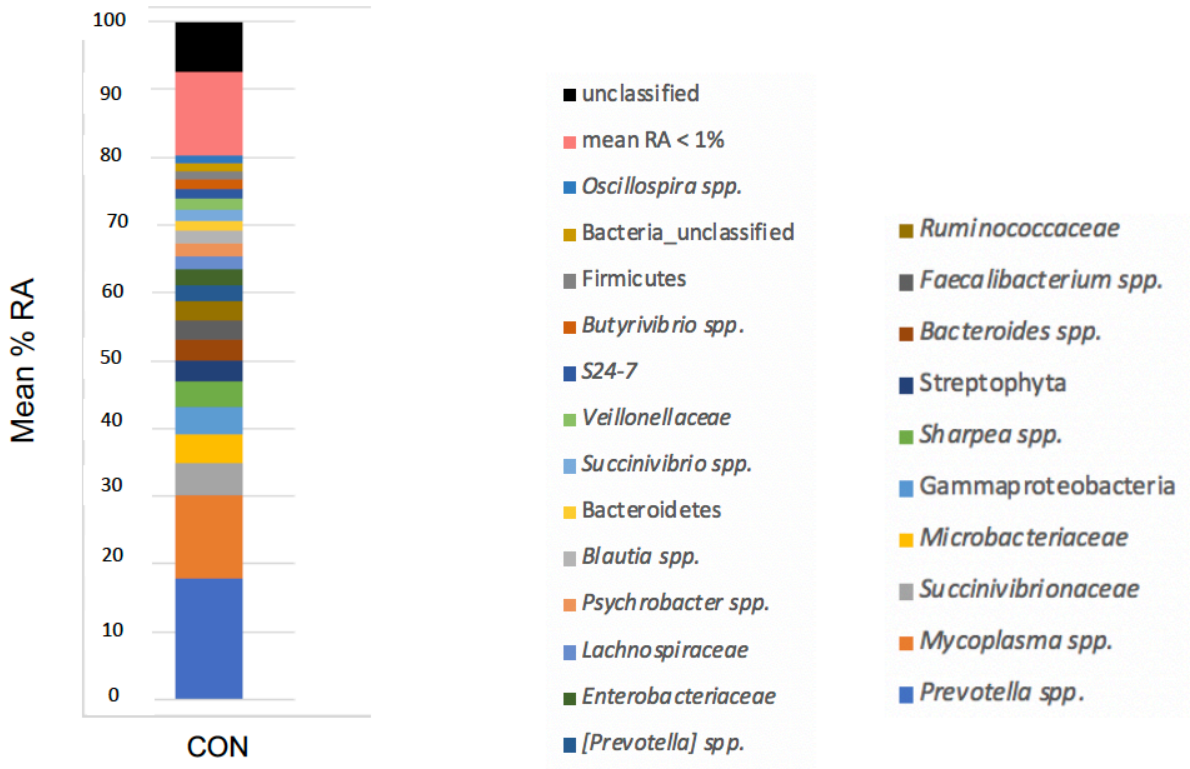
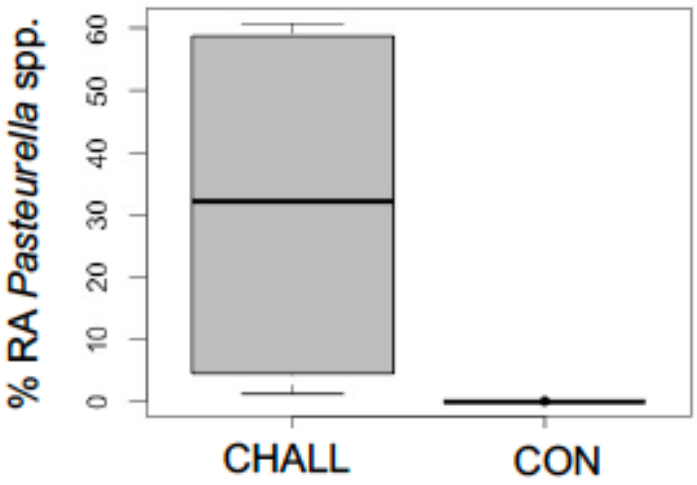


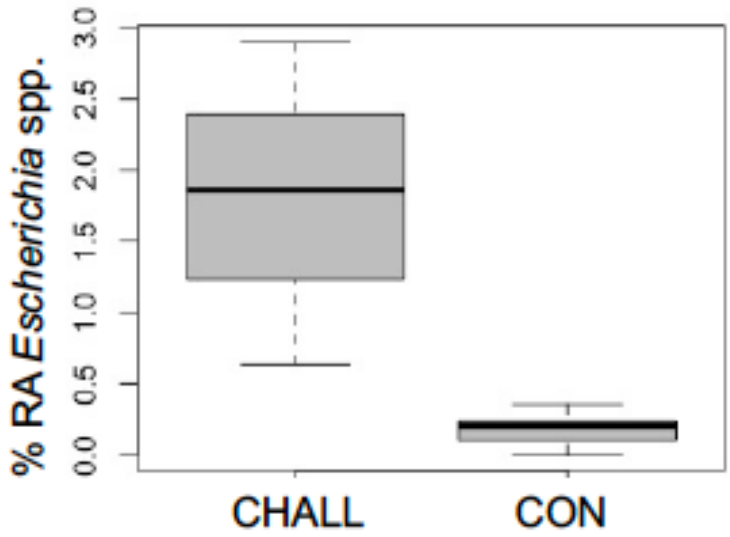
FIGURE 4.2 (a.) Percent mean relative abundance (RA) of dominant phyla identified in CHALL and CON calves. (b.) Percent mean RA of dominant genera in CHALL calves. (c.) Percent mean RA of dominant genera in CON calves. The unclassified category refers to taxa that could not be resolved. The category mean RA <1 % refers to the sum of all resolved taxa with an average percent RA < 1.0.
CHALL = calves experimentally challenged with *Pasteurella multocida* (n = 4); CON = calves not exposed to bacterial challenge (n = 5)

FIGURE 4.3 Percent relative abundance of genera significantly different between CHALL and CON lung samples.

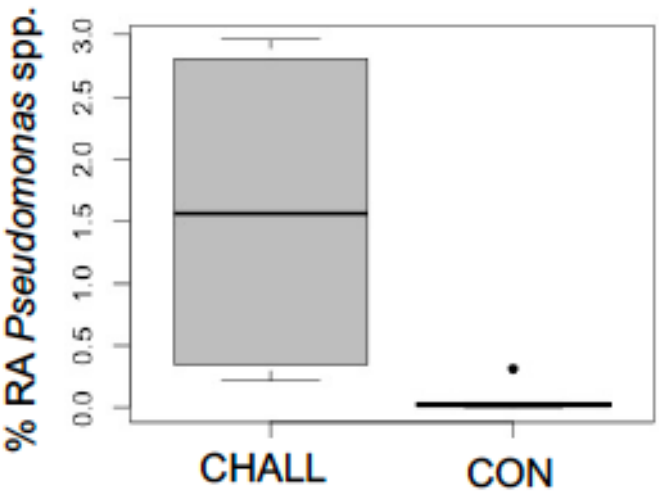
(a.) $P = 0.01$



(b.) $P = 0.01$



(c.) $P = 0.03$



(d.) $P = 0.03$

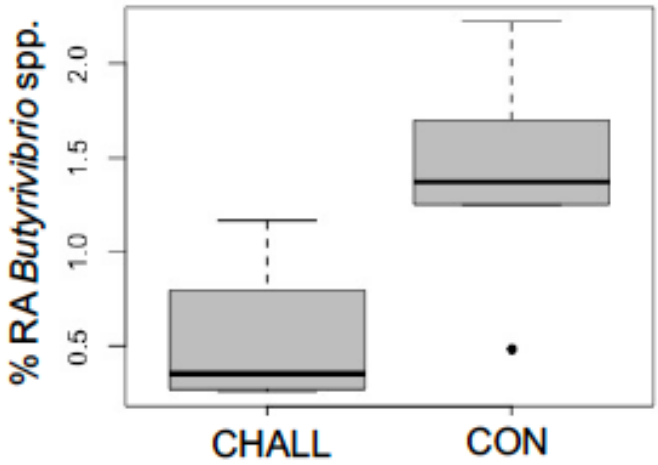
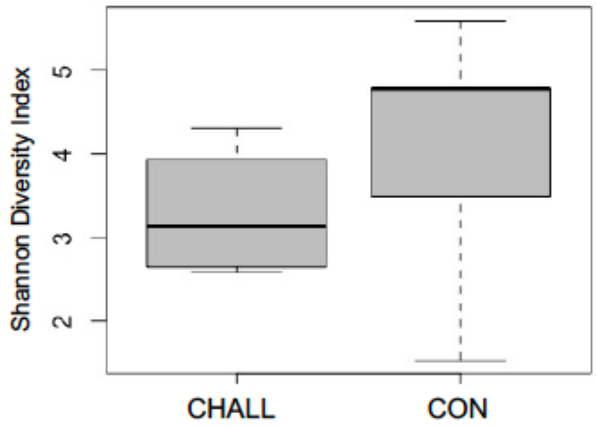


FIGURE 4.3 Boxplots depicting the minimum, Quartile 1, median, Quartile 3 and maximum percent relative abundance values for (a.) *Pasteurella* spp., (b.) *Escherichia* spp., (c.) *Pseudomonas* spp., and (d.) *Butyrivibrio* spp. among CHALL and CON calves. Outliers are depicted by closed circles. *P* values are the result of Kruskal Wallis tests.
CHALL = calves experimentally challenged with *Pasteurella multocida* (n = 4); CON = calves not exposed to bacterial challenge (n = 5)

FIGURE 4.4 Alpha diversity metrics in lung samples of CHALL and CON calves

(a.) $P = 0.40$



(b.) $P = 0.73$

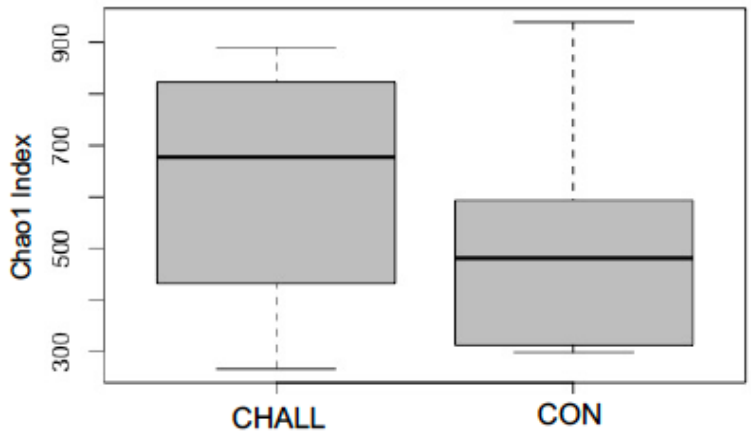
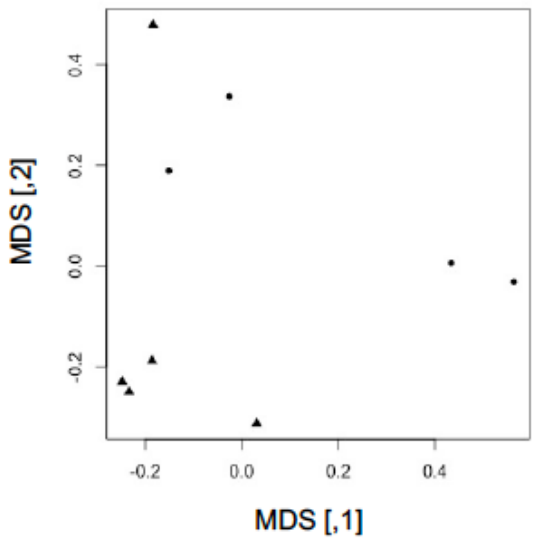


FIGURE 4.4 Boxplots depicting the minimum, Quartile 1, median, Quartile 3 and maximum values of Shannon diversity index (a.) and Chao1 index (b.) in CHALL and CON lung samples. The *P* values are the result of a Student's t test in (a.) and a Wilcoxon rank sum test in (b.) CHALL = calves experimentally challenged with *Pasteurella multocida* (n = 4); CON = calves not exposed to bacterial challenge (n = 5)

FIGURE 4.5 Metric multidimensional scaling plots of the Bray-Curtis dissimilarity metric in CHALL and CON lung samples

(a.)



(b.)

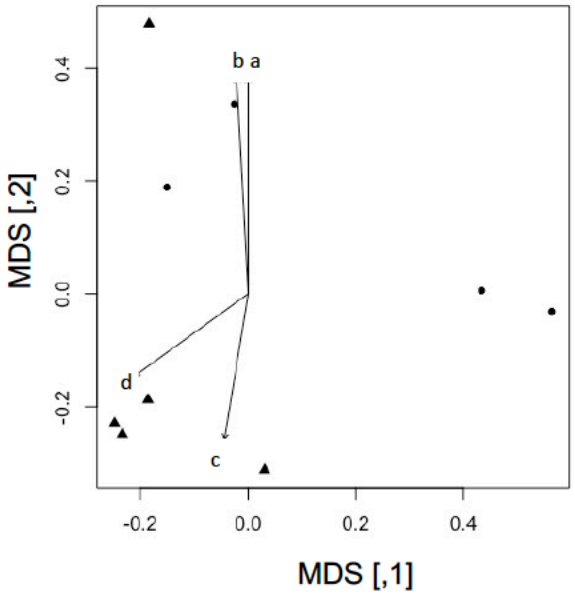


FIGURE 4.5 Metric multidimensional scaling (MDS) plots of the Bray-Curtis dissimilarity metric in CHALL and CON lung samples. (a.) closed circles = CHALL lung samples, (b.) closed triangles = CON lung samples. (b.) Vectors included in MDS plot; a=*Pasteurella* spp., b= *Escherichia* spp., c= *Pseudomonas* spp., d = *Butyrivibrio* spp.
CHALL = calves experimentally challenged with *Pasteurella multocida* (n = 4); CON = calves not exposed to bacterial challenge (n = 5)

CHAPTER 5. CONCLUSIONS AND FUTURE RESEARCH

5.1 DISCUSSION AND LIMITATIONS

Bovine respiratory disease (BRD) has a multifactorial pathogenesis and host susceptibility depends on the integrity of the respiratory microbiota as well as the immune system (Zeineldin et al., 2019). Further understanding of the intercommunication between host, microbiota and disease pathogenesis will require an integrative biological approach that examines all the molecular networks that may have impact on prevention and treatment strategies (Lawless et al., 2014). The data presented in this thesis demonstrate inroads into further understanding of how changes within the mucosal microbiota of the respiratory tract affect disease severity and also how they may impact host transcription of endogenous microRNAs (miRNA) that have epigenetic control of immune gene expression.

In Chapter 2, the results presented demonstrate that the composition of the respiratory microbiota may differ between calves with clinical signs consistent with BRD and calves with lung consolidation. Reduced nasopharyngeal (NP) bacterial species abundance within the NP was associated with clinical BRD; however, there was no significant association between NP microbial diversity and lung consolidation. The association between decreased upper respiratory tract microbiota diversity and clinical BRD is consistent with results from studies in feedlot cattle (Timsit et al., 2018; McMullen et al., 2019). A higher relative abundance (RA) of *Pasteurella* spp. within the NP was also associated with more severe lung consolidation, which emphasizes the importance of *Pasteurellacea* as pathobionts: resident colonizers of the NP that can facilitate lung pathology under certain conditions (Thomas et al., 2019; Zeineldin et al., 2019). Additionally, calves at 3 wk of age had a higher RA of *Mycoplasma* spp. within the NP compared to calves at 6 wk of age; the RA of *Mycoplasma* spp. was not associated with clinical

respiratory disease or lung lesions. The effects of early *Mycoplasma* spp. carriage and BRD risk require further investigation. The effect of previous antibiotic therapy on the composition of the NP microbiota was examined in this study; however, only a trend was identified in beta diversity and differences in genera were difficult to interpret due to the small number of calves that had been previously treated.

Overall, the results of Chapter 2 provide evidence of the utility of lung US in qualifying the case definition of BRD when evaluating the respiratory microbiota. One significant limitation of this study is its external validity. Calves were enrolled in this study from one farm and the NP microbiota was sampled at one point in time. Given the design of the larger GWAS protocol, sampling was not randomized and a few calves were sampled at both 3 wk and 6 wk of age. Additionally, the antibiotic treatment variable was treatment history collected retrospectively from DairyComp. The lack of randomization and independence of some of the 3 wk and 6 wk samples may have biased the results. It would also have been of interest to extend the sampling period until weaning and analyze longitudinal data. Results from a study in feedlot cattle suggest that the association between decreased NP microbial diversity and clinical BRD persist over time despite temporal variability in the overall NP microbial composition (Holman et al., 2015). It would be useful to know whether this pattern continues throughout the pre-weaning period in dairy calves.

In Chapter 3, an experimental challenge model with ampicillin-sensitive *Pasteurella multocida* was utilized to determine the effects of infection on the upper and lower respiratory microbiota. An additional objective of the challenge was to determine the effect of ampicillin on the respiratory microbiota following the challenge. A small subset of calves that was excluded from the challenge was also included to determine what effect anatomic site had on the

composition of the respiratory microbiota. Expanding on the results from Chapter 2, increased NP microbial diversity, as measured by species abundance, pre-challenge was associated with less disease severity, as measured by cumulative respiratory score, 14 days following experimental challenge regardless of ampicillin or placebo treatment. There was also a tendency identified for an association between higher pre-challenge NP species abundance and decreased cumulative lung ultrasound score following the challenge.

Dominant genera within the microbiota of the NP, NP tonsil, lymph node and lung microbiota all included bacteria associated with the oral cavity and the gastrointestinal tract (GIT) (e.g. *Prevotella* spp., *Bacteroides* spp., *Faecalibacterium* spp., *Blautia* spp., and unclassified families of *Ruminococcaceae* and *Lachnospiraceae*). Increased RA of these bacteria in the pre-challenge NP were each associated with a delay in the development of bronchopneumonia following *P. multocida* challenge; in contrast, higher RA of *Mycoplasma* spp. increased the speed of lesion development. The impact of GIT-associated bacteria, likely sourced from the oral cavity, ruminal regurgitation and microaspiration, is virtually unknown in dairy calves. While anaerobes, such as *Lachnospiraceae, Ruminococcaceae* and *Faecalibacterium* spp., are unlikely to proliferate at high levels within an aerobic environment, they may exert temporary anti-inflammatory effects or promote stability within the mucosal microbiota (Frank et al., 2007; Fujimoto et al., 2013). Microaspiration-derived bacteria, such as *Prevotella* spp., have been shown to induce lower respiratory tract inflammation within the human respiratory tract (Segal et al., 2013, 2016). Given the constant eructation of ruminants, microaspiration of oral and ruminal bacteria may have consistent contact with the respiratory tract in calves. The presence of GIT-associated bacteria within all anatomic sites of the

respiratory microbiota in this study necessitates further investigation of intercommunication between the respiratory and gastrointestinal microbiome in the context of BRD.

NP diversity was significantly affected by the bacterial challenge; however, ampicillin therapy had no significant effect on diversity in the NP, NP tonsil, lymph node or lung samples. The effect of ampicillin only significantly increased the NP RA of *Pasteurella* spp. and decreased the NP RA of *Prevotella* spp.; no other effects of antibiotic therapy on the respiratory microbiota were identified. This is in contrast to the results of studies in feedlot cattle that have documented an association between parenteral antibiotic therapy and decreased NP microbial diversity (Timsit et al., 2017; Holman et al., 2018). The evaluation of the effect of antibiotic therapy on the respiratory microbiota was likely limited by the dose range and dosing schedule. Finally, results of this study showed that although the overall phyla and genera are similar within the NP, NP tonsil, lymph node and lung, the relative distributions of taxa are different at each anatomic site. Surprisingly, *Pasteurella* spp. was identified as a dominant genus in all anatomic sample sites except for the NP tonsil. This indicates a potential difference in the surface microbiota collected by NP swabs and the microbiota adherent to NP tonsillar tissue. Additionally, diversity was not significantly different in each sample site; this is in contrast to patterns observed in feedlot cattle in humans and feedlot cattle of decreasing NP diversity from the NP to the lungs (Dickson et al., 2015; Zeineldin et al., 2017). The evaluation of the effects of sample site on the respiratory microbiota was likely affected by the small sample size (n = 9).

Aside from a small sample size used for analyzing the exclusion calves, the sample collection time points did not allow for detection of subtle changes in microbiota composition immediately following challenge and antibiotic therapy. Daily deep NP swabs and samples of the lower respiratory tract (i.e. bronchoalveolar lavage) may have allowed for more precise

measurement of changes in diversity metrics due to bacterial challenge and ampicillin therapy. An additional limitation was the postmortem tissue sample collection technique; samples may have not been representative of the bacterial population at each sample site.

There is also a possibility that tissue samples could have been contaminated since they were collected at postmortem. This was avoided as much as possible by only opening the thorax and using aseptic technique to collect the samples; the esophagus and distal GIT were not incised during necropsy examinations and sample collection.

The changes within the lung microbiota due to bacterial challenge with *P. multocida* were further explored in the context of the host response in Chapter 4. It is well known that the mammalian immune system plays an important role in maintaining the homeostasis of resident microbial communities; however, the mechanisms by which intercommunications between the host microbiota and the immune system occur are not well understood (Hooper et al., 2012). There is recent, exciting evidence to suggest that endogenous microRNA (miRNA) expression may be directed by changes to the microbiota composition (Edwards et al., 2019). These small RNAs function to repress messenger RNA expression; target genes of miRNA often involve the host immune system. Edwards et al. (2019) found a positive correlation between the RA of *Lactobacillu*s pp. within the vaginal microbiota of humans and has-miR-193b, which inhibits cell proliferation and subsequently protects against *Chlamydia trachomatis* infection.

In Chapter 4, we analyzed a subset of calves from the *P. multocida* challenge study to evaluate lung DE of miRNAs in dairy calves with and without induced pneumonia. Differential expression of miRNAs that were upregulated in challenged calves included miRNAs that have been shown to be potent host immune system regulators (bta-miR-21-5p) as well as previously identified miRNAs associated with ruminant pulmonary infection (bta-miR-21-5p, bta-149-5p,

bta-miR-146b). Interestingly, the target gene prediction analysis of identified miRNAs in this study showed a majority of targets in proteins involved in zinc or metal binding and transport. Zinc plays a crucial role in modulating the immune system in response to pathogens; when availability is altered the survival and proliferation of immune cells is decreased (Shankar and Prasad, 1998; Bonaventura et al., 2015). Interestingly, increased heavy metal resistance in the NP bacteria of dairy calves diagnosed with BRD has been documented previously (Gaeta et al., 2017). It is plausible that the disruption of the lung microbiota in the challenged calves of our studymay have impacted miRNA DE and thus affected immune cell function via zinc-binding proteins. The association between host immune cells, zinc transport and the respiratory microbiota in the context of BRD pathogenesis requires further investigation. From the miRNAs that were highly DE or had a high average read count, respectively, bta-miR-212 and bta-miR-10b were correlated with the RA of *Pasteurella* spp. The miRNA bta-miR-212 was positively correlated with *Pasteurella* spp. and bta-miR-10b was negatively correlated with bta-miR-10b. The RA of *Escherichia* spp. was also negatively correlated with bta-miR-10b; the RA of *Butyrivibrio* spp. was positively correlated with bta-miR-10b. Both miRNAs had predicted target genes associated with metal or zinc binding, which may indicate intercommunication with the host immune system.

The most important limitation of this study was the small sample size (n = 9). Although the experimental challenge did significantly increase the RA of *Pasteurella* spp. in lung tissue 14 days following challenge, the diversity metrics were not significantly different between challenged and control calves. Additionally, only 4 dominant genera were significantly different in RA between the two groups: *Pasteurella* spp., *Escherichia* spp., *Pseudomonas* spp., and *Butyrivibrio* spp. A larger sample size and greater difference in the lung microbiota

composition, which may have been the case immediately following the experimental challenge, might have illustrated more correlations of miRNA DE in challenged calves compared to control calves. Additionally, the target gene analysis has inherent false positives that may affect the prediction of functional annotation categories (Pinzón et al., 2017). It would be of interest to perform RNA-seq on the lung transcriptome of calves to determine which messenger RNA transcripts are DE; the DE of immune gene targets that utilize zinc would facilitate further understanding of the impact of miRNAs on the host immune system during *P. multocida* infection. Quantitative PCR of highly DE miRNAs identified in this study would also provide further evidence of an association between pulmonary microbiota changes and miRNA DE.

5.2 FUTURE RESEARCH

One of the most exciting aspects of Next-generation sequencing is the increasing availability of bioinformatic software platforms, which are easily accessible in free statistical programs, to improve processing and interpretation of large, complex data sets. These programs allow for clinical translation of sequencing data, which connects molecular mechanisms to clinical disease (Mangul et al., 2019). With this abundance of computational tools, however, there is a need to standardize methods of analysis to improve external validity and facilitate the comparison of microbiota sequencing data among different populations of animals. For example, there is variability in how researchers interpret amplicon sequencing data; research groups have utilized software programs that infer speciation from 16S rRNA data with denoising software (e.g. DADA2) (Timsit et al., 2018; McMullen et al., 2019). However, different packages have returned variable results with the same raw data, likely due to identifying false positive sequencing variants (Nearing et al., 2018). Further research that attempts to standardize a bioinformatic workflow, by comparing sequencing variants from denoising software to whole

genome sequencing results, will allow for more comparisons of the respiratory microbiota across multiple populations of calves; this will improve external validity of results. Standardized bioinformatic workflows would also facilitate investigations of potential respiratory probiotics.

The data presented in this thesis highlight the complexity of the mucosal microbiota, which likely involves intercommunication between the respiratory and gastrointestinal system (i.e. the gut-lung axis), as was shown in Chapter 3. In humans, it is generally understood that the gastrointestinal tract microbiota and lung microbiota engage in direct inter-compartment crosstalk (Enaud et al., 2020). Although the majority of these connections are likely made through regurgitation and swallowing of respiratory secretions, there are likely indirect intercommunications via host immune modulation (Trompette et al., 2014). In pre-weaning dairy calves, enteric disease and pneumonia are often clinical co-morbidities; this indicates a potential relationship between the intestinal and respiratory resident bacterial populations. The gut microbiota was shown to protect mice against induced pneumococcal pneumonia; disruption of the microbiota led to increased bacterial dissemination, inflammation and increased mortality in mice (Schuijt et al., 2016). In further studies evaluating risk factors of and prevention strategies against BRD that involve the respiratory microbiota, samples from the upper and lower respiratory microbiota as well as the gastrointestinal tract should be analyzed to better understand the plausible gut-lung axis in dairy calves. Direct-fed microbials could have significant beneficial effects on both the gastrointestinal and respiratory tract health of dairy calves.

As was demonstrated in Chapter 4, disruption of the lung microbiota is associated with DE of miRNAs that may have significant effects on host immune regulation and subsequent susceptibility to disease. Further understanding of this interconnection between dysbiosis and

potential immune cell modulation requires additional sampling of the upper respiratory tract microbiota. The relative abundances of dominant taxa are likely distinct between the upper and lower respiratory tract, as was demonstrated in Chapter 3. The evidence from Chapters 2 and 3 support the idea that the composition of the nasopharyngeal microbiota has a significant effect on the severity of respiratory disease. Continued progress in the understanding of epigenetic mechanisms during the context of commensal bacterial disruption, such as DE of miRNAs, may facilitate the development of sensitive biomarkers that could indicate dysbiosis of the respiratory tract prior to the development of lung lesions and/or clinical respiratory signs.

Finally, the entirety of the host respiratory tract includes resident fungi and viruses that likely interact with the respiratory microbiota to maintain community stability (Enaud et al., 2020). Previous metagenomic studies in respiratory secretions of dairy calves and beef cattle have identified characteristic virome profiles associated with BRD diagnosis (Ng et al., 2015; Zhang et al., 2019). Thus, further understanding of how the mucosal microbiota impacts BRD development and severity in dairy calves likely requires the simultaneous analysis of the virome and potentially, the mycobiota. Further understanding of the host respiratory microcommunities of bacteria, viruses and fungi will improve our assessment of host susceptibility and unique risk factors. This information may allow for tailored BRD prevention strategies, such as oral probiotics, that reduce the need for antibiotic therapy and improve dairy calf health and welfare.

5.3 REFERENCES

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