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Human airway organoids for bacterial-host interaction studies: methods, insights and translational promise

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ABSTRACT

Background The global burden of bacterial respiratory infections, now among the leading causes of mortality worldwide, has been exacerbated by the rise of antimicrobial resistance. This has highlighted significant gaps in current management strategies, underscoring the need for deeper insights into bacterial pathogenesis.

Methods This review assesses the utility of various experimental models for achieving human relevance and explores recent innovations in human airway organoid (HAO) technology for modelling bacterial infections.

Results Human airway organoid models offer a promising solution by merging the biological relevance of animal models with the versatility of cell cultures. Over the last decade, advancements in HAO technology have revolutionised our understanding of infection pathogenesis and expanded the scope of infection biology.

Conclusions We highlight the transformative discoveries made using HAOs and their potential to enhance healthcare management for bacterial respiratory infections.

INTRODUCTION

Bacterial infections have emerged as a global health crisis and are now the second leading cause of mortality worldwide, with lower respiratory tract infections responsible for more than 2 million deaths in 2019.¹ The escalating threat of antimicrobial resistance (AMR) further worsens clinical outcomes and highlights major gaps in current management strategies.² Mechanistic insights from human airway organoids (HAOs) can accelerate host-directed therapies and vaccine design for bacterial respiratory infections. HAOs provide a biologically relevant system that recapitulates key structural, molecular and functional features of the human airway,³ enabling more precise investigation of bacterial-host interactions. Over the last decade, advances in HAO technology have expanded opportunities for mechanistic discovery, pathogenesis modelling and translational research. Here, we review recent progress in HAO-bacterial models, outline their methodological strengths and limitations and highlight emerging applications that can guide the development of more effective strategies to combat bacterial respiratory disease.

HUMAN AIRWAY ORGANOID

Increasing prevalence of airway infections, chronic respiratory disease and AMR underscores the need

for biologically relevant models that mimic the structure and function of the airway epithelium and the pathophysiology of infected sites. A comprehensive understanding of the human respiratory system's structure is crucial for developing advanced models. The human respiratory system develops from the anterior foregut endoderm, giving rise to tracheal and lung progenitors. Epithelial progenitors differentiate into various cell types lining the conducting airway, from the nasal cavity to terminal bronchioles (figure 1A).⁴ The alveoli, specialised structures for gas exchange, primarily consist of alveolar type 1 and alveolar type 2 epithelial cells (figure 1A).

Traditional models, such as immortalised cell lines, offer an attractive solution but lack complexity (figure 1B), failing to mimic key biological processes like cell polarisation and differentiation.⁵ Primary cell lines, though closer to native tissues, are limited by their low expansion potential and cellular senescence.⁶ Animal models, despite being more complex, fall short of recapitulating human-specific disease mechanisms due to anatomical differences and limited pathogen tropism,⁷ and they struggle to assess chronic infections,⁸ necessitating alternative solutions.

HAOs have emerged as a promising alternative, bridging the gap between simpler *in vitro* models and more comprehensive, yet sometimes less reproducible, animal models (figure 1B). These self-organising systems mimic certain features of the structure, function and genetic characteristics of the airway epithelium.⁹ Organoids can be generated from either pluripotent stem cells (PSCs) or adult stem cells (ASCs) (figure 1C),¹⁰ each offering distinct advantages. PSC-derived organoids with mesenchymal components (figure 1C) are highly modifiable but often fail to fully mimic adult tissue,¹¹ while ASC-derived organoids retain better maturation and region-specific characteristics.¹² Patient-derived HAOs preserve individual genomic and transcriptomic landscapes,¹³ making them ideal for studying bacterial-host interactions, barrier integrity and ciliary function. However, challenges remain, including the lack of standardised metrics for barrier integrity and ciliary beat frequency, as well as limitations in long-term coculture stability.¹⁴ Most HAOs are generated as basal-out (BO) structures, with basal cells facing outward towards the culture medium, while ciliated cells and other epithelial cells orient towards the inner lumen of the organoids, simulating polarised epithelium.¹⁵ When studying polarity-dependent infections,



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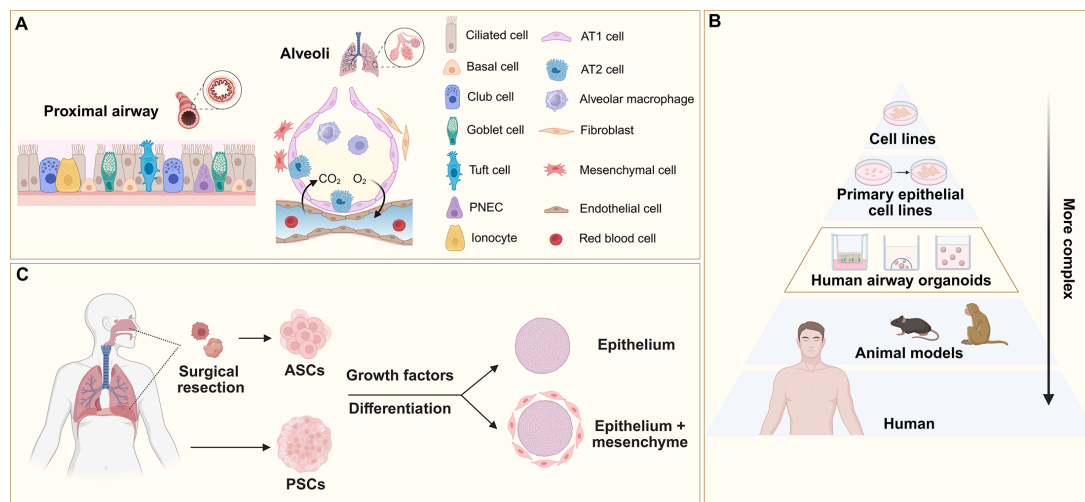


Figure 1 Key physiological features of the human airway and its relevant biological platforms. (A) The human airway system has two distinct structures: the proximal airway and the alveoli. The proximal airway contains basal cells, ciliated cells, club cells, goblet cells, tuft cells, pulmonary neuroendocrine cells (PNECs) and pulmonary ionocytes. The alveoli are mainly composed of alveolar type 1 (AT1) cells and alveolar type 2 (AT2) cells, endothelial cells, alveolar macrophages, mesenchymal cells and fibroblasts. (B) The current models used for the study of bacterial infection. Complexity increases from cell lines, primary epithelial cell lines, human airway organoids, to animal models. (C) Establishment of organoids derived from adult stem cells (ASCs) and pluripotent stem cells (PSCs). With appropriate growth factors and differentiation conditions, ASC-derived organoids lack mesenchymal components, while PSC-derived organoids have both epithelial and mesenchymal cells.

researchers often reverse the polarity to form apical-out (AO) organoids.¹⁶ Air-liquid interface (ALI) cultures are generally included within the broader definition of organoids.^{16–22} Unlike traditional ALI models, ALI-derived HAOs are self-assembled from airway stem cells, capable of differentiating to mimic the key structural, functional and genetic characteristics of human airway, expanding the potential of HAO models for investigating the pathogenesis of respiratory diseases and epithelium-specific infection-induced changes. These advancements in HAO technology enable pathophysiological studies of cellular mechanisms and diversity, providing powerful tools for investigating respiratory infections and developing new therapies.³

GENERATION OF HAO-BACTERIAL COCULTURE MODELS

HAOs are promising tools for studying bacterial-host interactions, but their closed structure and BO orientation limit bacterial contact with epithelial apical surfaces (figure 2). To address these limitations, researchers have developed several methods such as microinjection, organoid fragmentation, ALI culture and AO organoids (figure 3). Key factors in model selection include the host cell type, bacterial polarity preferences, mucus accumulation and experimental conditions. Careful optimisation of these models will improve our understanding of respiratory infections and support the development of effective therapies.

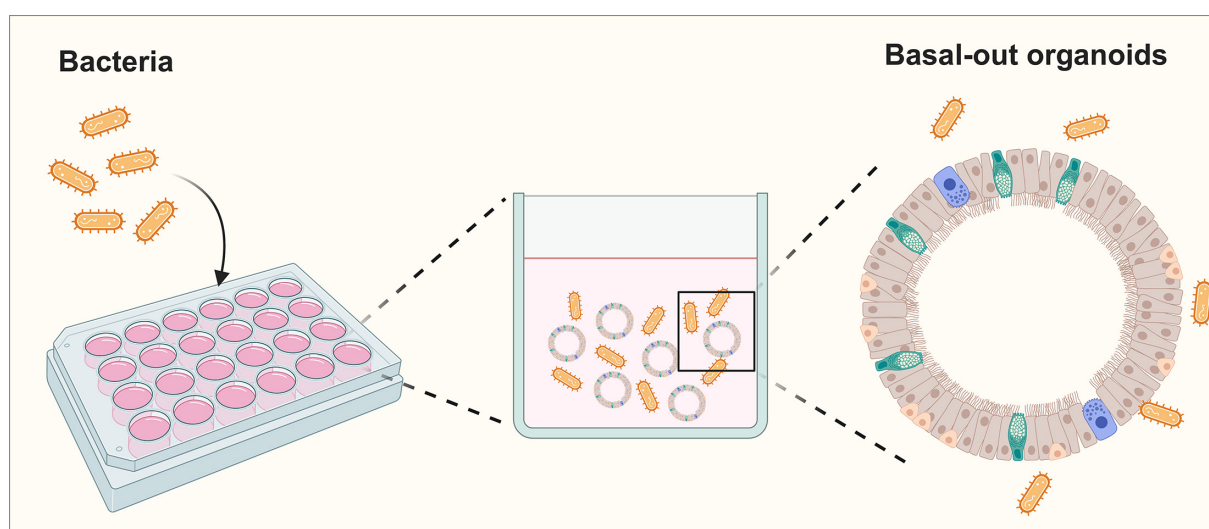


Figure 2 The basal-out organoids and bacteria coculture model. The closed and basal-out structure limits the accessibility of apical surfaces of epithelial cells to interact with bacteria.

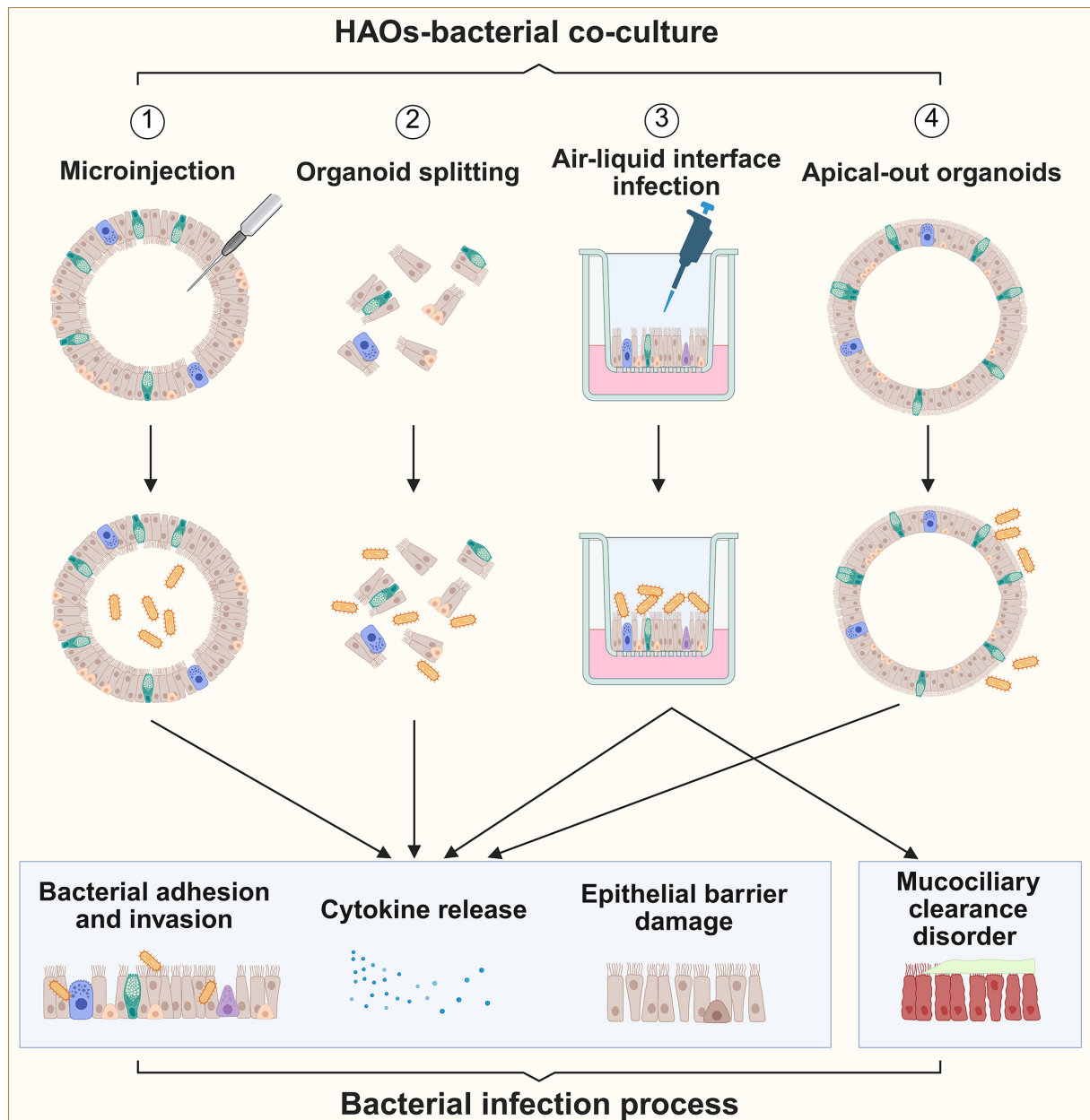


Figure 3 Methods using human airway organoids (HAOs) to study bacterial-host interactions. HAOs can be effectively exposed to bacteria in several ways, including microinjection, organoid fragmentation, air-liquid interface (ALI) culture and generation of apical-out organoids. A combination of appropriate coculture models is used to investigate various bacterial infection processes. For example, the ALI model is particularly well suited for assessing mucociliary clearance (MCC) disorder, while all four models enable the study of bacterial adhesion and invasion, cytokine release and epithelial barrier damage.

Microinjection

Microinjection has emerged as a widely used technique for introducing microbes into the lumen of HAOs,²³ creating a host-microbial interface at the apical side (figure 3) and offering precision and effectiveness. The closed structure of three-dimensional (3D) organoids creates a decreasing oxygen gradient, making it particularly suitable for studying infections with obligate or facultative anaerobes, such as *Klebsiella pneumoniae* and *Mycobacterium abscessus* (MAB).¹³ More complex coculture systems incorporating immune cells enable the study of early infection stages.²⁴ However, researchers must consider potential trace oxygen leakage into the luminal microenvironment, which may affect long-term cocultures with obligate anaerobes and infection efficiency of facultative anaerobes. Fluorescent bacteria aid

in visualising injections and evaluating antibiofilm therapies,¹³ though forming stable biofilms within organoids remains challenging due to bacterial type, injection volume and organoid culture condition. Several studies have focused on the standardisation and optimisation of this technique, including methods for calculating optimal injection volumes²⁵ and strategies for repeated microinjections.²⁶

While promising, microinjection requires specialised equipment, is labour-intensive and limits high-throughput applications. Variability in organoid cell numbers and potential bacterial leakage complicate results. Optimising techniques, standardising protocols and improving equipment are necessary to enhance precision and scalability for studying host-pathogen interactions.

Fragmentation

Fragmentation involves linearising 3D organoids into a two-dimensional system (figure 3) through mechanical blowing or enzyme digestion, followed by coincubation with bacteria before re-embedding in extracellular matrix (ECM).²⁷ While simple to perform and requiring no specialised tools, this technique disrupts organoid structure and cellular polarisation, overlooking initial bacterial-host interactions. Regenerated organoids may lack infected cells, as they arise from uninfected stem cells when bacteria target differentiated cells.⁵ Fragmentation may trigger non-specific immune responses due to the resulting interactions between bacteria and the basal side of HAOs,²⁸ disrupting natural infection dynamics. It also impairs tight junctions and weakens the epithelial barrier, which can influence bacterial persistence and tolerance during infection.²⁹ Additionally, infection efficiency varies across bacterial strains, especially in mixed infections.

Air-liquid interface

ALI culture is widely adopted to cultivate HAOs, exhibiting distinct morphological and epithelial barrier characteristics compared with submerged cultures.³⁰ It offers several advantages for studying bacterial infections by providing direct access to cells (figure 3). A common practice using ALI cultures involves removing unadhered or uninternalised bacteria after initial exposure to the apical layer of the organoids. For example, Prince *et al* introduced *Mycoplasma pneumoniae* to the apical side, washing off after 4 hours, leaving 2% bacteria associated with bronchial epithelial cells.³¹ The ALI model also allows for the simultaneous assessment of target cell preference for pathogen infection by adding bacteria or other derivatives to both the apical and basal sides. It offers a versatile platform for investigating key processes such as mucociliary clearance, epithelial barrier integrity (via transepithelial electrical resistance (TEER)),³² fluorescein isothiocyanate (FITC)-dextran³³ or FITC-inulin³⁴ measurements) and goblet cell function (via harvesting the mucus secreted on the apical layer). Furthermore, the ALI model facilitates studies of cell-cell communication during inflammation by incorporating endothelial cells, fibroblasts and various immune cell subpopulations into the basal layer. However, a main drawback of this model is the difficulty in scaling up due to the relatively large culture area of the transwell inserts, which requires a substantial amount of starting material for the epithelial compartment.

AO organoids

Growth reversed polarity culturing technique (AO organoid) is an alternative to microinjection, rendering the apical layer of the epithelium more accessible (figure 3). The epithelial polarity of HAOs can be reversed by removing ECM and maintaining the organoids in suspension on low-adhesion plates,³⁵ allowing high-throughput applications and screening.³⁵ Li *et al* successfully developed HAOs with an AO polarity that can proliferate and differentiate, revealing the important role of matrix metalloproteinases in determining the destiny of epithelial cells.³⁶ AO organoid system provides great promise for studying the airway epithelium's inflammatory response to bacterial infection and interaction of aerobic bacteria or bacterial metabolites with airway epithelium. However, the potential of AO organoids for studying bacterial-host interaction is tempered by challenges like poorly controlled differentiation and uncertainty regarding the complete polarity reversal's impact on host response.³⁷

As illustrated here, each of the models discussed offers distinct advantages for studying pathogen location, surface interactions

and immune responses. BO organoids are ideal for investigating basal interactions and bacterial invasion into deeper tissue layers, though they restrict the study of apical pathogens. In contrast, the ALI model is particularly well suited for examining apical interactions, such as pathogen adhesion, biofilm formation and mucus clearance (figure 3).^{18 38} AO organoids are useful for studying apical pathogen entry, adhesion and immune mediator secretion (figure 3), although maintaining their functionality during prolonged infections can pose challenges.³⁹ Additionally, microinjection and fragmentation techniques provide more flexibility in manipulating organoid structure and pathogen interactions,^{27 40} but they require careful optimisation to preserve organoid integrity and ensure reproducibility. Regarding the collection of secreted components like cytokines, immune mediators and mucus from the apical surface, the ALI model has a clear advantage, enabling easy tracking of immune responses and epithelial function. In contrast, other coculture models require more complex methods, such as sampling culture medium or analysing intracellular secretions,⁴¹ often needing specialised techniques like labelling secretory particles. By considering these factors, researchers can select the most appropriate coculture model to effectively study host-bacterial dynamics.

CURRENT LANDSCAPE OF THE APPLICATION OF HAO-BACTERIAL MODELS

The application of HAO models in studying host-pathogen interactions has seen significant progress, particularly in viral research involving pathogens such as SARS-CoV-2 and influenza virus.⁴² In contrast to the relatively well-established gastrointestinal organoid-bacterial coculture models,⁴³ the development of HAO-bacterial models has progressed more slowly due to the challenges of isolating, expanding and differentiating airway epithelial stem cells into a functional pseudostratified epithelium with ciliated and goblet cells. Table 1 summarises the host-pathogen mechanisms, disease characteristics and therapeutic insights gained from HAO-bacterial models.

Pseudomonas aeruginosa

Pseudomonas aeruginosa (PA) is a Gram-negative pathogen associated with diseases such as chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), bronchiectasis, lung cancer and ventilator-associated pneumonia.^{44–46} Its resistance to treatment is attributed to low outer membrane permeability, numerous efflux pumps and biofilm formation,^{47 48} which protect it from antimicrobial treatments and immune responses.⁴⁹ The ALI model is ideal for visualising biofilm formation.²² Our laboratory used this model to study PA-HAO interaction, employing dual-species transcriptome sequencing, TEER measurements and biofilm biomass assays to investigate quorum sensing (QS) mechanisms that regulate biofilm formation and immune evasion.²² A recent HAO-ALI study revealed QS-regulated genes in PA, including those involved in bacterial adhesion and biofilm formation (*pprB*), as well as phosphatase and phosphodiesterase activity (*eddA*),⁵⁰ suggesting an alternative approach to target QS. The ALI model excels in studying biofilm interactions but struggles with examining the initiation of biofilm formation. Additionally, patient-derived HAOs provide a more disease-specific perspective on host-pathogen interactions.⁵¹ For example, Chan *et al* developed HAOs derived from patients with COPD, with AO polarity, to study disease-related features such as goblet cell hyperplasia and ciliary abnormalities, providing new insights into PA's impact on inflammation in COPD.⁵²

Table 1 Summary of literature evidence on host-pathogen mechanisms, disease characteristics and therapeutic insights gained from HAO-bacterial models

Bacterial species	Coculture models	Insights	References
PA	ALI	Bacterial QS plays a crucial role in biofilm formation, disruption of barrier function and subversion of host immune responses.	22
	ALI	QS-regulated genes in PA involved in bacterial adhesion and biofilm formation (<i>pprB</i>), as well as phosphatase and phosphodiesterase activity (<i>eddA</i>).	50
	AO	PA-induced inflammatory and stress responses are significantly enhanced in COPD-derived HAOs.	52
	ALI	PA infection influences the reaction of bronchial epithelial cells to HRV infection.	53
	ALI	PA isolates from patients with CF as triggers of acute airway inflammation and neutrophil primary granule exocytosis, both independently and in combination with HRV.	54
	ALI	Type 6 secretion system activities promote preferential invasion of goblet cells, while type 3 secretion system activities by intracellular bacteria induce goblet cell death and expulsion.	55
	AO	Epithelial-derived inducible nitric oxide synthase plays a critical role in the host defence against PA infection.	56
	Microinjection	Targeting host cell lipid peroxidation could be a potential strategy to counteract the virulence mechanism mediated by microbial phospholipases.	57
	ALI	T2R bitter taste receptors play a critical role in eradicating PA infections in patients with CF.	58
Mycobacteria	Microinjection	Boosting of the nuclear factor erythroid 2-related factor 2 (NRF2)-NAD(P)H:quinone oxidoreductase 1 (NQO1) axis could serve as a potential host-directed strategy to improve MAB infection control in CF-derived HAOs.	13
	Microinjection	ASC-derived HAOs can be used to decipher very early events of mycobacteria infection in human settings, thus offering new avenues for fundamental and therapeutic research.	24
	Microinjection	Classic anti-TB drugs, such as rifampicin and bedaquiline, are effective in treating MTB-infected HAOs and macrophages.	23
	AO	MTB infection significantly alters the cell death patterns of HAOs and modulates the signal transduction pathways in PBMCs.	64
NTHi	ALI	The inflammatory response to NTHi can occur at both the basolateral and apical layers after contact with airway epithelium.	69
	ALI	NTHi releases OMVs during an infection of 1–10 days, which bind to host cell membranes.	70
	ALI	Inflammation-induced changes in vitamin D metabolism impair host's airway defence.	71
	ALI	After RV infection, NTHi is more prone to cause secondary infections.	34
	ALI	Targeting nitric oxide with antibiotics enhances killing of NTHi in biofilms in patients with PCD, suggesting a novel therapeutic approach.	72
	ALI	Structural modifications of macrolides can help clear intracellular NTHi, reducing its impact in the airways of patients with COPD.	73
SP	ALI	Influenza infections induce metabolic shifts in SP, promoting its transition from colonisation to a more invasive disease state.	75

Continued

Table 1 Continued

Bacterial species	Coculture models	Insights	References
	Microinjection	ESC-derived HAOs can model key events in SP interaction with the lung, including adherence, internalisation and the initial alveolar innate response.	14
	ALI	SP enhances the production of antimicrobial and inflammation-related cytokines in HAOs.	76
	AO	COPD-derived HAOs show a stronger proinflammatory response to SP infection, highlighting their potential for evaluating anti-inflammatory or antimicrobial therapies.	52
SA	ALI	SA infection disrupts the airway epithelium, with increasing severity over time, while pretreatment with vitamin D alleviates the damage.	78
	ALI	SA colonisation robustly induces epithelial cell production of IL-1 family cytokines, suggesting inflammasome signalling activation.	76

ALI, air-liquid interface; AO, apical-out; ASC, adult stem cell; CF, cystic fibrosis; COPD, chronic obstructive pulmonary disease; ESC, embryonic stem cell; HAO, human airway organoid; HRV, human rhinovirus; IL-1, interleukin 1; MAB, *Mycobacterium abscessus*; MTB, *Mycobacterium tuberculosis*; NTHi, non-typeable *Haemophilus influenzae*; OMV, outer membrane vesicle; PA, *Pseudomonas aeruginosa*; PBM, peripheral blood mononuclear cell; PCD, primary ciliary dyskinesia; QS, quorum sensing; RV, rhinovirus; SA, *Staphylococcus aureus*; SP, *Streptococcus pneumoniae*; TB, tuberculosis.

The HAO system has advanced research into the synergistic effects between PA and other pathogens. Endres *et al* demonstrated how PA infection influences HAO response to human rhinovirus (HRV),⁵³ while Laucirica *et al* developed an ALI model and showed that PA isolates from patients with CF trigger acute inflammation and neutrophil activation.⁵⁴ Recent research has uncovered that PA invades goblet cells, breaching the epithelial barrier from within via coordinated virulence factors in the ALI model.⁵⁵ Specifically, type 6 secretion system activities promote preferential invasion of goblet cells, while type 3 secretion system activities by intracellular bacteria induce goblet cell death and expulsion.

The HAO-bacterial model holds potential for identifying targeted microbial interventions. Grubwieser *et al* used HAOs with an AO polarity to examine early PA infection and the role of inducible nitric oxide synthase in host defence.⁵⁶ Bagayoko *et al* found that the PA effector ExoU exploits phospholipid peroxidation to promote cell necrosis by microinjection, suggesting a potential therapeutic target.⁵⁷ Other studies, such as those by Kuek *et al* and Qi *et al*, explored strategies like bitter taste receptors and sphingosine supplementation to combat PA infections.^{58–59} With PA classified as a critical pathogen by WHO, research into multidrug-resistant PA using HAO models is still emerging.⁶⁰ HAOs provide a unique opportunity to investigate biofilm formation on living tissue surfaces—an essential factor in chronic infections and antibiotic resistance.⁴⁹ They also allow investigation of host-driven resistance mechanisms, such as mucus production, antimicrobial peptides, pH environments and oxygen tension, to assess their impact on antibiotic efficacy and bacterial resistance. Together, these capabilities underscore the importance of HAO-bacterial coculture models in advancing AMR research and treatment strategies.

Mycobacteria

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) remains one of the deadliest pathogens globally, with early detection complicated by MTB's deep lung residence.⁶¹ Non-tuberculous mycobacteria like MAB also pose significant risks.⁶² Recent advancements include the construction of MTB-infected and MAB-infected HAOs via microinjection, with studies showing MTB's viability in the HAO lumen for up to 21

days,²⁴ a model difficult to replicate in animals.⁶³ Coculturing mycobacteria-infected HAOs with macrophages revealed macrophages migrating to the basal edge of HAOs, underscoring the importance of immune cells in MTB studies.²⁴ Kim *et al* reported that MTB can infect epithelial cells and macrophages in induced PSC (iPSC)-derived HAOs, with infections treated by rifampicin and bedaquiline.²³ Zhang *et al* revealed that MTB infection altered HAO cell death patterns and modulated the signal transduction in peripheral blood mononuclear cells (PBMCs) using AO-polarised HAO-PBMC cocultures.⁶⁴ Additionally, HAOs derived from patients with CF are proving useful for evaluating host-directed strategies¹³ and new antimicrobial compounds targeting MAB.⁶⁵ Despite these advances, key questions remain regarding mycobacteria entry and adhesion, epithelial immune evasion, adaptation to lung luminal niches (such as mucus content, hypoxia and oxidative stress), regulation of mucociliary function and epithelial barrier integrity, and biofilm formation.

Non-typeable *Haemophilus influenzae*

Non-typeable *Haemophilus influenzae* (NTHi) is a Gram-negative opportunistic pathogen found in the respiratory mucosa and associated with lung diseases.⁶⁶ Pathogen-recognising receptors on both apical and basal surfaces make cells susceptible to infection from both directions.^{67–68} Therefore, studying NTHi infection requires a model that enables investigation of both cellular polarities, with ALI organoid model being widely used for this purpose. Studies have shown NTHi induces inflammation at both cell surfaces.⁶⁹ Ren *et al* established long-term NTHi infection using ALI models, revealing that NTHi releases outer membrane vesicles that bind to host cell.⁷⁰ Schrupf *et al* found that inflammation-induced changes in vitamin D metabolism impair airway defence, increasing the risk and progression of NTHi infection.⁷¹ Viral infections weaken the epithelial barrier, facilitating secondary bacterial infections. For instance, HRV infection enhances NTHi's ability to cause secondary infections.³⁴ Coculture models of patient-derived primary airway epithelial cells with NTHi show promise for treating COPD and primary ciliary dyskinesia.^{72–73} These studies highlight the crucial role of HAO models in studying bacterial-host interactions, viral-bacterial synergism and NTHi-related respiratory infections.

Streptococcus pneumoniae

Streptococcus pneumoniae (SP), part of the respiratory flora, can cause invasive diseases in immunocompromised individuals.⁷⁴ D'Mello *et al* used the ALI model to study SP-host interactions, demonstrating that influenza infections trigger metabolic shifts in SP, enhancing its invasiveness.⁷⁵ Sempere *et al* modelled SP's interaction with alveolar epithelial cells using embryonic stem cell-derived HAOs through microinjection,¹⁴ but ethical concerns have led to a shift towards iPSCs for future HAO development, advancing SP pathogenesis research and treatment strategies. Boyd *et al* conducted the first coculture study with HAOs on ALI, demonstrating that SP enhanced antimicrobial and inflammation-related cytokine production.⁷⁶ COPD-derived HAOs with AO polarity induced a stronger proinflammatory response to SP, underscoring their potential for precision evaluation of anti-inflammatory or antimicrobial therapies.⁵²

Staphylococcus aureus

Staphylococcus aureus (SA), particularly methicillin-resistant SA, causes significant morbidity and mortality.⁷⁷ Using nasal organoids cultured in the ALI model, our laboratory found that SA infection disrupts the airway epithelium, with vitamin D treatment alleviating damage.⁷⁸ Boyd *et al* used HAOs on ALI to demonstrate that SA colonisation robustly induced interleukin 1 cytokine production, suggesting inflammasome activation.⁷⁶ Although SA infection has been studied in skin,⁷⁹ intestinal⁸⁰ and synovial organoids,⁸¹ research using HAOs is limited, highlighting a critical need for further studies on SA pathogenesis. Biofilm-associated bacteria, like SA, evade host immune responses and show enhanced antibiotic tolerance, complicating treatment. Biofilm formation is closely linked to AMR, as it creates a physical barrier and harbours tolerance mechanisms that reduce antibiotic effectiveness.⁸² The biofilm matrix can sequester antimicrobial agents, further aiding bacterial survival.⁸² As such, the application of HAO technology in elucidating the complex interplay among biofilm formation, immune evasion and AMR in SA infection remains a promising avenue for further research.

IMPROVEMENTS OF THE HAO-BACTERIAL MODEL RESEARCH

HAOs, which recapitulate airway mucociliary biology, are pivotal in understanding respiratory diseases and host-pathogen interactions. Despite advances in HAO-bacterial coculture techniques, consistently establishing bacterial infections in HAOs remains elusive. This review highlights advancements in HAO-bacterial cocultures, key for studying bacterial invasion while maintaining tissue architecture. However, existing models often oversimplify bacterial diversity and host complexity. Future development will focus on modelling chronic infections, bacterial diversity, integrating immune cells and vascular structures and enhancing multiorgan communication to better replicate human disease conditions (figure 4).

Chronic infections and bacterial diversity in modelling

Currently, coculture models primarily focus on host-bacteria interactions within 24 hours, overlooking chronic infections (figure 4), which are clinically relevant. For example, studies on SP and PA have observed bacterial adhesion and host immune responses within hours, but chronic infection modelling remains limited.^{14 56} Developing stable chronic infection models requires long-term bacterial propagation while maintaining cell integrity.⁸³ Strategies like low bacterial inoculum, daily cell washing

and adding antibiotics help prevent excessive cell death and bacterial overgrowth, as shown in Endres *et al*'s 16-day chronic PA infection model.⁵³ Similarly, Iakobachvili *et al* demonstrated the MTB viability for 21 days within HAOs through microinjection, while MAB actively replicated for 7 days, supporting chronic infection modelling in HAOs.²⁴ Incorporating biofilm formation, a hallmark of chronic and refractory pulmonary infections, is critical for these models.

Current research typically explores single bacterial infections,⁸⁴ neglecting coinfections common in clinical settings (figure 4). Moreover, the airway microbiome—an intricate and dynamic ecological system—plays an increasingly recognised role in chronic respiratory disease.⁸⁵ In this regard, ALI models offer a valuable platform for identifying pathobionts that may be overlooked by conventional disease models.⁸⁶ Future models should integrate dynamic microenvironments, biofilm formation and bacterial communities to better replicate chronic pulmonary infections. Key considerations include target cell type, bacterial infection preferences (apical vs basal interactions), high-throughput capabilities, support for prolonged infection and necessary cellular and bacterial parameters. Oxygen levels, mucus accumulation, cell debris and metabolites in the culture system also need careful management, as they significantly affect bacterial colonisation and pathogenicity.

Integration of immune cells and vascular structures

Immune cell-pathogen interactions are crucial in respiratory infections, influencing pathogen clearance or triggering a cytokine storm, causing tissue scarring and necrosis. HAO technology integrates epithelium, immune cells and bacteria (figure 4), offering significant potential, but the triculture model remains underexplored. Coculturing immune cells with HAOs is a developing method for studying these interactions.⁸⁷ Most macrophage-based triculture models focus on mycobacterial infections, as macrophages serve as the natural host cell.⁸⁸ These models show macrophages migrating towards HAOs and phagocytosing pathogenic material,²⁴ but they fail to fully replicate the *in vivo* environment, as macrophages cannot access HAO lumen. Kim *et al* observed that macrophages phagocytose MTB but die within 96 hours, allowing MTB to spread to epithelial cells. Anti-TB drugs reduced MTB survival and inflammation, aiding drug delivery system development.²³ Zhang *et al* found MTB reshaped signalling dynamics between PBMCs and AO-polarised HAOs, with *TNFSF15* and *BDNF* as key mediators.⁶⁴ HAO-neutrophil triculture is scarce, likely due to neutrophils' short survival time and rapid functional changes *in vitro*.⁸⁹ These studies underscore the potential of HAO systems to recapitulate airway diseases, including those encountered in complex and heterogeneous disorders such as bronchiectasis, with chronic inflammation, mucus hypersecretion, structural airway damage and host-microbe interactions, where infection plays a pivotal role.⁹⁰

Additionally, incorporating vascular structures into HAO models (figure 4) is essential for enhancing the physiological relevance of host-pathogen interactions, but remains challenging. Vascularisation strategies include coculturing organoids with vascular cells, combining lineage-specific organoids with vascular organoids, co-differentiating stem cells, the use of organoid-on-a-chip technology and 3D bioprinting.⁹¹ Recent studies, such as Qadir *et al*'s work on iPSC-derived lung progenitor cells and endothelial cells, have advanced vascularised HAO models, better representing inflammatory responses and tissue interactions.⁹² Tan *et al* generated HAOs by combining bronchial

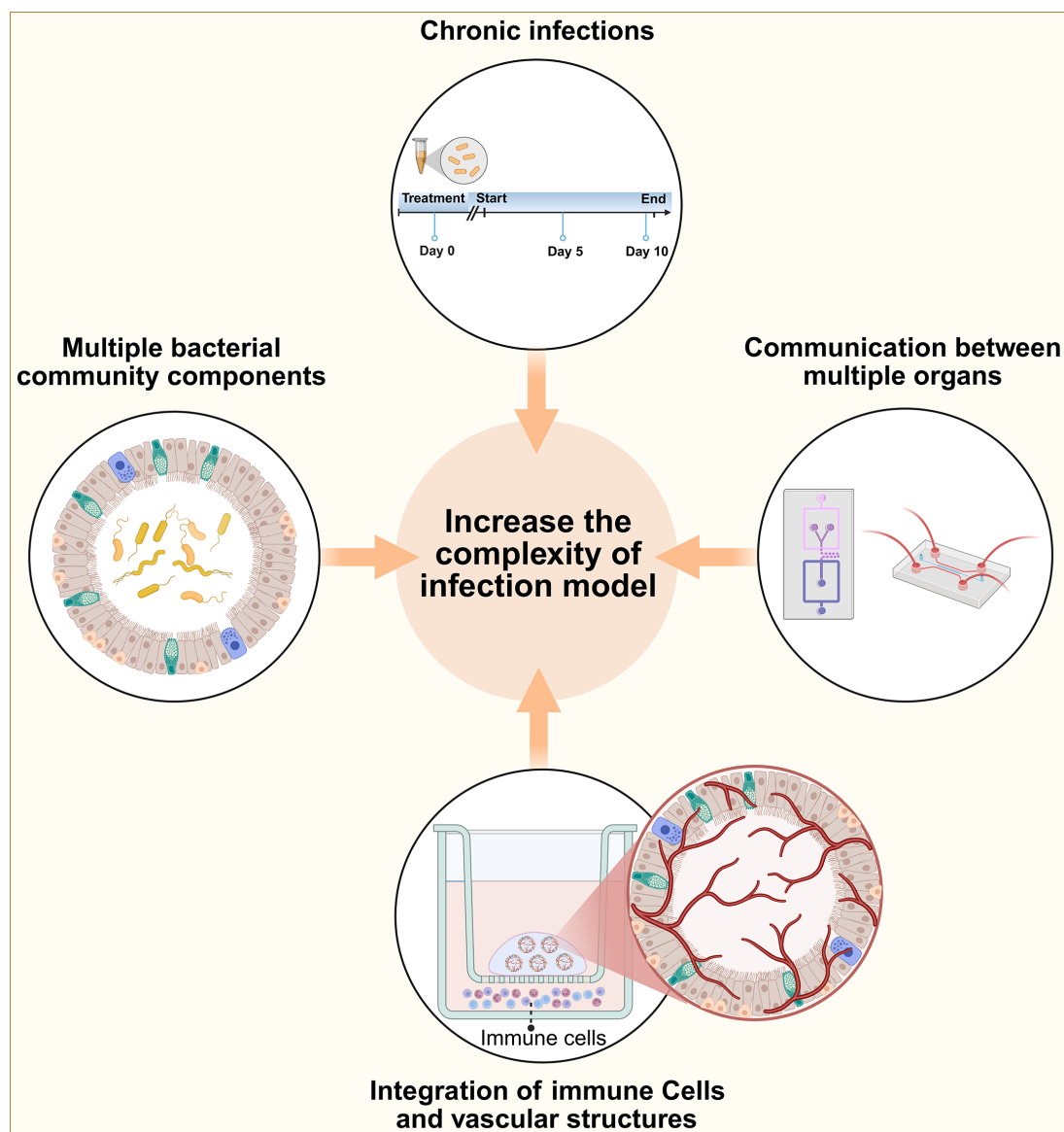


Figure 4 Methods to increase the relevancy of infection models. The simulation of chronic infections, the interactions of diverse bacterial components, the introduction of immune cells and vascular structures and communication between multiple organs can be taken into account in future research to establish more relevant cocultures.

epithelial cells, lung fibroblasts and endothelial cells, forming invasive tubular structures that mimic branching morphogenesis and enable transplantation.⁹³ The application of vascularised HAO technology in infectious disease research is still in its early stages, explaining the limited literature on the topic. While omitting immune and vascular components simplifies laboratory set-up, it compromises physiological relevance by overlooking key cell-cell and cell-bacteria interactions.⁸⁴ Integrating immune cells and vascular structures in HAO systems (figure 4) will be crucial for studying these interactions in both homeostatic and pathological conditions, potentially leading to new therapeutic approaches.

Enhancing multiorgan communication

Traditional pathogen infection studies in organoids often focus on localised epithelial responses, but infections typically involve multiple organs. HAO models simulating responses across organs, like the upper and lower respiratory tracts or along the gut-lung axis, are crucial.⁹⁴ Multiorgan chips, integrating several

organ systems on a microchip (figure 4), offer a promising approach for studying these complex interactions.^{95 96} Shpichka *et al* highlighted that integrating multiorgan systems with microinjection platforms can replicate coinfections and microbiota imbalances, offering advantages such as ease of standardisation, high reliability and the ability to isolate external factor interference, aiding the development of targeted therapies.⁹⁷ However, challenges like system complexity, difficulty in read-out, limited scalability and throughput constraints hinder their widespread use in infectious disease research. Additionally, single-cell transcriptomic and epigenomic analyses can help elucidate microbial tropism and intercellular signalling, setting new standards for the field.

CONCLUSIONS

The advancement of HAO cultures enhances our understanding of bacterial interactions within the airway microenvironment. By integrating additional cell types and mimicking respiratory pathophysiology, HAOs are evolving into more sophisticated

models for studying respiratory infections. These models offer patient-specific insights, such as cystic fibrosis transmembrane conductance regulator (CFTR)-mutant HAOs for personalised therapies, and enable high-throughput drug screening, antibiotic development and vaccine design. HAOs have the potential to replace animal models, addressing ethical concerns and providing more human-relevant data. To maximise their clinical potential, standardising HAO protocols, ensuring reproducibility and facilitating organoid biobanking are essential. These efforts will accelerate HAO-based personalised medicine and improve clinical outcomes in respiratory infections.

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