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MINI-REVIEW | *Translational Physiology*

Recent advances in understanding the ecology of the lung microbiota and deciphering the gut-lung axis

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Willis KA, Stewart JD, Ambalavanan N. Recent advances in understanding the ecology of the lung microbiota and deciphering the gut-lung axis. *Am J Physiol Lung Cell Mol Physiol* 319: L710–L716, 2020. First published September 2, 2020; doi:10.1152/ajplung.00360.2020.—A rapidly expanding new field of lung research has been produced by the emergence of culture-independent next-generation sequencing technologies. While pulmonary microbiome research lags behind the exploration of the microbiome in other organ systems, the field is maturing and has recently produced multiple exciting discoveries. In this mini-review, we will explore recent advances in our understanding of the lung microbiome and the gut-lung axis from an ecological perspective.

bacteria; bronchopulmonary dysplasia; fungi; microbiome; mycobiome

INTRODUCTION

Pneumonia has been one of the oldest foes of mankind and continues to be a deadly threat to the current day. Since 1881, when Louis Pasteur and George Sternberg simultaneously discovered *Streptococcus pneumoniae* (8), the isolation of microbial organisms from the lung has been durably linked with disease in the minds of clinicians and researchers. Pulmonary homeostasis has therefore been assumed to demand sterility.

However, the healthy lung is not sterile. Microbes live in the most extreme environmental niches on earth and likely did so long before humans existed, so it should not be surprising that they can live in the relatively less challenging environment of the lung. The relatively recent development of culture-independent techniques for the identification of microbes has allowed for an explosion of microbiome-related and ecological research. Yet the nonsterility of the lung challenges two centuries of pulmonary biology, which may explain why, even in 2007, the lungs were not sampled in the Human Microbiome Project (9), a critical oversight that has severely hampered this field of research. When amplicon sequencing was eventually applied to the lungs a relatively low abundance community has been consistently demonstrated across organisms and throughout the lifespan (9).

In contrast to the research involving the gut microbiome, a relative paucity of data exists exploring the physiologic implications of the respiratory microbiome, but this is beginning to change. Here, we will focus on recent developments in understanding the lung microbiota and the interactions between the lungs and the intestinal microbiota. We will explore recent advances in techniques for understanding the microbiome and suggest how they might be used to study the lung microbiota using a robust ecological framework. Finally, we will highlight recent advances in the study of the gut-lung axis.

ADVANCES IN ECOLOGICAL INFERENCE AND ITS FUTURE IN THE LUNG

Advancements in technology and theoretical microbial ecology have the potential to unlock a greater understanding of the association between pulmonary health and the variation in community structure. Likewise, the combination of these data with new statistical methods have uncovered ecological patterns in diverse ecosystems that may be relevant for mammalian host-microbiome studies. Identifying patterns of microbial diversity in the lung has numerous potential applications, such as exploiting disturbances in community structure to promote health (42). Common terms in microbial ecology are defined in Table 1.

Applying ecological theory may assist in understanding the lung microbiome. Within the healthy lung, microbial communities are often conceived as randomly and continuously mixing (panmictic) throughout the organ (17). The healthy lung may be helpful as a model for microbiome studies when examined across taxonomic, phylogenetic, and functional domains. Unique aspects of lung physiology provide singular challenges. Flow of microbes in the lungs occurs in two directions, via both the mucosa and airflow. The composition

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Table 1. *Common terms in microbial ecology*

Term	Definition
Alternative stable state	Ecosystems capable of existing under different community structures while maintaining the same or equivalent functions
Assembly	The study of how ecological processes shape observed patterns in microbial communities
Community structure	The composition of taxa in a community including the number of species and their relative abundances
Disturbance	The cause of a change in community structure or function such as a cough or routine of antibiotics
Diversification	Generation of new genetic variation through horizontal gene transfer
Diversity	Variation observed in an ecosystem at taxonomic, phylogenetic, or functional levels
Functional redundancy	A characteristic of an organism where certain taxa contribute in equivalent ways to an ecosystem function
Historical contingency	The legacy effect of a microorganism from a previous point in time
Migration	Introduction of new taxa
Niche	A specific environmental condition that selects for specific organisms
Panmictic	Random mating within a potential breeding population (horizontal gene transfer)
Phylogenetic	A suite of analyses that takes into account the evolutionary history of a community as species are not easily defined in microorganisms
Resilience	Capacity to return to a previous state after a disturbance event
Resistance	Ability to not be affected by disturbance
Selection	The sorting of microbial taxa by environmental conditions (e.g., moisture or pH)
Spatial variation	Differences in community structure/function in three-dimensional space
Stochastic changes	Variation in community structure/function that cannot be explained
Succession	Gradual changes in community structure over time due to a changing environment
Taxonomy	Names of a species or group of species, often referred to as a "taxon," plural "taxa"

of deeper branches of the lower respiratory tract are therefore a function of migration, replication, mechanical elimination, local mucosal and airway immune modulation.

Community assembly is thought to be determined by processes of elimination, immigration and growth within communities (33) as well as selection, diversification, and stochastic changes in microbial species abundance (44). These processes are likely not uniform in the healthy lung and this difference may be exaggerated in pulmonary disease. Diversification, for example, takes place over large generational scales (14), and thus has a less immediate effect than immigration/elimination. Recent evidence has also emphasized that community structure is often historically contingent on previous states of community composition and taxon abundance (20, 30).

The addition of functional data about gut microbial communities may expand our understanding of the lung microbiota. While most studies focus on taxonomic community composition, studies have begun to incorporate functional analysis of gene expression, proteins and metabolites which are equally, if not more, important than community composition. The incorporation of functional traits could further elucidate how these microbial communities interact with humans and each other.

Likewise, advances in molecular identification using metagenome-assembled-genomes and chromosome conformation capture (meta3C) (31) have increased the number of distinct microbial taxa by ~30% (37). This uncharacterized microbial mass may help characterize unexplained variation in the community structure-lung health relationship. This technique was recently applied to study of the lung of patients with cystic fibrosis and uncovered previously unknown functional microbial diversity (46).

Functionally redundant ecosystems are more resistant and resilient to shifts in structure caused by disturbance events (4), such as a diseased state and maintain consistent symbiosis. Changes in community structure influence microbiome function; however, in microbiomes where multiple taxa complete similar functions, removal of one microbe would not greatly hinder the ecosystem function but shift the community structure to an alternative stable state (26). While the gut is known to exhibit functional redundancy (26), it is currently unclear to what magnitude this is present, if at all, in the healthy and diseased lung. Understanding functional redundancy in lung microbial communities, which may be easier than far more dense and diverse gut communities, could advance our knowledge of what constitutes meaningful differences in microbial communities between healthy individuals or disease states.

Lung microbiomes also exhibit ecological succession, the process of change in the structure of an ecological community over time. The fetal lung may harbor detectable microbial DNA as early as the first trimester (3); however, the sterility of the womb during normal pregnancy is contested (15) and remains an open question. It should be noted that these studies investigated bacteria and did not test the presence of archaea, fungi, viruses, or functional activity. Microbial communities may seed the infant as early as five minutes after birth (19) and is largely structured by mode of delivery feeding choices, and the perinatal use of antibiotics (41). By the time an infant is one year old, lung microbial communities have generally stabilized in community structure (1). The potential roles of lung microbiome ecological succession in disease development or lung physiology remain mostly unexplored.

New analysis techniques borrowed from landscape ecology have been applied to understand spatial variation in animal microbiomes, allowing researchers to advance beyond simple taxonomic analysis of amplicon sequencing. Application of these techniques to the lung microbiome may yield a more mechanistic understanding of host-microbe interactions. Selective pressures (e.g., moisture, pH) largely shape community structure of the microbial communities adhered to an organ's surface (47). Spatial variation in community structure influences interactions between members of the microbiome (50). Neighboring microbes are more likely to transfer nutrients and signaling molecules, however, current next generation sequencing techniques are unable to detect such relationships. The ability to detect spatial relationships would also allow the application of additional ecological theories such as biological market theory which borrows economic principals to explain the successful habitation of microbes in ecosystems (45).

In addition to the lung, the gut also exhibits considerable spatial variation in microbial populations (49). In general, a gradual increase in microbes is detected moving distally from the stomach towards the colon (49), but multiple functional niches likely exist within each anatomical site and within each respective microbial community. This raises an important caveat: many studies of the gut microbiome utilize stool to characterize the microbiota, and while a large number of microbes are swept into the fecal stream, the intestinal mucosal layer protects some populations, most notably the immunologically significant segmented filamentous bacteria, from identification using this technique (23).

Microbes outside of the lung may also be relevant for clinical outcomes; the influence the atmospheric microbiome has on colonization of the newborn lung likewise is unknown. Exposure to diverse taxa during childhood may be associated with lower risks of asthma (9). Near-surface microbial communities also vary significantly by the surrounding environment (40) and their three-dimensional configuration (24). Characterizing variation of microbial diversity in room air may aid the development of models of lung colonization, successional development and pulmonary disease.

It is becoming increasingly clear that dysbiosis and disease states are characterized by a shift in microbial diversity. Combining analyses of community structure at taxonomic, phylogenetic, and functional levels may enhance the ability to predict disease state (11). Likewise, as these models become more accurate, precision medicine may employ statistical and machine learning models to diagnose disease and forecast recovery. A trend in microbial ecology has emerged wherein models incorporate likelihood and probabilities into prediction and classification problems. These approaches rely on Bayes' theorem, in which the probabilities of a hypothesized outcome (e.g., a diagnosis) are updated based on given evidence and are more sensitive to subtle effects missed by more common frequentist statistical tests (2).

THE GUT-LUNG AXIS

While the mature respiratory and gastrointestinal systems constitute vastly different environments and functions, they share structural similarities and a common embryonic origin. It is perhaps not surprising then that evidence is emerging of a connection between these two systems. Termed the gut-lung axis, this influence of the gut microbiota on the function of the respiratory system is a rapidly expanding area of intense interest (Fig. 1).

Building on preclinical animal models and observational human cohorts, a role for the gut microbiome in modulating the development of asthma has been relatively well established over the last several years. As recently reviewed (9), asthma is a heterogeneous disease composed of multiple endotypes, each with different contributions from the lung or gut microbiomes. A key study by Finlay et al. (6) used human data from the CHILD (Canadian Healthy Infant Longitudinal Development) cohort. They identified four key potential bacterial genera that

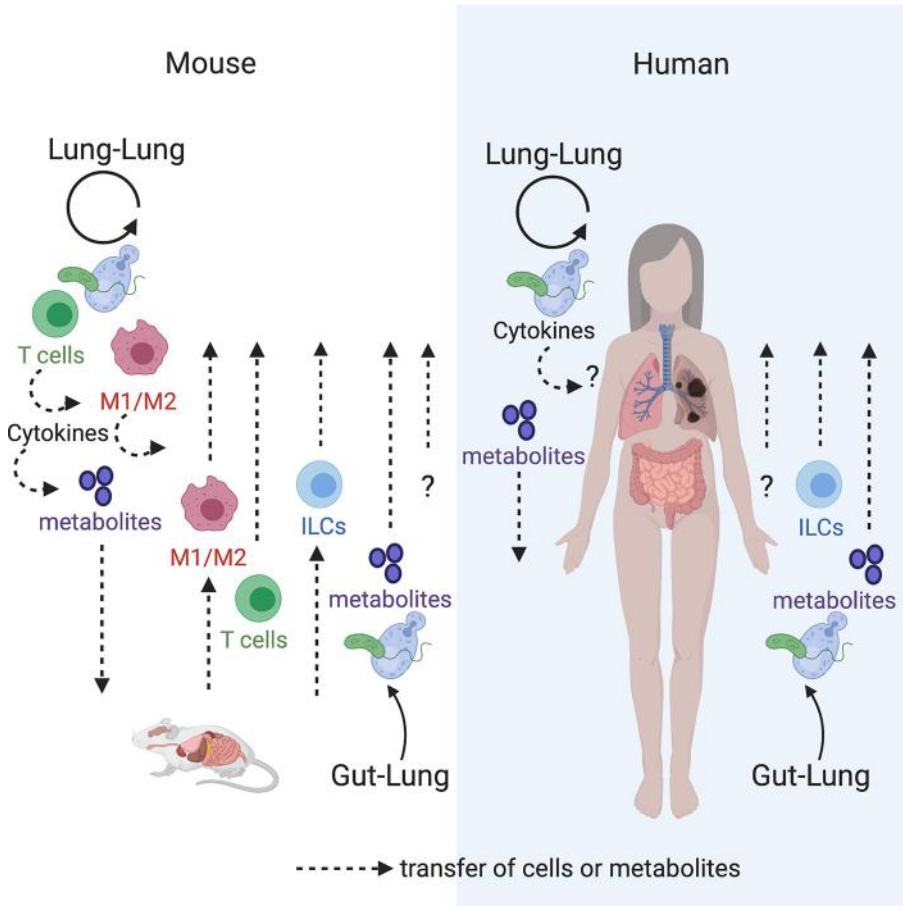

Fig. 1. Simplified cellular mechanisms involved in interactions between the lung and gut microbiomes and the host, several of which remain to be characterized in humans. Some identified mechanisms include T cells, macrophage polarization (M1/M2), innate lymphoid cells (ILCs), cytokines, and metabolites including lipopolysaccharides, small chain fatty acids, histamine, oxylipins, and prostaglandins. Dashed lines indicate the movement of cells or metabolites between arising from the gut or descending from the lung. [Figure created with BioRender.com.]

were decreased in infants who developed asthma and were associated with increased acetate in the urine metabolome. On a more mechanistic level, they were able to show a likely link between small chain fatty acid production by colonic bacteria using an ovalbumin exposure mouse model in germ-free mice colonized with stool from an asthmatic infant. Supplementation with these four missing genera reduced pulmonary inflammation. In addition, small chain fatty acid supplementation to pregnant mice prevented pulmonary inflammation, possibly by inducing pulmonary T regulatory cells in the resulting offspring.

The gastrointestinal tract microbiota is the most well studied host-associated ecosystem for a variety of reasons including abundance of microbes and ease of sample collection. While there are numerous potential mechanistic connections between the gut and the lung, the most intense focus has been on alterations of systemic immunity. However, the relative contributions of lung and gut microbiome to the immunologic tone of the lungs remains an unanswered question. This question often arises because interventions that alter the gut microbiota, such as systemic antibiotics, often also alter the lung microbiota. Schuijt et al. (39) used such an antibiotic-depleted microbiome model to identify these mice had an increased susceptibility to pneumonia. To overcome these limitations, researchers often use gnotobiotic (microbe-free animals colonized with a specific microbiome) or fecal microbiota transfer models to suggest causality. While not without limitations, Schuijt et al. (39) were able to show that fecal microbiota transfer from healthy mice to antibiotic-depleted mice ameliorated their increased susceptibility to pneumonia. Their evidence also suggests that this effect was mediated by alterations in lipid metabolism in alveolar macrophages induced by the gut microbiota.

Other recent studies have identified additional microbial metabolites with potential to modulate inflammation in the lungs. Histamine-secreting bacteria are increased in people with asthma, however, when ovalbumin-exposed mice were supplemented with histamine, pulmonary inflammation was actually reduced (10). Administration of 12,13-diHOME, an oxylipin, decreased pulmonary T regulatory cells and increased inflammation in a cockroach antigen exposure model (28).

In an antibiotic-depleted pneumonia model, Deshmukh et al. (22) were able to identity a critical developmental window in newborn mice where the gut microbiota was required for education of the education of IL-22$^+$ type 3 innate lymphoid cells (ILC3s) and subsequently a mature response to bacterial pneumonia. ILCs are a recently discovered form of innate immune cells that function as the innate counterparts to T cells (36). ILC3s, which express IL-22 and IL-17 and are therefore analogous to T helper 17 cells, coordinate mucosal immunity primarily at the intestinal mucosal barrier. In newborn mice, a critical developmental period exists where ILC precursors are educated by the intestinal microbiota and then traffic to the lung and influence susceptibility to bacterial pneumonia. In the lung, alveolar fibroblast-derived insulin-like growth factor 1 (IGF1) promotes their further development into mature ILC3s (34). Similar developmental windows have also been identified in asthma (6).

In adult mice, Ashley et al. (7) in an elegant series of experiments were able show a potentially causative role for hyperoxia-induced alterations in the lung and gut microbiota in oxygen-induced lung injury in mice. Using a series of mice with different starting microbiomes, germ-free and antibiotic-depleted mice they showed that alterations in the lung and gut microbiota, including an increase in *Staphylococcus* and elimination of the *Erysipelotrichaceae* family, preceded lung injury and variations in microbial communities correlated with differences in cytokines in bronchoalveolar lavage. They also confirmed similar findings in adult mice that our group recently demonstrated in newborns, namely that germ-free mice are protected from injury by hyperoxia (18) and that antibiotic exposure can alter susceptibility to oxygen-associated lung injury (48).

Dickson et al. (16), recently used the variability in the lung and gut microbiomes between healthy adult mice sourced from different vendors to explore their relationship with cytokine expression in bronchoalveolar lavage. They identified significant differences in the concentrations of IL-1α and IL-4 which correlated with lung bacterial community composition more strongly than gut communities.

To date most studies have focused on the bacterial component of the microbiome (bacteriome). However, the microbiome is an interkingdom mixture of bacteria, archaea, fungi, and viruses interacting with the host and each other. While continuous colonization of the human gut by fungi remains controversial, fungal dysbiosis in the human gut microbiome may be a key biomarker of risk to develop asthma (5). That fungi could influence pulmonary inflammation should not be surprising as fungal cell wall components are common human house dust allergens. In preclinical models, overgrowth of fungi from the genus *Candida* following antibiotic exposure was linked to either T helper 2 cell-mediated inflammation (*C. albicans*) (32) or PGE_2-mediated polarization of pulmonary macrophages (*C. parapsilosis*) (25). In addition to antibiotics, antifungals also induce T helper 2 allergic inflammation in a mouse house dust mite exposure model (29). These changes were then recapitulated in mice with defined microbiome of eight bacterial species and additional dysbiotic fungal communities without exposure to antibiotics. Moreover, recent work by Arrieta and colleagues suggests that intestinal fungi play a more important role in host immune development than the bacterial microbiome (43).

THE GUT-LUNG AXIS IN NEWBORN LUNG DISEASE

The recent developments in asthma and pneumonia have led researchers to also begin exploring whether the gut microbiota can influence hyperoxia-induced lung injury and repair. Several observational studies have examined the airway microbiome in newborn infants and subsequently the potential relationship to the development of bronchopulmonary dysplasia (BPD) (27), a common chronic lung disease of premature infants. However, to date, as highlighted by a recent systematic review (35), methodological differences and a lack of data regarding the gut-lung axis means that the issue of whether the microbiome shapes BPD remains an open question.

A recent, multicenter observational study of preterm infants from the United Kingdom found that the lung and gut microbiota of intubated infants diverged to a greater extent in infants that eventually develop BPD than those that did not (21). Looking more granularly at the bacterial load of individual infants they also appreciated episodic links between IL-6 and

IL-8 in bronchoalveolar lavage concentrations that corresponded with increases in bacterial load. Further evidence of a potential mechanistic link between the composition of the gut microbiota and the development of BPD is provided by another recent observational study that noted differences in immune gene expression that correlated with differences in the gut microbiome between infants with and without BPD (38). Overall, these intriguing studies open the door for more mechanistic work methodologically capable of producing more evidence for a causal role of the microbiome.

As recently reviewed in *American Journal of Physiology-Lung Cellular and Molecular Physiology* (13), recent efforts to characterize the lung microbiota and understand the role of the gut-lung axis in preclinical animal models of BPD have also begun to emerge. Building on the clinical observation that a more prolonged duration of antibiotic exposure was linked with an increased risk of developing BPD and severe BPD (12), we recently developed a preclinical model of perinatal antibiotic exposure that allowed us to interrogate the effects of maternal administration of antibiotics on lung injury (48). In mouse offspring born to mothers exposed to antibiotics before and after pregnancy, and to a lesser extent in mothers exposed only during pregnancy, hyperoxia produced durable alterations in lung architecture, particularly in expansion of the interalveolar septa. While this study suggests a potential role for the intestinal microbiota to influence pulmonary development, further mechanistic work using perhaps fecal microbiota transfer or gnotobiotic models is required to more solidly link the gut microbiome changes to alterations in pulmonary architecture and quantify (if any) a contribution of the intrinsic lung microbiota. In addition, in the same issue of *American Journal of Physiology-Lung Cellular and Molecular Physiology*, we also examined the impact of exposure to hyperoxia in mice devoid of a microbiome (axenic or 'germ-free') and showed the somewhat surprising result that germ-free mice were partially protected from lung injury (18). The adaptive and innate immune systems of germ-free mice are markedly abnormal due to the absence of the microbiota, which may highlight the importance of the host immune system to understanding neonatal lung injury and repair.

Indeed, Deshmukh et al. (34) have recently published one potential avenue by which the gut microbiota may influence the pulmonary innate immune response in BPD. Building on their work exploring the role of ILC3s in defense against newborn pneumonia (22), they were also able to show that exposure to hyperoxia reduced the development of pulmonary ILC3 cells in newborn mice, likely due to a reduction in expression of IGF1 in pulmonary fibroblasts. They also showed that human infants with BPD had reduced expression of IGF1 in bronchoalveolar lavage fluid. This is a particularly noteworthy finding, as it may help explain why young survivors of BPD have an increased susceptibility to pneumonia (34).

SUMMARY

Our understanding of the interactions between the intrinsic lung microbiota and the extrinsic gut microbiota on pulmonary physiology remains in its infancy. However, considerable advances have been made in the last several years. Application of ecological theory, more advanced analysis techniques, including visualization and spatial analysis, as well as more mechanistic work in in vitro and ex vivo model systems hold promise for unraveling the multitude of mechanistic interactions between the human host and their microbial cotravelers.

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DISCLAIMERS

N.A. is an Editorial Board Member of *AJP-Lung*. His participation complies with the American Physiological Society requirements for recusal from review and discussions of authored works.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.A.W. prepared figure; K.A.W. and J.D.S. drafted manuscript; K.A.W., J.D.S., and N.A. edited and revised manuscript; K.A.W., J.D.S., and N.A. approved final version of manuscript.

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