Respiratory Bacterial Microbiota in Cattle
From Development to Modulation to Enhance Respiratory Health

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KEYWORDS
- Microbiome • Bovine respiratory disease • 16S rRNA sequencing • Metagenome
- Alternative to antimicrobials

KEY POINTS
- The respiratory bacterial microbiota is dynamic, changing significantly during periods of increased risk for bovine respiratory disease.
- The respiratory microbiota is inhabited predominantly by 5 bacterial phyla: Proteobacteria, Firmicutes, Tenericutes, Actinobacteria, and Bacteroidetes; the relative abundance of each differs by animal age and production system.
- Upper respiratory tract and lower respiratory tract microorganisms differ in diversity and composition. The nasopharyngeal microbiota contributes the most to the lower respiratory microbiota and thus should be the primary target for sampling or modulation strategies.
- Composition of the respiratory microbiota is associated with respiratory health; increased abundances of respiratory \textit{Lactobacillus} and/or \textit{Lactococcus} are associated with good respiratory health.
- Intranasal application of selected \textit{Lactobacillus} strains modifies the composition of the nasopharyngeal microorganisms in cattle and can provide colonization resistance against opportunistic bacterial pathogens such as \textit{Mannheimia haemolytica}.

\textsuperscript{a} Vet Clin Food Anim 36 (2020) 297–320
https://doi.org/10.1016/j.cvfa.2020.03.001
vetfood.theclinics.com
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INTRODUCTION

Over the past decade it has become clear that mammals live in symbiosis with their abundant resident microbes.1 Advances in culture-independent techniques (eg, 16S ribosomal RNA [rRNA] sequencing) have enabled detection and quantification of bacterial species that are difficult or impossible to detect by culture-based methods (Box 1).2 These advances in the field of molecular techniques, in particular metagenomics, have led to the definition of the animal microbiota, a term that refers to the complex microbial ecosystems in and on bodies of animals.1

Like other body sites, the respiratory tract of cattle is colonized by a variety of different bacterial microbiotas directly after birth.3 Composition and diversity of these microbiotas have been recently associated with respiratory health in cattle.4,5 More specifically, airway microbiotas enriched with known beneficial bacteria, such as Lactobacillus, have been associated with good respiratory health,6,7 whereas microbiotas enriched with known bacterial pathogens, such as Mycoplasma bovis, Mannheimia haemolytica, or Pasteurella multocida, have been associated with bovine respiratory disease (BRD).4,6

Investigating the role of the respiratory microbiota in health and disease is a relatively new, rapidly developing field of research that provides new opportunities for the prevention and treatment of BRD.8 This review summarizes current knowledge regarding composition of the respiratory bacterial microbiota in dairy cattle and beef cattle and its relationship with the development of BRD. Approaches to modulate the respiratory bacterial microbiota to promote enhanced heath (eg, probiotics, bacteriophages, and prebiotics) also are discussed.

COMPOSITION OF THE BACTERIAL RESPIRATORY MICROBIOTA IN HEALTHY CATTLE

The diversity of bacteria on earth is vast, comprising 55 phyla.9 The cattle respiratory tract is inhabited predominantly by 5 of these phyla (Proteobacteria, Firmicutes, Tenericutes, Actinobacteria, and Bacteroidetes [Table 1]), which underlines its suitability for the growth of only a limited number of bacteria. This diversity is largely due to the biophysical properties of respiratory mucosal surfaces, that is, temperature, moisture, and pH.10

The composition of the airway microbiotas evolves over time due to a variety of selection pressures, which further influence the colonization process of the respiratory tract, including (Fig. 2) (1) endogenous forces, such as mucus, IgA, and innate/adaptive immune recognition,10 and (2) exogenous forces, such as the maternal vaginal microbiota, environmental biodiversity,11 diet,12 infection,4,6 stressful events (weaning, transportation, and commingling)13–15 and parenteral antibiotics.16,17 Unfortunately, to date, no study has described the composition of the developing airway microbiotas across the life span of either dairy cattle or beef cattle.

A systematic review of the literature (performed in PubMed on December 12, 2019; key words [respiratory] AND [cattle] AND [microbiota OR microbiome]) revealed that

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<th>16S rRNA sequencing</th>
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<td>Typically, short segments of the 16S rRNA that include hypervariable regions are sequenced for bacterial classification in microbiota studies (Fig. 1). Therefore, composition of the respiratory microbiota often is reported at the phylum, family, or genus level but not at the species level because only a small proportion (30%–50%) of these short 16S rRNA sequences can be classified as OTUs beyond the genus level.</td>
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most published studies have focused on the respiratory microbiota of postweaned beef cattle (n = 16), with only a limited number of studies describing the composition of the nasopharyngeal microbiota in preweaned dairy calves (n = 3)\(^3\),\(^4\),\(^18\) or beef calves (n = 2).\(^19\),\(^20\) Furthermore, these studies focused on the upper respiratory tract (URT), with only 6 studies reporting the composition of the lower respiratory tract (LRT) microbiota (sampled by transtracheal aspiration\(^6\),\(^11\),\(^13\),\(^21\) or bronchoalveolar lavage [McMullen C, Alexander TW, Leguillette R, et al. Topography of the respiratory tract bacterial microbiota in feedlot beef calves, submitted for publication]\(^22\)).

**The Nasopharyngeal Microbiota from Birth to 1 Month of Age in Dairy Calves**

Colonization of the airways in dairy calves begins immediately after birth and evolves quickly during the first weeks of life.\(^3\) Abundance of bacteria in the nasopharynx (measured by the number of 16S rRNA gene copies) increases significantly from birth to 14 days of age and then either decreases slightly until day 35\(^4\) or remains the same until day 42.\(^23\) The interval from birth to day 14, therefore, is highly critical for microbial establishment\(^23\) and can predispose calves to a healthy state or pneumonia/otitis during the first weeks of age (discussed later).
The nasopharyngeal microbiota of preweaned dairy calves is dominated by Proteobacteria, especially at 3 days and 14 days of age, when this phylum can represent up to 70% of total bacterial diversity. After day 14, however, the diversity (combined richness and evenness) of the nasopharynx increases, with other phyla becoming more abundant (including Tenericutes, Firmicutes, Actinobacteria, and Bacteroidetes). The most abundant bacterial genera in the nasopharynx of dairy calves are *Mannheimia*, *Moraxella*, *Mycoplasma*, *Psychrobacter*, and *Pseudomonas*. Relative abundances of these genera change over time, with the relative abundance of *Moraxella* decreasing between day 14 and day 35 and the relative abundances of *Mannheimia* and *Mycoplasma* concurrently increasing substantially.

In dairy calves, composition of the nasopharyngeal microbiota is highly influenced by the maternal vaginal microbiota. In 81 dairy cow-calf pairs, 73%, 76%, and 87% of the bacteria detected by next-generation sequencing (ie, operational taxonomic units [OTUs]) were shared between the maternal vaginal microbiota and the calf nasopharyngeal microbiota at 3 days, 14 days, and 35 days of age, respectively. The most abundant shared bacterial genera in the dam vaginal and calf nasopharyngeal samples across all sampling days were *Mannheimia*, *Moraxella*, *Bacteroides*, *Streptococcus*, and *Pseudomonas*. The significant overlap between the 2 microbiotas was attributed to the transfer of maternal microbes to the neonate at birth via the vaginal canal. *Mannheimia* was found to be relatively more abundant in the vaginal microbiota of dams whose calves did not develop pneumonia and/or otitis compared with the microbiota of dams whose calves did develop disease. Therefore, it appears that the prepartum higher abundance of *Mannheimia* in the vagina of dairy cows may confer a protective effect on the health of the respiratory tract and middle ear of their progeny.

The Nasopharyngeal Microbiota from Initial Vaccination to Preconditioning or Weaning in Beef Cattle (ie, Preweaned Beef Cattle)

The nasopharyngeal microbiota changes significantly between initial vaccination (approximately 40 days of age) and preconditioning (approximately 130 days of age) or weaning (approximately 150 days of age) in beef calves. At initial vaccination, the diversity of the nasopharyngeal microbiota is low, with a high abundance of bacteria from the phylum Actinobacteria (more specifically, from the *Promicromonosporaceae* and *Microbacteriaceae* families). Nasopharyngeal diversity then increases, with higher proportions of Tenericutes, Proteobacteria, and Firmicutes at the time of preconditioning or weaning. At the genera level, relative abundances of *Mycoplasma*, *Moraxella*, and *Psychrobacter* were higher at weaning than at initial vaccination. Although some commonalities of evolution among calves exist, groups of preweaned calves that were raised on different farms evolved differently (even when managed similarly), implying that factors other than age (eg, environment and contact with older animals) have important roles in development of the nasopharyngeal microbiota.

The Nasopharyngeal Microbiota from Weaning to the First Weeks on Feed in Beef Cattle (ie, Postweaned Beef Cattle)

The structure of the nasopharyngeal microbiota evolves significantly from weaning at the ranch to 40 days to 60 days after entrance to a feedlot. The largest shift occurs between departure from the ranch of origin and the first 7 days on feed, with a sharp increase in diversity of the nasopharyngeal microbiota during this short interval. For example, the number of bacterial taxons (ie, OTUs) almost doubled (100 OTUs
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<tr>
<td>Postweaned feedlot beef calves</td>
<td>Cross-sectional; 2 populations (BRD, n = 82; healthy, n = 82)</td>
<td>Proteobacteria (69.3%), Tenericutes (22.5%), Firmicutes (3.3%), Actinobacteria (2.3%), Bacteroidetes (2.3%)</td>
<td>Mycoplasma (22.2%), Moraxella (19.5%), Histophilus (19.0%), Psychrobacter (9.8%), Mannheimia (6.3%), Pasteurella (4.4%), Pseudomonas (1.8%)</td>
<td>McMullen et al, 2019</td>
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<td>Longitudinal; 4 populations (BRD at entry, n = 22; BRD at diagnosis, n = 22; healthy at entry, n = 44; healthy at diagnosis, n = 10)</td>
<td>Proteobacteria (34.8%), Firmicutes (18.6%), Actinobacteria (17.2%), Bacteroidetes (12.1%), Tenericutes (11.2%), Fusobacteria (1.2%)</td>
<td>Moraxella (10.9%), Mycoplasma (10.7%), Acinetobacter (9.7%), Rathayibacter (5.0%), Promicromonaspora (4.4%), Mannheimia (4.1%), Solibacillus (3.5%), Clostridium (3.3%), Corynebacterium (3.8%), Pasteurella (1.9%)</td>
<td>Zeineldin et al, 2017</td>
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<td>Longitudinal; 2 populations of 30 calves sampled at the ranch (d 0), at feedlot entry (d 2), and on d 7 and d 28 after entry</td>
<td>Tenericutes (41.1%), Proteobacteria (31.8%), Firmicutes (4.6%)</td>
<td>Mycoplasma (40.8%), Moraxella (18.7%), Pasteurella (6.8%), Mannheimia (3.8%)</td>
<td>Stroebel et al, 2018</td>
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<td>Longitudinal; 1 population of 4 calves</td>
<td>Proteobacteria (68.9%), Firmicutes (19.2%)</td>
<td>At entry: Pseudomonas (23.7%), Shewanella (23.5%), Acinetobacter</td>
<td>Holman et al, 2015</td>
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<tr>
<td>1</td>
<td>Longitudinal; 1 population of 30 calves sampled at the ranch (d 0), at feedlot entry (d 2), and on d 40 after entry</td>
<td>Tenericutes (53.2%), Proteobacteria (34.7%), Firmicutes (4.2%), Bacteroidetes (3.7%), Actinobacteria (3.4%)</td>
<td>NR</td>
<td>Timsit et al, 2016</td>
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<td>2</td>
<td>Longitudinal; 2 populations (treated with NORS [n = 10] or tilmicosin [n = 10] at entry) sampled at entry, and on d 1, d 5, and d 10 after entry</td>
<td>Tenericutes (92.8%), Proteobacteria (5.9%), Firmicutes (0.6%), Actinobacteria (0.6%), Bacteroidetes (0.1%)</td>
<td>NR</td>
<td>Timsit et al, 2017</td>
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<td>3</td>
<td>Cross-sectional; 3 populations (controls, n = 5; medium selenium, n = 6; high selenium, n = 5)</td>
<td>Proteobacteria (31.7%), Bacteroidetes (27.5%), Firmicutes (24.3%), Actinobacteria (7.1%), Tenericutes (4.4%)</td>
<td>NR</td>
<td>Hall et al, 2017</td>
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<td>4</td>
<td>Longitudinal; 1 population of 13 calves sampled at the ranch (d 0), at feedlot entry (d 2) and on d 5 and d 12 after entry</td>
<td>Proteobacteria (36.1%), Firmicutes (20.1%), Tenericutes (19.3%), Actinobacteria (12.7%), Bacteroidetes (8.6%)</td>
<td>NR</td>
<td>Amat et al, 2019</td>
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At d 60: *Staphylococcus* (20.8%), *Mycoplasma* (14.9%), *Mannheimia* (10.4%), *Moraxella* (9.4%)
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<tr>
<th>Study</th>
<th>Population Description</th>
<th>Microbiota Composition</th>
<th>Author(s)</th>
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<tr>
<td>Preweaned and postweaned beef calves</td>
<td>Longitudinal; 3 populations of 40 calves sampled at initial vaccination, weaning and on d 40 after entry at feedlots</td>
<td>Proteobacteria (27.5%), Actinobacteria (25.9%), Tenericutes (24.3%), Firmicutes (13.5%), Mycoplasma (24.1%), Lactococcus (10.7%), Moraxella (7.4%), Histophilus (6.78%), Pasteurella (6.0%)</td>
<td>McMullen et al, 19 2018</td>
</tr>
<tr>
<td>Preweaned dairy calves</td>
<td>Longitudinal; 1 population of 81 calves sampled at 3 d, 14 d, and 35 d of age</td>
<td>Proteobacteria (52.1%), Firmicutes (23.0%), Bacteroidetes (11.4%), Tenericutes (3.4%)</td>
<td>Lima et al, 3 2019</td>
</tr>
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**Abbreviation:** NR, data not reported; NORS, nitric oxide releasing solution.

*a Only studies reporting the general composition of the nasopharyngeal microbiota are presented. Data from Refs. 2,3,12–14,19,24,25,31,39
vs 200 OTUs) in the nasopharynx of 13 beef steer calves during the 48-hour interval from leaving ranch of origin to on-arrival processing at a feedlot.²⁴

The nasopharyngeal microbiota stabilizes after the first week on feed, with diversity either remaining the same or slightly decreasing until 40 days to 60 days on feed.¹⁴,¹⁵,¹⁷ The nasopharyngeal microbiota also becomes more homogeneous among cattle during this interval.¹⁵,²⁵ For example, at day 0 prior to transport, 76 OTUs were shared in the nasopharyngeal microbiota of 14 Angus-Herford cross heifers, whereas at 5 days and 12 days after arrival at a research feedlot, there were 373 and 274 OTUs, respectively, that were shared among these cattle.¹⁵

Numerous factors can explain significant shifts in the structure of the nasopharyngeal microbiota around cattle marketing (Fig. 2). First, transportation and adaptation to a feedlot environment have a major effect on the nasopharyngeal bacterial community.¹³–¹⁶ By sampling the nasopharynx of preconditioned calves that were transported directly to a research feedlot, Holman and colleagues¹⁵ (2017) determined that presence and relative abundance of numerous bacteria changed significantly before (day 0) and after (day 2) transportation, with a higher abundance of Acinetobacter and Streptococcus and a lower abundance of Pasteurella and Bacillus after transportation. This shift in the nasopharyngeal microbiota occurred even in the absence of commingling or change in diet, because cattle were fed the same diet at both locations and were kept separate from other cattle housed at the feedlot.

Adaptation to new diets and mixing cattle from multiple origins also alter the structure of the nasopharyngeal microbiota in cattle.¹²,¹³ For example, recently weaned beef calves fed selenium-biofortified alfalfa hay had a higher bacterial diversity in their nasal cavities compared with healthy controls.¹² Concerning the impact of mixing cattle on the nasopharyngeal microbiota, a minimum duration and frequency of contact among cattle is needed for horizontal transmission of commensal and pathogenic bacteria to occur. Commingling cattle for 24 hours at an auction market did not significantly affect the diversity or the composition of the nasopharyngeal bacteria in 2 groups of 15 recently weaned beef calves.¹³ This suggests that being held for 24 hours at an auction market was not enough time to allow bacterial transfer or that the environment of the auction market was not conducive to interanimal bacterial transfer.
Finally, parenteral antibiotics given at or soon after arrival to control BRD (ie, metaphylaxis) modifies diversity and composition of the nasopharyngeal microbiota.\textsuperscript{13,16,17} A single parenteral injection of either oxytetracycline or tulathromycin at feedlot placement altered the nasopharyngeal microbiota in comparison with cattle receiving only in-feed antibiotics for up to 60 days postadministration; oxytetracycline significantly reduced relative abundance of \textit{Mannheimia} from feedlot entry to 60 days postarrival and cattle given either oxytetracycline or tulathromycin had a significantly lower relative abundance of \textit{Mycoplasma} at day 60 compared with those given only an in-feed antibiotics.\textsuperscript{17} Effects of parenteral oxytetracycline and tulathromycin on the nasopharyngeal microbiota were most important at days 2 and 5 post-treatment.\textsuperscript{18} At that time, both oxytetracycline and tulathromycin appeared to confer some protection against \textit{Pasteurella} spp colonization in the nasopharynx.

The nasopharyngeal microbiota of postweaned beef cattle often is dominated largely by Proteobacteria and Tenericutes, with lower proportions of Firmicutes, Actinobacteria, and Bacteroidetes (see Table 1). Of the dominant genera, \textit{Mycoplasma}, \textit{Moraxella}, \textit{Acinetobacter}, \textit{Psychrobacter}, \textit{Mannheimia}, \textit{Pasteurella}, and \textit{Corynebacterium} are identified most frequently. There is considerable variability, however, in composition of the nasopharyngeal microbiota among groups of cattle and even among individual cattle within a group.\textsuperscript{13,19} Furthermore, as discussed previously, the nasopharyngeal microbiota evolves significantly from entrance to the first weeks on feed. Therefore, it is difficult to identify a so-called normal microbiota in feedlot cattle.

In summary, the nasopharyngeal microbiota of beef cattle changes significantly between weaning and the first weeks on feed. This evolution may explain why beef cattle are more susceptible to BRD during the first 40 days to 60 days on feed,\textsuperscript{26} because an unstable microbiota is less resistant to colonization by pathogens.\textsuperscript{27}

### Composition of the Lower Respiratory Tract Microbiota

The LRT, previously thought to be sterile, is now known to harbor a unique microbiota (Fig. 3).\textsuperscript{6,11,13,21,22} Characterization of the tracheal\textsuperscript{6,11,13} and bronchial\textsuperscript{22} microbiotas in cattle revealed that these bacterial communities are distinct from nasal and nasopharyngeal microbiotas. Bacterial communities in the LRT are less rich and less diverse than the URT microbiotas,\textsuperscript{8,11} consistent with URT being directly exposed to ambient airborne microbial communities. Furthermore, some bacteria, such as \textit{Mycoplasma}\textsuperscript{6,11,13} and \textit{Pasteurella}\textsuperscript{11,22} typically are enriched in the LRT compared with the URT.

Despite differences between URT and LRT microbiotas, most bacterial genera identified in the LRT also are present in the URT (McMullen C, Alexander TW, Leguillette R, et al. Topography of the respiratory tract bacterial microbiota in feedlot beef calves, submitted for publication).\textsuperscript{11,22} This is explained by the fact that, in healthy animals, bacterial composition of the LRT is determined more by a constant flow (immigration and elimination) of transient bacteria originating from the URT than replication of resident bacteria.\textsuperscript{26} In humans, the bacteria reaching the lung primarily originate from the oropharynx and the mouth.\textsuperscript{28} In cattle, however, the nasopharynx seems to be the primary source of bacteria for the LRT. In a recent study by the authors’ team (McMullen C, Alexander TW, Leguillette R, et al. Topography of the respiratory tract bacterial microbiota in feedlot beef calves, submitted for publication), which compared bacterial communities of 17 locations across the respiratory tract, the lung microbiota was more compositionally similar to the nasopharynx than any other URT microbiota, including the mouth, oropharynx, palatine tonsils, or nostrils. Consequently, the nasopharyngeal microbiota should be the primary target for sampling strategies and the
INFLUENCE OF THE BACTERIAL MICROBIOTA ON RESPIRATORY HEALTH

Composition, diversity, and stability of the respiratory microbiota can play a role in either predisposing cattle to BRD or providing protection against colonization and/or proliferation of bacterial pathogens in the respiratory tract (also known as colonization resistance) (see Fig. 2). The principal niche in the URT to modulate in order to promote good respiratory health (ie, prebiotics, probiotics, and bacteriophages).

**Fig. 3.** Mean relative abundance of bacteria present at greater than or equal to 1% abundance at the genus level of different URT and LRT sampling locations in 15 healthy beef steer calves. URT, upper respiratory tract; LRT, lower respiratory tract. (From McMullen C, Alexander TW, Leguillette R, et al. Topography of the respiratory tract bacterial microbiota in feedlot beef calves. submitted for publication; with permission).
Role of the Composition of the Microbiota on Respiratory Health

Primary bacteria involved in BRD are *M haemolytica*, *P multocida*, *H somni*, and *Mycoplasma bovis* (Table 2). Although these bacteria are opportunistic in nature, cattle with them in their respiratory tract are at higher risk of developing BRD. For example, recently weaned beef cattle positive for *M haemolytica* on deep nasopharyngeal swabs at feedlot entry were more likely (odds ratio 1.7; 95% CI, 1.1–2.4) to be affected with BRD within 10 days after arrival than cattle negative for this bacterium. Furthermore, in 174 dairy calves, relative abundance of *Mannheimia* and *Mycoplasma* was higher at days 14 and/or 28 in the nasopharynx of calves that subsequently developed BRD versus those that remained healthy. Therefore, limiting colonization of opportunistic bacterial pathogens in the respiratory tract can reduce the prevalence of BRD in cattle.

Based on recent 16S rRNA sequencing, presence of bacteria other than *Pasteurellaceae* or *Mycoplasma bovis* in the respiratory tract also may predispose cattle to BRD (see Table 2). For example, *Moraxella* was enriched in the nasopharynx of calves that developed BRD. Furthermore, a bacterium from the Leptotrichiaceae family was more abundant among postmortem lung tissue samples from dairy calves that died from BRD compared with lesion-free lung tissue of clinically healthy calves. These findings indicate that there may be other bacterial species with the potential to be secondary BRD pathogens that the veterinary community is unaware of. Before implementing mitigation strategies against them, however, further research is needed to confirm a causal relation between their presence in the respiratory microbiota and BRD.

Comparison of the respiratory tract microbiota of healthy calves with those that developed BRD revealed the presence of specific commensal bacteria in the respiratory tract that can confer protection against the disease (see Table 2). For example, cattle having a higher relative abundance of *Lactobacillaceae* and *Bacillaceae* in their nasopharynx at feedlot entry were less likely to develop BRD during the first 60 days on feed. Furthermore, in a comparison of the nasopharyngeal and tracheal microbiotas of 60 feedlot cattle with BRD to 60 healthy pen-mates, tracheal microbiota of healthy cattle was enriched with *Mycoplasma dispar*, *Lactococcus lactis*, and *Lactobacillus casei*.

Commensal bacteria can confer resistance against colonization and proliferation of opportunistic bacterial pathogens through several mechanisms. First, resistance can be provided through occupation of an otherwise vacant respiratory niche. As a result, invading pathogens have to compete for adhesion receptors and nutrients. For example, numerous lactic acid bacteria (LAB) had greater adhesion to bovine bronchial epithelial cells than *M haemolytica*. Commensals in the nasopharynx also can directly inhibit growth of bacterial pathogens by modifying their environment (ie, production of lactic or acetic acid) or producing antimicrobial molecules (eg, bacteriocins and hydrogen peroxide). Finally, commensals can enhance colonization resistance against pathogens via immune stimulation of the host and modulation of mucosal inflammation. For example, *Streptococcus salivarius* inhibited inflammatory responses in human bronchial epithelial cells (ie, down-regulation of the nuclear factor κB pathway) and promoted host microbe homeostasis.

New knowledge that commensal bacteria are not mere bystanders but have a role in maintaining respiratory health in cattle has led to advent of respiratory probiotics (discussed later).

Role of the Overall Diversity and Stability of the Microbiota on Respiratory Health

Because biodiversity correlates to efficiency of nutrient utilization by a community, a more diverse bacterial community is, in theory, more likely to resist colonization by
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<tr>
<td>Postweaned feedlot beef calves</td>
<td>Cross-sectional: 2 populations (dead calves with lung lesions at necropsy,  n = 15; non-BRD related mortality,  n = 3)</td>
<td>BAL collected at necropsy</td>
<td><em>M</em> <em>haemolytica</em>, <em>Mycoplasma bovis</em>, and <em>H</em> <em>somni</em> were relatively abundant (&gt;5%) in most but not all BRD samples. Other relatively abundant genera (&gt;1%) included <em>Acinetobacter</em>, <em>Bacillus</em>, <em>Bacteroides</em>, <em>Clostridium</em>, <em>Enterococcus</em>, and <em>Pseudomonas</em>. <em>Mycoplasma bovis</em> was not detected in non-BRD lung samples.</td>
<td>Klima et al, 58 2019</td>
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<td>Cross-sectional: 2 populations (BRD,  n = 82; healthy pen-matched,  n = 82)</td>
<td>DNS</td>
<td>Bacterial communities differed between BRD and CTRL groups. Relative abundance of <em>H</em> <em>somni</em>, <em>M</em> <em>haemolytica</em>, <em>Mycoplasma bovis</em>, or <em>P</em> <em>multocida</em> did not differ between BRD and CTRL groups. The proportion of samples that contained <em>Mycoplasma bovis</em> was higher, however, in the BRD group (43.90%) compared with the CTRL group (18.29%). Richness was lower in cattle with BRD.</td>
<td>McMullen et al, 39 2019</td>
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<td>Cross-sectional over 5 wk after feedlot entry: 4 populations (calves sampled in 2015: BRD,  n = 25 [5 pooled samples]; healthy,  n = 30 [6 pooled samples]) and calves sampled in 2016: BRD,  n = 8 [16 pooled samples]; healthy,  n = 38 [10 pooled samples])</td>
<td>NS and DNS</td>
<td>Bacterial communities differed between BRD and CTRL groups only in 2016 (not in 2015). In 2016, <em>Psychrobacter</em> was more abundant in calves with BRD compared with CTRL in weeks 4 and 5 after feedlot entry, whereas <em>Moraxella</em> was greater in calves with BRD compared with CTRL throughout all 5 wk after feedlot entry.</td>
<td>McDaneld et al, 32 2018</td>
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</table>
Cross-sectional: 2 populations (BRD, n = 60; healthy pen-matched, n = 60) DNS and TTA Bacterial communities present within the airways clustered into 4 distinct metacommunities that were associated with sampling locations and health status. Metacommunity 1, enriched with Mycoplasma bovis, M haemolytica, and P multocida, was dominant in the nasopharynx and trachea of cattle with BRD. In contrast, metacommunity 3, enriched with Mycoplasma dispar, Lactococcus lactis, and Lactobacillus casei, was present mostly in the trachea of CTRL cattle. Metacommunity 4, enriched with Corynebacterium, Jeotgalicoccus, Psychrobacter, and Planomicrobium, was present in the nasopharynx only. Metacommunity 2, enriched with H somni, Moraxella, and L lactis, was present in both BRD and CTRL cattle. Richness and diversity were lower in the trachea and nasopharynx of cattle with BRD.

Longitudinal; 4 populations (BRD at entry, n = 22; BRD at diagnosis; n = 22; healthy at entry, n = 44; healthy at diagnosis, n = 10) DNS Bacterial communities differed between BRD and CTRL groups. At the phylum level, Proteobacteria was higher in BRD calves vs CTRL (32.12% vs 16.32%). Actinobacteria (38.20% vs 16.58%) and Fusobacteria (3.86% vs 0.03%) were higher in CTRL. At the genus level, Acinetobacter (12.54% vs 2.16%), Solibacillus (3.71% vs 0.02%), and Pasteurella (2.38% vs 0.03%) were higher in BRD. Mycoplasma and Moraxella were numerically higher in BRD (but P > .05). Rathayibacter (20.09% vs 3.96%) was higher in CTRL. No difference in bacterial diversity and
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### Preweaned dairy calves and their dams

| Longitudinal: 1 population sampled at 3 d, 14 d, 28 d, and 35 d of age. During the study period, calves had BRD (n = 16), otitis media (n = 28), or BRD and otitis media (n = 5) or remained healthy (n = 32). | DNS and vaginal swabs (for the dams) | The relative abundance of *Mannheimia* was significantly higher at d 14 in animals that eventually developed BRD than in calves that remained healthy. The genera *Porphyromonas* and *Campylobacter* were relatively more abundant in the vaginal microbiota of dams whose progeny developed disease, and *Mannheimia* and *Caloramator* were relatively more abundant in the vaginal microbiota of dams whose progeny remained healthy. | Lima et al.\(^4\) 2019 |

### Abbreviations:
- BAL, bronchoalveolar lavage; CTRL, control; DNS, deep nasal swab (≥20 cm long); NS, nasal swab (<20 cm long); S1, serotype 1; TTA, transtracheal aspiration.

Data from Refs. \(^3,4,6,7,11,18,31–33,39,58\)
pathogens. For example, in children, colonization of the URT by acute otitis media pathogens (Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis) was associated with lower levels of diversity in the URT microbiota. In cattle, diversity of the URT and LRT was lower in cattle with BRD than in their healthy pen-mates. Because overgrowth of pathogens in the respiratory tract could lead to a loss of diversity, however, it is difficult to determine whether reduced diversity predisposes cattle to BRD or is merely a consequence of proliferation of bacterial pathogens preceding clinical BRD. Therefore, additional longitudinal studies investigating the role of the microbiota diversity in respiratory health are needed.

A lack of stability in the URT microbiota during the first year of life has been associated with an increased risk of URT disease (such as otitis media) in human infants. Perhaps disturbances in the bovine nasopharyngeal microbiota observed around the first month of age in dairy calves and during weaning/marketing in beef cattle predispose to colonization and/or proliferation of bacterial pathogens in the respiratory tract. Impact of microbiota stability on respiratory health in cattle, however, has not been reported.

MODULATION OF THE BACTERIAL RESPIRATORY MICROBIOTA TO PROMOTE HEALTH

Currently, modulation of the respiratory microbiota to promote health is based primarily on the use of parenteral antimicrobials in cattle. This therapeutic strategy is effective in reducing URT colonization by M haemolytica or P multocida and thus typically decreases incidence of BRD for 2 weeks to 3 weeks after administration. Parenteral antimicrobials, however, also significantly disrupt microbial interactions among bacterial communities of the respiratory tract (Amat S, Timsit E, Workentine M, et al. Intranasal administration of bacterial therapeutics induces longitudinal modulation of the nasopharyngeal microbiota in post-weaned beef calves, submitted for publication). These communities then can become potentially more permissive to colonization by exogenous bacteria or proliferation of endogenous ones (Amat S, Timsit E, Workentine M, et al. Intranasal administration of bacterial therapeutics induces longitudinal modulation of the nasopharyngeal microbiota in post-weaned beef calves, submitted for publication). For example, on-arrival mass medication with tulathromycin was followed by rapid horizontal spread of a tulathromycin-resistant strain of M haemolytica. Furthermore, parenteral administration of antimicrobials such as tulathromycin or oxytetracycline increased abundance of resistant genes, such as erm(X), sul2, tet(M), msr(E), and tet(H), in the nasopharyngeal microbiota.

Fortunately, other approaches to modulate the respiratory microbiota and promote health (eg, probiotics, prebiotics, and bacteriophages) have potential as viable alternatives to parenteral antimicrobials.

Definition, Mechanisms of Action, and Possible Application Route of Probiotics

Probiotics are defined as “live microorganisms that when administered in adequate amounts, confer a health benefit to the host.” It is noteworthy that a higher abundance in healthy animals compared with sick animals is not enough for a strain to be designated as probiotic. A causative relationship with health promoting effects also should be demonstrated.

New probiotic postulates (based on Koch’s postulates) have been recently suggested in development of next-generation probiotics. They are defined as follows:
1. The microorganism is present in high abundance in healthy animals and decreased abundance in those suffering from a disease.

3. The microorganism can be isolated from healthy animals and grown in pure culture.

3. The cultured organism should promote health when introduced into a diseased animal.

4. Because probiotics are, by definition, administered as live organisms, it should be possible to reisolate these microorganisms from the healthy experimental host and confirm that they are identical to the original specific causative agents.

Based on these postulates, numerous LAB are potential candidates for next-generation probiotics.

Probiotics promote respiratory health through 3 main mechanisms (Fig. 4). First, they can have a direct antimicrobial action against bacterial respiratory pathogens by producing antimicrobial molecules, for example, lactic and acetic acid, bacteriocins, and hydrogen peroxide, in their microenvironment. Second, probiotics can enhance the epithelial barrier by, for example, stimulating production of mucin or antimicrobial peptides (ie, defensins, lysozymes, and cathelicidins). Finally, administration of probiotics also can modulate host immune responses (both innate and adaptive immunity) by interacting with host pattern recognition receptors of the mucosa. For example, probiotic bacteria can modulate maturation of dendritic cells toward an anti-inflammatory interleukin (IL)-10 profile or stimulate regulatory T-cell activity to control overt inflammatory conditions. In addition, they can modulate cytokine production and stimulate B-cell and antibody production (IgA and IgG). Numerous other immunomodulatory effects of probiotics have been described, but a complete review of these mechanisms is outside the scope of this article (consult reviews by Lebeer and colleagues [2010] and van den Broek and colleagues [2019] for additional information). Most reports on effects of probiotics on host immunity are from humans or mice and not cattle.

Traditionally, probiotics have been administered by an oral route. Orally applied probiotics could benefit the URT via systemic immune effects. They do not, however, have a direct antimicrobial action against bacterial respiratory pathogens, and they do not affect the URT’s local immune response. Conversely, nasal application of probiotics has the advantage of promoting more direct contact of the applied organisms with the respiratory tract mucosa and microbiota. Furthermore, by using the nasal route, probiotics do not have to survive transit through the gastrointestinal tract (especially the rumen). Therefore, the next logical step is to design probiotics that can be applied intranasally.

**Intranasal Administration of Probiotics in Cattle**

To investigate whether nasal application of probiotics can modulate the respiratory microbiota to promote health in cattle, the authors’ team first selected in vitro probiotic strains originating from the nasopharynx of healthy cattle with properties that are important for URT probiotics (discussed later). Then, these probiotics strains were administered to dairy calves and beef calves (Amat S, Timsit E, Workentine M, et al. Intranasal administration of bacterial therapeutics induces longitudinal modulation of the nasopharyngeal microbiota in post-weaned beef calves, submitted for publication) to investigate their health-promoting effects.

For selection of probiotic strains, the authors used a stepwise approach. Bacteria isolated from the nasopharynx of healthy cattle for their ability to inhibit *M haemolytica* (178 isolates from 12 genera). Subsequently, abilities of selected isolates were evaluated to adhere to bovine turbinate (BT) cells (n = 47), compete against *M haemolytica*
for BT cell adherence (n = 15), and modulate gene expression in BT cells (n = 10). *Lactobacillus* strains had the strongest inhibition against *M. haemolytica*, with 88% of isolates having inhibition zones ranging from 17 mm to 23 mm. All isolates tested in competition assays reduced *M. haemolytica* adherence to BT cells (32% to 78%). Among the 84 bovine genes evaluated, selected isolates slightly upregulated expression of IL-8 and IL-6. After ranking isolates for greatest inhibition, adhesion,
competition, and immunomodulation properties, 6 *Lactobacillus* strains from 4 different species were selected as the best URT probiotic candidates: *L amylovorus*, *L buchneri* (2 strains), *L curvatus*, and *L paracasei* (2 strains). The authors primarily focused on LAB because these bacteria have a long history of safe use (eg, generally recognized as safe). Other bacteria, however, such as *Mycoplasma dispar*, also could have some probiotic properties, because they were present in higher abundance in healthy animals versus sick animals.

Health-promoting effects of the 6 selected *Lactobacillus* strains were first evaluated in dairy calves. For this evaluation, 1-week-old to 3-week-old dairy calves received either an intranasal cocktail of the 6 probiotic strains (3 × 10⁹ colony-forming units [CFUs] per strain; n = 12) 24 hours prior to an intranasal *M haemolytica* challenge (3 × 10⁸ CFUs), or only phosphate buffered saline (PBS) prior to challenge (control group; n = 12). Nasal swabs were collected over the course of 16 days after probiotic inoculation. Probiotic strains were reisolated up to 13 days after inoculation, with variation existing among strains and calves. Their administration significantly reduced nasal colonization by *M haemolytica*. It also modified composition and reduced the diversity of the nasal microbiota and altered interbacterial relationships among the 10 most abundant genera. This study demonstrated, for the first time, that intranasal probiotics developed from bovine nasopharyngeal *Lactobacillus* could reduce nasal colonization by *M haemolytica* in dairy calves.

In a second study, the authors investigated the health promoting effects of the same probiotic cocktail in beef cattle (Amat S, Timsit E, Workentine M, et al. Intranasal administration of bacterial therapeutics induces longitudinal modulation of the nasopharyngeal microbiota in post-weaned beef calves, submitted for publication). In that study, on arrival at the feedlot, newly received beef steers either received (1) an intranasal cocktail of the 6 strains (3 × 10⁹ CFUs per strain; n = 20); (2) intranasal PBS (negative control; n = 20); or (3) parenteral tulathromycin (Draxxin [Zoetis (Kirkland, Ontario, Canada)]) (positive control; 2.5 mg/kg; n = 20). Nasopharyngeal swabs were collected for up to 42 days post-treatment. Nasopharyngeal colonization by probiotics was most apparent at day 2 postinoculation; however, administration of probiotics modified composition and reduced the diversity of the nasopharyngeal microbiota for up to 42 days. Compared with PBS and probiotics, parenteral tulathromycin decreased bacterial load in the nasopharynx and increased abundance of the antibiotic-resistant gene *msr*(E). There were no significant effects among treatments on relative abundance of *M haemolytica*, *P multocida*, or *H somni*. This second study demonstrated that a unique intranasal inoculation of probiotics could modify the nasopharyngeal microbiota for up to 42 days in postweaned beef cattle. Unfortunately, it did not provide useful information on potential health promoting effects of the probiotic cocktail, because the disease challenge was very low, with only 5 of the 60 calves diagnosed with BRD during the study period (3 in the PBS group and 2 in the probiotic group).

In summary, a single intranasal administration of 6 selected *Lactobacillus* strains modified the nasopharyngeal microbiota of dairy cattle and beef cattle and provided colonization resistance against *M haemolytica*. Additional research is needed to define the optimal dose and duration of application of this probiotic cocktail to maximize health benefits as well as to further confirm its health promoting effect (ie, can it reduce incidence of BRD after administration, which is the outcome of interest for the cattle industry). Perhaps a single inoculation is not enough to prevent BRD. In human studies, intranasal probiotics to prevent otitis media in infants typically are given multiples times over a few days or weeks; for example, 10 days per month over 2 consecutive months or twice daily for 10 days. Furthermore, it is noteworthy that probiotics
have different abilities to colonize and influence a particular individual. For example, it has been reported that transient colonization by probiotic strains is highly variable in the lower gastrointestinal tract of humans, with some humans being more permissive to colonization and others being resistant.\textsuperscript{50} It, therefore, is possible that the composition of the probiotic cocktail should be adapted to animal age and production system, that is, preweaned dairy calves, preweaned or postweaned beef cattle, and veal calves.

**Other Strategies: Bacteriophages and Prebiotics**

Bacteriophage therapy is another way to modulate the bacterial structure of the respiratory microbiota.\textsuperscript{51} Bacteriophages are viruses that infect bacteria. They either can be stably integrated into bacterial genomes (lysogenic phase) or can replicate and lyse bacteria, releasing virus particles (lytic phase).\textsuperscript{52} Bacteriophages are highly specific and typically infect only 1 bacterial species, serotype, or strain. Their inoculation in the nasopharynx of cattle, therefore, can remove bacterial respiratory pathogens without having an impact on the commensal flora.\textsuperscript{51} Furthermore, bacteriophages can amplify exponentially after administration and thus do not always need multiple administrations. Bacteriophages with lytic properties against *M haemolytica* have been isolated and characterized.\textsuperscript{53} Unfortunately, to the authors’ best knowledge, there are no published data on their use to remove *M haemolytica* from the nasopharynx of cattle. The increasing prevalence of antibiotic resistance in *M haemolytica* and *P multocida* isolated in the United States,\textsuperscript{43,54} Canada,\textsuperscript{29} and Europe\textsuperscript{55} nevertheless is creating an impetus to further investigate bacteriophage therapies in cattle.

Prebiotics are nonviable substrates that serve as nutrients for beneficial microorganisms harbored by the host, including administered probiotic strains and indigenous (resident) microorganisms.\textsuperscript{56} Commonly studied prebiotics include fructooligosaccharides, galacto-oligosaccharides, inulin, and resistant starch.\textsuperscript{57} Administration of prebiotics alone or in combination with probiotics in the URTs of cattle could promote the selective growth of bacteria considered beneficial, for example, *Lactobacilli*. Unfortunately, to date, prebiotics have been used solely to selectively enhance the growth of beneficial bacteria in the digestive tract, and further research is needed before recommending their use for modulating the respiratory tract microbiota of cattle.

**SUMMARY**

The respiratory tract of cattle is colonized by complex bacterial ecosystems also known as bacterial microbiotas. These microbiotas evolve over time and are shaped by numerous factors, including maternal vaginal microbiota, environment, age, diet, parenteral antimicrobials, and stressful events (eg, transportation and commingling). The resulting microbiota can be diverse and enriched with known beneficial bacteria (ie, *Lactobacillus* and *Lactococcus*) that can provide colonization resistance against opportunistic bacterial pathogens or, on the contrary, with bacterial pathogens, such as *M haemolytica*, *P multocida*, *H somni*, or *Mycoplasma bovis*, predisposing cattle to respiratory disease. Beneficial bacteria promote health through 3 main mechanisms: (1) direct antimicrobial action against bacterial pathogens, (2) enhancement of the epithelial barrier, and (3) modulation of the host immune response. Among beneficial bacteria, *Lactobacillus* are of particular interest because they generally are regarded as safe. Intranasal inoculation of a cocktail of 6 *Lactobacillus* strain modified the structure of the nasopharyngeal microbiota over a few weeks in beef calves and dairy calves and provided colonization resistance against *M haemolytica*. That the
respiratory microbiota can be modulated by nonantimicrobial approaches to promote health creates new potential strategies for prevention and treatment of BRD.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. John Kastelic for editing the article.

DISCLOSURE

Dr E. Timsit is an Innovation Scientist at Ceva Animal Health and is responsible for early phases of drug discovery and development. None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the article. This article reflects the views of the authors and should not be construed as representing the views of Ceva Animal Health.

REFERENCES


