

Review

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Review

Preterm Infants' Airway Microbiome: A Scoping Review of the Current Evidence

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Abstract: The aim of this scoping review was to investigate and synthesize existing evidence on the airway microbiome of preterm infants, to outline the prognostic and therapeutic significance of these microbiomes within the preterm population, and identify gaps in current knowledge, proposing avenues for future research. We performed a scoping review of the literature following the Arskey and O'Malley framework. In accordance with our inclusion criteria and the intended purpose of this scoping review, we identified a total of 21 articles. The investigation of the airway microbiome in preterm infants has revealed new insights into its unique characteristics, highlighting distinct dynamics when compared to term infants. Perinatal factors, such as mode of delivery, chorioamnionitis, the respiratory support, and antibiotic treatment, could impact the composition of the airway microbiome. The 'gut-lung axis', examining the link between the lung and gut microbiome, as well as modifications in respiratory microbiome across different sites and over time, has also been explored. Furthermore, correlations between the airway microbiome and adverse outcomes, such as bronchopulmonary dysplasia (BPD), have been established. Additional research in neonatal care is essential to understand early colonization of infants' airways and explore methods for its optimization. The critical opportunity to shape long-term health through microbiome-mediated effects likely lies within the neonatal period.

Keywords: preterm infants; lung microbiome; airway microbiome

1. Introduction

The airway microbiome is defined as the collective genome of the microbial community that colonizes the respiratory tract [1]. Until recently, the infant airway was thought to be sterile. It is now known that the respiratory tract is colonized by microbiota, with reports of as many as 10-100 bacterial cells per 1000 human cells, but, at present, the normal composition of the neonatal respiratory microbiome is not fully understood [2]. The lung microbiome observed at birth undergoes changes during the first week of life, leading to the establishment of one or more dominant organisms by Day of Life (DOL) 7, which continue to evolve over the subsequent months of postnatal life. [3] There is limited understanding of the mechanisms governing the development and progression of the airway microbiome in premature infants. The environment around the fetus, including the amniotic fluid, placenta and vagina, has a distinct microbiome that could potentially be introduced into the newborn's respiratory tract before or during birth. While it was previously believed that infants delivered vaginally acquire maternal vaginal flora, and those born via cesarean section are primarily colonized with maternal skin flora, it now seems that all fetuses experience initial colonization during the prenatal period. [3,4] There are several factors that can influence the composition and development of the respiratory microbiome, especially in preterm infants. These factors include the mode of delivery, the environmental microbiome surrounding the fetus, early exposure to prenatal and postnatal antibiotics and steroids, sepsis, use of mechanical ventilation or other respiratory support devices, feeding and nutrition, and the concomitant intestinal microbiota

development [5]. Indeed, both the gut and the respiratory tracts share common embryonic origin, and there appears to be a connection between the development of the gut and lung microbiomes (gut-lung axis) [6,7]. The composition of the oral and lung microbiome may also be affected by the administration of oropharyngeal colostrum to the buccal mucosa of preterm infants [8–10].

The maturation of early life microbiota during the perinatal period plays a key role in shaping the immunological, neural and physiological development of infants, ultimately affecting their overall health [5]. Lungs and airways microbial colonization normally occurs in the prenatal or postnatal period and can be involved in normal immune and structural development. The interplay between commensal bacteria and lung epithelial cells, along with leukocytes, triggers the activation of toll-like receptors, leading to the stimulation of dendritic cells and the initiation of TH1- and TH17-mediated responses. This promotes normal immune tolerance. In contrast, disrupting or eliminating this normal commensal microbiota can potentially trigger atypical inflammatory reactions, which could contribute to the development of bronchopulmonary dysplasia (BPD) [3,5]. Airway dysbiosis could lead not only to the development of BPD, but also to a higher incidence of asthma and pneumonia in childhood. [11]

Considering all that has been said so far, the objectives of this review are to (1) research and summarize the available evidence regarding the airway microbiome of preterm infants, (2) describe the prognostic and therapeutic role of these microbiomes in the preterm population, (3) identify knowledge gaps in this topic and thus suggest considerations for future research. As such, we conducted a scoping review of the relevant articles in the subject of lung microbiome in preterm infants.

2. Materials and Methods

The study protocol of this review was developed following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Extension for Scoping Review (PRISMA-ScR) guidelines[12] and following the methodological guidance reported in Arksey and O'Malley and Levac 'papers[13,14].

The systematic literature search was performed in July 2023 (and updated in November 2023) on the following databases: Web of science, Cinahl, Ovid Medline, and Embase. Search strategies and keywords for each database are reported in **Supplementary Materials**. Additional studies were identified by screening the reference lists of the full-text screened articles.

Given that scoping reviews are made in order to “map”, summarize and clarify emerging evidence about a topic, they typically have broader inclusion criteria. Thus, we included in our review any type of human study (randomised controlled trial (RCT), prospective and retrospective cohort studies, case reports) reporting on airways microbiota of infants born prematurely (before 37 weeks' gestation) firstly samples during the neonatal period. We only included those studies using culture-independent molecular techniques and reporting at least one alpha- or beta-diversity metric or a microbial profile. We did not focus on any particular outcome, in order to get a broader view about the role of airways microbiota on the pathophysiology of prematurity, on preterm outcomes and possible therapeutic interventions. We included studies regardless of publication date. We excluded animal studies, ongoing studies and non-english written studies, narrative and systematic reviews.

Two authors independently screened all titles and abstracts obtained from the searches and assessed the full texts of all the potentially eligible studies. Conflicts were solved by discussion or by consultation with a third author. Afterward, two independent reviewers extracted study characteristics using a predefined charting form that was further refined. The relevant information extracted were author, year, country of publication, article type, study design, participant characteristics, type of microbiome, intervention, and outcomes. One author audited quality, the sources of evidence, and data charting to confirm the validity of the results.

3. Results

A total of 1123 references were retrieved through the search stage, instead 1 more eligible article was found by screening the reference lists, giving a final records count of 769 after duplicate removal.

After title, abstract and full-text screening, we included 21 studies, the characteristics of whom are reported in **Table 1**. Please see **Figure 1** for the flow diagram based on PRISMA.

The studies were published between April 2010 and November 2023. 8 studies were conducted in the US, 4 in China, 4 in the UK, 3 in Germany, 1 in Italy, 1 in Australia and 1 in Switzerland and New Zealand.

3.1. Airway microbiome differences between term and preterm infants

Some of the included studies described differences between the preterm and term infants' airway microbiota. Pattaroni et co-authors [15] demonstrated that gestational age has a strong effect on airway microbiota composition. In fact, studying tracheal aspirates of 26 preterm and 19 term-born intubated infants, they found that the samples could be divided into three microbiota clusters: one dominated by *Staphylococcus*, one by *Ureaplasma* genera and one characterized by a balanced composition including *Streptococcus*, *Neisseria*, *Prevotella*, *Porphyromonas*, *Veillonella* and *Fusobacterium* genera (the latter called "mixed"). Thus, they found that none of the samples collected from infants born before 30 weeks' gestation (22 babies) clustered into the mixed profile. Otherwise, the samples of term infants mostly fell into the 'mixed' cluster. In 2022 McDavid and colleagues [16] confirmed the postmenstrual age (PMA) as a central factor influencing mucosal microbiota. They conducted a longitudinal study evaluating the nasal and gut microbiome of 122 preterm and 80 full term infants from birth until 12 months of age, evaluating a total of 1748 nasal swabs, and they found that lower gestational ages (Gas) were associated with Community State Types (CST) dominated by *Staphylococcus*, that later diverged to more niche-specific taxa such as *Streptococcus* and *Corynebacterium*.

Similarly, Grier et al [17] showed that infants born prematurely and term-born are initially colonized by microbiota that exhibit a distinct profile, which then tend to converge towards a similar pattern around 50 weeks PMA.

3.2. Airway microbiome temporal and anatomical modification

Another aspect that the included studies focused on was to describe the longitudinal changes of the respiratory microbiota of the preterm infants in the different parts of the respiratory tract (nose, throat, trachea, lungs) and in relation to other anatomical sites (mouth, gut, skin).

Grier et al [17] in 2018 described and compared patterns of development of the nose, throat and gut microbiota from birth through the first year of life of 82 preterm and full term infants. They collected weekly samples from the preterm infants during hospital stay, monthly samples after their discharge and from full term neonates; in case of acute respiratory illness the sample was excluded. They clustered the samples into CSTs, which indicate approximate microbiota composition. Thus, they found that the CSTs of the three sampled sites are very different: the variability of taxa is low at the pharyngeal level (with a predominance of *Streptococcus* or *Staphylococcus*), increases at the nasal level (*Streptococcus* and *Corynebacterium* ranging from 5 to 50%) and is highest at the intestinal level (average abundance of each taxa of 1%).

These patterns change during the first year of life in a similar order in all infants, evolving from simpler to more diverse patterns within each body site. In particular, they showed how in the earliest stages after birth both gastrointestinal and respiratory microbiota contain high levels of *Staphylococcus*, which decreases beyond 39 weeks' PMA. Thereafter, a transition to anaerobic germs occurs at the intestinal level, while at the respiratory level an aerobic microbiota remains, dominated by *Streptococcus* (both in the nose and throat), with an emergence of *Corynebacterium*, *Alloiococcus*, *Moraxella* and *Veillonella* in the nose, and *Veillonella* and *Neisseria* in the throat.

A strong association between microbiota CSTs across body sites, in particular between CSTs of the nose and gut and taxa in the throat was shown by Grier et al [17]. As the association cannot be entirely explained by time and by direct transmission between different sites, they hypothesized a possible systemic coordination during early life development.

Similar observations were made by Gallacher and colleagues [16] regarding the temporal evolution of the microbiota. In 2020 they analyzed in a large prospective study a total of 1102 samples

(nasopharyngeal aspirates (NPA), tracheal aspirate fluids (TAF), bronchoalveolar lavage (BAL) and stool) from 55 mechanically ventilated preterm infants (median gestational age (GA) 26.0 weeks). They found that bacterial load was lower soon after birth (day 1-3) in the upper airways (with only 1.1% and 6.7% of TAF and NPA respectively successfully sequenced) than in the bowel (with 35.7% of success rate in stool samples). Bacterial load reached a peak around day 4-7 in BAL and TAF, slightly later (8-14 days) in NPA and stool. They showed Proteobacteria and Firmicutes being the most abundant phyla in all samples, with Tenericutes (*Ureaplasma* and *Mycoplasma*) also present in the respiratory samples. During the first month of life TAF and BAL microbiota pattern remained stable (with a relative abundance of Proteobacteria), instead Proteobacteria abundance in NPA and stool increased from 15-30% to 46-55% respectively. *Staphylococcus* was the dominant genus in nearly 30% of all samples. Nevertheless, they did not find any significant pattern over time and all anatomical sites sampled showed their own distinct pattern of bacteria community.

Another small prospective study conducted in 2021 [3] evaluated 80 samples of oral and tracheal aspirates from 42 intubated preterm infants. Oral samples were collected at DOL 3±1, tracheal samples at DOL 3±1 and 7±1. They found *Staphylococcus*, *Halomonas*, *Ureaplasma*, *Streptococcus*, *Bacillus*, *Nesterekonina*, *Schlegelella*, *Escherichia*, *Thermobacillus*, and *Acinetobacter* being the predominant general in overall oral and tracheal aspirates. Among samples, *Shlegelella* has been found to be statistically more abundant in the early tracheal aspirates than in early oral and late tracheal samples. No other differences were found in the relative abundance of the other genera. Lastly, alpha diversity in the trachea DOL 3 was statistically higher than that of saliva DOL3 and successively decreased to DOL 7, with the most prevalent genera approximating the early oral composition by DOL 7.

Some studies analyzed the microbial characteristics of the intestine and lung for exploring the mechanism of the gut-lung axis. Yang et al. [18] collected and analyzed stool and pharyngeal secretion samples on DOL 1 and on DOL 28 from 13 appropriate for gestational age (AGA) preterm infants. They observed that at DOL 1, there was no statistically significant difference in microbial composition between the intestine and pharynx, in contrast to what was observed on DOL 28. The pharyngeal microbiota was primarily composed of *Ureaplasma*, *Bacteroides*, and *Fusobacterium* on DOL 1, and *Streptococcus* and *Rothia* on DOL 28. In contrast, the gut microbiota predominantly consisted of *Unidentified Enterobacteriaceae*, *Ralstonia*, *Streptococcus*, *Fusobacterium*, and *Ureaplasma* on DOL 1, and of *Unidentified Clostridiales*, *Klebsiella*, *Unidentified Enterobacteriaceae*, *Enterobacter*, and *Streptococcus* on DOL 28. The microorganisms identified in both intestine and pharynx were *Ureaplasma* and *Fusobacterium* on DOL 1, and *Streptococcus* on DOL 28. Similarly, Young et al. [19] reported that microbiomes of different sites (oral and endotracheal secretions, breast milk and stool) in extremely preterm infants were most similar immediately after birth and diverged longitudinally over the first 60 days of life. Throughout the entire study period and across all sites, four dominant taxa were consistently noted: *Enterococcus*, *Enterobacteriaceae*, *Staphylococcus*, and *Escherichia*.

In 2023, Yao et al. [20] conducted a study on 10 twin pairs (20 cases) and 20 singleton preterm infants, collecting endotracheal suction after birth and stool samples from the first fetal stool. They found that the infants did not exhibit significant differences in gut or respiratory bacterial colonies, suggesting that the diversity and richness of the initially colonized bacteria were similar between twins and singletons. Bacterial colonies were more abundant and homogeneous in the gut than in the airways, as shown by alpha-diversity analysis; the dominant intestinal phylum and genera were *Proteobacteria* and *Acinetobacter* and *Comamonas*, respectively. In contrast, the most abundant phylum and genera in the airways were Firmicutes and *Streptococcus* and *Ralstonia*, respectively.

3.3. Airway microbiome and the immune-system

Among the studies we included in this scoping review, four evaluated the possible association between airway microbiome and local inflammatory response, other two investigated instead the connections with immunological system development and the risk of sepsis.

About inflammatory status of the airway, Gallacher et al [16] in 2020 measured IL-6 and IL-8 in all samples and found that they were undetectable in NPA and stool, instead they were present in high concentration in the lower airways samples (BAL and TAF) in which sequencing was successful

(sufficient bacterial load). Afterward, they evaluated temporal changes in the latter interleukin levels and showed that each patient has episodic increases of IL-6 and IL-8 corresponding to increased bacterial load in the samples within 24 hours and to the presence of predominant taxonomic unit such as *Acinetobacter*, *Enterobacteriaceae*, *Mycoplasma* and *Ureaplasma*.

One year later Brewer et al [3] quantified leukocyte activation by Polymerase Chain Reaction (PCR) in tracheal aspirates collected on DOL 3 of preterm infants, quantifying the expression of inflammatory genes (CD66, IL1b, ICAM1). They found a higher expression of IL1b in the samples with lower bacterial diversity. Instead levels of expression of the latter genes were not associated with the need for longer intubation. Furthermore, Lohman et al. [1] reported that there were significant differences in the levels of IL-1 β , IL-10, and tumor necrosis factor- α on endotracheal aspirate samples of premature infants exposed or not to chorioamnionitis. Levels of lipoteichoic acid (LTA) and lipopolysaccharide (LPS) were not statistically higher in infants exposed to chorioamnionitis, and also the levels of all other biomarkers did not differ significantly.

An interesting study by Wagner et al. [21] assessed the airway response in mechanically ventilated preterm infants by examining 12 proteins in tracheal aspirates collected at 7 days of age. Infants were categorized into three clusters based on protein profiles: Cluster 1 had high levels of most proteins, Cluster 2 showed a mix of high proinflammatory and low anti-inflammatory proteins, and Cluster 3 had low levels of all proteins. Associations were found between these biomarker clusters and antenatal factors. Additionally, subtle differences in airway microbiota were observed among the clusters, such that Cluster 3 infants (more exposed to pre-eclampsia) were more likely to be dominated by *Staphylococcus*, with a relative absence of *Ureaplasma*, and Cluster 1 (more exposed to antepartum haemorrhage) exhibited the highest levels of *Streptococcus* and *Gemella*.

Regarding sepsis, in a prospective multicenter cohort study, Dos Anjos Borges et al. [22], who analyzed the distribution of taxa in pharyngeal swabs between early onset sepsis (EOS) and non-EOS neonates, observed that taxa with higher relative abundance in non-EOS cases included common skin inhabitants such as *Staphylococcus epidermidis* or *Cutibacterium acnes*.

Lastly, a recent study [23] demonstrated that preterm and term infants' airway microbiota and immune system development are strictly correlated, constituting an age-independent microbiota-T cell axis. In particular, McDavid and co-authors found that early T cell phenotypic differentiation predicts later microbiota, and T cell functional maturation follows microbiota exposures. Thus, they explored abnormal developmental paths in newborn T cells and microbiota, revealing their association with respiratory issues in infancy. The study suggests that prenatal exposure to antibiotics or infections disrupts these trajectories, impacting respiratory microbial colonization and predicting the risk of respiratory compromise within the first year of life.

3.4. Airway microbiome and perinatal factors

A number of potential clinical determinants of the airway microbiome (such as mode of delivery, antibiotic exposure, sex, chorioamnionitis) were analyzed by many of the included studies. Gallacher et al [16] found that preterm infants delivered vaginally had a significantly higher abundance of Gram-negative genera (*Acinetobacter*) and *Pseudomonas* and *Mycoplasma* in TAF samples, and *Serratia* in NPA. Instead cesarean section gave increased *Staphylococcus* in NPA and TAF samples. *Acinetobacter* presence was significantly higher in infants recruited by one of the two recruiting sites which was having an outbreak of this bacteria, showing the great influence of the environment. No differences instead were noted between sex.

Similarly, Pattaroni et al [15] found that *Ureaplasma* genera was only seen in tracheal aspirates of vaginally born preterm neonates and in a single case of emergency cesarean section with membrane rupture, instead *Staphylococcus* was predominant in the samples of babies born by cesarean section. Moreover, they demonstrated that in the preterm population delivery mode has the highest impact (28%) on variability of the microbiota composition. Differently, in the term-born group this factor loses its effect and the postnatal age explains the 14% of microbiota variance. Rosenboom et al. [24] reported that the significant impact of birth mode on the airway microbiome at one week postpartum was no longer detectable one month postpartum.

In contrast, Lal et al. [25] highlighted that there were no statistically significant differences in the respiratory microbiome based on the mode of delivery (cesarean or vaginal delivery). Payne and colleagues [26] didn't find a correlation between the mode of delivery and the rate of isolation of *Mycoplasma* and *Ureaplasma spp*, but denaturing gradient gel electrophoresis (DGGE) revealed that microbes were more commonly found in nasogastric aspirate (NGA) samples from preterm infants delivered vaginally, compared to those delivered by cesarean section.

Tirone et al. [27] demonstrated that, while the vaginal microbiota of mothers with spontaneous Preterm Birth (SP^{PTB}) showed a significant difference in alpha diversity, marked by a decrease in *Lactobacillus* and an increase in *Proteobacteria* abundance, there were no significant differences in alpha and beta diversity between the neonatal bronchoalveolar lavage fluid (BALF) samples of SP^{PTB} group and the Medically Indicated Preterm Birth (MI^{PTB}) group. Regarding chorioamnionitis, also Lohman et al. [1] reported that there were no statistically significant differences in bacterial diversity in endotracheal aspiration samples from newborn with and without exposure to chorioamnionitis, although they noted a trend toward decreased diversity in those infants who had been exposed. In contrast, Lal et al. [25] noted a reduction in *Lactobacillus spp* expression in infants who were exposed to chorioamnionitis.

Grier and co-authors [17] reported that samples taken during antibiotic therapy had a global suppression of bacterial growth in TAF and NPA, but no differences in alpha diversity with respect to those taken when not on antibiotics. Lal et al. [25] observed that the airway microbiome did not differ between infants born to mothers who received prenatal antibiotics and those born to mothers who did not. In addition, sixty percent of the Lohmann et al [1] cohort received 48 hours of empiric iv ampicillin and gentamicin, and there were no significant differences in Shannon's index in the initial samples between infants receiving empiric antibiotics and those not, similarly as said by Pattaroni et al [15]. Also at 15 months of age, the influence of antimicrobial therapy was not detectable [24].

In one study [3] the administration of oropharyngeal colostrum (OPC) to intubated premature infants was not correlated to any differences on bacterial composition nor on inflammatory activity of oral and tracheal samples. Nevertheless, a delayed first administration was associated with a trend toward decreased alpha diversity and increased IL1b.

3.5. Airway microbiome functionality

Using the Predictive Functional Profiling of microbial communities (PICRUSt) which analyzes the functional metagenome based on marker gene data, researchers [17] tried to predict the functions of microbiota in the rectum, nose, and throat over a baby's first year. They analyzed top pathways in different community state types (CSTs) at each site. Early CSTs in all three sites showed a distinct function enriched in pathways like lipid, purine, and pyrimidine metabolism, crucial for neonatal growth and colonization. Energy metabolism in early and late CSTs was linked to the pentose phosphate pathway and later diversified with exposure to different diets. Two-component signal transduction pathways suggested increased communication within the microbiota community and with the host. Comparing functions in initial and later CSTs in the nose and gut revealed emerging pathways, indicating the development and diversification of microbial communities in response to environmental factors and changes in energy sources.

3.6. Airway microbiome and BPD

In our literature review, about half of the included studies examined the role of the respiratory microbiome in the development of BPD.

In 2010, Payne et al [26] described the airway microbiome of fifty-five preterm infants requiring mechanical ventilation by analyzing endotracheal and nasogastric aspirate (ETA, NGA) samples using a culture-independent approach, denaturing gradient gel electrophoresis (DGGE) profiling. In addition, they used species-specific PCRs for *Mycoplasma* and *Ureaplasma spp* and correlated microbial findings and perinatal risk factors to the development of BPD. They discovered that bacterial sequences associated with coagulase-negative *Staphylococcus species* (*S. haemolyticus* and *S. epidermidis*)

were the most prevalent. *Fusobacterium nucleatum* was the most frequently identified organism in NGA samples, while in ETA samples, it was *Staphylococcus haemolyticus*. Additionally, *Mycoplasma hominis* was detected through PCR analysis in 9%-13% of ETA samples, and *Ureaplasma spp* in approximately 50%. They noted a significant correlation between the identification of *Ureaplasma spp* on ETA samples and poor outcome for BPD. The presence of this microorganism was also correlated with a more prolonged period of mechanical ventilation.

A small observational study performed by Mourani and colleagues [28] in 2011 investigated the bacterial composition of the respiratory tract using pyrosequencing in 10 preterm infants (gestational age < 28 weeks, birth weight 500-1250 g) who had been mechanically ventilated for at least 21 days and later developed BPD. They collected tracheal aspirate samples from each patient at 4 time points (within 72 hours of birth, and at 7, 14, 21 days of life). Mourani et al. found that in the first 72 hours after birth, the majority of tracheal aspirates (8/10 samples) had an undetectable bacterial load. Within the first week of life, all samples collected contained a detectable bacterial load (>70 copies/reaction) and all were successfully amplified for bacterial identification. Almost all samples (96.9%) with bacterial detection had a dominant organism, most commonly either *Staphylococcus spp* and *Ureaplasma spp*. *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Escherichia coli* were the other dominant organisms identified in tracheal aspirate samples. Bacterial load, Shannon's alpha-diversity index and evenness were not associated with BPD severity or infant age. Stressman et al. [29], in a study regarding fourteen endotracheal secretions samples from eight mechanically ventilated preterm infants at risk of developing BPD, reported *Staphylococcus aureus*, *Enterobacter spp*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa*, *Streptococcus spp*, and *Ureaplasma spp* as the most common bacterial sequences. Due to the limited sample size (3/8 infants with BPD), they were unable to make a comparison of the airway microbiota between infants with and without BPD.

Also Wagner et al. [30] in 2017 examined the relationship between the airway microbiota and the severity of BPD in mechanically ventilated preterm infants (gestational age \leq 34 weeks, birth weight 500-1250 g) using 16S rRNA sequencing. Microbiome analysis was performed on tracheal aspirate samples of 152 infants (51, 49, and 52 with mild, moderate, and severe BPD, respectively) collected at enrollment (intubation), 7, 14, and 21 days of age. The cross-sectional analysis conducted on 7 days of life samples did not reveal a significant correlation between microbiome composition and the subsequent severity of BPD. *Staphylococcus* (68%) and *Ureaplasma* (18%) spp were predominant in the microbiome, but Shannon Diversity, bacterial load, and the relative abundance of individual taxa showed no strong associations with BPD status. In contrast, a longitudinal analysis of airway microbiome composition over time demonstrated that preterm infants who eventually developed severe BPD experienced greater turnover in bacterial communities with age, acquired less *Staphylococcus* in the initial days after birth, and had higher initial levels of *Ureaplasma spp*.

On the other hand, Xu et al. [31] demonstrated that the Shannon index of tracheal aspirate was significantly lower at birth (Day 1) and on Day 7 after birth in premature infants with BPD compared to those without BPD. The disparity was more pronounced on Day 1 and exhibited a negative correlation with the severity of BPD. Additionally, they observed a significant difference in the bacterial composition of the airway microbiome at birth among the four groups (mild BPD, moderate BPD, severe BPD, non-BPD), with a less distinct difference on Day 7 after birth. At the phylum level, Proteobacteria dominated the airway microbiome of all infants on day 1 and 7, with no significant differences in composition among the four groups. However, at the genus level, the composition of the airway microbiome differed significantly among the four groups on day 1. *Stenotrophomonas* was more abundant in BPD compared to non-BPD, and its abundance positively correlated with the severity of the disease.

Lohman et al. [1] were the first to analyze the airway microbiome of premature infants and investigate its correlation with bacterially mediated inflammation and the onset of BPD through 16S rRNA sequencing. They enrolled twenty-five patients with gestational age ranging from 24 to 32 weeks who underwent endotracheal intubation and mechanical ventilation in the first 24 hours of life; ten of them developed BPD. Longitudinal tracheal aspirate samples were collected at the time of intubation (within 24 hours of delivery) and at days of life 3, 7, and 28, if the infants were still

intubated at those time points. Lohman and colleagues found that infants with BPD had significant differences in airway microbiome diversity compared to infants without BPD. Specifically, at the time of intubation, premature infants who developed BPD had lower bacterial diversity than those who did not develop BPD, as estimated by the number of observed species and the Shannon diversity index. This finding is supported by Tirone et al. [27] who also observed a slight reduction, although not statistically significant, in Shannon diversity index in BALF samples from infants with BPD. Tirone and colleagues did not identify a characteristic microbiological signature linked to BPD development at either the genus and phylum level. In contrast, Lohman et al. [1] discovered distinct microbiome evolution patterns between infants with BPD and those without BPD, as revealed by their phylum-level analysis. The airway microbiome of infants with BPD exhibited an elevated presence of Firmicutes phyla alongside a reduction in Proteobacteria phyla, whereas infants without BPD showcased a comparatively diverse and stable microbiome. While *Acinetobacter* remained the predominant genus in both groups, its relative abundance decreased over time in the BPD group, with a concurrent rise in *Staphylococcus* and *Klebsiella* spp.

Lal et al. [25] also conducted a study on the airway microbiome using 16S rRNA sequencing. They analyzed tracheal aspirate specimens obtained from extremely low birth weight (ELBW) and full term (FT) infants who underwent intubation and mechanical ventilation within the first six hours of life (due to respiratory distress syndrome, asphyxia and prenatal depression, or surgical indications). Tracheal aspirate samples were collected at the time of intubation and subsequently whenever tracheal suctioning was clinically indicated. The findings of this study indicated that the airway microbiota in infants with BPD showed decreased diversity and abundance (both alpha and beta diversity) and differed significantly from that of preterm infants soon after birth and full term infants at a similar post-menstrual age. In contrast to Lohman et al.'s study [1], preterm infants with BPD exhibited an airway microbiome characterized by increased Proteobacteria and decreased Firmicutes and Fusobacteria compared to newborn full term infants matched for PMA. Among Proteobacteria, the most abundant in infants with BPD were *Enterobacteriaceae*. *Lactobacillus* was found to be statistically significantly less abundant in the early airway microbiome of infants who later developed BPD, but the overall abundance of *Lactobacillus* was low. Furthermore, Lal et al. reported that the airway microbiome of both ELBW and FT infants at birth showed a predominance of Firmicutes and Proteobacteria on the first day of life, along with the presence of Actinobacteria, Tenericutes (including *Ureaplasma* spp), Bacteroidetes, Cyanobacteria, Fusobacteria, and Verrucomicrobia. During the first 60 days of life, they noted a decrease in Firmicutes and increase in abundance of *Proteobacteria* in the respiratory tract of ELBW.

In the study of Brewer et colleagues [3] the decrease in bacterial alpha diversity in trachea samples of intubated premature infants on DOL 3 to 7 was not associated with the diagnosis of BPD. Neither timing of OPC administration influenced the incidence of BPD.

Concerning the nasal microbiome in premature infants and its correlation with the development of BPD, we have identified a recent study conducted by Xu et al. [32] in 2022. This prospective observational study involved 28 premature infants (gestational age ≤ 30 weeks and birth weight 700-1550 g), divided into 13 infants with BPD and 15 controls. PCR amplification and 16S rDNA sequencing were performed on nasal swabs collected from the anterior nares at 1 and 3 weeks of postnatal age (5-7 DOL and 15-21 DOL, respectively). Xu and colleagues did not find a difference in nasal microbial diversity between preterm infants with BPD and controls. They identified Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria as dominant phyla, and *Muribaculaceae*, *Escherichia*, *Staphylococcus*, and *Lachnospiraceae* as dominant genera in the nasal microbiome of all groups. The expression of *Prevotella* increased, and *Caulobacter* decreased in the BPD group at both time points. *Prevotella* and *Caulobacter* were correlated with the severity of BPD. Additionally, *Prevotella* increased in infants on invasive mechanical ventilation, while *Caulobacter* decreased at both week 1 and week 3.

Recently, Selway et al. [33] discovered that the microbial diversity observed on buccal swabs during the first week of life (PT1) in preterm infants who developed BPD was lower compared to those infants who did not develop BPD, with higher relative abundance of *Corynebacterium* (11.3% in

BPD-patients, 0.9% in not-BPD). Furthermore, they analyzed the diversity and composition of the microbiome in tracheal aspirate from premature infants requiring intubation. The most dominant amplicon sequence variants were identified as *Paenibacillus*, *Staphylococcus*, *Erythrobacteraceae bacterium K-2-3*, *Streptococcus anginosus*, *Ureaplasma*, *Burkholderiaceae*, and *Streptococcus agalactiae* sequences. *Ureaplasma* has been associated with an increased risk of developing BPD and was also identified as dominant taxa in buccal swabs (PT1) from infants with BPD who did not receive intubation.

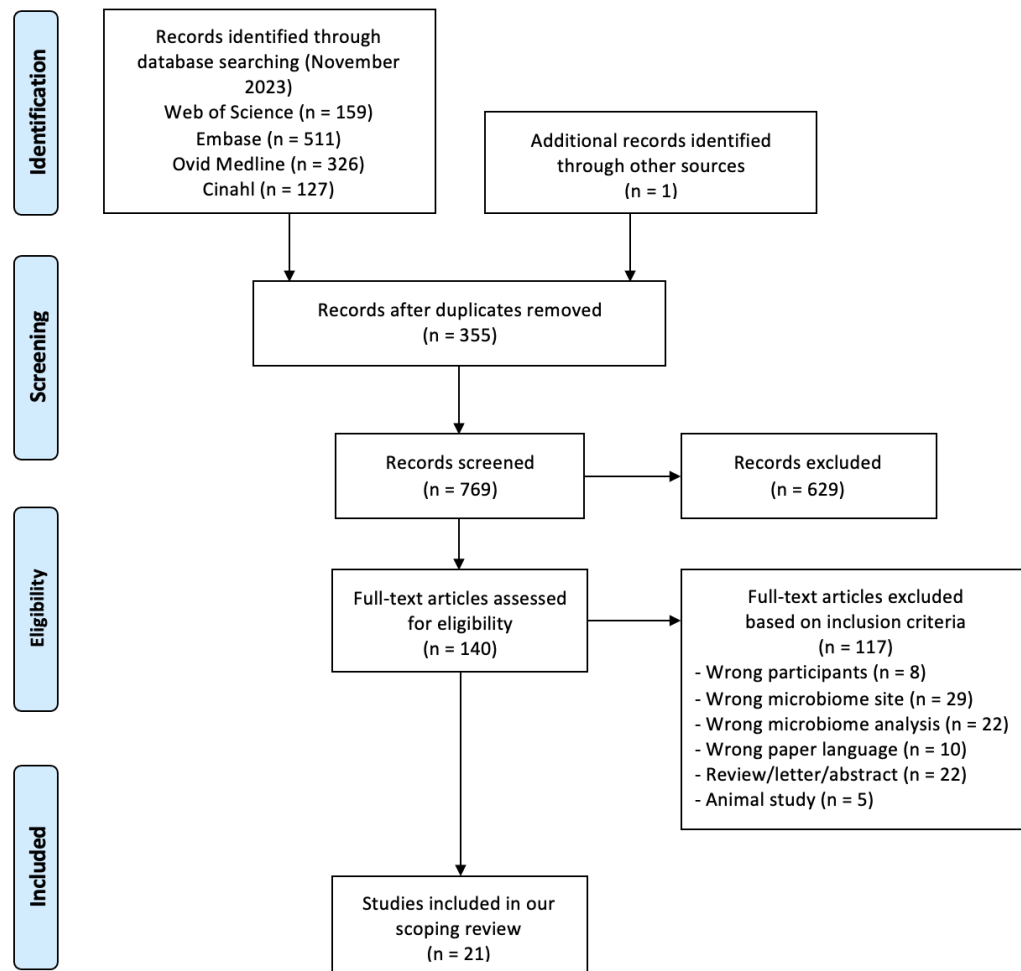


Figure 1. PRISMA 2009 research method flow diagram.

Table 1. Characteristics of included studies.

Study	Country	Population	Microbiome site	Aim
Brewer 2021 [3]	USA	GA \leq 32 wk, birth weight (BW) < 2000 g and intubated for respiratory distress syndrome	Tracheal aspirates (TA) on days 3-7, oral aspirate DOL 3	Oral vs tracheal microbiota during the first week of life, effects of OPC administration on microbial diversity or leukocyte inflammatory activity in the lung
dos Anjos Borges 2023 [22]	Germany	GA \leq 34 wk born from mothers with preterm premature rupture	vaginal swab - umbilical cord blood (UC), rectal swabs (RE), and pharyngeal swabs	Correlation between vaginal PPRM mothers' microbiome and pharyngeal/umbilical cord/meconium/rectal neonatal microbiota

Study	Country	Population	Microbiome site	Aim
		of membranes (PPROM)	(PH) at delivery-meconium sample prior to 48 h of life	
Gallacher 2020 [16]	UK	GA \leq 32 wk and intubated	TAF, BAL and NPA daily during the first week then twice weekly or until extubation, stool weekly until 28 DOL	Temporal modification of bacterial communities, gut-lung axis. Association between the bacterial communities and lung inflammation.
Grier 2018 [17]	USA	Preterm and full-term infants	Rectal, nasal, throat swabs weekly during admission, monthly after discharge until 1 year of corrected age (CA)	Temporal modifications of the early microbiota and associations of microbiota across body sites. Differences between term and preterm infants' microbiota.
Lal 2016 [25]	USA	ELBW preterm infants, full-term infants	TA at intubation and at clinically indicated tracheal suctioning	Differences in preterm and FT infants' airway microbiota and association with risk of developing BPD
Lohman 2014 [1]	Texas, USA	GA \leq 32 wk and intubated in the first 24 h	TA at intubation and 3, 7, and 28 DOL	BPD
McDavid 2022 [23]	USA	GA 23-42 wk	Nasal swab or rectal swab weekly or monthly in the first year of life	Correlation between immune system, microbiota and respiratory morbidity
Mourani 2011 [28]	Colorado, USA	GA \leq 34 wk, BW 500–1250 g, mechanical ventilation (MV) for at least 21 days	TA within 72 hours of birth, and 7, 14, and 21 DOL	BPD
Pattaroni 2018 [15]	Switzerland and New Zealand	GA 23-41 wk with endotracheal intubation for elective surgery or respiratory support	TA at postnatal age 1 day - 1 year	To identify the early-life bacterial colonization pattern of the lower airways in relation to the maturing immune system during the first year of life.
Payne 2010 [26]	UK	BW < 1300 g requiring MV for at least 24 h	Gastric fluid aspirate, Endo-TA (ETA) within 24 hours of life	BPD
Rosenboom 2023 [24]	Germany	GA 24-33 wk, full-term infants	Oropharyngeal swabs at week 1 of life, 1-9-15 months of life	Longitudinal development of the airway metagenome of preterm infants during the first 2 YOL.

Study	Country	Population	Microbiome site	Aim
Selway 2023 [33]	Australia	Preterm infants, full-term infants, adult	Buccal swab 2-12 DOL and 36 wk GA, TA at intubation	Compare preterm-term infant-adult microbiome and BPD/sepsis
Stressman 2010 [29]	UK	GA \leq 31 wk, intubated at birth and received surfactant therapy	ETA in the first week of life	to investigate the use of culture-independent molecular profiling methodologies to identify potential etiological agents in neonatal airway secretions
Tirone 2022 [27]	Italy	GA \leq 30 wk intubated in the first 24 hours of life and their mothers	Mothers' vaginal swab, newborns' BAL fluid at 1-3-7 DOL and meconium specimen	Relationship between maternal vaginal microbiota and neonatal lung and intestinal microbiota; association between neonatal lung/meconium microbiota and BPD
Xu 2022a [31]	China	GA $<$ 34 wk and intubated in the first 24 hours	TA day 1 and 7	Mild, moderate, severe BPD and no BPD
Xu 2022b [32]	China	GA \leq 30 wk	Nasal swab 5-7 DOL and 15-21 DOL	BPD
Yang 2021 [18]	China	GA 26-32 wk	Stool samples and pharyngeal swabs 1-28 DOL	Gut-lung axis
Yao 2023 [20]	China	GA 32-35 wk	Endotracheal suction at birth and stool specimen from the first fetal stool	Diversity and community structure in the bacterial microbiome of the airways and intestines of preterm twin and singleton neonates
Young 2020 [19]	UK	GA $<$ 26 wk	Oral and endotracheal secretions, stool, and breast milk over the first 60 days of life	Characterize the development of microbiota
Wagner 2017 [30]	USA	GA \leq 34 wk and BW 500-1250 g and MV	TA at enrollment, 7, 14, and 21 DOL (+/- 48 hours)	BPD
Wagner 2019 [21]	USA	GA \leq 34 wk and BW 500-1250 g and MV	TA at 7 +/- 48 h DOL	Relationship between prenatal events, postnatal airway host response and microbiota, and clinical outcomes

4. Studies Excluded after Full-Text Screening

Quite a few articles were excluded after the full text screening because the analysis of the airway microbiome was performed without using culture-independent molecular techniques and without reporting at least one alpha- or beta-diversity metric or a microbial profile [34]. Similarly, several studies were excluded because they exclusively analyzed the presence and quantity of specific organisms in samples from the airways, subsequently correlating them with certain outcomes, without examining the respiratory microbiota as a whole [35,36].

Three studies were excluded, although they were relevant to our scoping review, because the full text was not available, or they were just congress abstracts. (1) The aim of the study by Gallacher and colleagues [37] was to investigate the microbial colonization of the lungs in intubated preterm infants at risk of developing BPD, and to correlate these findings with pro-inflammatory cytokines, through 16S rRNA sequencing on bronchoalveolar lavage samples. (2) Deshpande et al. [38] studied the gut and tracheal microbiome in preterm infants requiring mechanical ventilation who received probiotics. They showed that routine probiotic administration may alter the respiratory microbiota, and consequently respiratory outcomes in preterm neonates. (3) Gorlanova et al. [39] compared nasal microbiota of preterm and term infants at mean 45 weeks postmenstrual age and evaluated the impact of environmental factors on the nasal microbiota in preterm infants, reporting that nasal microbiota composition is associated with preterm birth.

We had to exclude from our literature review, another interesting study [40] on the nasopharyngeal microbiota in preterm infants from birth until six months of age and the impact of antibiotics and hospitalization on it, as only the pre-print and no-peer reviewed version was available.

Several studies were excluded because, despite focusing on premature infants' airway microbiota, the samples used to characterize the microbiome were collected at non-neonatal age. Particularly, (1) Rofael et al. [41] characterized the airway microbiome in young adults born extremely premature, both with and without neonatal BPD, in comparison to matched term-born controls. They conducted gene sequencing on induced sputum from 92 individuals aged 19 years belonging to the EPICure study (51 extremely preterm and 41 controls) and reported a significant dysbiosis in the airway microbiome associated with extremely preterm birth, which correlated with forced expiratory volume in the 1st second (FEV1). (2) Perez et al. [42] conducted a study on the nasopharyngeal microbiome of preterm and full-term infants aged between 6 months and 2 years. The taxonomic profile obtained in samples from preterm infants differed slightly from the one of the full-term controls. They found *Moraxella* and *Burkholderia* being predominant, mixed with *Streptococcus*, *Neisseria*, *Staphylococcus*, *Janthinobacterium*, *Haemophilus* and the within-group heterogeneity very high. In full-term instead, *Streptococcus* was predominant, with low dissimilarities within-group. At the phylum level, preterm infants showed an increase in Proteobacteria (large group of Gram-negative bacteria) and a decrease in Firmicutes (Gram-positive bacteria such as *Clostridium* and *Lactobacillus*). To assess the functionality of the respiratory microbiota in modulating airway inflammation and respiratory symptoms during viral infections in preterm infants, samples were obtained during acute infection and at a follow-up visit. Upon comparing the paired samples, Perez and colleagues found that the preterm airway microbiota was less resilient during rhinovirus (RV) infection, exhibiting a high within-group dissimilarity in preterm infants at baseline, which gets higher in RV samples. This suggests an "unstable" microbiome prone to widespread changes in bacterial diversity during minor viral illnesses.

5. Discussion

The exploration of the airway microbiome in preterm infants has recently emerged with novel insights into its distinctive characteristics, contrasting dynamics with term infants, its correlation with adverse outcomes such as BPD, and thus potential therapeutic interventions.

About the distinctive features of preterm infants' airway microbiome, our analysis highlights notable differences between the airway microbiota of preterm and term infants. Studies consistently report that gestational age plays a pivotal role in characterizing the early microbial colonization [15–

17,23]. In fact, gestational age at birth, rather than postnatal age, appears to influence the characteristics of the airway microbiota at birth. Preterm birth at lower gestational ages seems to result in reduced diversity in the microbiota, which is dominated by *Staphylococcus*. Later it has been noted a convergence of microbiota profiles around 50 weeks PMA in both preterm and term-born infants, suggesting a developmental trajectory towards a shared pattern [17]. However, significant disparities persist during the early stages, emphasizing the influence of prematurity on the initial microbial landscape.

Many studies found an association between nearest and farthest anatomical sites of sampling microbiome and found strong interconnections [3,17–20]. Similarly, a recent scoping review [43] provides valuable insights into the oral microbiome of preterm infants, complementing our focus on the airway. The review emphasizes the interconnectedness of oral and airway microbiomes, hinting at potential seeding reservoirs for respiratory microbes. This interplay adds a layer of complexity to our understanding and underscores the need for a comprehensive approach in studying the microbial landscape of preterm infants.

Our scoping review, in addition to broadening the scope of analysis, updates the systematic review by Pammi and colleagues [2], that examined available evidence up to 2017 regarding the airway microbiome and BPD in preterm neonates. In our study, a significant portion of the reviewed literature delves into the association between the airway microbiome and the development of BPD in preterm infants. Findings collectively suggest a complex interplay between microbial diversity, composition, and the risk of BPD. Notably, infants diagnosed with BPD exhibit distinct microbial evolution patterns, marked by lower diversity at the time of intubation [1,25,27,31] and variations in phylum-level abundance [1,26,30,33]. The prevalence of specific genera, such as *Staphylococcus* and *Ureaplasma*, stands out as potential indicators of BPD risk. The observed microbial signatures offer valuable insights into the intricate relationship between early microbial colonization and the subsequent respiratory health of preterm infants. The potential impact of dysbiosis in determining an outcome such as BPD is supported by other longer-term or broader-spectrum evidence, such as the evidence of dysbiosis correlated with reduced FEV1 in preterm adults [41], or associated with cystic fibrosis, lower respiratory tract infections and asthma [44,45].

Understanding the impact of perinatal factors on the airway microbiome is crucial for contextualizing our findings. Delivery mode emerges as a key determinant, with vaginally born preterm neonates displaying different microbial profiles compared to those delivered via cesarean section. The influence of antibiotics and antifungals on microbiota composition, although explored to a varying extent across studies, adds another layer of complexity. Interestingly, our review did not reveal consistent patterns regarding the effect of antibiotic exposure on microbial diversity, suggesting multifaceted interactions between antibiotics, microbial communities, and respiratory outcomes.

Identifying potential therapeutic interventions to modulate the airway dysbiosis in preterm infants emerges as a critical avenue for further research. Current evidence suggests a correlation between early T cell phenotypic differentiation, microbiota exposures, and the risk of respiratory compromise. This age-independent microbiota-T cell axis highlights the potential impact of prenatal exposures, such as antibiotics or infections, on disrupting developmental trajectories. Exploring interventions to manipulate the airway microbiome, such as probiotics or targeted antibiotics, could offer avenues for mitigating the risk of adverse respiratory outcomes. However, the intricate balance required for such interventions necessitates careful consideration, given the evolving nature of the microbiome during early life.

In conclusion, our comprehensive review sheds light on the nuanced dynamics of the airway microbiome in preterm infants, emphasizing its role in influencing respiratory outcomes, unraveling the complexities of microbial colonization, understanding its correlation with BPD, and identifying therapeutic strategies represent crucial steps towards improving the long-term respiratory health of preterm infants.

6. Limitations, Knowledge Gaps, and Directions for Future Research

There were some limitations to the studies that we reviewed.

In the examination of endotracheal aspiration across multiple studies, with the exception of one [20], the airway microbiota was consistently sampled through endotracheal tubes. Consequently, the ability to draw conclusions concerning the evolution of the airway microbiome in free-breathing infants is limited (such as in preterm infants on continuous positive airway pressure or with a high-flow nasal cannula). An additional concern lies in the potential for endotracheal tube aspiration to overemphasize bacteria that colonize the endotracheal tube itself, rather than accurately representing the microbial composition of the broader airway or lungs. This introduces a potential bias in the interpretation of the airway microbiome dynamics in the studied population.

Another potential limitation concerns studies that analyze the microbiome in correlation with the development and severity of BPD. On one hand the definition of BPD is still controversial and therefore, the use of different definitions may compromise the comparison of results across various studies. On the other hand, although significant, the clinical studies mentioned earlier are inherently observational, thus they do not furnish any evidence supporting a causal relationship between alterations in the airway and lower respiratory microbiomes and the clinical course of affected infants. For this reason, recently there has been an emerging interest in preclinical studies analyzing the role of microbiome in lung development [46]. One of the latest studies for example confirmed the role of airway dysbiosis on the risk of developing more severe BPD phenotype by influencing the expression of local antioxidant response, which could be ameliorated by supporting colonization with *Lactobacillus* [47]. Thus, more studies are mandatory on this argument to better understand targets of future microbiome-based therapeutics for respiratory disease.

In the analysis of the neonatal respiratory microbiome, advancements such as the utilization of 16S rRNA sequencing, a method employed in the majority of included studies, have significantly enriched our understanding. However, it is crucial to acknowledge a limitation in these methodologies, as they do not distinguish between viable and dead bacteria [3]. On the other hand, a metagenomic approach employing shotgun sequencing offers increased granularity in sample composition analysis. This approach enables the identification of bacterial species or strains and provides information on antimicrobial resistance mutations. Further, RNA metagenomic profiling can offer additional insights into microbial activity and the metabolism of microbiomes [48,49]. Future studies on the newborn airway microbiome that integrate meta-taxonomic methods (such as 16S rRNA targeted sequencing) and metagenomic approaches would yield additional evidence, potentially enhancing treatment approaches for adverse respiratory outcomes, such as BPD and asthma [44].

The evolving landscape of research in preterm infants' airway microbiome presents exciting opportunities for innovative investigations and potential therapeutic interventions. Characterizing the lung microbiome in premature infants opens avenues for novel treatments aimed at preventing dysbiosis. Strategies such as oral or inhaled probiotics, prebiotics, dietary manipulation, judicious use of antibiotics, therapies targeting proinflammatory bacteria, and the development of bacterially derived drugs for inflammation modulation and bacterial overgrowth are all promising areas for exploration. Recognizing that bacterial colonization likely initiates in-utero, addressing maternal dysbiosis through probiotics and prebiotics during pregnancy emerges as a prospective strategy. Moreover, delving deeper into the gut-lung axis and exploring the role of extra-pulmonary microbes in respiratory disease development stands as an imperative avenue for further investigation [50,51].

This holistic approach to understanding and influencing the airway microbiome in preterm infants holds great potential for advancing both preventive and therapeutic interventions in neonatal respiratory health.

7. Conclusions

The exploration of the airway microbiome in preterm infants represents a dynamic and evolving field with far-reaching implications for neonatal health. The synthesis of current literature highlights the intricate interplay between microbial colonization, respiratory outcomes, and potential

therapeutic interventions. As we navigate through the complexities of the preterm airway microbiome, it becomes evident that future research should delve deeper into the mechanistic underpinnings of dysbiosis and its impact on respiratory health. Addressing methodological challenges, incorporating advanced technologies, and fostering collaborative, multi-center studies will be essential in advancing our understanding. The promising avenues of intervention, from probiotics to targeted antibiotic therapies, underscore the potential to shape and modulate the airway microbiome for improved neonatal outcomes. With these insights, the trajectory of research in preterm infants' airway microbiome not only contributes to the refinement of clinical practices but also lays the foundation for personalized and effective strategies in promoting respiratory health in this vulnerable population.

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