Trends in Microbiology

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Review

Contribution of the Mucosal Microbiota to Bovine Respiratory Health

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Recognizing the respiratory tract as a dynamic and complex ecosystem has enhanced our understanding of the pathophysiology of bovine respiratory disease (BRD). There is widespread evidence showing that disease-predisposing factors often disrupt the respiratory microbial ecosystem, provoking atypical colonization patterns and a progressive dysbiosis. The ecological factors that shape the respiratory microbiota, and the influence of these complex communities on bovine respiratory health, are a rich area for research exploration. Here, we review the current status of understanding of the bovine respiratory microbiota, the factors that influence its development and stability, its role in maintaining mucosal homeostasis, and ultimately its contribution to bovine health and disease. Finally, we explore the limitations of current research approaches to the microbiome and discuss potential directions for future research that can help us better understand the role of the respiratory microbiota in the health, welfare, and productivity of livestock.

Highlights

Next-generation sequencing technology has allowed a wider exploration of respiratory microbiota in cattle.

Identification of site-specific microbiota within the bovine respiratory tract provides clinical insight into the potential role of microbiota in different ecological niches.

The successful interaction and communication between the host immune system, mucosal epithelium, and resident microbiota are crucial for providing colonization resistance against translocating microbes.

There are still major challenges in bovine respiratory microbiome research that need to be addressed.

The Bovine Respiratory System and Its Microbiota

Bovine respiratory disease (BRD) is a major health and economic problem, particularly in recently weaned and newly transported feedlot calves [1]. Despite decades of research on disease control, BRD and its consequences remain the main causes of cattle morbidity, mortality, welfare concerns, and production loss [2]. From an anatomical and physiological perspective, cattle have a complex respiratory system that can be broadly divided into the upper respiratory tract (URT) and the lower respiratory tract (LRT) [3]. The URT includes paired nasal cavities, paranasal sinuses, nasal passages, nasopharynx, oropharynx, tonsils, and the upper portion of the larynx. The LRT includes the lower portion of the larynx, trachea, bronchi, bronchioles, and alveoli. The fundamental functions of the respiratory tract are oxygen and carbon dioxide exchange, maintenance of acid–base balance, and to warm, humidify, and filter inhaled air [3]. The airway mucosal epithelium acts as a front line of defense against respiratory pathogens by producing functional molecules that initiate multiple innate and adaptive immune mechanisms that are crucial for lung defense mechanisms [4–6].

While respiratory defenses have been traditionally attributed to various structural and functional aspects of the epithelium and mucosa-associated immune cell population, there has been recent curiosity about the potential contribution of mucosal microbial populations and host–**microbiota** (see Glossary) interactions to respiratory tract immune defense and host health [7]. The term '**microbiome**' was first proposed in 2001 and was defined as 'the ecological community of commensal, symbiotic, and pathogenic microorganisms living within a particular environment' [8]. The mammalian microbiome includes all members of the microbiota (bacteria, archaea, fungi, viruses, and eukaryotes), their genomes, and the surrounding environment [9]; each member of the microbiota colonizes different organ site(s) and occupies specific biogeographical niche(s), such as the rumen [10], intestine [11,12], vagina [13], uterus [14], URT [15], and LRT [16,17].

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Previously, research on microbiota, driven by next-generation sequencing, has focused primarily on microbes in the gastrointestinal tract and their metabolic and immune functions [18]. However, a number of recent research studies have used metagenomic sequencing to survey the bovine respiratory microbiota (Table 1). Despite the clear differences in design and study populations in these studies, the overall results are harmonious and they support the notion that the respiratory microbiota is of paramount importance to bovine respiratory health. Given that respiratory microbial phenotypes (microbiota) and genotypes (microbiome) are important to health, an understanding of microbial succession in the respiratory tract, and its dysregulation in disease states, could provide crucial understanding of the pathophysiology of respiratory infections [19,20].

Here, we review the current status of our understanding of the bovine respiratory microbiota, the factors that influence its development and stability, its role in maintaining mucosal **homeostasis**, and ultimately its role in BRD pathophysiology. Although bovine respiratory microbiota homeostasis is still a relatively emerging field of research, the evidence to date suggests that optimization of equilibrium in the mucosal microbial ecosystem may be a useful approach for reducing the incidence and severity of microbial-related disease. Because little information is available on the role of viruses, phages, and fungi in the cattle microbiome, our discussion in this review focuses on the important role of bacteria in bovine respiratory health. Importantly, we also explore the potential limitations of current research approaches to study the microbiome and discuss potential directions for further research that will help us better understand the role of the respiratory microbiota in the health, welfare, and productivity of livestock.

Genomic Tools Used to Study the Bovine Respiratory Microbiota

Previous studies of microbial populations in the bovine respiratory tract have focused primarily on culture-based techniques, focusing on the main microbes that are readily cultured [21]. While these methodologies have been useful, they are reductionist methods and do not offer information on uncultured microbes, that are likely dominant at these locations, and thus, provide only a limited understanding of the complexity of these clinically critical microbial ecosystems [21]. Therefore, microbiology research has transitioned from culture-based methods to a more holistic approach of investigating the entire *in vivo* microbial population. This has been accomplished by employing various molecular techniques to quantify the microbial composition of the respiratory tract in cattle [22]. Immunohistochemistry and fluorescent *in situ* hybridization have been applied to demonstrate the presence and characteristics of organisms in clinical respiratory specimens [23]. In addition, real-time qPCR is also one of the most common techniques used for amplifying and quantifying specific DNA or RNA sequences in host or environmental samples [24]. While these techniques identify specific bacterial species, and evaluate similarities and dissimilarities in the microbial composition, they do not provide direct molecular sequence data. Furthermore, only previously classified bacteria can be identified using these techniques, since each assay is generally designed to detect a narrow range of bacterial taxa [25]. These limitations have led to the advancement and extensive adoption of high-throughput sequencing of the 16S rRNA marker gene as a common phylogenetic gene for profiling microbes present in a specific host or environment [26]. The 16S rRNA gene is pervasive between prokaryotes and contains many highly conserved domains conjointly with nine variable and hypervariable regions [27]. Current sequencing technologies are used to isolate microbial DNA from a single specimen, and generate a large number of short-sequence reads in a single assay run. To date, most high-throughput sequencing studies in cattle have been based on the Illumina platform [28] and 454 pyrosequencing [29]. The available sequencing platforms are additionally capable of generating more robust information on the microbiota functional profiles. For instance, whole-genome sequencing (shotgun **metagenomics**) has superseded 16S metataxonomics because it has the capacity

Glossary

Colonization resistance: a direct or indirect mechanism whereby the microbiota protects the host from foreign pathogens or pathobiont colonization.
Community structure: the composition and abundance of microbial species in the same community.
Dysbiosis: a change in, and disturbance of, microbiota from the steady-homeostatic state composition within a microbial ecosystem.
Homeostasis: healthy balance of all members of the microbiota within their specific environment.
Metabolomics: an analytical approach used to determine all the metabolites and metabolite profile(s) in any given strain or single tissue.
Metagenome: the collection of genomes and genes from the members of a microbiota. that through shot-gun sequencing of DNA.
Metagenomics: a method used for analyzing the **metagenome** and gaining information on the potential function of the microbiota, the microbiota composition and what those microbes are doing.
Metaproteomics: large-scale characterization of the entire protein complements of environmental or clinical samples at a given point in time.
Metatranscriptomics: high-throughput sequencing of the entire gene expression of microbiota within an ecosystem to obtain whole-gene expression profiling and regulation of complex microbial communities.
Microbial diversity: the broad variety and distribution of microorganisms in a microbiota in a single population or sample (alpha- diversity) or between two populations or samples (beta diversity).
Microbiome: the collection of microorganisms and their genomes in a particular environment. The respiratory microbiome refers to the microbiome of the respiratory tract.
Microbiota: a community of microorganisms that live on or within the host. The bovine respiratory microbiota refers to all the microorganisms found in the cattle respiratory tract.
Operational taxonomic unit (OTU): the operational proxy of a taxon that classifies groups of closely related individuals. The OTU taxonomic definition is based on nucleotide identity (usually 97% for 16S rRNA).

to generate a deeper and more comprehensive view of the composition, structure, and metabolic functions of the microbiota [30]. This information is crucial for a better understanding of the whole microbial ecosystem and allows researchers to illuminate the uncultured and novel microorganisms. Furthermore, equivalent advancement in mass spectrometry techniques has led to improved approaches for detecting the activity of the microbiota, including protein products (**metaproteomics**), gene expression (**metatranscriptomics**), and metabolic profiles (**metabolomics**). Combining the sequence information with these multi-omics techniques may be the cornerstone for improving our understanding of the intricate respiratory microbial niche and its association with livestock health and disease in the future.

Developmental Dynamic of the Bovine Respiratory Microbial Ecosystem

The development of the respiratory tract structure and function in cattle is a complex multistage process that can be divided into three sequential periods [31]. First is the development of the bronchial and lung epithelium which occurs in the embryonic period, beginning in the fourth week of gestation [32]. Second, throughout the fetal period, the preliminary structures for gas exchange are established with ramified bronchiolar development. Finally, during the postnatal period, the alveoli and lungs are both developed with a capacity for full, functional gas exchange. In parallel with this sequential, anatomic growth of the respiratory tract, the gradual colonization of microbes takes place throughout gestation, and during the periparturient period, thereby establishing the respiratory microbiota in early life. The composition, trajectory, and stability of the developing microbiota are impacted by many intrinsic and extrinsic components that may play a role in respiratory tract morphogenesis [33,34].

In contrast to the persistent assumption that neonates are born in a microbially germ-free state, the recent use of culture-independent methods has shown that microbial DNA is present in the amniotic fluid [35,36], thus increasing the likelihood that respiratory tract growth during pregnancy occurs in the presence of microbial populations. In swine, the microbiota of piglets following delivery resembles that of the sow and depends on the route by which the pig is delivered [37]. The succession of microbial colonization after birth is a complex process, and is strongly shaped by several host and environmental factors, such as colostrum ingestion, housing, and dietary composition [20]. In people, various microorganisms are detectable in the URT of healthy neonates during the first few hours of life [38]. Similarly, bovine respiratory microbiota colonization occurs shortly after birth [39] and is subsequently influenced by multiple factors, including diet [40], host genetics, age [41], antimicrobial use [42], vaccinations, season, different management strategies during the production cycle, and the surrounding environment [43,44] (Figure 1).

The process of weaning has a pivotal influence on the respiratory microbial composition of calves, particularly when associated with other environmental stressors [40,43,45]. The URT microbiota in cattle appears to develop rapidly from weaning to feedlot arrival, and from arrival to day 40 post-arrival [43]. Following arrival at the feedlot, routine processing procedures, such as ear tagging, weighing, vaccinating, and prophylactic anthelmintic and antimicrobial treatments, have both short- and long-term impacts on the respiratory microbial populations [43,46]. Stress, viral infection, and dietary changes may explain the URT **dysbiosis** that occurs from arrival at the feedlot until weaning [43]. The different stressors encountered at the feedlot were shown to impair host defenses which in turn, reduced containment of bacterial pathogens in the URT [47–50]. Viral infection has also been shown to contribute to the development of nasopharyngeal dysbiosis through interference with epithelial function and impairment of host mucosal immune defenses [47]. For instance, bovine respiratory viruses, including BoHV-1, BRSV, and BPiV-3, replicate in the airway mucosal epithelium, causing inflammation and thereby facilitating subsequent adhesion and colonization of pathogenic microbes [51]. Unfortunately, the full impact of viral infections

Table 1. Summary of the Existing Metagenomic Studies on Cattle Respiratory Microbiota

Study population	Age of cattle	Sample type	Sequencing platform and 16S rRNA gene hypervariable region	Overall sequence analysis	Data availability accession number	Refs
Crossbred beef-breed steer (*n* = 128) and heifer (*n* = 36) calves	Not specified	Deep nasal swab	Illumina Miseq (V4)	A total of 7 240 915 reads were obtained across all samples with an average Phred quality score of 35.9. After processing, a total of 4 988 778 reads remained across all samples with an average coverage of 30 419 reads (range 16 121–41 849) per sample. From these sequences, 2700 unique sequence variants (SVs) were identified across all samples.	None	[98]
120 mixed-breed beef steers at high risk of developing respiratory disease	Weaned calves, comingled and auction-market-derived population	Transtracheal aspirations and deep nasal swabs	Illumina Miseq (V4)	A total of 20 577 564 sequences resulted from all samples. The median number of sequences per sample was 86 502 ± 17 868 (range 43 316–162 699). After filtering, the SVs table contained 513 SVs with a total of 19 713 248 reads. Median number of sequences per sample was 84 331 ± 18 792 (range 36 998–161 888).	None	[71]
60 Angus-cross beef heifers	The population studied from weaning at their ranch of origin to 28 days after entrance into a feedlot	Deep nasal swabs and transtracheal aspirates	Illumina Miseq (V4)	The raw SV table contained 16 764 SVs with a total of 16 206 284 reads across all samples. The median number of sequences per sample was 46 477 ± 8943, with a minimum of 15 515 and maximum of 71 866. After filtering, the SV table contained 587 SVs with a total of 14 527 137 reads. The median number of sequences per sample was 42 307 ± 10 969 with a minimum of 11 603 and maximum of 70 719. Taxonomic analysis revealed a total of 12 bacterial phyla in deep nasal swabs, and 13 phyla in transtracheal aspirates.	None	[99]
30 feedlot cattle	Not specified (≥60 days on feed	Deep nasopharyngeal swab	Illumina Miseq (V4)	A total of 1376 OTUs representing 266 genera were identified among all NP samples.	PRJNA394129	[56]
22 calves (17 males and 5 females) post-weaned calves	5 to 14 months	Deep nasal swabs and transtracheal aspirates	Illumina Miseq (V3–V4)	After quality trimming and pair merging, the total read count was 3 482 819, with an average read length of 407 ± 80 bp. The total reads classified in the OTU table was 1 645 584, divided in 526 932 from the nasal swab samples (median 24 072, min–max 159–167,198) and 1 118 652 from the tracheal aspirate fluid samples (median 53 494 min–max 189–143,886).	None	[72]

Table 1. (continued)

Study population	Age of cattle	Sample type	Sequencing platform and 16S rRNA gene hypervariable region	Overall sequence analysis	Data availability accession number	Refs
8 feedlot cattle	6–8 months	Deep nasopharyngeal swab and bronchoalveolar lavage	Illumina Miseq (V3–V4)	A total of 298 875 sequences resulted from all samples. The number of sequences per sample ranged from 2419.0 to 48 092.0 and comprised 195 OTUs across all samples.	PRJNA323521	[17]
135 Charolais feedlot cattle	6–8 months	Deep nasopharyngeal swab	Illumina Miseq (V1–V3)	A total of 1 297 074 sequences were recovered. The mean sequence per sample was 91 567 and comprised 562 OTUs (97% identity cutoff) across all samples. Taxonomic analysis revealed a total of 15 different bacteria phyla, and 165 bacterial genera, across all samples.	PRJNA318938	[46]
16 Holstein heifer calves (10 healthy and 6 diagnosed with BRD) selected from a larger cohort of 174 animals	Day 1 of life until 65 days of life (age of weaning)	Deep nasopharyngeal swab	MiSeq Illumina (whole-genome sequencing-shotgun metagenomics)	The overall sequence analysis revealed a total of 21 155 233 sequences (mean number of sequences per sample: 661 101; median: 638 404; range: 383 190–994 172). Taxonomic analysis revealed 5 predominant phyla and 20 dominant genera across all the samples.	MG-RAST From 4678806.3, to 4678836.3	[30]
20 Angus-cross heifers	Recently weaned, studied at the first 40 days after arrival at the feedlot	Deep nasal swabs	Illumina MiSeq (V3–V4)	A total of 7 211 407 sequences were obtained across all samples. Sequences were clustered into 1201 OTUs across all samples and 172 OTUs were retained after filtering low-abundance OTUs. Taxonomic analysis revealed a total of eight phyla across all samples.	None	[42]
32 beef calves and 6 dairy calves	0–12 months	Cranial lung lobe tissue and mediastinal lymph node	Illumina MiSeq (V3–V4)	Overall, a total of 115 bacterial OTUs were identified. 72 OTUs were identified to genus level. Additionally, 32 OTUs could only be identified as far as family level, 7 OTUs could only be identified to order, 2 to class level, and 2 only to phylum level.	SRP080306	[16]
45 Angus beef calves (17 heifers and 28 steers)	6.5 to 9 months (222 ± 6.4) days	Nasal swabs	Illumina MiSeq (V4)	A total of 137 749 sequences were yielded from all analyzed samples (n = 16; mean ± SD, 70 859 ± 10 178). Rarefaction analysis was performed at a depth of 7214 sequences.	SRP090121	[40]
30 Angus-beef steers	Weaning age 196 ± 21 days of age	Deep nasopharyngeal swabs	Illumina MiSeq (V3)	A total of 10 494 168 sequences were obtained across all samples, with a total length of 175 bases per read postprocessing, and an average coverage of 111 640 sequences per sample. Taxonomic analysis revealed a total of 16 phyla across all samples.	None	[43]

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Table 1. (continued)

Study population	Age of cattle	Sample type	Sequencing platform and 16S rRNA gene hypervariable region	Overall sequence analysis	Data availability accession number	Refs
14 beef heifers (Angus × Hereford heifers)	8 months	Nasopharyngeal swab	Illumina MiSeq (V4)	A total of 3 868 199 archaeal and bacterial 16S rRNA gene sequences, with an average length of 260 bp remained after primer removal, quality-filtering, and chimera-checking. These sequences were clustered into 6381 OTUs. These OTUs were assigned to 28 different phyla and 478 genera across all samples.	PRJNA296393	[100]
174 Holstein calves	3 to 35 days	Deep nasopharyngeal swabs	Illumina MiSeq (V4)	A total of 63 638 904 were obtained across all samples. The average coverage was 91 567, the SD was 58 425, and the range was 1 423–657, 375 numbers of reads per sample. Taxonomic analysis revealed eight predominant phyla and 30 dominant genera across all the samples.	None	[85]
5 feedlot calves were selected from 5000 cattle upon entry to feedlots and again from the same animals at 60 days	Not specified	Nasopharyngeal swab	Microarray Phylochip (full-length 16S rRNA gene)	A total of 275 unique OTUs (97% sequence similarity) were detected among all samples. The number of OTUs identified in each sample was highly variable and ranged from 20 to 210. Taxonomic analysis revealed a total of 22 different bacteria phyla, and 64 bacterial families, across all samples.	None	[15]

on cattle respiratory microbiota has not been investigated because the cattle in most published studies had been previously vaccinated against the most common BRD viruses.

Several studies have demonstrated the beneficial impact of simple management interventions on nasopharyngeal **microbial diversity** in postweaned calves and the association of such practices with respiratory health during this important phase of disease susceptibility in the production cycle. For instance, the shift in gut microbiota that results from dietary changes during the early postweaning period may also contribute to changes in the respiratory tract microbiota [40]. Feeding weaned calves selenium-biofortified alfalfa hay for 9 weeks in a preconditioning program, prior to feedlot entry, favorably altered nasal microbial communities [40]. While the mechanistic pathways by which the gut microbiota affects the respiratory microbiota have not been fully elucidated in cattle, the roles of micro-aspiration, inhalation of bacterial, and direct mucosal dispersion have been well described in other species [52].

While antimicrobials are a commonly employed management strategy in the control and prevention of respiratory disease in many species, they have also been shown to impact resident, mucosal, microbial communities at various life stages [53]. In children, antimicrobial administration decreases the relative abundance of beneficial microbiota in the healthy URT, and increases the incidence of subsequent respiratory tract infection [54,55]. Similarly, in feedlot cattle, parenterally administered antibiotics temporally affect the URT microbiota [56]. The prophylactic

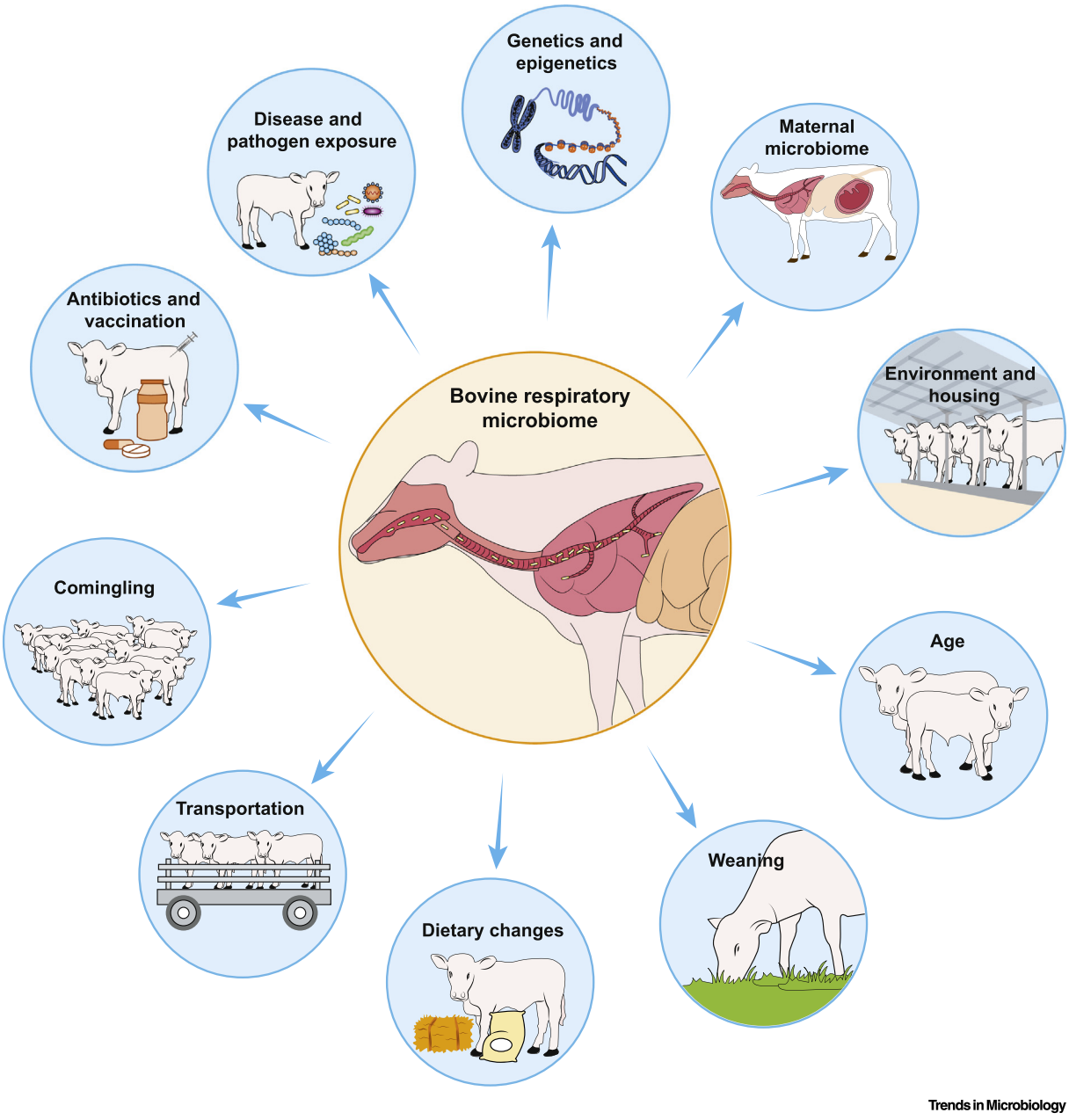

Figure 1. Factors Affecting the Development of the Bovine Respiratory Microbiota. Airway microbiota developments are highly dynamic and are shaped by various host and environmental factors, including host genetics, mode of delivery, diet and the microbiota of the mother, environmental housing, weaning, feeding type, transportation, comingling, antibiotic treatment, vaccination, and pathogen exposure.

administration of either parenteral tilmicosin or nasal nitric oxide-releasing solution (NORS) in high-risk calves within a day of arrival significantly impacted the nasopharyngeal microbiota [42]. These antimicrobial-related perturbations in nasopharyngeal microbial composition bridged the first 10 days of feedlot acclimation and were associated with an observation of fewer Pasteurellaceae culture-positive cattle in the tilmicosin-treated group than in the NORS-treated group. Because nasopharyngeal Pasteurellaceae colonization has been implicated as an

important prerequisite for BRD onset [57], these relative differences in colonization between these prophylactic measures may explain why NORS is inferior to tilmicosin for controlling BRD in high-risk populations [58].

The URT Microbiome in Cattle and Respiratory Homeostasis

The significance of the mucosal microbiota composition in promoting mucosal health has been recognized for several years in different biogeographic locations (skin, gastrointestinal and reproductive tracts), but its importance in the respiratory tract has been demonstrated only relatively recently [59]. Understanding the nature and inherent variation in the composition of the URT microbial community in healthy individuals is necessary before conclusions can be made regarding its role in susceptibility to respiratory disease [60]. Recent research suggests that the bovine URT microbiota is rich and diverse, and shows substantial variability between individuals [15,46]. The high animal-to-animal variation in the URT microbiota is associated with multifactorial components (host, environmental, and dietary) [61], and can provide significant challenges for data interpretation in studies based on small numbers of experimental subjects.

Although studies on respiratory microbiota in cattle have mainly relied on characterizing the microbiota structure, the role of these communities in health and production outcomes is less well understood. It is clear from studies of other species that microbial populations are crucial in modulating host immune defenses; this, in turn, influences respiratory homeostasis. The successful communications between the mucosal microbiota and their host immune system (Figure 2) are essential in regulation of mucosal immunity and maintenance of metabolic homoeostasis [62]. While the detailed structure and function of the respiratory mucosal immune system [6] is beyond the scope of this review, a better understanding of the nature of interaction

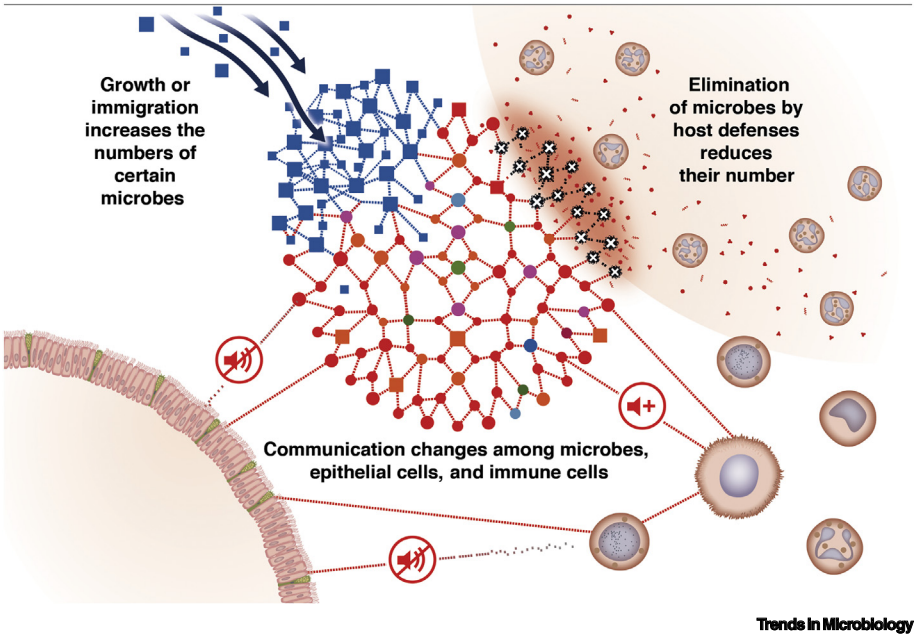

Figure 2. Interaction between the Host and the Mucosal Microbiota in the Bovine Respiratory Tract. The microbial signaling and communication among microbes, mucosal epithelial cells, and immune cells is necessary for promoting the host's immune system. The successful interaction between these components lead to reduce the number of invading pathogens through recruitment of immune cells, an increase in the host's defense mechanism, and an increase the numbers of certain beneficial microbes.

between mucosal microbiota, epithelium, and immune cells is essential in determining the factors that shape the resident microbial population.

The first line of defense of the respiratory tract is the nasopharynx mucosal layer, which physically entraps inhaled particles, including unwelcome microbes, and facilitates their clearance from the respiratory tract towards the nasal and oral cavities, through an escalator-like ciliary action. The surface mucus also contains antimicrobial peptides, glycoproteins, and IgA which, in combination with cellular immune effectors, play an important role in regulating the microbial ecological homeostasis between commensals, symbionts, and pathobionts at the mucosal surface [63,64].

The second line of defense is the mucosal epithelium, which produces antimicrobial peptides that contribute to effective barrier functions [65]. In addition to the respiratory tract epithelial cells, luminal and mucosal surface macrophages and dendritic cells express various specific receptors (innate pattern-recognition receptors) that play a crucial role in recognition and subsequent clearance of potential pathogens [65,66]. Additionally, network signaling and communication between resident microbes and mucosal epithelial cells is crucial for the recruitment of immune cells from other regions. Together, these three-way communications ensure the maintenance of epithelial health, the regulation of inflammation, and, ultimately, the **community structure** of local residential, microbial populations.

Several groups have conducted research on human and animal models to identify the signaling pathways of the respiratory microbiota and their mechanism in regulating host immune response. In a study of healthy adults, members of specific respiratory microbiota (*Veillonella* and *Prevotella*) were linked with increased lymphocyte numbers in respiratory specimens [67]. In addition, the higher frequency of Proteobacteria was associated with systemic inflammation in patients with acute respiratory distress [63]. A recent study in a mouse model revealed that members of the Bacteroidetes taxon decreased lung inflammation and provided the necessary defense against respiratory pathogens [68]. Although these findings support the concept that the balanced microbiota structure in the URT is important in mucosal immunity and respiratory tract homoeostasis, definitive studies are warranted to confirm the biological and clinical relevance of these processes in cattle health.

The LRT Microbiota in Cattle

In contrast to the other mucosal sites, specially the URT, the LRT in healthy populations has been considered sterile using culture-dependent and conventional molecular techniques; however, technological advancement in next-generation technologies has revealed that the healthy LRT is not sterile but instead contains complex and diverse microbial communities [67,69,70]. While the composition of the URT microbiota in feedlot cattle has been thoroughly described, only a few published studies have described the LRT microbiota in feedlot cattle [16,17,71–73].

In healthy feedlot cattle, the bacterial communities in the LRT differ from those found in the URT, suggesting the presence of self-sustaining microbial communities in each biogeographic location [17,72]. Despite a self-sustaining LRT microbiota, next-generation sequencing has revealed interesting information on the association between the presence of particular members of microbiota in the URT and LRT in feedlot cattle [17]. Associations between microbiota members can be either a positive relationship (mutualism) or a negative relationship (antagonism) [34]. The complex directionality and interaction between these populations is likely taxon-specific or even strain-specific, compatible with the community structure concept. The correlations of the presence and abundance of certain taxa between the URT and LRT are compatible with the notion of a mutualistic interaction between microbiota at these two ecological niches in the bovine respiratory tract [17], and aligns well with the current dogma in human medicine [69,74]. The difference in the

composition of the microbiota in the URT and LRT raises interesting questions in regard to the mechanisms of **colonization resistance** at different locations, and to the potential source of microbial communities along the respiratory tract. In healthy calves, the URT and LRT microbiota were characterized by the dominance of a *Mycoplasma* population followed by *Moraxella*, *Sphingomonas*, *Corynebacterium*, *Aggregatibacter*, and *Psychrobacter* in the URT, and *Pasteurella*, *Bacteroides*, *Mannheimia*, and *Ureaplasma* in LRT samples [72]. Similarly, in healthy people, the LRT and URT microbiota was dominated by the same populations, including *Staphylococcus*, *Moraxella*, *Streptococcus*, and *Haemophilus*, but lacked other typical microbiota of the URT, such as *Corynebacterium* [75]. Culture-independent microbial studies in the adult human have revealed that the oropharynx is the main source of lung microbiota [76], while, in neonates, the source of microbiota colonization is more likely to be both the URT and oropharynx [75]. To date, the role of the gut microbiota in LRT microbial community colonization has not been equivocally corroborated [76] and more work is needed in this area of study.

The composition of LRT bacterial communities is not uniform or consistent; there is substantial microbial variation between different lung fields [77]. At the present time, spatial microbial diversity in healthy cattle lungs is unknown but certainly warrants additional exploration because there is a clear anatomico-physiological predilection for respiratory disease in certain lung regions. Studies in sheep have suggested that the nature and extent of bacterial community variation between different anatomical locations in the lungs vary significantly between individuals [78]. This supports the notion that local physicochemical factors are significant in airway microbiota selection and in maintaining respiratory homeostasis (Figure 3). Understanding the nature and influence of

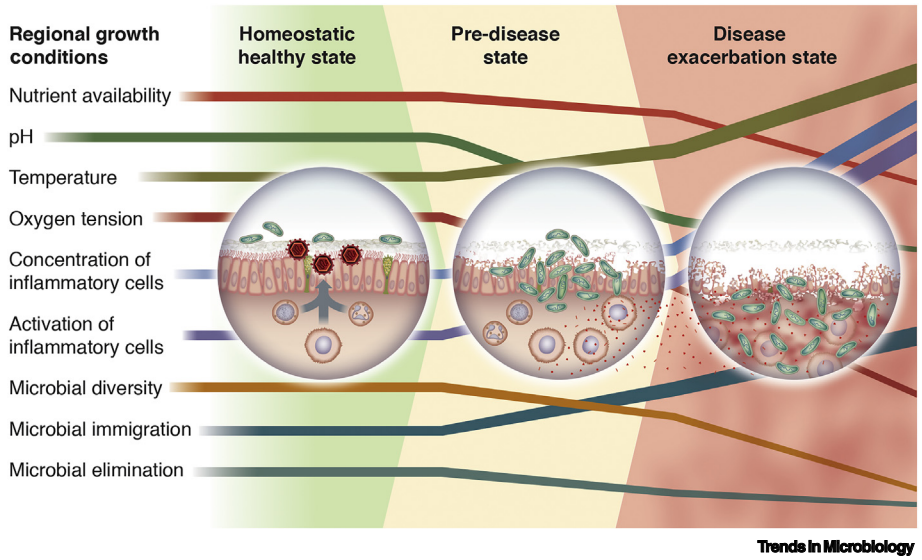

Figure 3. Hypothetical Model of Different Ecological Factors That Shape the Lower Respiratory Tract (LRT) Microbial Ecosystem. This figure shows the potential mechanistic explanations for the ecological determinant of LRT microbial dysbiosis and homeostasis. The composition of the LRT microbial ecosystem is determined by different factors, including local airway regional growth conditions, microbial immigration to the airway, microbial diversity, and microbial elimination from the airway. In the homeostatic healthy state, respiratory microbial communities are compartmentalized within the lumen through exclusion by the mucus and neutralization by the antimicrobial peptides. During the pre-disease state, the partial loss of mucosal barrier function results in bacterial dysbiosis, colonization of pathogens across the mucosal epithelium, release of proinflammatory cytokines, and chemokine activation. During the disease exacerbation state, loss of respiratory mucosal barrier function results in microbial translocation across the mucosal epithelium, impaired respiratory microbial clearance, exacerbation of respiratory inflammation, and loss of tolerance to the self-immune response. Line thickness emphasizes the direction of change.

these factors will prove essential in understanding the variability and functional relevance of the lung microbiota, and in designing strategies to optimize respiratory health. Together, these physiological and microbial factors can impact the regulation of respiratory microbiota towards a homeostatic ecosystem that, at equilibrium, is resistant to colonization by pathogens, or, conversely, an unstable ecosystem that favors infection. In the stable, homeostatic state, respiratory microbial communities are compartmentalized within the lumen through exclusion by the mucus and neutralization by the antimicrobial peptides. Individuals in this healthy state are highly resilient to perturbation (disruptions to homeostasis). The second or intermediate state is termed the 'pre-disease' state in which individuals are in an unstable state, sensitized to perturbation, and existing at the very edge of normal function. During the pre-disease state, partial loss of mucosal barrier function results in bacterial dysbiosis, colonization of pathogens across the epithelium, release of proinflammatory cytokines and chemokines, and activation of local immune cells. This can be viewed as a reversible state and a great place to intervene to restore normal health. The 'disease' state occurs when the unstable pre-disease state undergoes decline, or a downward transition, into clinical deterioration. During the disease state, there is often detectable mucosal damage and an accompanying loss of the respiratory mucosal barrier, which can result in microbial translocation across the epithelium. Individuals in this disease state are also in a somewhat stable condition, and can be considered as resilient and robust in their pathological state. As such, this state is not readily reversible. Together the epithelial damage and impaired respiratory microbial clearance, interfere with local immune regulation which thereby exacerbates respiratory inflammation, with a subsequent decline in to clinical disease.

The Role of Respiratory Microbiota Dysbiosis in BRD

BRD is a multifactorial syndrome involving many pathogens and is influenced by a combination of factors, including the host, the surrounding environment, and management practices [1,79,80]. Extensive research indicates that the primary microbial taxa involved in BRD are *Mannheimia haemolytica*, *Histophilus somni*, *Pasteurella multocida*, *Trueperella pyogenes*, *Mycoplasma bovis*, *Arcanobacterium pyogenes*, *Mycoplasma dispar*, *Ureaplasma diversum*, and *Mycoplasma bovirhinis* [81]. These microbial species are considered commensal inhabitants in the respiratory tract and are commonly found in both healthy and sick cattle [82]. Under specific conditions (e.g., mucosal, metabolic, or neuroendocrine stress and viral infections) these taxa can proliferate in the URT and invade the lung via inhalation [47,83]. While host immunity is important in controlling pathogenic overgrowth in the URT, recent evidence suggests that the respiratory microbiota is crucial in determining respiratory health and preventing colonization of respiratory pathogens 'colonization resistance' on the LRT mucosal surface [84]. The commensal microbiota directly inhibits the growth of bacterial pathogens, likely through the use of all available nutrients, modification of local environment, the invoking of antimicrobial molecule production, and modulation of mucosal inflammation. Several studies have revealed associations between the URT microbiota and BRD development in cattle [30,46,84,85]. Altered respiratory microbiota associated with BRD are shown in Table 2. This alteration further supports the theory that microbial dysbiosis relates to overall bovine health and demonstrates the need for future research to better understand these complex ecosystems.

According to previously published studies, BRD-affected calves experience dominance of a particular pathobiont or pathogen(s) [30,46,84]. This scenario is referred to as 'microbial domination', which is thought to be associated with eventual respiratory infection [86]. In BRD-affected calves the predominant bacterial populations in URTs were *Moraxella*, *Streptococcus*, *Haemophilus, Prevotella*, and *Neisseria* [84]. Other microbial species identified in BRD-affected calves included *Pseudomonas* [30], *Acinetobacter*, *Solibacillus*, and *Pasteurella* [46]. Differences in taxa between healthy and BRD-affected feedlot calves can be accompanied by variations in richness and

Table 2. Changes in the Respiratory Microbiota Associated with Bovine Respiratory Disease (BRD) in Cattle

Case definition of BRD	Sample collection	Main findings	Refs
BRD was defined when the animal showed ≥ one visual respiratory sign (depression, nasal and/or ocular discharge, cough, or dyspnea), abnormal lung sounds detected at auscultation, a rectal temperature ≥40°C, and a serum haptoglobin concentration ≥0.25 g/l.	Transtracheal aspirations and deep nasal swabs were collected from the animals that showed signs of respiratory disease and from pen-matched healthy control calves.	The main finding of this study was that the airway microbiota was clustered into four different meta-communities based on sampling location and health status. The trachea and nasopharynx of diseased claves were dominated by *Mycoplasma bovis, Mannheimia haemolytica*, and *Pasteurella multocida.* In contrast, the trachea of healthy steers was dominated by *Mycoplasma dispar, Lactococcus lactis* and *Lactobacillus casei*, while the nasopharynx of healthy steers was dominated by *Corynebacterium, Jeotgalicoccus, Psychrobacter*, and *Planomicrobium.* Additionally, some of the sick and healthy calves showed a high relative abundance of *Histophilus somni, Moraxella*, and *L. lactis* – but that was primarily detected in one feedlot. Based on alpha diversity analysis, there was lower bacterial richness and evenness in diseased calves compared with their healthy pen-mates.	[71]
BRD was defined when two or more of the following clinical signs were detected: cough, rectal temperature >39.5°C, respiratory rate >40 breaths/min, increased cranioventral lung sounds, or wheezes.	Deep nasopharyngeal swabs were collected at 14 and 28 days of life from calves that remained healthy throughout the preweaning period (n = 10) and calves that showed clinical signs of BRD preweaning (n = 6).	At the phylum level, there was a significant difference in Bacteroidetes relative abundance between groups. At the genus level, there were differences between groups for *Pseudomonas* spp. The most important genera related to respiratory diseases, such as *Mycoplasma* spp., *Mannheimia* spp., and *Pasteurella* spp., were detected, but with no statistically significant difference between the groups. At the species level, there was a significant difference between groups of calves in the relative abundance of *Pseudomonas fluorescens*. The relative abundance of some functional features tended to be numerically increased in samples from BRD calves compared with healthy calves. Analysis of resistance to antibiotics and toxic compounds revealed differences in cobalt-zinc-cadmium resistance between BRD and healthy calves.	[30]
BRD lesions were defined as macroscopic consolidation or abscessation of lung tissue present on the cranial lobes of the lung.	Cranial lung-lobe tissue samples were collected postmortem from beef calves (n = 32) and dairy calves (n = 6). Mediastinal lymph node tissue was also collected from 32 of these animals. In addition, 20 lungs (cranial lobe) and 20 corresponding mediastinal lymph node tissue samples were collected from clinically healthy calves.	The most abundant OTUs in the postmortem lungs and lymph nodes of the calves which died from BRD were classified as Leptotrichiaceae, *Mycoplasma,* Pasteurellaceae, and *Fusobacterium.* The most abundant OTUs in the postmortem lung tissue samples from dairy calves which died from BRD were classified as Leptotrichiaceae, *Fusobacterium, Mycoplasma, Trueperella* and *Bacteroides.* Leptotrichiaceae, *Mycoplasma* and Pasteurellaceae showed higher abundance in lymph node tissue samples collected from fatal cases of BRD in dairy calves, compared with clinically healthy calves without lung lesions.	[16]
BRD was defined when clinical signs of respiratory disease were detected (anorexia, nasal discharge, change in respiratory pattern, rectal temperature ≥40°C, and Whisper lung score ≥3).	Deep nasopharyngeal swabs were collected from any calves showing clinical signs of BRD, just prior to treatment (n = 22). In addition, clinically healthy, pen-matched controls calves (n = 10) were sampled at the same time.	At the phylum level, there was a statistically significant change in the relative abundance of Proteobacteria, Actinobacteria, and fusobacteria between the clinically healthy calves, and BRD affected calves. At the genus level, in BRD affected calves, there was a statistically significant predominance of	[46]

Table 2. (continued)

Case definition of BRD	Sample collection	Main findings	Refs
		Acinetobacter, Solibacillus and *Pasteurella.* Discriminant analysis revealed that the nasopharyngeal microbiota in feedlot calves at entry, and in BRD-affected calves, were also distinct from pen-matched healthy calves. There were no statistically significant differences in microbial diversity or richness between the groups.	
BRD was defined when two or more of the following clinical signs were detected in a calf: cough, rectal temperature >39.5°C, respiratory rate >40 breaths/min, increased cranioventral lung sounds or wheezes.	Deep pharyngeal swabs were collected from 174 Holstein heifer calves on days 3, 14, 28, and 35 of life.	The relative abundance of the bacterial genera *Mannheimia, Moraxella*, and *Mycoplasma* was significantly higher in diseased versus healthy animals, and the total bacterial load of newborn calves at day 3 was higher for animals that developed BRD than for healthy animals. The relative abundance of *Mannheimia* and *Moraxella* at day 14 in calves diagnosed with pneumonia was significantly higher when compared with healthy calves. Similar results were observed for *Mannheimia* and *Mycoplasma* at day 28. Bacterial diversity indexes did not differ significantly when comparing health statuses, regardless of the age time point.	[85]
Based on antibiotic treatment history, cattle were randomly selected for the current study. Cattle in the BRD group were diagnosed and treated by veterinarians. The specific criteria for BRD diagnosis were not mentioned in this study.	Nasopharyngeal swab samples were collected from approximately 5000 cattle upon entry to feedlots and again from the same animals at 60 days on feed. Feedlot calves were randomly selected and assigned to two groups: (i) those treated for BRD while placed in feedlots (n = 5), and (ii) the control group (not treated for BRD while placed in feedlots, n = 5).	At the phylum level, the relative abundance of Actinobacteria was lower in cattle treated for BRD. At the family level, there was a greater relative abundance of Lactobacillaceae, Micrococcaceae, Lachnospiraceae, Lactobacillaceae, and Bacillaceae in healthy cattle compared with BRD-affected cattle. Cattle diagnosed with BRD had significantly less bacterial diversity and fewer OTUs in their nasopharynx at both sampling times. All entry samples of cattle diagnosed with BRD had BRD-associated bacteria (*Mannheimia haemolytica or Pasteurella multocida*), although 3/5 healthy cattle were also positive for *M. haemolytica* at this time point.	[84]

diversity of URT microbiota that can be traced back to disparities at feedlot entry [46]. It is likely that multiple stressors associated with recently arrived cattle and the adjustment to the feedlot ultimately impact the mucosal immune status and respiratory tract microbial health [87]. Hypothetically, instability in the documented URT microbiota may explain why cattle are most susceptible to BRD during the first few weeks after feedlot arrival. Therefore, understanding the intrinsic and extrinsic factors that affect the postentry maturation of the respiratory microbiota will be important in determining whether management strategies can encourage and sustain the microbial ecology of the respiratory tract during this important and determinative production phase.

Potential Pitfalls in Respiratory Microbiome Studies

The use of sequencing technologies and bioinformatic analyses in microbiome research have illuminated the presence and importance of previously unidentified and unculturable microbial populations in the healthy and diseased mammalian respiratory tract and has allowed researchers to assess and compare the microbiota in different ecological niches. Despite the benefits of the available tools, the study of the respiratory microbiota presents unique technical challenges with key limitations which may distort the microbial composition and abundances

observed in sequence datasets [88]. One of these technical challenges is the real risk of contamination during sample handling and processing [89–91]. Contamination can originate from DNA sources other than the samples under study (contaminant DNA) or from DNA exchange between study samples (cross-contamination) [92]. Contaminant DNA can arise from many sources, including sample collection, laboratory equipment, DNA extraction kits, and amplification and sequencing reagents [93]. Cross-contamination can occur at multiple steps, including accidental DNA transfer to wrong tube or plates, aerosolization during pipetting, barcode cross-contamination, and residual amplicons from past sequencing runs [92]. Collectively, DNA contamination is dynamic and can seriously undermine cutting-edge work to identify and understand the importance of low-biomass respiratory microbiomes, particularly in the absence of uniform laboratory practices (sample-collection methods and storage, DNA-extraction methods, choice of 16S rRNA variable region, sequencing platforms, and bioinformatic pipelines) [34,94]. Additional sources of study unreliability and data inaccuracy in published experiments, that can potentially affect estimates of microbiota composition and frequency, are problems with amplicon length and the absence of mock communities in sequencing runs [95]. For instance, despite the need to detect low-level contaminants, most bovine respiratory microbiota studies have not reported using negative control samples in sequencing runs. This omission provides a source of uncertainty in studies reporting the presence of low-abundance, novel organisms in the respiratory tract. The risk of contamination during certain sampling methods of the respiratory tract also represent a technical challenge for microbiome studies. For instance, many published studies in the bovine field have used bronchoalveolar fluid samples to represent the LRT [17,96]. Passing an endoscope, bronchoscope, or bronchoalveolar sample tube through the URT introduces a substantial risk of sample contamination via carryover of pharyngeal microbiota [17] and yet it has been suggested that, despite the markedly divergent microbiota of the mouth and nose, the route of bronchoscope administration has no detectable influence on LRT microbiota [97].

In view of the significant, inherent technical difficulties associated with studying low-biomass microbiota, to ensure reliable and accurate sample analysis and data interpretation it is important to develop and utilize standard operating procedures (Figure 4) that can help to reduce all the possible sources of contamination, ensure the careful use of appropriate negative and positive controls during sampling and laboratory workflow, remove any artifactual **operational taxonomic units (OTUs)** postsequencing, and carefully interpret the contributions of any contamination during analysis. The combination of these risk-reduction measures will help researchers to continue to provide reliable, reproducible, and accurate data on microbial community profiles, and will enhance the validity and dependability of future respiratory microbiota research in cattle.

Concluding Remarks and Future Perspectives

Recent advances in sequencing technologies with culture-based techniques have opened up novel possibilities for characterizing the bovine respiratory microbiota and provide potential targets for investigating the associations between resident microbial communities and BRD development. Despite significant efforts being expended to discover these associations, this research field is only at the initial stages of revealing the mechanisms by which altered microbial communities disrupt respiratory homeostasis (see Outstanding Questions). The complexity of the respiratory ecosystem and methodological challenges have limited our understanding of the mechanisms of microbial colonization and symbiosis, and ultimately identification of the microbial contribution to respiratory mucosal health and resilience. Much of the work to date has analyzed the microbiota composition and diversity in the respiratory tract, and several authors have indicated that characterizing the airway microbiota functionally is the next step. In this

Outstanding Questions

What are the mechanisms of microbial acquisition and colonization in the LRT in cattle? It is necessary to understand the host's role in the selection of certain microbes and/or the abilities of the microbiota to communicate to the host's immune system and mucosal epithelium.

What is the functional potential of ubiquitous microbiota in the bovine respiratory tract? It is important to recognize that the functional role of ubiquitous microbiota in the respiratory tract will be specific to the ecological niche.

What is the directionality of BRD causation? In all available bovine respiratory microbiome studies to date, the respiratory microbiota in BRD-affected calves is altered, and it differed from that in the healthy control. It is still unclear whether the change in respiratory microbiota and colonization by pathogenic microbes result in respiratory disease or whether the altered respiratory tract environment and mucosal inflammation result in dysbiotic microbiota.

What are the consequences of other constituents within the respiratory microbiome (i.e., fungi, viruses, archaea) on the development of bovine respiratory disease? It is critical to understand how other members of the microbiome impact the development of bacterial infection.

What host factors drive alterations in microbiome composition during BRD infection? Screening for host genetic factors and immune response will likely be crucial for effective therapeutic strategy.

How can we overcome the technical challenges and limitations that impair the study of the respiratory microbiome? Developing reliable analytical methods that can reduce cross-contamination between samples and DNA contaminants will likely be crucial for the accurate estimation of microbiota composition and frequencies.

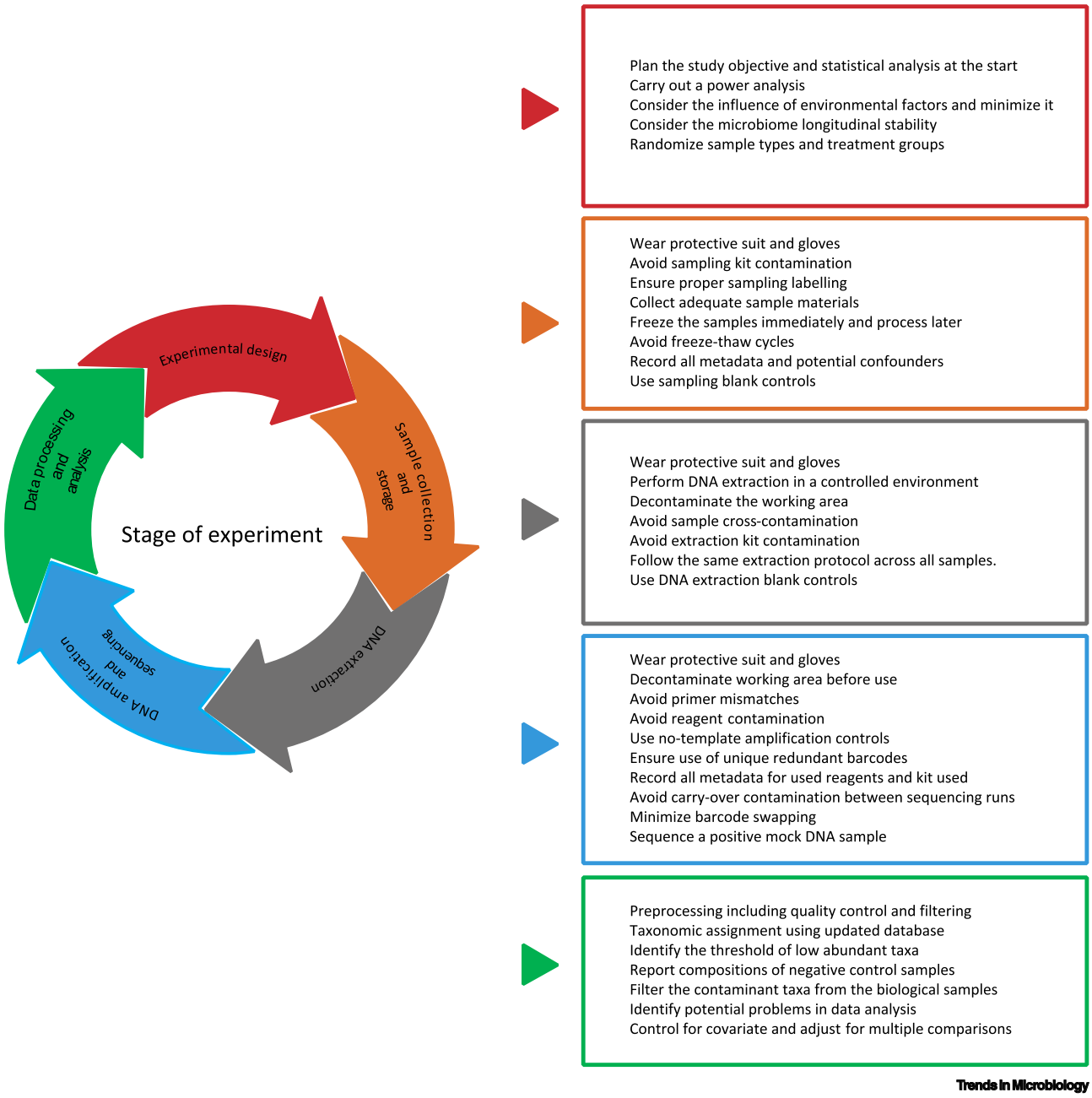

Figure 4. The Sequential Steps and Methods for Minimizing Contamination in Respiratory Low-Biomass Samples in a Microbiome Study.

context, 16S sequencing should be followed by complementary laboratory methods and approaches to verify the results before any definite conclusion can be made. Advanced multi-omic techniques [i.e., proteomic (proteins), transcriptomic (RNA), and metabolomic (metabolite) approaches] would provide further crucial insights into the role of airway microbiota in the respiratory health of cattle. Expanding shot metagenomic sequencing to target other members of the microbiome (e.g., viruses, archaea, and fungi) will also add further insight into how these elements

interact with respiratory microbiota. Finally, further research is required to uncover the ways in which respiratory tract microbial communities contribute to mucosal health and immune system modulation, which could lead to the development of appropriate alternative therapies in this field.

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