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Reviews in microbial pathogenesis

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Reviews in microbial pathogenesis

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Table of contents

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Editorial: Reviews in microbial pathogenesis

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Editorial on the Research Topic
Reviews in microbial pathogenesis

Microbiology is a fast-growing research field. The rapid expansion of this field is driven by ongoing discoveries of emerging pathogens, microbial adaptation, resistance mechanisms and technological advancements. While these trends will likely continue to drive new discoveries and novel solutions for infectious diseases, the overwhelming volume of research publications makes it difficult for scientists to keep pace with the latest findings. Additionally, access to cutting-edge research can be limited by paywalls, creating barriers for both early-career researchers and those in resource-limited settings.

The present Research Topic, focusing on reviews in microbial pathogenesis, includes 16 publications (15 peer-reviewed articles and 1 erratum), including 72 authors, to address these challenges. By providing open-access, comprehensive reviews and accessible summaries of recent findings, this Research Topic offers a valuable platform for the researchers to navigate key advancements in the field of microbiology, emphasizing important directions for future research and offer insights into emerging trends and methodologies.

The review by Mohammad et al. addressed the role of lipoproteins (Lpps) in the pathogenesis of *Staphylococcus aureus*. *S. aureus* produces Lpps that contribute to metabolism, are essential for the survival of bacteria and are important for tissue invasion. Additionally, they are capable of affecting disease pathogenesis by modulating the immune response (Mohammad et al., 2020; Nguyen and Götz, 2016) via binding to Toll-Like Receptors 2 (TLR2), leading to activation of innate immune responses (Hashimoto et al., 2006). Binding of these molecules to TLR2 causes rapid migration of innate immune cells, including monocytes/macrophages and neutrophils (Mohammad et al., 2019, 2021). Also, Lpps can stimulate interferon γ producing T cells (Saito and Quadery, 2018), although there is limited effect on B cell activation (Mohammad et al., 2019). The *in vitro* and *in vivo* effects of Lpps are believed to be mediated by interaction with TLR2-dependent neutrophil recruitment (Mohammad et al., 2019). It was demonstrated that neutrophils and macrophage recruitment is facilitated by the release of chemokines such as MIP-2, KC, MCP-1 and MPO (Mohammad et al., 2021). The role of TLR2 in the pathogenesis of Lpps-induced pathology is also supported by the TLR2-knockout mouse model (Schmaler et al., 2009).

In another review, Schwermann and Winstel summarize the functional diversity of *Staphylococcus* surface proteins, which play a crucial role in host interaction and pathogenesis. *Staphylococcus* expresses several surface proteins, including clumping

factor B (ClfB), fibronetin-binding protein B (FnBPB), and iron-regulated surface determinant protein A (IsdA), which bind to human loricrin receptor on epithelial cells. This binding facilitates initial adhesion and invasion of host cells (Clarke et al., 2009; da Costa et al., 2022; Mulcahy et al., 2012), particularly on skin and nasal surfaces. Bacterial surface proteins also contribute to immune evasion and persistence (Kim et al., 2010). For example, Clumping factor A(ClfA), collagen adhesin and protein A (SpA) are key factors in the pathogenesis of septic arthritis (Josefsson et al., 2001; Palmqvist et al., 2002; Xu et al., 2004). ClfA is also involved in biofilm formation, which protects *S. aureus* from phagocytosis (Dastgheyb et al., 2015). The essential role of *S. aureus* surface proteins in infections make them attractive targets for the development of novel therapeutics. Immunization with IsdA or IsdB has been shown to reduce the virulence of *S. aureus* (Kim et al., 2010). Additionally, anti-SpA monoclonal antibodies demonstrated therapeutic potential in mouse abscess models (Cheng et al., 2011). Overall, this strongly indicates that targeting specific surface proteins could be a viable strategy for controlling *S. aureus* infections.

Acosta-Espana and Voigt shed light into the differences between entomophthoromycosis and mucormycosis. Fungi, causing entomophthoromycosis and mucormycosis, were initially placed into the class *Zygomycetes* (Voigt et al., 1999), including *Entomophthorales* and *Mucorales*. In 2007, the phylum *Zygomycetes* was replaced by *Mucoromycota and Zoopagomycota* (Spatafora et al., 2016). However, the old terms are still used in many publications, creating confusion about fungal species identification. The authors summarize information on *Basidiobolales*, *Entomophthorales*, and *Mucorales* to address this confusing issue and make it clear and easy for clinical researchers to use the updated fungal taxonomy. The authors state that current taxonomy identifies the classes *Mucoromycota* (order *Mucorales)*, *Zoopagomycota* (order *Entomophthorales* [*Conidiobolus* spp.]) and *Basidiobolales* (*Basidiobolus* spp.). Instead of the term "zygomycosis", more defined terms should be used, such as: 1. Infection with *Mucorales* should be referred to as mucormycosis; 2. Infection with *Basidiobolus* spp. as basidiobolomycosis; 3. *Conidiobolus* spp. as conidiobolomycosis. These fungal infections have differences in pathogenesis as mucormycosis is diagnosed primarily in patients with impaired cellular immunity [Center for Disease and Prevenstion (CDC), n.d.], while basidiobolomycosis and conidiobolomycosis occur in immunocompetent patients (Geramizadeh et al., 2015; Kundu and Chakraborty, 2023; Spatafora et al., 2016). Clinical presentations of these infections differ as well. Patients infected with *Mucorales* species have rapid spread with angioinvasion and necrosis [Center for Disease and Prevenstion (CDC), n.d.]. In contrast, the slow progression of clinical symptoms is characteristic of *Basidiobolus* spp. and *Conidiobolus* spp. (Raghavan et al., 2020). The diagnosis is based on epidemiologic, clinical, imaging, histopathologic, microbiologic, and molecular data, followed by the confirmatory report of a fungal culture.

A review by Mlynek and Bozue addressed the impact of phase variation and biofilm formation in *Francisella tularensis*. *F. tularensis* causes tularemia, a zoonotic disease often transmitted through contact with rabbits (Ellis et al., 2002). There are two primary subspecies: *F. tularensis subsp. tularensis* (Type A) and *F. tularensis subsp. holarctica* (Type B), both can be transmitted to humans (Larson et al., 2020). Different species within the *Francisella* genus exhibit varying capabilities to form biofilms. Subspecies of *F. tularensis* tend to form less defined structures compared to *Francisella novicida* (Mahajan et al., 2011; Margolis et al., 2010). These differences are partly due to genetic variations. For example, *F. novicida* retains a functional cyclic-di-GMP system (c-di-GMP), which is absent in *F. tularensis* (Kingry and Petersen, 2014). The *wbt* locus in *F. tularensis*, which contributes to O antigen synthesis, contains genes that are not present in *F. novicida* (Kingry and Petersen, 2014). GMP stimulates biofilm formation by upregulating genes encoding extracellular polysaccharides (Hickman et al., 2005). Additionally, the O antigen contributes to biofilm formation in *F. tularensis* isolates (Champion et al., 2019). Biofilms enhance bacterial persistence by protecting against host defenses and antibiotic treatment. Notably, biofilm formation has been shown to reduce susceptibility of *F. tularensis* to ciprofloxacin (Siebert et al., 2020), further complicating treatment efforts.

The role of *Francisella* peptidoglycan biosynthesis enzymes in morphology, pathogenesis and treatment of infection is discussed in the review by Bachert and Bozue. The bacterial cell wall is constantly remodeling in response to environmental changes and cell division. Peptidoglycan (PG) remodeling is a coordinated process involving several enzymes. PG biosynthesis begins with the formation of a lipid II precursor (Egan et al., 2020). The precursor is subsequently polymerized with penicillin-binding proteins (PBPs). Many organisms encode multiple PG enzymes with redundant function (Lee et al., 2017; van Heijenoort, 2011). Interestingly, this is not a characteristic of *F. tularensis* (Kijek et al., 2019). There are currently five carboxypeptidases and two lytic transglycosylases known in *Francisella* (Sauvage et al., 2008). They all have distinct roles in cell morphology (Spidlova et al., 2018; Zellner et al., 2021) and contribute to the immunomodulating activity of this bacterium (Nakamura et al., 2021). This suggests that PG enzymes could be used as a therapeutic target specifically against this organism.

Approaches for identifying bacterial effector kinases are summarized in the review by Louis et al. Many pathogens encode proteins with sequence homology to eukaryotic kinase domains (Anderson et al., 2015; Moss et al., 2019; Navarro et al., 2007). Some of these bacterial kinases can phosphorylate host cell proteins to manipulate signaling pathways, thereby promoting bacterial replication and survival within the host (Park et al., 2019; Tegtmeyer et al., 2017). However, understanding the role of these kinases in the pathogenesis of bacterial disease is limited, primarily due to insufficient knowledge of their target host proteins. Improved identification of host targets for bacterial kinases could pave the way for the development of novel antimicrobial therapeutics that disrupt these critical interactions.

Manipulation of host signaling pathways by *Neisseria gonorrhoeae* is discussed by Walker et al. The mucosal epithelium serves as the primary portal of entry for *N. gonorrhoeae* (Quillin and Seifert, 2018). During colonization, bacterial pili facilitate

cell-to-cell contact with the epithelium, while Opa proteins further promote adherence. Gonococci pili bind to several host receptors, including α1β1 and α2β1 integrins in the male urethral epithelium (Edwards and Apicella, 2005). In contrast, gonococcal pili can also bind to the complement receptors, CD46 and CR3, as well as the I-domain containing integrin receptors (Edwards et al., 2001). The interaction between microbial Opa protein and the CEACAM family of receptors on neutrophils contributes to the clinical manifestation of gonococcal infection (Sarantis and Gray-Owen, 2012). The CEACAM-Opa interaction promotes the colonization of cervical epithelial cells by suppressing exfoliation (Yu et al., 2019). Furthermore, CEACAM-Opa inhibits Th1/Th2 lymphocyte responses while promoting a pro-inflammatory Th17 lymphocyte phenotype (Feinen et al., 2010; Liu et al., 2012). Another key gonococcal protein, PorB, increases calcium influx, which is required to reduce lysosome counts in infected cells (Hopper et al., 2000). PorB also reprograms macrophages (Mosleh et al., 1998) to create a more favorable environment for survival. Additionally, PorB can bind to soluble C4b-binding protein (C4BP) and factor H of complement (Ram et al., 1998, 2001), aiding immune evasion. Understanding the mechanisms employed by *N. gonorrhoeae* to evade immune clearance and promote intracellular replication is essential for the development of vaccines and therapeutics.

The importance of interaction between host and microbial neuraminidases (NA) in the pathogenesis of viral and bacterial co-infection of respiratory epithelium is discussed by Escuret and Terrier. Pathogens infecting epithelial cells of the respiratory tract such as influenza viruses use NA and hemagglutinin (HA) to enter the cell. Bacteria can also express NA for adherence and invasion of epithelial cells (Vimr and Lichtensteiger, 2002). Intriguingly, during viral-bacterial co-infections, viral NA can remove sialic acids that typically mask bacterial adhesion receptors, thereby facilitating bacterial colonization (Peltola and McCullers, 2004). This synergistic effect enhances the severity of respiratory infections (Wren et al., 2017). Given the pivotal role of NA in viral and bacterial interactions, they present attractive targets for developing preventive and therapeutic strategies aimed at mitigating co-infection severity.

Jin et al. discussed the advancements in the understanding of mechanisms of *Bartonella* pathogenesis. Endothelial cells are the primary target for *Bartonella* species (Deng et al., 2012). The bacterium uses α-enolase or phosphopyruvate hydratase to activate plasmin and promote extracellular matrix degradation (Díaz-Ramos et al., 2012). The *Bartonella* BadA protein can activate hypoxia-inducible factor-1 and secrete pro-angiogenic cytokines (Kempf et al., 2001, 2005). It can also provide resistance to complement killing (Deng et al., 2012). BadA and Vomp proteins also facilitate immune evasion by antigen variations (Linke et al., 2006). To evade the immune repose, *Bartonella* produces LPS, an antagonist of the TLR4 receptor (Malgorzata-Miller et al., 2016). Still, many aspects of *Bartonella*'s pathogenesis remain unknown, requiring the development of novel *in vivo* and *in vitro* methods.

Recent data on pathogens causing sepsis are summarized in the review by Gatica et al. A diverse group of pathogens that belong to the normal microflora (*Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Streptococcus pyogenes*) can cause sepsis (Gouel-Cheron et al., 2022). Sepsis risk increases with age, compromised immunocompetence and comorbidities. Also, each microbe has a unique set of virulence factors facilitating adhesion, penetration and replication. Therefore, approaches for diagnosis and treatment will differ for each type of sepsis. The traditional use of antibiotics led to the development of drug-resistant strains, making therapeutic options limited. Therefore, searching for new approaches for diagnosing and treating sepsis remains a pressing medical issue.

In the review by Ayesha et al. the role of *Legionella pneumophila* outer membrane vesicles (OMVs) in interaction with the host is discussed. *L. pneumophila* secretes OMVs containing proteins, toxins, nucleic acids and antibiotic-resistance enzymes (Flesher et al., 1979). OMV cargo delivered to eukaryotic cells can inhibit innate protection against bacteria. For example, it was shown that proteins delivered by OMVs can inhibit the fusion of legionella-containing phagosomes and lysosomes (Fernandez-Moreira et al., 2006). Also, OMVs can inhibit the production of pro-inflammatory cytokines by macrophages (Jung et al., 2016). The ability of OMVs to deliver the cargo could be used to develop vaccines and deliver drugs.

The role of infection in Kawasaki vasculitis is discussed in the review by Wang et al. Environmental factors were suggested to play a role in the disease pathogenesis (Chang et al., 2020). However, the seasonal nature of outbreaks suggests an infectious etiology of Kawasaki vasculitis (Valtuille et al., 2023). Multiple DNA and RNA viruses and bacterial pathogens were suggested as causing Kawasaki vasculitis (Guo et al., 2022; Huang et al., 2020; Kafetzis et al., 2001; Xiao et al., 2020). Having many microbes linked to Kawasaki vasculitis could indicate that the disease is multifactorial, where multiple factors contribute to the disease pathogenesis.

The role of lipolytic enzymes in the pathogenesis of *Mycobacterium tuberculosis* is discussed in the review by Lin et al. There are four types of lipolytic enzymes in *M. tuberculosis* (Mtb) based on specificity to a substrate (Dedieu et al., 2013; Delorme et al., 2012) The first class contains lipases hydrolyzing water-insoluble long-chain carboxylesters like long-chain triglycerides (TAG). Esterases are in the second group, which hydrolyze small water-soluble carboxylesters. The third group includes phospholipases. The last four groups contain cutinases, which digest carboxylesters. Lipases digest lipids in the extracellular matrix, promoting Mtb tissue penetration (Nazarova et al., 2017). Also, Mtb lipases digest lipids to release energy and survive inside the cells (Kumari et al., 2020). Mtb lipases could be used as a disease biomarker (Low et al., 2009) or could be a target for novel therapeutics (West et al., 2011).

The interaction between microflora and cervical cancer progression is discussed by Amaris et al. Cervical cancer is ranked as the most common cancer in women (Arbyn et al., 2020). *Fusobacterium* spp., *Peptostreptococcus* spp., *Campylobacter* spp., *and Haemophilus* spp., as potential biomarkers for cervical cancer progression (He et al., 2022; Wu et al., 2021; Zhou et al., 2022). Additionally, *Alloscardovia* spp., *Eubacterium* spp., *and Mycoplasma* spp. were identified in HPV-positive cervical cancer (Gao et al., 2013), while *Methylobacterium* spp. were more often detected in HPV-negative carcinomas.

Animal models of *Klebsiella pneumonia* infection of the mucosa are summarized by Assoni et al. Multiple factors should be considered when selecting an animal model: site of infection, type of immune response and susceptibility of an animal. Mice and rats were the most used to study *K. pneumonia* respiratory tract infection (Ferreira et al., 2019; van der Weide et al., 2020). A rabbit model was used to study empyema caused by *K. pneumonia* (Shohet et al., 1987). More recently, cynomolgus macaques were used to study the pathogenesis and immune response to *K. pneumonia* (Liu et al., 2022). This model provides an inside look at the immune response to this microbe. The Zebrafish model was used to study neutrophil and macrophage reaction to *K. pneumonia* (Zhang et al., 2019). *K. pneumonia* can colonize different niches, which makes it challenging to select an appropriate animal model. Careful considerations should be taken before selecting a model to study *K. pneumonia* infection.

In conclusion, this Research Topic provides a collection of reviews covering pathogenic mechanisms of some important microbial pathogens of the section Infectious Agents and Disease. This Research Topic will be of interest for researchers, healthcare providers and infection control officials.

Author contributions

AC: Writing – original draft, Writing – review & editing. DP: Writing – original draft, Writing – review & editing. SK: Writing – original draft, Writing – review & editing.

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Staphylococcus aureus lipoproteins in infectious diseases

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Infections with the Gram-positive bacterial pathogen *Staphylococcus aureus* remain a major challenge for the healthcare system and demand new treatment options. The increasing antibiotic resistance of *S. aureus* poses additional challenges, consequently inflicting a huge strain in the society due to enormous healthcare costs. *S. aureus* expresses multiple molecules, including bacterial lipoproteins (Lpps), which play a role not only in immune response but also in disease pathogenesis. *S. aureus* Lpps, the predominant ligands of TLR2, are important for bacterial survival as they maintain the metabolic activity of the bacteria. Moreover, Lpps possess many diverse properties that are of vital importance for the bacteria. They also contribute to host cell invasion but so far their role in different staphylococcal infections has not been fully defined. In this review, we summarize the current knowledge about *S. aureus* Lpps and their distinct roles in various infectious disease animal models, such as septic arthritis, sepsis, and skin and soft tissue infections. The molecular and cellular response of the host to *S. aureus* Lpp exposure is also a primary focus.

KEYWORDS

lipoproteins, lipopeptides, *Staphylococcus aureus*, infection, immunity, TLR2, host-pathogen interactions, metabolic fitness

Introduction

The Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) is mostly known as being associated with dreaded antibiotic-resistant infections, and rightly so, *S. aureus* plays a much broader role in human diseases. On the one hand, *S. aureus* colonizes nearly half of the human population, permanently or intermittently, as a commensal bacterium (Wertheim et al., 2005). On the other hand, *S. aureus* is able to rapidly manifest its highly pathogenic traits as soon as it invades our body, and frequently causes severe clinical infections in humans, such as osteomyelitis, infective endocarditis, infectious arthritis, metastatic abscess formation and device-related infections (Edwards

and Massey, 2011; Tong et al., 2015). It is also well known as the leading cause of bloodstream infections (Edwards and Massey, 2011). However, the molecular bases of *S. aureus* transition from commensal to pathogen remain elusive. Thus, gaining a greater understanding of its virulence mechanisms and interaction with the host is of vital importance in order to combat infectious diseases by *S. aureus*.

Staphylococcus aureus is a very resourceful pathogen (Lowy, 1998) as it possesses an immense arsenal of virulence factors, which enable the bacterium to thrive as an opportunist in humans. By biological function, virulence factors can be categorized as toxins, enzymes, immune evaders, as well as adhesins (Jin et al., 2021). By origin, they are divided to a capsular polysaccharide (O'Riordan and Lee, 2004), bacterial surface proteins (Foster et al., 2014; Jin et al., 2021), cell wall components (Lowy, 1998; Xia et al., 2010), and extracellular toxins (Xu and McCormick, 2012). Each virulence factor may have multiple biological functions. The perfect combination of those bacterial components helps staphylococci to adhere to host cells/tissues, resist engulfment of phagocytes, lyse the leukocytes, escape the immune killing, and finally cause the systemic and focal infections in different organs.

In the context of septic arthritis that is mainly caused by *S. aureus*, numerous virulence factors of *S. aureus* as well as various host factors targeted by the bacterium have been widely studied lately (Fei et al., 2011, 2022; Ali et al., 2015a,b,c; Mohammad et al., 2016, 2019, 2020; Na et al., 2016, 2020; Baranwal et al., 2017; Fatima et al., 2017; Jarneborn et al., 2020). These, among other important *S. aureus* virulence factors, have been extensively reviewed elsewhere (Mohammad, 2020; Jin et al., 2021).

Among the wide array of bacterial molecules that *S. aureus* exhibits are the lipoproteins (Lpps), which represent a major class of surface proteins in this opportunistic pathogen (Nguyen and Götz, 2016). Thus far, up to 70 Lpps have been detected in *S. aureus*, and the number of Lpps vary within various *S. aureus* genomes (Shahmirzadi et al., 2016). It is now widely believed that Lpps, peptidoglycan (PGN; Krause, 1975; Hrsak et al., 1979; Muller-Anstett et al., 2010; Volz et al., 2010; Schaffler et al., 2014) and bacterial excreted DNA/RNA (Miyake et al., 2018) are the main immune stimulators. By contrast, lipoteichoic acid (LTA), previously mistakenly considered an immunostimulant because of its contamination with Lpps (Hashimoto et al., 2006a,b; Zahringer et al., 2008), is not a TLR2 agonist. Unfortunately, in many review articles and textbooks it is still described as such.

It is well known that Lpp maturation is of critical importance for pathogenicity, inflammation, and immune signaling (Stoll et al., 2005; Nguyen et al., 2015; Nguyen and Götz, 2016). Lpps also play an essential role in the bacterial survival under infectious conditions due to their broad range of functions, including nutrient- and ion acquisition (Schmaler et al., 2009; Nguyen and Götz, 2016; Shahmirzadi et al., 2016; Nguyen et al., 2020). Lately, *S. aureus* Lpps have been shown to display important, but also differential roles in various inflammatory or infectious *in vivo* settings. Such host–pathogen interactions are the main focus of this review article.

Bacterial lipoproteins

Staphylococcus aureus Lpps consist of a lipid and a protein moiety. The lipid part is covalently linked to a cysteine residue in the N-terminal region, enabling anchoring of Lpps to the outer leaflet of the bacterial cytoplasmic membrane (Nguyen and Götz, 2016). Furthermore, the triacylated fatty acid structure of the lipid moiety is incorporated into the membrane, while the protein portion protrudes toward the cell wall and beyond (Nguyen and Götz, 2016; Shahmirzadi et al., 2016). In contrast to those of *S. aureus* and other Gram-positive bacteria, Lpps of Gram-negative bacteria are also lipid-anchored to the inner leaflet of the outer membrane (Braun and Rehn, 1969).

The lipid portion of Lpps in *S. aureus* serves as a microbe-associated molecular pattern (MAMP) component and alerts the innate immune system through detection by pattern recognition receptors (PRRs), mainly TLR2, in host cells (Nguyen and Götz, 2016; Nguyen et al., 2017). Despite the fact that the lipid moiety is embedded in the membrane, a minor proportion of mature Lpps in *S. aureus* tend to be released from the membrane, enter the cell wall, and parts of the lipidated structures can be exposed on the cell surface (Stoll et al., 2005). The lipid modification is an absolute requirement for the activation of the host immune signaling, as Lpps lacking the lipid structure display no such stimulatory activity (Stoll et al., 2005; Nguyen and Götz, 2016). Thus, the lipid moiety functions as an important danger signal to the host (Nguyen and Götz, 2016; Nguyen et al., 2017). Consistently, Lpps and/or lipopeptides are the predominant ligands of TLR2 (Aliprantis et al., 1999; Brightbill et al., 1999; Hashimoto et al., 2006a).

Staphylococcus aureus mutant strains deficient in pre-Lpp lipidation (Δ*lgt* mutant) are less virulent than their parental strains due to reduced pathogenicity (Schmaler et al., 2009, 2010; Nguyen and Götz, 2016; Mohammad et al., 2020, 2021). The various outcomes of Lpp originating from *S. aureus* are summarized in Tables 1, 2, reviewed in Mohammad (2020).

Abbreviations: Lpps, Lipoprotein(s); *lpl*, lipoprotein-like lipoprotein genes; Lpl1, lipoprotein-like protein 1; *lgt*, diacylglyceryl transferase enzyme encoding gene; *lsp*, signal peptidase II encoding gene; *lns*, lipoprotein *N*-acylation transferase system; *lnt*, apolipoprotein *N*-acyltransferase; LTA, lipoteichoic acid; PGN, peptidoglycan; TSST-1, Toxic shock syndrome toxin-1; EVs, extracellular vesicles; MAMP, microbe-associated molecular pattern; PRRs, pattern recognition receptors; TLR2, Toll-like receptor 2; IL, interleukin; KC, keratinocyte-derived chemokine; MCP-1, monocyte chemoattractant protein 1; MIP-2, macrophage inflammatory protein-2; MPO, myeloperoxidase; PAI-1, plasminogen activator inhibitor-1; TF, tissue factor; *S. aureus*, *Staphylococcus aureus*.

TABLE 1 *Staphylococcus aureus* lipoproteins and their distinct role in various *in vitro* settings.

Cell types	Species/compound	Outcome	References
Human cells			
– MonoMac6, – Pulmonary epithelial cell line (A549), – Umbilical vein endothelial cells	*S. aureus* Δ*lgt*	Impaired production of IL-1, IL-6, and MCP-1	Stoll et al. (2005)
MonoMac6	*S. aureus* Δ*lgt*	Diminished levels of TNFα and IL-10	Stoll et al. (2005)
MonoMac6	*S. aureus* Δ*lpl*	Attenuated induction of TNF and IL-6	Nguyen et al. (2015)
THP-1	Heat-killed *S. aureus* Δ*lgt*	Lower production of TNF, IL-1β and IL-8	Kang et al. (2011)
– MonoMac6 – TLR2-transfected HEK293 cells	Purified *S. aureus* SitC	Induction of TNF and IL-6 expression	Muller et al. (2010)
Whole blood	*S. aureus* Δ*lgt*	Impaired proliferation	Bubeck Wardenburg et al. (2006)
Blood serum	*S. aureus* Δ*lgt*	No difference	Bubeck Wardenburg et al. (2006)
HeLa cells	*S. aureus* Δ*lpl*	Increased cell invasion frequency	Nguyen et al. (2016)
Murine cells			
Peritoneal macrophages	*S. aureus* Δ*lgt*	Impaired TLR2-MyD88-mediated cytokine production of IL-1, IL-6, IL-10 and TNF	Schmaler et al. (2009)
Peritoneal macrophages	Purified *S. aureus* SitC	TLR2-MyD88-mediated induction of TNF and IL-6 expression	Kurokawa et al. (2009)
Keratinocytes	Purified *S. aureus* SitC	Induction of TNF and IL-6 expression	Muller et al. (2010)
Peritoneal macrophages	– Purified *S. aureus* Lpp – Synthetic lipopeptides	TLR2-mediated induction of MIP-2, KC, and MCP-1 with a quick and dose-dependent release	Mohammad et al. (2019)
– Peritoneal macrophages – Splenocytes	– Purified *S. aureus* Lpp – Synthetic lipopeptides	TLR2-mediated induction of TNFα	Mohammad et al. (2019)
Peritoneal macrophages	– Purified *S. aureus* Lpp – Synthetic lipopeptides	TLR2-mediated induction of PAI-1, but not TF	Mohammad et al. (2021)
– Peritoneal macrophages – Splenocytes	*S. aureus* Δ*lgt* Extracellular vesicles	Impaired TLR2-mediated production of MIP-2, TNFα and IL-6	Kopparapu et al. (2021)
Whole blood	*S. aureus* Δ*lgt*	No difference	Mohammad et al. (2019)
Activated macrophages	*S. aureus* Δ*lgt*	Impaired proliferation	Bubeck Wardenburg et al. (2006)
Blood serum	*S. aureus* Δ*lgt*	Downregulated expression of IL-6 and KC, but not MCP-1	Mohammad et al. (2020)
Peritoneal macrophages	Purified *S. aureus* Lpp + GFP-expressing *S. aureus*	No impact on phagocytosis capacity	Mohammad et al. (2019)
Bone marrow-derived dendritic cells	*S. aureus* Δ*lgt*	Impaired TLR2-MyD88-mediated expression of B-cell activating factor	Im et al. (2020)
Bone marrow-derived dendritic cells	Synthetic lipopeptides	Induced TLR2-MyD88-mediated expression of B-cell activating factor	Im et al. (2020)
HeLa	Purified *S. aureus* Lpp – without lipid moiety	Extended G2 phase cycle	Nguyen et al. (2016)
HaCaT	Purified *S. aureus* Lpp – without lipid moiety	Increased host cell invasion *via* activation of Hsp90 receptor	Tribelli et al. (2020)
Others			
Bovine mammary epithelial cells	*S. aureus* Δ*lgt*	Impaired TLR2-mediated production of TNF, IL-6, and CXCL8	Liu et al. (2022)

Lpp, lipoproteins; Δ*lgt*, deletion mutant of preprolipoprotein diacylglyceryl transferase; Δ*lpl*, deletion mutant of lipoprotein-like lipoprotein genes; GFP, green fluorescent protein.

Biosynthetic pathway of *Staphylococcus aureus* Lpps

As an important part of the bacterial cell envelope homeostasis, lipidation of proteins naturally occurs as a posttranslational molecule reformation process, which ultimately leads to the formation of mature Lpps in both Gram-positive and Gram-negative bacteria (Buddelmeijer, 2015). Lpp modifications occur within the cytoplasmic membrane of the bacteria and involve the activity of the diacylglyceryl transferase (Lgt) and the

TABLE 2 *Staphylococcus aureus* lipoproteins and their distinct role in different *in vivo* animal models.

Site/organ – administration	Species/compound	Outcome	References
Murine models			
Knee – intra-articular	Purified *S. aureus* Lpp	– Bone destruction ↑	Mohammad et al. (2019)
Knee – intra-articular	*S. aureus* Δ*lgt*	– Knee swelling ↑ – Bacterial load ↑	Mohammad et al. (2019)
Knee – intra-articular (co-injection)	Purified *S. aureus* Lpp + *S. aureus*	– Bone destruction ⇩ – Bacterial load ⇩	Mohammad et al. (2019)
Knee – intra-articular	– Purified *S. aureus* Lpp – Synthetic lipopeptides	– Bone resorption ↑	Schultz et al. (2022)
Femur – intraperitoneal	Synthetic lipopeptides	– Bone resorption ↑	Kim et al. (2013)
Knee – intra-articular	*S. aureus* Δ*lgt* Extracellular vesicles	– Virulence ⇩	Kopparapu et al. (2021)
Septic arthritis – intravenous	*S. aureus* Δ*lgt*	– Virulence ⇩ – Arthritis severity- no effect	Mohammad et al. (2020)
Sepsis – intravenous	*S. aureus* Δ*lgt*	– Virulence ⇩	Schmaler et al. (2009)
Sepsis – intravenous	*S. aureus* Δ*lgt*	– Virulence ↑	Bubeck Wardenburg et al. (2006)
Sepsis – intravenous	*S. aureus* Δ*lsp*	– Virulence ⇩	Bubeck Wardenburg et al. (2006)
Sepsis – intravenous	Synthetic lipopeptide pretreatment + methicillin-resistant *S. aureus* (MRSA)	– Bacterial load ⇩ – Survival ↑	Huang et al. (2017)
Skin – subcutaneous	Purified *S. aureus* Lpp	– Skin inflammation ↑	Mohammad et al. (2021)
Skin – subcutaneous	*S. aureus* Δ*lgt*	– Virulence ⇩	Mohammad et al. (2021)
Skin – epicutaneous	*S. aureus* Δ*lpl*	– Virulence ⇩	Nguyen et al. (2015)
Skin – intradermal	Purified *S. aureus* Lpp	– Virulence ↑	Saito and Quadery (2018)

Lpp, lipoproteins; Δ*lgt*, deletion mutant of preprolipoprotein diacylglyceryl transferase; Δ*lpl*, deletion mutant of lipoprotein-like lipoprotein genes; Δ*lsp*, deletion mutant of prolipoprotein signal peptidase.

signal peptidase (Lsp). In Gram-negative bacteria a third enzymatic step takes place that is catalyzed by the *N*-acyltransferase (Lnt), as described elsewhere (Nakayama et al., 2012; Buddelmeijer, 2015; Nguyen and Götz, 2016; Nguyen et al., 2020).

Lacking the *lnt* gene, *S. aureus* has been considered to produce only diacylated Lpps. However, with the development of gas chromatography–mass spectrometry (GC–MS) analysis, *S. aureus* was found to produce diacylated (Tawaratsumida et al., 2009) and triacylated Lpps (Kurokawa et al., 2009) depending on the environmental conditions (Kurokawa et al., 2012). Interestingly, in our previous studies, we identified SitC as triacylated Lpp in *S. aureus* (Nguyen et al., 2017) while Lpl1 from *S. aureus* SA113 was shown to exist both in a diacylated and triacylated form (Schultz et al., 2022). Recently, it was found that the *N*-acylation of Lpps in *S. aureus* and most likely many other Firmicutes, which lack *lnt*, is mediated by the two enzymes LnsA and LnsB (Gardiner et al., 2020). Figure 1 illustrates the Lpp biosynthesis pathway in *S. aureus*.

The *Staphylococcus aureus* Lpp function

Lpps are characteristically divided into two functional entities, whereby the protein moiety serves and maintains the bacteria with its metabolic nutrition and function, whereas the lipid moiety has a key role in anchoring the protein into the bacterial membrane as well as in pathogenicity (Shahmirzadi et al., 2016; Nguyen et al., 2020).

The importance of *S. aureus* Lpps can be studied in numerous ways. Firstly, by inhibiting their maturation by mutating the specific catalytic enzymes, Lgt and Lsp. In the Δ*lgt* mutant the cysteine residue remains unmodified, hence preventing lipidation (Stoll et al., 2005). In the Δ*lsp* mutant, the first lipid modification of the Lpp is initiated; however, the signal peptide remains intact rather than cleaved (Nguyen and Götz, 2016). This ultimately leads to a disturbed balance within the Lpp biosynthetic machinery and may result in improper accumulation of immature Lpp.

Secondly, the significance of bacterial Lpps can also be investigated by isolation and purification of specific Lpps from the bacteria of interest, or thirdly, by using synthetic lipopeptides that resemble the lipid moiety structure of bacterial Lpp, such as Pam_3CSK_4 (triacylated lipid form) or Pam_2CSK_4 (diacylated lipid form). With regard to purified *S. aureus* Lpps, many pathogenic *S. aureus* strains harbor a genomic island, termed νSaα (Kuroda et al., 2001; Diep et al., 2006; Baba et al., 2008), that possesses highly conserved genes such as the lipoprotein-like cluster (*lpl*; Baba et al., 2008;

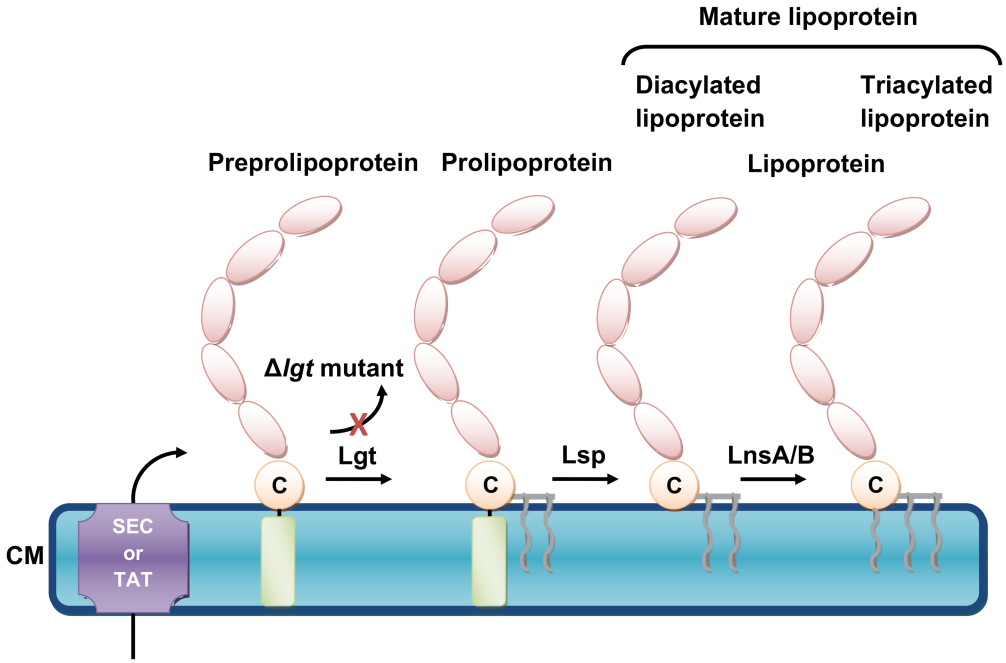

FIGURE 1
The biosynthetic pathway of *Staphylococcus aureus* lipoproteins. *S. aureus* lipoproteins are synthesized as preprolipoprotein precursors, which comprise an N-terminal signal peptide sequence (depicted as light-green cylinder), and are translocated across the cytoplasmic membrane (CM) by either the general secretory (Sec) or twin arginine translocation (TAT) pathways. The first enzyme, the preprolipoprotein diacylglyceryl transferase Lgt enables the transfer of a diacylglyceryl moiety to the indispensable cysteine residue (depicted as a beige circle with the letter, C), which forms a prolipoprotein. This lipid modification is followed by the second enzyme, the prolipoprotein signal peptidase Lsp., which cleaves the signal peptide and generates a mature diacylated lipoprotein. A third enzyme is required in order to form a mature triacylated lipoprotein. This lipid acylation is catalyzed by lipoprotein *N*-acylation transferase system LnsA/B. When *lgt* is deleted (Δ*lgt* mutant), the maturation of lipoproteins is inhibited and lipidation no longer occurs.

Nguyen et al., 2015) encoding, among others, for the model lipoprotein Lpl1. The latter is denoted Lpl1(+sp) or Lpl1(−sp) depending on whether it carries or not the lipid moiety (Nguyen et al., 2015). Lately, Lpl1 has been extensively utilized in numerous studies to assess the importance of Lpps (Nguyen et al., 2015, 2016, 2017, 2018; Kumari et al., 2017; Mohammad et al., 2019, 2021; Tribelli et al., 2020; Kopparapu et al., 2021; Schultz et al., 2022). Figure 2 illustrate the structures and entities of Lpps as well as synthetic lipopeptides in *S. aureus*.

The host immune response to Lpps in infections with *Staphylococcus aureus*

Despite its immense repertoire of virulence factors, upon host invasion, *S. aureus* alerts the host's immune system and a battle between the host and the pathogen is immediately initiated. As mentioned, TLR2 serves as a critical receptor for Lpp and also recognizes synthetic lipopeptides (Aliprantis et al., 1999). Some of the innate and adaptive immune responses as well as complications that arise upon host recognition of *S. aureus* Lpps are briefly discussed below.

Staphylococcus aureus Lpps and innate immunity

It is now well established that purified *S. aureus* Lpps trigger a rapid influx of innate immune cells, such as monocytes/macrophages and neutrophils, when injected in murine knee joints (Mohammad et al., 2019). A similar outcome with rapid recruitment of inflammatory cells was also observed in the skin model, as subcutaneous injection of *S. aureus* Lpps resulted in infiltration of neutrophils and monocytes/macrophages and induced skin lesions in mice (Mohammad et al., 2021). Intriguingly, leukocyte depletion by cyclophosphamide treatment was shown to diminish the Lpp-induced effect in the skin model. While PBS-treated control mice upon infection with *S. aureus* Newman parental strain, expressing Lpps, displayed more severe skin lesions and higher bacterial burden than mice infected with the derivative mutant strain Δ*lgt*, which lacks Lpp expression, the leukocyte depleted mice exhibited no such effect (Mohammad et al., 2021). This suggests that the effect induced by *S. aureus* Lpp expression in the skin model is dependent on the presence of leukocytes. Upon depletion of monocytes/macrophages in the purified *S. aureus* Lpp-induced local murine knee arthritis model, the arthritogenic and bone

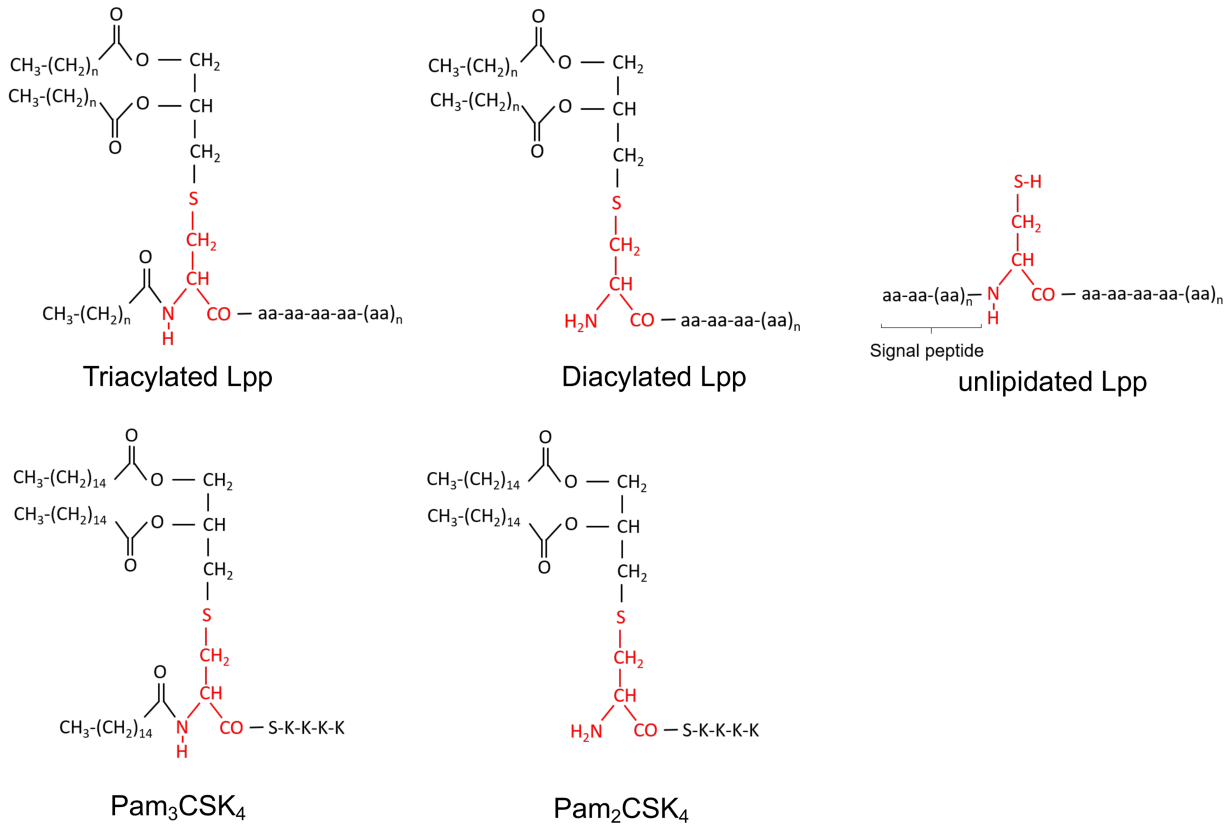

FIGURE 2
Chemical structure of *Staphylococcus aureus* Lpps (triacylated, diacylated and unlipidated lipoproteins) and synthetic peptides (Pam_2CSK_4 and Pam_3CSK_4). Cystein is marked in red.

destructive effect was abolished, whereas neutrophil depletion played a minor role (Mohammad et al., 2019). This indicates that monocytes/macrophages are the main culprit behind the development of local knee arthritis in response to purified Lpps. Importantly, when purified Lpps and live *S. aureus* were co-injected into murine knee joints, bacterial eradication occurred. This was mediated by monocytes/macrophages and neutrophils, since depletions of these phagocytes resulted in aggravated disease severity and increased bacterial burden in local knee joints (Mohammad et al., 2019). Although *S. aureus* Lpps are known as potent stimulators of nitric oxide synthase and mediate nitric oxide production in mouse macrophages (Kim et al., 2015), our previous data showed that anti-nitric oxide synthase treatment had no impact on the bacterial clearance in knee joints (Mohammad et al., 2019).

TLR2 interacts differently with bacterial Lpps/lipopeptides depending on their lipid chain structure, although the downstream signaling events are conserved (Schenk et al., 2009). The N-terminal cysteine modification in bacterial Lpps (diacylated or triacylated structures) ultimately dictates the type of TLR2 hetero-complexes (with TLR1 or TLR6) that will form and lead to receptor activation (Gardiner et al., 2020). The elicited innate immune signaling, in turn, further modulates the host's immune response and the inflammatory reactions (Nguyen and Götz, 2016). Importantly, the construction of *S. aureus* Δ*lgt* mutants along with improved methods for extraction and purification of Lpps (Stoll et al., 2005), have clearly proved that TLR2 is a major receptor exclusively activated by Lpps/lipopeptides (Bubeck Wardenburg et al., 2006; Hashimoto et al., 2006a,b). The TLR2 sensing ability varies considerably between different bacterial species (Nguyen and Götz, 2016). Nevertheless, among the immense range of virulence factors staphylococci dispose, Lpps are still considered as the main immunobiologically active components (Hashimoto et al., 2006a,b; Nguyen and Götz, 2016). It may seem paradoxical in bacterial evolution that Lpps evoke a cell-mediated immune response, specifically through TLR2-MyD88 signaling, thus initiating a battle with the host immune system. However, this skillful bacterium is known to utilize various strategies in order to escape immune recognition (de Jong et al., 2019; Cheung et al., 2021). A good example is the release of the staphylococcal superantigen-like protein 3 (SSL3), which is a TLR2 antagonist (Koymans et al., 2015). SSL3 prevents TLR2 dimerization with its co-receptors by forming a complex that partially closes off the pocket in TLR2, which consequently inhibits the binding between Lpps and TLR2 (Koymans et al., 2015).

Staphylococcus aureus Lpps and adaptive immunity

In our previous work we showed that T-cells play a minor role in the severity of arthritis that follows intra-articular injection of *S. aureus* Lpl1 in the murine knee model, as CD4+ and CD8+ T-cell depletion as well as CTLA4-Ig treatment gave rise to similar outcomes in the treated groups and in the control group (Mohammad et al., 2019).

In *S. aureus*-induced skin infection model, a recent study showed that purified Lpps caused skin inflammation, accompanied by interferon γ producing T cell accumulation (Saito and Quadery, 2018). CD4+ T-cells have been suggested to offer protection against secondary *S. aureus* skin and soft tissue infections (Montgomery et al., 2014). However, it was recently demonstrated that skin tissues from mouse auricle upon challenge with purified *S. aureus* Lpps did not increase the migration levels of T-cells or NK cells in both C57BL/6 wild-type and TLR2 deficient mice (Mohammad et al., 2021).

In the case of Lpp-induced joint inflammation, intra-articular injection of *S. aureus* Lpps did not trigger the influx of either B- or T-cells (Mohammad et al., 2019). In *S. aureus* skin infection, B-cells are known to produce antibodies that are directed against *S. aureus* virulence factors, and thus mediate important immune responses against the pathogen (Krishna and Miller, 2012). However, in the context of the *S. aureus* Lpp-induced skin infection model, subcutaneous injection of the *S. aureus* Lpp-expressing strain induced the same effects with regard to the severity of the skin lesions and bacterial burden in mice deficient in B- and T-cells (SCID mice), as in Balb/c control mice (Mohammad et al., 2021). These findings indicate that the adaptive immunity plays a minor role in the Lpp-induced effects in murine skin infection.

Lipoproteins and host immune response

Importance of *Staphylococcus aureus* Lpps in iron acquisition and metabolic fitness

Lpp maturation is a necessity for many bacterial species in their battle against the host (Kovacs-Simon et al., 2011). Investigation of the pathogenic properties of Lpps has mostly been conducted through implementation of *lgt* or *lsp* mutants in various *in vitro* and *in vivo* settings. Important parameters, such as the ability to acquire ions and nutrients as well as bacterial survival and bacterial proliferation, have thus been studied to a large extent. Lpp maturation has been shown to be of fundamental importance in iron acquisition, stimulating *S. aureus* bacterial growth, as the *S. aureus* SA113 Δ*lgt* mutant strain exhibited growth defects compared to its parental strain under nutrient-restricted conditions (Stoll et al., 2005). In line with these results, we demonstrated that the Δ*lgt* mutant had impaired growth compared to its parental strain in nutrient-poor conditions, while the Δ*lgt* mutant and parental strain had similar growth rates in nutrient-rich conditions (Mohammad et al., 2020). Schmaler et al. demonstrated that upon repletion of iron sources, the SA113 Δ*lgt* mutant strain that had been grown in iron-depleted conditions exhibited impaired iron-dependent bacterial growth compared to its parental strain (Schmaler et al., 2009). Acquisition of iron is a pervasive feature employed by pathogens such as *S. aureus* to improve their survival and proliferation (Hammer and Skaar, 2011; Haley and Skaar, 2012). It is not surprising that among proposed 67 Lpp of *S. aureus* USA300, a highest number of Lpp are involved in iron transport (Shahmirzadi et al., 2016). It has been experimentally proved that *S. aureus* uses 5 transport systems to obtain iron from the environment including FhuCBG Fe chelator system with the involvement of 2 Lpp FhuD1 and FhuD2 (Sebulsky et al., 2000, 2004; Sebulsky and Heinrichs, 2001), SirABC iron regulator system with Lpp SirA (Heinrichs et al., 1999; Dale et al., 2004), IsdCDEF heme uptake system with Lpp IsdE (Mazmanian et al., 2002; Grigg et al., 2007), SstABCD siderophore transport system with Lpp SstD (Morrissey et al., 2000) and FepABC iron transport system with Lpp FepA (Biswas et al., 2009).

Overall, Lpps in *S. aureus* contribute to provide the bacteria with adequate amounts of iron under infectious conditions. As iron is an essential resource for *S. aureus* to survive and thrive, especially in the iron-depleted conditions of the infected human body (Hammer and Skaar, 2011; Sheldon and Heinrichs, 2012), inadequate maturation of Lpps can be detrimental for this pathogenic bacterium as the maintenance of the metabolic activity and fitness is threatened. The largest iron reservoir in the host is represented by the heme iron, which serves as the preferred iron source for *S. aureus* (Skaar et al., 2004). As *S. aureus* seeks ways to acquire sufficient iron uptake during an infection, the host innate immune system employs defensive mechanisms by limiting the iron availability to the invading bacteria, functioning as one of the primary host defense responses during infection (Haley and Skaar, 2012; Cassat and Skaar, 2013). *Staphylococcus aureus* can therefore utilize Lpps as an efficient iron transporter in order to circumvent this nutritional immunity, thus increasing its chances of survival and consequently of causing disease. The loss or disturbances of iron-regulated systems, including Lpp maturation, during *S. aureus* infections are strongly associated with attenuated virulence (Torres et al., 2006; Pishchany et al., 2014), which further implies the critical role iron serves in the pathogenesis of diseases this bacterium causes.

Staphylococcus aureus Lpps – *in vitro* effects

The use of *S. aureus* Δ*lgt* mutants as well as purified Lpp compounds and synthetic lipopeptides have demonstrated the

important role of Lpps in triggering the release of cytokines and chemokines as a response of the host immune system.

Synthetic lipopeptides are known to potently stimulate the secretion of various cytokines in human monocytes and macrophages (Hoffmann et al., 1988; Kreutz et al., 1997). In contrast to the parental strain, the *S. aureus* Δ*lgt* mutant displays reduced immune-stimulatory abilities in various human cells, such as the human monocytic cell line (MonoMac6), the human pulmonary epithelial cell line (A549), and in human umbilical vein endothelial cells, with consequent impaired production of pro-inflammatory cytokines and chemokines, including IL-6, IL-8, and monocyte chemoattractant protein 1 (MCP-1; Stoll et al., 2005). Furthermore, both TNFα and IL-10 levels were diminished over time in MonoMac6 cells (Stoll et al., 2005). In another human monocytic cell line, referred to as THP-1 cells, cell stimulation with heat-killed *S. aureus* Δ*lgt* mutant was associated with lower production of TNF, IL-1β and IL-8, as compared to those cells that were stimulated with the heat-killed *S. aureus* parental strain (Kang et al., 2011). Also, *S. aureus* Δ*lgt* mutants were associated with impaired TLR2-MyD88-mediated cytokine production (IL-1, IL-6, IL-10 and TNF) in mouse peritoneal macrophages, whereas the *S. aureus* parental strain induced early and strong cytokine release (Schmaler et al., 2009). Purified *S. aureus* SitC is well known to induce TNF and IL-6 expression in murine peritoneal macrophages in a TLR2-MyD88-dependent manner (Kurokawa et al., 2009). The release of such pro-inflammatory cytokines was also demonstrated in human monocytes and mouse keratinocytes, and in TLR2 expressing HEK cells (Muller et al., 2010).

We showed that stimulation of peritoneal macrophages with purified *S. aureus* Lpl1 induced a quick and dose-dependent release of the neutrophil chemoattractant, macrophage inflammatory protein-2 (MIP-2) and keratinocyte-derived chemokine (KC) as well as the monocyte chemoattractant, MCP-1 (Mohammad et al., 2019). These strong and rapid effects were observed already 4 h after stimulation and were dependent on the lipid- and not the protein moiety. In fact, purified Lpl1(+sp) induced similar levels as lipopolysaccharide, which served as the positive control, while Lpl1(−sp), lacking the lipid moiety, was only capable to exhibit similar stimulation levels as the negative medium control in terms of secretion of the assessed chemokines (Mohammad et al., 2019). These findings were only observed in the peritoneal macrophage supernatants collected from the C57BL/6 wild-type and not in the TLR2 deficient mice, and were thus mediated through the PRR TLR2 (Mohammad et al., 2019). The importance of *S. aureus* Lpp and its lipidation was further verified by the fact that the triacylated synthetic lipopeptide Pam_3CSK_4 induced similar MIP-2, KC and MCP-1 levels to the purified Lpl1(+sp) compound (Mohammad et al., 2019). In addition to the neutrophil- and monocyte chemoattractant chemokines, the pro-inflammatory cytokine, TNFα, was also induced in a TLR2-dependent manner upon Lpl1(+sp) and Pam_3CSK_4 stimulation of both mouse peritoneal macrophages and splenocyte cultures (Mohammad et al., 2019). Overall, our findings indicate that *S. aureus* Lpps are potent immune stimulators that induce rapid release of important chemokines and cytokines from immune cells exclusively *via* TLR2.

With respect to bacterial growth, our data revealed that SA113 parental strain and Δ*lgt* mutant strain proliferated similarly in mouse whole blood during a 2 h incubation (Mohammad et al., 2019), which are not in agreement with previous findings in human whole blood (Bubeck Wardenburg et al., 2006). However, parameters such as the assessed time points, different *S. aureus* strains (SA113 vs. Newman), and the different sources of whole blood (mouse vs. human) might explain these discrepancies. Nevertheless, in the model of systemic infection, the expression of mature *S. aureus* Lpp was responsible for a more systemic inflammatory status, whereby the Newman parental strain was associated with higher levels of IL-6 and KC, but not MCP-1 (Mohammad et al., 2020). No differences between the parental strain and the *lgt* deficient mutant strain were observed in TLR2 knockout mice, suggesting that the cytokine response was dependent on TLR2 (Mohammad et al., 2020). Although *S. aureus* Newman parental strain had higher bacterial proliferation in the presence of activated murine macrophages compared to the Newman Δ*lgt* mutant strain, the phagocytic capacity of macrophages was not affected by the Lpp expression (Bubeck Wardenburg et al., 2006). These results are in agreement with our data whereby purified *S. aureus* Lpl1 did not influence the phagocytic capacity of macrophages (Mohammad et al., 2019).

It was very recently demonstrated that *S. aureus* Lpps are the culprits behind the pro-inflammatory property of *S. aureus* extracellular vesicles (EVs) *in vitro*. Upregulated levels of MIP-2, TNFα and IL-6 were observed upon stimulation of murine peritoneal macrophages with Lpp-carrying EVs but not with Lpp-deficient ones, since EVs isolated from *S. aureus* Δ*lgt* mutant lacked the capacity to induce any immune stimulation (Kopparapu et al., 2021). A similar outcome was observed in an *in vitro* splenocyte stimulation setting (Kopparapu et al., 2021). As expected, this pro-inflammatory response to *S. aureus* Lpp EVs was shown to be TLR2-dependent (Kopparapu et al., 2021).

Gardiner et al. studied the importance of *lnsA/B* in the immune-stimulatory effects of *S. aureus* using HEK-TLR2 cells, and demonstrated that the expression of IL-8 increased ~10 times upon deletion of either of the two genes, *lnsA* or *lnsB* (Gardiner et al., 2020). This suggests that an intact *lnsA/B* system is advantageous for *S. aureus* to evade TLR2 immune recognition. Furthermore, various staphylococcal species possess varying lengths of the *N*-acylation at the N termini of the cysteine residue within the lipid moiety of Lpps: the opportunistic pathogens *S. aureus* and *S. epidermidis* carry a long-chain *N*-acylated fatty acid, while the non-commensal, non-pathogenic *S. carnosus* (Biswas et al., 2009; Rosenstein and Götz, 2013) carries a short-chain *N*-acylated fatty acid, which corresponds to a heptadecanoyl fatty acid and an acetyl fatty acid, respectively (Nguyen et al., 2017). Nguyen et al.

revealed that *S. carnosus* was capable to induce 10-fold higher TLR2-mediated cytokine responses compared to *S. aureus* and *S. epidermidis* (Nguyen et al., 2017). Furthermore, both TNFα and IL-8 secretion were strongly upregulated by *S. carnosus* in MonoMac6 and HEK-TLR2 cells in comparison to several *S. aureus* strains, including SA113, HG003, and the MRSA strain, USA300 (Nguyen et al., 2017). This concept was further proved as the Lpp SitC that was purified from *S. carnosus* triggered higher TLR2-mediated induction of IL-8 than SitC that was extracted from *S. aureus* (Nguyen et al., 2017). This was also confirmed in human monocyte-derived dendritic cells whereby the levels of various pro-inflammatory cytokines were elevated in a similar manner (Nguyen et al., 2017). Overall, these findings are in line with previous reports that showed that different modifications of the lipid moiety trigger diverse TLR2 activations (Armbruster et al., 2019; Gardiner et al., 2020). Interestingly, the expression of other *S. aureus* components, more precisely capsular polysaccharide, can mask *S. aureus* Lpps and attenuate the recognition of Lpps and TLR2 activity (Hilmi et al., 2014).

It was recently demonstrated that Lpl alters the cell cycle in HeLa cells delaying the G2/M phase transition, which consequently leads to increased cell invasion (Nguyen et al., 2016). This effect was shown to be mediated by the secretion of cyclomodulin (Nguyen et al., 2016), which functions as bacterial toxin disturbing the regular course of the host cell cycle (Taieb et al., 2011). Cyclomodulin seems to be utilized by *S. aureus* not only to increase host invasion but also to induce bacterial proliferation within the host cells (Alekseeva et al., 2013). The factors that contribute to the effects of Lpl on host cells were recently addressed by Nguyen et al. (2018). In fact, it was revealed that not only the growth phase of *S. aureus* differentially affects the observed phenotypes but also that the host contributes to such effects since bacterial invasion frequency was higher in TLR2 expressing cells (Nguyen et al., 2018).

Nguyen et al. showed that deletion of the entire *lpl* operon in the *S. aureus* USA300 strain resulted in attenuated induction of pro-inflammatory cytokines in human monocytes, macrophages and keratinocytes, compared to the parental strain expressing the *lpl* gene cluster (Nguyen et al., 2015). To verify the observed phenomenon, the *lpl* cluster was cloned into another *S. aureus* strain, HG003, which naturally lacks *lpl* genes, and this resulted in increased production of the pro-inflammatory cytokines (Nguyen et al., 2015). The purified lipidated Lpp, Lpl1, was further shown to evoke a TLR2-dependent response (Nguyen et al., 2015). A recent study revealed that the cluster of *lpl* proteins in *S. aureus* was upregulated in a MRSA strain during sub-inhibitory exposure to β-lactam antibiotics (Shang et al., 2019). This suggests that *S. aureus* Lpps, more specifically, Lpl has a virulent nature resulting in enhanced pathogenicity of MRSA. *Staphylococcus aureus* Lpps and their differential roles in various *in vitro* settings are summarized in Table 1.

Staphylococcus aureus Lpps – *in vivo* effects: The role in virulence and pathogenicity during host invasion

Besides their vital role in maintaining and upregulating the fitness of the bacteria (Shahmirzadi et al., 2016), bacterial Lpps possess a variety of key functions, some of which serve critical roles during infectious and inflammatory conditions. In most, but not all cases, maturation of Lpps has been strongly associated with enhanced pathogenic invasion, bacterial survival and immune activation (Nguyen and Götz, 2016; Shahmirzadi et al., 2016).

Intra-articular injection of *Staphylococcus aureus* Lpps causes joint inflammation

The importance of IL-1 in Lpl1-induced synovitis was recently assessed in mice through treatment with the IL-1 receptor antagonist (anakinra). It was shown that IL-1 did not play a major role in the induction of synovitis in a local arthritis model (Mohammad et al., 2019). The importance of TNF has also been studied in the context of *S. aureus* Lpp exposure. We explored whether TNF inhibition had any beneficial effects in the model of purified Lpp-induced synovitis by treating the mice with anti-TNF treatment (etanercept). TNF was indeed partially involved in modulating the arthritogenic effects in local *S. aureus* Lpl1-induced knee arthritis (Mohammad et al., 2019).

Decreased arthritis severity is closely associated with lower levels of IL-6 in local joints, suggesting that IL-6 is an important cytokine for maintenance of septic arthritis (Mohammad et al., 2019). We also demonstrated that *S. aureus* Lpps trigger the quick release of KC and MIP-2 in local tissues including knees and skin with enhanced influx of phagocytes, consequent inflammation and tissue damages (Mohammad et al., 2019, 2021).

We reported that *S. aureus* Lpps play various roles in different murine models. In a mouse model of *S. aureus* septic arthritis, Lpps gave rise to pronounced arthritogenic effects in both NMRI and C57BL/6 wild-type mouse strains, whereas TLR2 deficient mice displayed no signs at all following intra-articular knee joint challenge with the purified Lpl1(+sp) (Mohammad et al., 2019). The arthritogenic properties were thus mediated through TLR2 but also *via* the lipid portion of Lpp, since Lpl1(−sp), comprising only of the protein moiety, lacked the ability to cause any knee joint swelling. In addition to the observed long-lasting macroscopic arthritic effect in the Lpl1(+sp) group, which was detected already within 24 h after injection, Lpl1 contributed to local knee synovitis in a dose-dependent fashion. The histological sections revealed that Lpl1 induced synovitis even when administered at the nanogram level, indicating that *S. aureus* Lpps are highly potent. Intriguingly, when challenging the mice with an Lpl1 dose at the microgram level, all joints developed bone erosions within 10 days after injection (Mohammad et al., 2019).

Prior to this study, we reported that murine knee joints, challenged with antibiotic-killed *S. aureus*, displayed severe bone erosion and long-lasting arthritis (Ali et al., 2015c). This aspect is indeed very clinically relevant since patients suffering of septic

arthritis are likely to develop irreversible permanent joint destruction, even after successful bacterial eradication through the standard treatment procedure (Goldenberg, 1998). Since the *S. aureus* cell wall components were responsible for causing this inflammatory effect in the joints, partially *via* TLR2 (Ali et al., 2015c), this prompted us to study the involvement of *S. aureus* Lpps in the host reaction. We were able to show that Lpl1 served as one of the inducers, and also elucidated the cellular mechanism by showing that monocytes/macrophages were the responsible cell type mediating the Lpl1-induced effect in the local knee joints through TLR2-dependent responses (Mohammad et al., 2019). This further strengthens the concept that the disease severity of septic arthritis is at least partially mediated by an exaggerated immune response that is triggered by specific bacterial components, such as Lpp (Mohammad et al., 2019), which is in agreement with previous reports (Deng et al., 1999; Ali et al., 2015c).

Intra-articular injection of *Staphylococcus aureus* Lpps causes focal bone resorption

Recently, we also demonstrated in a local knee arthritis model that *S. aureus* Lpp induced bone resorption in NMRI mice, an effect that was mediated through its lipid moiety and that was dependent on monocytes/macrophages (Schultz et al., 2022). Moreover, when challenging the mice with synthetic lipopeptides through intra-articular knee joint injection, the diacylated lipid moiety, Pam_2CSK_4, was more potent in inducing bone resorption than the triacylated lipid moiety, Pam_3CSK_4 (Schultz et al., 2022). In fact, we have recently showed that Lpl1(+sp), isolated from *S. aureus* SA113 strain, contains both diacyl and triacyl lipid-moieties (Schultz et al., 2022). A previous study demonstrated that intraperitoneal injection of synthetic lipopeptides, resembling the lipid structure of Lpps, were associated with severe bone loss in the femurs of mice (Kim et al., 2013). This further suggests that lipidated Lpps play a potent pathogenic role in the bone of mice independent of the route of administration.

Recently, we revealed that upon intra-articular knee joint injection of mice, Lpp-carrying EVs displayed pathogenic capacities by giving rise to more severe macroscopic arthritis as well as synovitis in two different mouse strains (NMRI and C57BL/6 wild-type mice), and that such effect was mediated by monocytes/macrophages *via* TLR2 (Kopparapu et al., 2021).

Staphylococcus aureus Lpps play distinct role in local- and hematogenous septic arthritis, and sepsis

In a local knee septic arthritis model triggered by intra-articular injection of living bacteria, the SA113 Δ*lgt* mutant strain, lacking Lpp expression, resulted in more pronounced knee joint swelling in comparison to its parental strain. In addition, this was accompanied by increased bacterial load as well as elevated IL-6 levels in the local infected knee joints (Mohammad et al., 2019). Conversely, in a mouse model of *S. aureus*-induced sepsis, inoculation with the same strain resulted in a lower bacterial burden in the knee joints of mice (Schmaler et al., 2009). Another study demonstrated that *S. aureus*, deficient in Lpp expression, causes bacterial immune evasion and lethal infections in a murine sepsis model (Bubeck Wardenburg et al., 2006). This clearly suggests once more that Lpps implement different behavior strategies in different animal models, depending on the transmission route, the assessed time points, and the examined organs. Indeed, in our well-established murine model of hematogenous septic arthritis, intravenous inoculation with the *S. aureus* Newman parental strain resulted in higher bacterial persistence in kidneys of both C57BL/6 wild-type and TLR2 deficient mice (Mohammad et al., 2020). In fact, this corroborates previous reports whereby more pronounced bacterial burden in different organs, including kidneys, was demonstrated in mice after infection with SA113 parental strain compared to the Lpp-deficient Δ*lgt* mutant strain, independent of TLR2 and MyD88 signaling, in a sepsis model (Schmaler et al., 2009). Moreover, a previous study demonstrated that increased bacterial persistence occurred in kidneys of Balb/c mice when infected with the USA300 MRSA parental strain in comparison to its Δ*lpl* mutant strain, lacking the *lpl* gene cluster (Nguyen et al., 2015). Furthermore, in a murine sepsis model, it was recently demonstrated that mice pre-treated with the synthetic lipopeptide Pam_3CSK_4 had decreased bacterial burden and increased survival following infection with a *S. aureus* MRSA strain (Huang et al., 2017).

The role of *Staphylococcus aureus* Lpps in skin infections

Defects in the skin barrier, due to breaches or abrasions in the skin tissue, enable *S. aureus* to penetrate into the damaged site and enter the underlying tissue, thus establishing a skin infection. It consequently proliferates on site, and release different bacterial components and toxins, causing symptoms. Directly after infection, leukocytes are rapidly recruited, and antimicrobial peptides are upregulated in the site of infection. Invasion into the host cells is one of the effective strategies for *S. aureus* to survive from the innate immune killing. Undoubtedly, the νSaα specific *lpl* cluster contributes to bacterial invasion into human keratinocytes as significantly less Δ*lpl* mutant bacteria were found intracellularly in cultured keratinocytes compared with its intact parental strain. Moreover, the described effect could be reversed by the complemented mutant (Nguyen et al., 2015). The *in vitro* effect of Lpl proteins was also confirmed by a murine skin invasion model using shaving and tape-stripping, as higher bacterial counts were found in mice that were epicutaneously infected with the USA300 parental strain as compared to the Δ*lpl* mutant (Nguyen et al., 2015). Interestingly, it was recently shown that unlipidated Lpl1 protein prompts *S. aureus* host cell invasion *via* direct interplay with the Hsp90 receptor (Tribelli et al., 2020).

In the skin invasion model, the epidermis was disrupted by tape-stripping. In contrast, subcutaneous injection of bacteria

bypassed the natural protection layers such as epidermis and dermis. However, a similar phenomenon was also observed when we subcutaneously infected the mice with the SA113- or the Newman parental strain, as these *S. aureus* Lpp-expressing strains induced fulminant bacterial growth in local skin of mice independent of host TLR2 signaling in contrast to their *lgt*-deficient mutant counterparts (Mohammad et al., 2021). This was accompanied by skin abscess formation and delayed wound healing in the local tissues of mice (Mohammad et al., 2021). Furthermore, upon subcutaneous injection with a complemented mutant strain, SA113Δ*lgt* (pRB474::*lgt*), used in order to validate the impact of *S. aureus* Lpps in the skin infection model, the inoculated mice displayed more severe skin lesions and higher bacterial loads in the mouse skin homogenates in comparison to the SA113Δ*lgt* mutant strain (Mohammad et al., 2021). In addition, the levels of leukocyte chemoattractants (MIP-2, KC and MCP-1) and myeloperoxidase (MPO) in those skin homogenates were shown to be upregulated (Mohammad et al., 2021).

In the murine skin model, it was also revealed that subcutaneous injection of purified *S. aureus* Lpps in mice was associated with enhanced levels of tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) in a lipid-moiety-dependent manner (Mohammad et al., 2021). Similarly, co-injection of purified *S. aureus* Lpp and live *S. aureus* bacteria deficient in Lpp expression also displayed upregulated expression of PAI-1, in a lipid-moiety-dependent fashion (Mohammad et al., 2021), suggesting that Lpp expression causes an imbalance of the coagulation/fibrinolysis hemostasis in the murine skin model. Importantly, in fibrinogen-depleted mice the Lpp-induced effects were fully abolished (Mohammad et al., 2021). This indicates that *S. aureus* Lpp expressing bacteria promote fibrous capsule formation upon skin infection by utilizing fibrinogen, thus shielding the bacteria from immune killing. This further shows that different *S. aureus* Lpp-expressing strains give rise to similar virulent characteristics in two different skin infection models. Purified *S. aureus* Lpps were shown to promote murine skin inflammation through activation of dendritic cells in an intradermal injection model (Saito and Quadery, 2018). Likewise, induced skin inflammation was also observed in our subcutaneous skin injection model (Mohammad et al., 2021).

The plausible mechanism for distinct roles of *Staphylococcus aureus* Lpps in different models

The cellular mechanism behind the lower bacterial load in the murine knee joints intra-articularly inoculated with Lpp-expressing *S. aureus* was elucidated using co-injection of live bacteria with purified *S. aureus* Lpp or synthetic lipopeptides. This resulted in bacterial eradication in the knee joints – a phenomenon that was mediated through TLR2-dependent responses with neutrophils acting as the main phagocytic cell engulfing the bacteria (Mohammad et al., 2019). In fact, enhanced levels of the neutrophil attracting chemokines, KC and MIP-2, were observed in the supernatants of knee homogenates (Mohammad et al., 2019). In the skin model, instead, co-injection of live bacteria with purified *S. aureus* Lpps resulted in opposite effects as the skin damage worsened by displaying more severe lesions and abscess frequencies, along with increased local bacterial persistence. This was associated with increased levels of the neutrophil chemoattractant chemokines, MIP-2 and KC, and the critical recruiter of monocytes/macrophages, MCP-1, as well as MPO in the skin homogenates of mice in a lipid-moiety-dependent manner (Mohammad et al., 2021). Our data indicate that not only purified *S. aureus* Lpps but also a mixture of Lpps and live bacteria are able to activate a powerful innate immune response in the model of local knee arthritis, whereas the same mixture plays a more beneficial role for the bacteria in the skin model. Elevated levels of the same chemokines were also observed in the skin homogenates upon subcutaneous injection with purified *S. aureus* Lpps exclusively, in a lipid- and a dose-dependent fashion through TLR2 (Mohammad et al., 2021), strongly suggesting that Lpps play a potent role in triggering local inflammatory responses in different organs.

In the hematogenous septic arthritis model, expression of Lpp in *S. aureus* increased mortality, weight loss and cytokine production, and decreased bacterial clearance independent of TLR2, indicating the important role of Lpp in bacterial fitness and virulence (Mohammad et al., 2020). As Lpp receptor, TLR2 plays a role in the host defense against infection, as TLR2 deficient mice infected with the Newman parental strain displayed enhanced arthritis symptoms as well as increased weight loss, mortality and bacterial burden in kidneys compared to the wild-type controls (Mohammad et al., 2020). This result was not so surprising since several reports previously showed that TLR2 deficient mice are significantly more susceptible to *S. aureus*-induced infections and display increased bacterial loads in different organs in comparison to their wild-type counterparts (Takeuchi et al., 2000; Miller et al., 2006; Sun et al., 2006; Stenzel et al., 2008; Schmaler et al., 2009).

In the local knee arthritis model, we demonstrated that the destructive arthritis caused by Lpp is TLR2-dependent (Mohammad et al., 2019), possibly due to an excessive inflammatory reaction. However, in the hematogenous septic arthritis model, we showed that the destructive arthritis caused by Lpp-expressing *S. aureus* was TLR2-independent. The multifunctionality of Lpps, i.e., in nutrition and fitness, bacterial survival and pathogenicity during host-interactions, or its ability to evade immune recognition or to trigger various immune responses upon invasion, are all of significant importance during infection with live *S. aureus* expressing Lpp.

A schematic illustration of the effects of *S. aureus* Lpp in hematogenous and local *S. aureus* arthritis models is shown in Figure 3.

Lpps have emerged as an important factor also during the pathogenesis of *S. aureus* systemic infections (Schmaler et al., 2009; Mohammad et al., 2020). Furthermore, another study indicated that *S. aureus* Δ*lgt* mutant was hypervirulent in contrast to its parental strain, in a murine sepsis model (Bubeck Wardenburg et al., 2006). Surprisingly, in the same

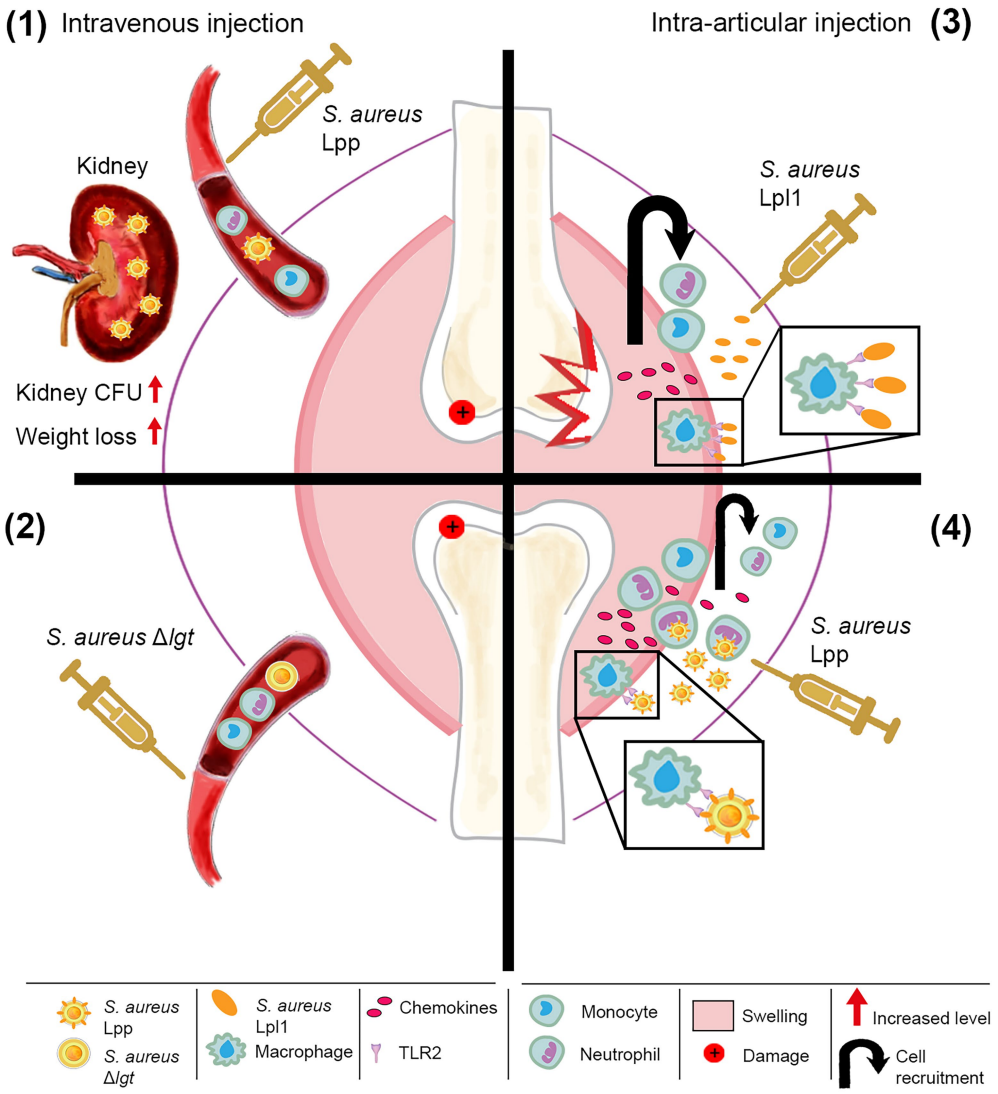

FIGURE 3
Schematic illustration of the effects of *Staphylococcus aureus* lipoprotein (Lpp) in hematogenous and local *S. aureus* arthritis models. **Left panel:** *S. aureus* parental strain, expressing Lpp (*S. aureus* Lpp), significantly aggravates systemic infection with increased mortality, weight loss, and bacterial burden in the kidneys **(1)** compared to the derivative *lgt* mutant strain, lacking Lpp (*S. aureus* Δ*lgt*) **(2)**. However, both *S. aureus* strains have similar outcomes with regard to bone erosion. **Right panel:** Lpp has dual effects in the local knee model. Intra-articular injection of purified Lpp (*S. aureus* Lpl1) induces rapid TLR2-dependent infiltration of phagocytes. Moreover, Lpl1 causes severe joint inflammation and bone erosions dependent on monocytes/macrophages through TLR2 **(3)**. In contrast, live *S. aureus* Lpps act as adjuvants, triggering recognition by TLR2 and subsequent neutrophil recruitment, leading to more efficient bacterial killing and diminished bone destruction **(4)**. CFU, colony-forming units.

article, the authors demonstrated that the *lsp*-deficient strain exhibited attenuated virulence, which is more in line with many other studies (Bubeck Wardenburg et al., 2006). One could speculate that these contradictory findings might be due to variations in animal species and age, bacterial dose or bacterial strain, duration of the course of infection, or that the generation of the *lgt* deletion mutant might have been performed differently in the reported studies, possibly underlying these divergent outcomes on lipidation and maturation of staphylococcal Lpp in murine staphylococcal sepsis. Nevertheless, from the majority of the previous studies we conclude that *S. aureus* Lpps are pathogenic in systemic and skin infections. *Staphylococcus aureus* Lpps and their differential roles in different *in vivo* models are summarized in Table 2.

Concluding remarks

Staphylococcus aureus Lpps play a differential role depending on the affected organ and route of injection, as described below.

Lpps display a dual function in local *S. aureus* arthritis models. On the one hand, purified Lpp, but not toxic shock syndrome toxin-1 (TSST-1) or PGN, induced chronic macroscopic arthritis. Intra-articular injection with Lpps induced rapid TLR2-dependent infiltration of monocytes/macrophages and neutrophils. Furthermore, *S. aureus* Lpps caused severe joint inflammation and bone erosions, which were mediated by monocytes/macrophages through TLR2. On the other hand, Lpp expression in *S. aureus* led to reduced bacterial burden in the arthritic knee joints. The observed phenomenon was due to Lpp acting as adjuvant and triggering recognition by TLR2 followed by subsequent neutrophil recruitment, leading to more efficient bacterial killing and diminished bone destruction.

Staphylococcus aureus Lpps were found to be prominent virulence factors independent of host TLR2 expression. Mice that were intravenously inoculated with the *S. aureus* Lpp-expressing parental strain succumbed more to the disease, had increased weight loss, and exhibited impaired bacterial clearance in their kidneys, than mice inoculated with the *S. aureus* Δ*lgt* mutant strain, lacking Lpp expression. Notably, the worst outcome was observed in mice lacking TLR2 and inoculated with the *S. aureus* parental strain, strongly indicating the protective role of TLR2 in hematogenous spread of *S. aureus*-induced septic arthritis. However, in contrast to the local septic arthritis model, *S. aureus* Lpps exhibited a limited role in bone erosion.

Staphylococcus aureus Lpps were associated with severe inflammatory response in the skin model. The observed skin lesions and inflammation were mediated through TLR2-dependent mechanisms. Lpp contributed to a similar influx of innate immune cells as observed in the local knee arthritis model with monocytes/macrophages as well as neutrophils being recruited to the local tissue. In addition, subcutaneous injection of *S. aureus* parental strain was associated with elevated bacterial burden in the skin biopsies and more severe skin lesions. Importantly, Lpp expression initiated the activation of the coagulation and inhibition of fibrinolysis, and resulted in enhanced local fibrin deposition and abscess capsule formation in murine skin infection, whereas depletion of leukocytes and fibrinogen resulted in the total abrogation of effects induced by *S. aureus* Lpp. Such findings indicate that *S. aureus* Lpp-expressing bacteria utilize a "lockdown" strategy, consequently preventing the bacteria from being killed by the immune system, which represents a novel bacterial immune evasion mechanism.

Overall, Lpp maturation contributed to staphylococcal immune evasion. An overview of the proposed functions of Lpps in *S. aureus* infections is summarized in Figure 4.

Future perspectives

As mentioned above, bacterial Lpps exist in two different forms depending on the lipid moiety, i.e., diacylated and triacylated Lpps. Our future aim is to further explore and compare the different lipid-moieties of the staphylococcal species *S. aureus* and *S. carnosus*, as well as the synthetic lipopeptides, Pam_3CSK_4 and Pam_2CSK_4, and investigate their role in the induction of bone erosion. To our knowledge, there are currently no studies available regarding the role of diacylated and triacylated Lpps on the induction of bone damage. We hypothesize that the diacylated Lpp structure is a more potent inducer of bone erosion. As outlined before, the degree of acylation of the lipid moiety impacts the immune response. Importantly, Nguyen et al. recently showed that the lipid-moieties of Lpps from different bacterial species significantly differ regarding their immune stimulatory activity (Nguyen et al., 2017). We also recently revealed that Lpps cause bone resorption in a mouse model of *S. aureus* septic arthritis, and that the diacylated lipid moiety, Pam_2CSK_4, was more potent in inducing bone resorption than the triacylated lipid moiety, Pam_3CSK_4 (Schultz et al., 2022). In addition, earlier studies conducted on skin resident cells demonstrated that di- but not triacylated Lpps suppressed the immune tolerance, a phenomenon that was mediated through IL-6 release, and the subsequent induction and accumulation of myeloid-derived suppressor cells (Skabytska et al., 2014).

Finally, it has recently been proposed that the combination of different staphylococcal MAMPs might exert an additive or possibly even a synergistic effect in immune stimulation (Nguyen and Götz, 2016). *Staphylococcus aureus* Lpps as well as PGN are known MAMPs of *S. aureus* (Nguyen and Götz, 2016). It is known that most staphylococcal infections are successfully promoted by the coordinated action of different virulence factors rather than a single virulence factor (Fournier and Philpott, 2005). In fact, co-stimulation of dendritic cells with PGN and synthetic lipopeptide enhanced immune stimulatory effects compared to PGN or lipopeptide stimulation alone (Schaffler et al., 2014). Therefore, we plan to determine whether Lpps and PGN act synergistically in staphylococcal skin infections.

As we have seen through the above studies, Lpp gave rise to different outcomes in different organs. What does this depend on? We speculate that the different clinical outcomes might be explained by the anatomic differences, composition difference of immune cells and distribution of blood vessels in the different organs. However, more detailed studies are warranted in the future to answer this question.

Author contributions

MM: wrote the manuscript. AA, M-TN, FG, RP, and TJ: critically revised the manuscript. Some content of this article is a part of the Ph.D. thesis of MM. All authors contributed to the article and approved the submitted version.

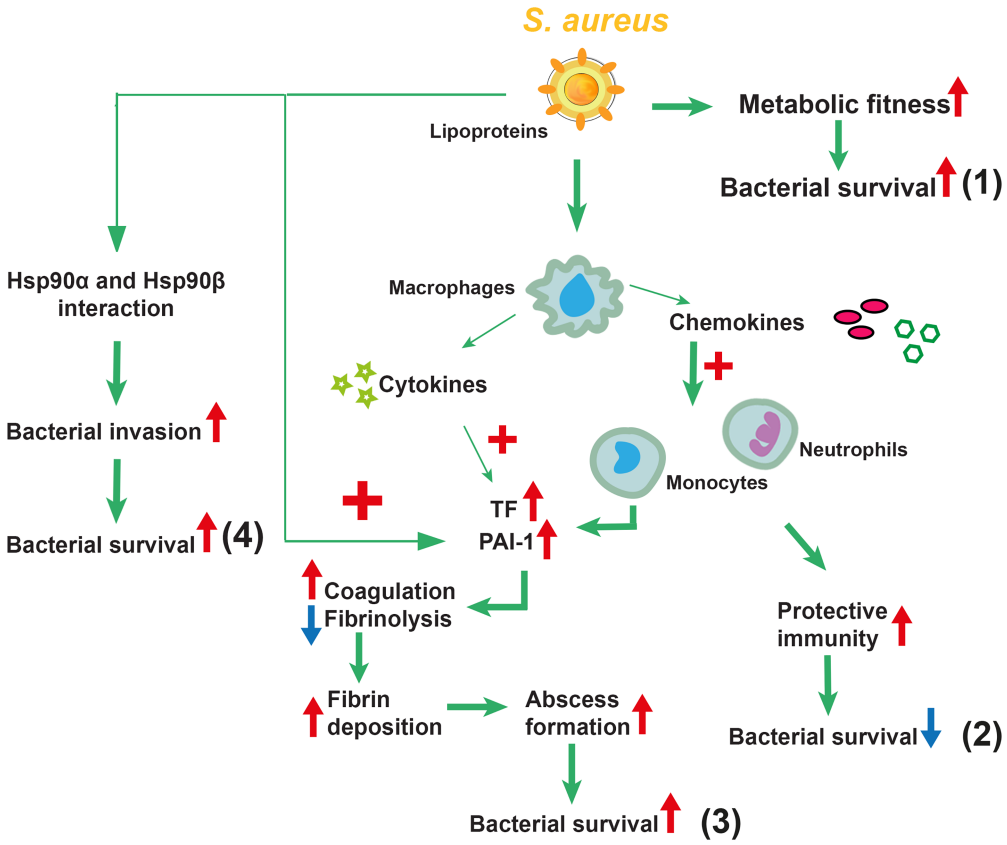

FIGURE 4
Overview of the proposed functions of lipoproteins (Lpps) in *Staphylococcus aureus* infections. *S. aureus* Lpps play a distinct role depending on the route of infection. Following systemic administration of Lpp-expressing *S. aureus*, increased metabolic fitness and increased bacterial survival are observed **(1)**. Local administration of *S. aureus* Lpps has dual effects depending on the affected organ. In the knee joint infection, Lpps have a protective role by triggering the innate immunity, leading to more efficient bacterial elimination with subsequent diminished bone destruction **(2)**. In skin infection, in contrast, Lpp-expressing *S. aureus* leads to increased abscess formation, facilitating the bacteria to evade the innate immunity and resulting in increased bacterial survival **(3)**. Furthermore, Lpp prompts *S. aureus* host cell invasion *via* direct interplay with the heat shock proteins Hsp90α and Hsp90ß, consequently leading to bacterial survival **(4)**.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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An old confusion: Entomophthoromycosis versus mucormycosis and their main differences

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Fungal diseases were underestimated for many years. And the global burden of fungal infections is substantial and has increased in recent years. Invasive fungal infections have been linked to several risk factors in humans which basically depend on the individual homeostasis of the patients. However, many fungi can infect even apparently healthy people. Knowledge of these pathogens is critical in reducing or stopping morbidity and/or mortality statistics due to fungal pathogens. Successful therapeutic strategies rely on rapid diagnosis of the causative fungal agent and the underlying disease. However, the terminology of the diseases was updated to existing phylogenetic classifications and led to confusion in the definition of mucormycosis, conidiobolomycosis, and basidiobolomycosis, which were previously grouped under the now-uncommon term zygomycosis. Therefore, the ecological, taxonomic, clinical, and diagnostic differences are addressed to optimize the understanding and definition of these diseases. The term "coenocytic hyphomycosis" is proposed to summarize all fungal infections caused by *Mucorales* and species of *Basidiobolus* and *Conidiobolus*.

KEYWORDS

basidiobolomycosis, conidiobolomycosis, zygomycosis, phycomycosis, mucoralomycosis, entomophthoramycosis

Highlights

- Systematic comparison between basidiobolomycosis (BM), conidiobolomycosis (CM) and mucormycosis (MM).
- World distribution of BM, CM and MM based on systematic review of case reports.
- Comprehensive pathophysiology of the role of virulence factors.

Introduction

Fungal diseases have been underestimated and neglected worldwide (Rodrigues and Nosanchuk, 2020). Yet the fungi that infect humans are silent killers or cause misery for millions (Denning, 0000). The Global Action Fund for Fungal Infections (GAFFI) estimates that 300 million people suffer from severe fungal infections and 25 million are at high risk for death or loss of vision (fungal keratitis) (Global Action For Fungal Infections, 2020). According to the World Health Organization, not all countries report data on fungal diseases, incidence, resistance, and public health impact are poorly known (World Health Organization [WHO], 2022). The impact on mortality from fungi is estimated at 1.5 million people per year (Bongomin et al., 2017). The attention is focused on the most common diseases such as cryptococcal meningitis, invasive candidiasis, *Pneumocystis jirovecii* pneumonia, invasive aspergillosis, fungal asthma, and fungal keratitis (Bongomin et al., 2017).

In any case, there are several fungal microorganisms that can infect humans. These need to be carefully studied in the light of the factors which contribute to the increased incidence of invasive fungal infections. These risk factors include treatment with immunomodulatory drugs, patients with HIV/AIDS, patients with oncohematological diagnoses, diabetes, COVID -19 pandemic, antifungal prophylaxis, etc., (Cornely et al., 2008; Limper et al., 2017; Peter Donnelly et al., 2020; Saud et al., 2020; Hoenigl et al., 2022; Thomas-Rüddel et al., 2022). However, fungi do not only affect patients with risk factors. For example, dermatophyte onychomycosis, conidiobolomycosis, basidiobolomycosis, coccidioidomycosis, fungal keratitis, etc., can also occur in patients with apparently normal immunity (al Jarie et al., 2011; Weiblen et al., 2016; Lipner and Scher, 2019).

The list of fungal species, which become pathogenic, is long. Mucormycosis associated with COVID -19 (CAM) gained worldwide attention in early 2021 (Banerjee et al., 2021; Pal et al., 2021; Skaria et al., 2022). This was a disfiguring superinfection caused by *Mucorales* with high mortality that occurred in patients with SARS-CoV-2 infection with multiple risk factors (Patel et al., 2020; Aranjani et al., 2021; Mathew et al., 2021; Biswal et al., 2022; Hoenigl et al., 2022). In past and still present times, the terms "zygomycosis" and "zygomycetes" were used which resulted in confusion that affects *Basidiobolus* spp. and *Conidiobolus* spp. that were included in the old taxonomic classification. This led to the abolition of the class Zygomycetes and the misuse of the term zygomycosis.

In the clinical field, the histopathology of the infected tissue plays a fundamental role in the diagnosis of patients infected with *Mucorales*, *Basidiobolus* spp. and *Conidiobolus* spp. since hyphae with sparse or non-septate hyphae (coenocytic) can be found in all of them. The occurrence of coenocytic hyphae was associated with zygomycosis for many years. However, these morphologically similar fungi had significant phylogenetic differences with dramatic consequences on the clinical outcome. The detailed differences are analyzed in this manuscript, and a summary is shown in **Figure 1**.

Definitions of terms and pathogens associated with entomophthoromycosis and mucormycosis

Formerly, the fungal species causing entomophthoromycosis and mucormycosis were assigned to the class *Zygomycetes* (Voigt et al., 1999). This class included the orders *Entomophthorales* and *Mucorales*. This classification primarily included microorganisms with frequent asexual reproduction by sporangia, sexual reproduction by zygospores, and the formation of non-septate (coenocytic) hyphae, to name a few. These definitions led to the diagnosis of zygomycosis in patients with hyphal-infected tissue with little or no septation. This term also included the subtypes referred to as mucormycosis and entomophthoromycosis. And entomophthoromycosis included the terms "conidiobolosis" and "basidiobolosis" (Costa, 2012).

The phylum *Zygomycota* was abandoned (Hibbett et al., 2007). It was replaced by the phylum *Mucoromycota* and *Zoopagomycota*, based on the phylogenetic analyzes of Spatafora et al. (2016). These changes were supported by a higher number of loci and taxa in molecular phylogenetic analyzes within members of the phylum *Zygomycota*. As a result, the clinical names for infections with these pathogens changed. The order *Mucorales* was included in the phylum *Mucoromycota*, and patients infected with *Mucorales* develop mucormycosis (Chibucos et al., 2016; Jeong et al., 2019). The order *Basidiobolales* and *Entomophthorales*, on the other hand, belong to the subphylum *Entomophthoromycotina*, so patients infected with these fungi suffer from entomophthoromycosis. More specifically, the *Basidiobolales* cause basidiobolomycosis and the *Entomophthorales* infection produce conidiobolomycosis (El-Shabrawi et al., 2014).

With the new classification of the *Mucorales* and *Entomophthorales*, the term zygomycosis became obsolete (**Figure 2**). However, much of the scientific literature, especially case reports, retained or confused the definitions. The pathogens of the order *Mucorales* (subphylum: *Mucoromycotina*) are associated with mucormycosis and belong to the genera *Rhizopus*, *Mucor*, *Lichtheimia*, *Cunninghamella*, *Apophysomyces*, *Rhizomucor*, *Saksenaea*, *Synchephalastrum*, *Thamnostylum*, *Cokeromyces*, *Actinomucor*, *Cokeromyces* (Acosta-España and Voigt, 2022). Entomophthoromycosis is caused by species of orders *Basidiobolales* and *Entomophthorales* which are *Basidiobolus* and *Conidiobolus*, respectively (both in the subphylum: Entomophthoromycotina). The main

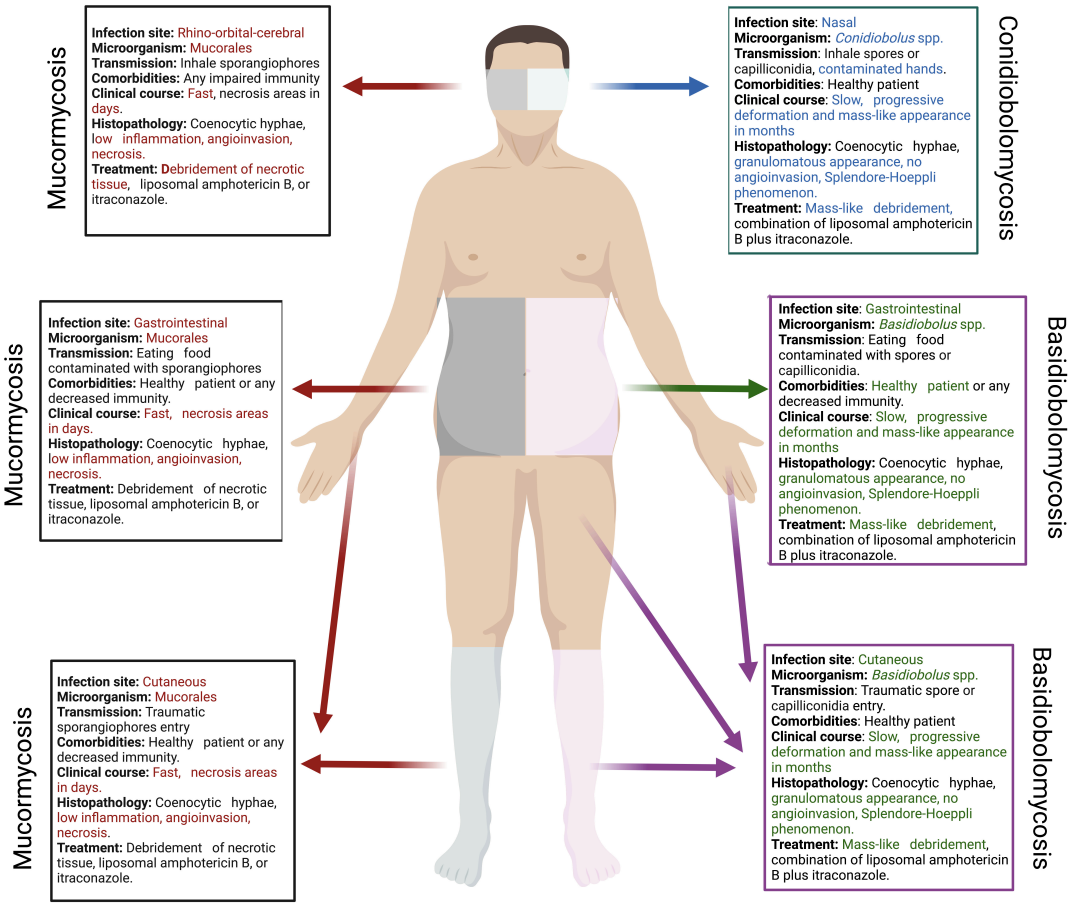

FIGURE 1
Main differences between basidiobolomycosis, conidiobolomycosis and mucormycosis. On the left, the main differences of the main forms of mucormycosis (red), conidiobolomycosis (blue) and basidiobolomycosis (green) are explained. This image was created with Biorender.com.

species causing basidiobolomycosis is *B. ranarum*, but cases of *B. meristosporus*, *B. omanensis*, and *B. haptosporus* have also been reported (Kamalam and Thambiah, 1984; Sanglard et al., 2017; Bshabshe et al., 2020; Al-Hatmi et al., 2021). Finally, conidiobolomycosis is caused by *Conidiobolus coronatus, Conidiobolus pachyzygosporus, Conidiobolus lamprauges,* and *Conidiobolus incongruus* (Walsh et al., 1994; Wüppenhorst et al., 2010; Kimura et al., 2011; Das et al., 2019; Stavropoulou et al., 2020).

Ecology and transmission of *Basidiobolales*, *Entomophthorales*, and *Mucorales*

Basidiobolales

The fungi that cause mucormycosis and entomophthoromycosis are saprotrophic microorganisms from the environment. *Basidiobolus* sp. was originally isolated from the gut contents of frogs (Feio et al., 1999; Gugnani, 1999). And frogs normally feed on insects. Therefore, this fungus has been isolated from the entire bodies of insects in various environments. Also, in the excreta or gut contents of amphibians and reptiles and other animals (Feio et al., 1999; Hung et al., 2020). In addition, the isolation from plant debris and soil has also been reported (Chetambath et al., 2010). Basidiobolomycosis infection occurs after a scratch or puncture from an insect, plant, or other object that carry fungi (Clark, 1968). The gastrointestinal tract has also been found to become infected after ingestion of soil, animal feces, or food contaminated with *Basidiobolus ranarum* (AlSaleem et al., 2013).

Entomophthorales

In a study over a 9-months in irrigated vegetable fields and citrus orchard soils *Conidiobolus coronatus* was the

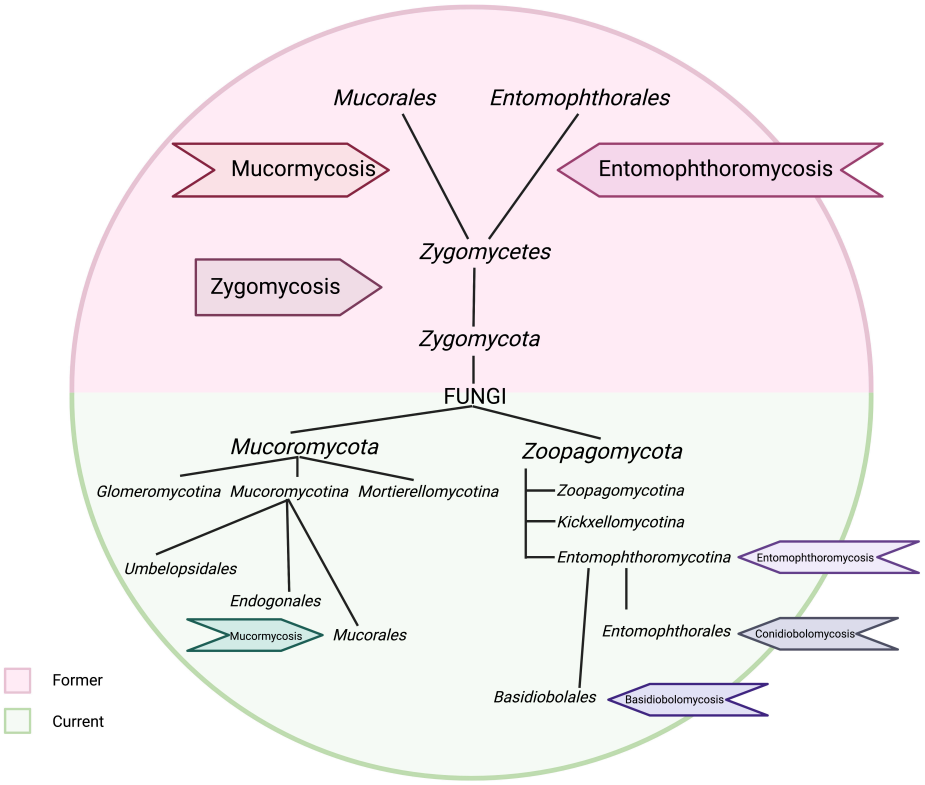

FIGURE 2
Taxonomy and terms used in mucormycosis and entomophthoromycosis. This image shows the old nomenclature for zygomycosis in the light purple section. The current taxonomic classification and terminology is indicated in the light green section. This image was created with Biorender.com.

predominant species, accounting for 31.4% of all fungal isolates (Ali-Shtayeh et al., 2002). This confirms that the ecological distribution of this fungus as a primary saprobe relies on soil humus, decaying plant material in hot and humid climates. Occurrence in insects, reptiles, amphibians, and mammalians like dogs, deers, horses, llamas, non-human primates, and sheep has been reported (Weiblen et al., 2016). Conidiobolomycosis may occur by inhalation of spores, insect bites, or insertion of dirty hands into the nostrils (Thomas et al., 2006). An association of conidiobolomycosis with gastrointestinal transmission was not found: An extensive literature search for case reports in Academic Google, ScienceDirect, PubMed, and Taylor and Francis revealed no human case reports to support the mechanism of gastrointestinal transmission.

Mucorales

Mucorales are found in a wide variety of organic substrates, including bread, vegetable matter (decomposing fruits), crop residues, soil, animal excrement, and compost piles (Hoffmann et al., 2013). Environmental studies have shown the presence of various species in buildings, contaminated medical material (tongue depressors, needles, etc.), ventilation systems, and flowerpots in hospitals (Ribes et al., 2000; Cornely et al., 2019; Biswal et al., 2022). Transmission occurs *via* entry of sporangiospores through the respiratory tract. This may allow inoculation in the naso-facial area or reach the lower respiratory tract. On the other hand, trauma with contaminated materials can lead to infection of the affected area. Transmission through contaminated food with concomitant intestinal infection has also been described (Lee et al., 2014).

Difference between risk factors and affected populations in basidiobolomycosis, conidiobolomycosis, and mucormycosis

Basidiobolomycosis

Basidiobolomycosis is known for its burden in countries of the African continent (El-Shabrawi et al., 2017). In any case, the infection has been reported in several countries

with tropical and subtropical climates (**Figure 3A**, Suppl. A). Pediatric patients are the most affected group (Gugnani, 1992). To a lesser extent, adolescents are also affected. Adults rarely contract the disease. In Uganda, the male-to-female ratio was reported 3:2, while a 3:1 ratio has been reported in Nigeria (Clark, 1968). The main risk factor is minor trauma related to insects or objects that carry the pathogen. The association between *Basidiobolus* spp. infection and comorbidity has not been described. On the other hand, a more invasive form of visceral basidiobolomycosis has been reported (Choonhakarn and Inthraburan, 2004).

Conidiobolomycosis

Conidiobolomycosis is distributed worldwide, especially in tropical and subtropical countries (**Figure 3B**, Suppl. B). Nigeria is the country with the highest number of reported cases. From various studies, affected individual's range in age from 20 to 50 years, but cases outside this age range have also been reported (Gupta and Soneja, 2019; Sigera et al., 2021). Studies show a different ratio between men and women. From a ratio of 4:1 in Gupta and Soneja to a ratio of 10:1 between men and women in Martinson and Clark (Martinson and Clark, 1967; Gupta and Soneja, 2019). Studies conducted in small populations with this disease show a lower male-to-female ratio (Chowdhary et al., 2010). It is particularly common in people who work or participate in outdoor activities (Martinson and Clark, 1967). However, disseminated infections with fatal outcome in a kidney transplant patient have been reported (Walker et al., 1992).

Mucormycosis

In mucormycosis, the picture is different. Patients often present comorbidities related in some way to immune deficit. Major comorbidities include diabetes mellitus (diabetic ketoacidosis), hematologic malignancies, Coronavirus disease 2019 (COVID-19), solid organ transplantation, hematopoietic stem cell transplantation, liver disease, corticosteroid use, and neutropenia. Of course, this infection can also occur in patients without concomitant diseases associated with severe trauma such as a car accident, surgery, etc. However, it may also be associated with minor trauma such as injection sites (Song et al., 2017; Patel et al., 2020; Hoenigl et al., 2022). The *Mucorales* infection has been reported from several countries, with a significant burden in United States (Suppl. C) and India (Prakash and Chakrabarti, 2021). The global distribution of mucormycosis based on case reports is shown in **Figure 3C**.

The infection can occur in any age group. The multicenter observational study in India by Patel et al. (2020) showed an age range of 35–58 years. Meanwhile, a systematic review and meta-analysis of case reports found a range of 39–61 years (Jeong et al., 2019). It is important to note that the pediatric population may also be affected. For example, intestinal infections have been reported in neonates (Sarin, 2010). In addition, cerebral rhino-orbital form has been described in children with comorbidities (Amanati et al., 2021; Masmoudi et al., 2021). The male-to-female ratio is about 2.2:1, with slight variations between reports (Song et al.; Jeong et al., 2019; Patel et al., 2020; Gupta and Ahuja, 2021).

Virulence and invasion factors of basidiobolomycosis, conidiobolomycosis, and mucormycosis

Basiobolomycosis

Virulence factors are summarizing all properties that microorganisms use to establish infection, multiply, and evade host immunity (Hogan et al., 1996; Cross, 2008). Several of these virulence factors have been described in fungi pathogenic to humans (Nosanchuk and Martinez, 2015). However, in the case of basidiobolomycosis and conidiobolomycosis, information is still scarce. Elastase, esterase, collagenase, and lipase were suspected as virulence factors in *Basidiobolus* spp. (Shaikh et al., 2016). On the other hand, the production of urease, *N*-acetyl-β-glucosaminidase, trypsin, lipase, lecithinase, gelatinase, collagenase, and elastase were confirmed as virulence factors in 10 isolates of *Basidiobolus ranarum* (Feio et al., 1999). Additionally, extracellular enzyme activities of proteases and lipases were evaluated *in vitro* whilst amylase, RNAse, and DNAse activities were missing in *Basidiobolus* (Okafor et al., 1987). Even though the *in vitro* production of extracellular enzymes may be crucial for the development of basidiobolomycosis, infection models are essential to confirm the activity of these enzymes as virulence factors.

Conidiobolomycosis

Conidiobolus coronatus was proven for the *in vitro* presence of extracellular lipases and proteases (Okafor et al., 1987) and for the production of collagenase, esterase, and elastase from pathogenic and saprophytic strains of *Conidiobolus coronatus* (Fromentin, 1978; Gugnani, 1992). In addition, the production of hydrolytic enzymes, including proteases and chitinases, has been demonstrated in insect cuticle infection models (Freimoser et al., 2003). *C. coronatus* also expressed a sequence tag (EST) (BQ622285) with similarity ($E = 4 \times 10^{-32}$) to a ferritin subunit (iron storage protein) (Freimoser et al., 2003). Components of iron uptake and iron storage are virulence factors that has been

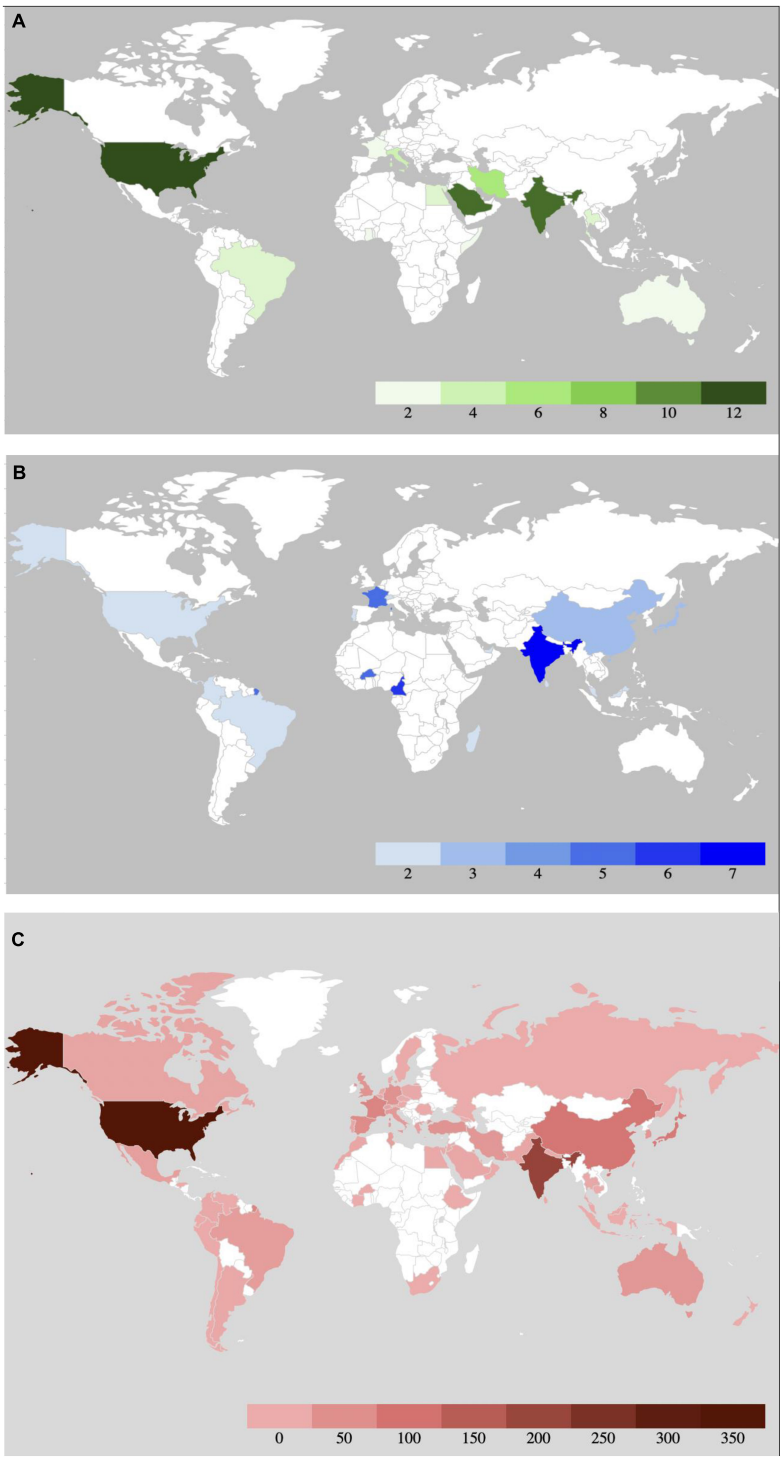

FIGURE 3
Global prevalence of infections by *Mucorales*, *Conidiobolus* spp. and *Basidiobolus* spp. based on a systematic review of case reports. **(A)** A systematic review of case reports with the term basidiobolomycosis and *Basidiobolus* from 1987 to 8/29/2022 was performed in PubMed. Cases with confirmed *Basidiobolus* spp. infections that allowed identification of the reporting country were included. Detailed information can be found in supplement A. **(B)** A systematic review of case reports with the term conidiobolomycosis and *Conidiobolus* spp. from 1978 to 8/29/2022 was performed in PubMed. Cases with confirmed *Conidiobolus* spp. infections that allowed identification of the reporting country were included. Detailed information can be found in supplement B. **(C)** A systematic review of case reports with the term mucormycosis between from 2000 to 8-22-2022 was performed in PubMed. Cases with confirmed *Mucorales* infections that allowed the identification of the reporting country were included. Detailed information can be found in supplement C. Systematic reviews were performed following the PRISMA 2020 criteria (Page et al., 2021).

studied in other fungal pathogens (Haas, 2012; Fourie et al., 2018; Martínez-Pastor and Puig, 2020).

In *Galleria mellonella* infected by *C. coronatus*, the autophagic pathway is induced under the influence of stress and reactive oxygen species (Kazek et al., 2020). In another study, *C. coronatus* was shown to kill larvae of *G. mellonella* by damaging hemocytes through the protein coronatin-2 (Boguś et al., 2017). In the case of conidiobolomycosis, most infection models have been performed on insects. It is likely that these virulence factors are also expressed in humans and contribute to pathogenesis. Invasion and destruction of infected tissue may be enhanced by enzyme production of *C. coronatus*.

Mucormycosis

In the case of mucormycosis, the picture is entirely different from the previous fungal diseases described. Several studies have been performed describing virulence factors in different species within the order *Mucorales* (Hassan and Voigt, 2019). Epithelial cells are critical in patients infected with mucormycosis. In infection models using mice, glucose-regulated protein 78 (GRP78) expression was shown to increase in the presence of elevated glucose and iron concentrations (Liu et al., 2010). Indeed, diabetic mice showed increased expression of GRP78 in epithelial cells of the paranasal sinuses, lungs, and brain. Liu et al. (2010) showed that *Rhizopus arrhizus* (formerly: *Rhizopus oryzae*) enhanced epithelial cell invasion and damage in a receptor-dependent manner. Later, Gebremariam et al. (2014) described that spore coat protein homologs (CotH) from *Mucorales* is the fungal ligand for GRP78. Reduction of CotH expression in a mutant strain of *R. arrhizus* demonstrated decreased virulence in diabetic mouse models of infection (Gebremariam et al., 2014).

Other host factors associated with mucoralean invasion include contact with the extracellular matrix proteins and platelet-derived growth factor receptor (PDGFR). Bouchara et al. (1996) showed that *R. arrhizus* spores adhere to laminin and collagen type IV. This is important in patients with diabetes, chemotherapy and other diseases that can cause epithelial damage. As a result of epithelial damage, extracellular matrix proteins are exposed that can serve as fungi ligands (Bouchara et al., 1996). PDGFs, on the other hand, are a family of receptors found on various cell types. They have been found to affect blood vessel formation and early hematopoiesis (Andrae et al., 2008). A variety of gliomas, sarcomas, and leukemias are associated with autocrine PDGF activation (Andrae et al., 2008; Demoulin and Montano-Almendras, 2012). Epithelial damage has been linked to PDGFs in mucormycosis. When PDGFs were inhibited, endothelial damage was decreased in a model of infection with *R. delemar* (Chibucos et al., 2016). In addition, the results of Chibucos et al. (2016) suggest that *Rhizopus* may induce angiogenesis pathways to support its hematogenous dissemination. Also a massive platelet activation was reported in mice infected with *Lichtheimia corymbifera* leading to angioinvasion, thrombosis and necrosis followed by dissemination (Schulze et al., 2017).

After penetrating the tissue. It is possible that these dormant spores of *Mucorales* germinate and swell in the tissue, which in turn produces rapidly growing hyphae as part of their natural life cycle. According to various studies, filamentous growth, and germination lead to angioinvasion, vascular thrombosis, and tissue necrosis in the host (Warkentien et al., 2012). For survival in infected tissues, *Mucorales* require the uptake of iron. *Mucorales* can take up iron *via* three pathways: high-affinity iron permeases, low molecular weight iron chelators (siderophores such as rhizoferrin and xenosiderophores such as deferoxamine), and extraction of iron from host hemoglobin *via* heme oxygenase (Thieken and Winkelmann, 1992; Stanford and Voigt, 2020). *R. arrhizus* showed a significant increase in growth in serum samples with exogenous increase in iron concentration (Artis et al., 1982). Another example of the contribution of iron in the infection process is the detection of mucormycosis in dialysis patients after receiving therapy with the xenosiderophore deferoxamine (Boelaert et al., 1993). Moreover, the application of the xenosiderophore deferasirox resulted in an increased lethality in mice (Spellberg et al., 2012). So, these siderophores need to be carefully and conservatively administered in patients with risk of mucormycosis.

Proteases produced by *Mucorales* contribute to shocking tissue damages by expansive necroses. *Rhizopus* alkaline protease enzyme (Arp) has been detected in *R. microsporus* var. *rhizopodiformis*, which plays a role in promoting the coagulation process in patients with mucormycosis (Spreer et al., 2006). Serine and aspartate proteases (SAPs) produce proteolytic activities aid in the colonization and penetration of tissues (Schwartze et al., 2014; Mandujano-González et al., 2016). *In vitro* studies show the presence of polygalacturonases, amylases, proteases, lipases, and laccases but their role as virulence factors was not confirmed by animal models yet (Alves et al., 2002; Geethanjali et al., 2020). Although, the proteases may obviously contribute to mucormycosis-induced tissue damage.

The susceptibility of *Mucorales* to antifungal agents must be considered because fluconazole (FLU) is used as first-line choice drug in patients with suspected fungal disease. But these mucoralean fungi are not susceptible to short-chain azoles such as FLU (Lee et al., 2013; Caramalho et al., 2017). The resistance to FLU in *M. circinelloides* has been linked to the RNA interference (RNAi) pathway (Calo et al., 2014). RNAi uses double-stranded RNA molecules to silence genes through translation or transcription and was previously known as contributor to co-suppression, post-translational gene silencing (PTGS), or silencing. Epimutant strains of *M. circinelloides* produce sRNAs specific for the drug target gene and transiently suppress its expression by mRNA degradation. After several passages in a drug-free medium, the epimutants re-express the

target gene and become sensitive to the antifungal drug again (Calo et al., 2014).

Phagocytic cells play a critical role in the clearance of pathogens. The macrophage-mediated response has been highlighted in various fungal pathogens such as *Cryptococcus neoformans*, *Candida albicans*, and others (Xu and Shinohara, 2017). The intracellular persistence of *R. arrhizus* spores in alveolar macrophages was widely demonstrated (Ghuman and Voelz, 2017; Andrianaki et al., 2018; López-Fernández et al., 2018). This intracellular survival is achieved by inhibition of phagolysosome formation by melanin on the spore surface in *R. arrhizus* (Andrianaki et al., 2018). Melanin is a pigment found in many organisms. This melanin has already been linked to the evasion of immunity by various mechanisms in fungi such as *Aspergillus fumigatus and C. neoformans* (Casadevall et al., 2000). In the case of *R. arrhizus* has a different structure from the 1,8-dihydroxynaphthalene (DHN) melanin found in *A. fumigatus* as it is eumelanin (Soliman et al., 2020). It inhibits LC3-associated phagocytosis (Andrianaki et al., 2018). On the other hand, through transcriptomics of the host and pathogen, studies on iron supplementation and genetic manipulation of the iron assimilation pathway of fungi. Iron restriction in macrophages was shown to regulate immunity to *Rhizopus* (Andrianaki et al., 2018).

Mucoricin is a cell-associated/secreted/shed is a ricin-like protein which is a carbohydrate binding lectin and highly potent toxin widely present in pathogenic mucoralean fungi (Soliman et al., 2021). The toxin has been associated with inhibition of protein synthesis and cell-destructive effects (apoptosis and necrosis), promotes inflammation and recruitment of polymorphonuclear cells, increases vascular permeability, and protects hyphae from phagocytosis in necrotic tissue. Treatment with IgG anti-mucoricin in models of intrathecal infection with *R. delemar* in mice decreased mortality compared to the control group (Soliman et al., 2021). In addition, in mice with pulmonary mucormycosis, the use of IgG anti-mucoricin protected the tissue from inflammation and infiltration with *R. delemar* (Soliman et al., 2021). A summary of virulence factors is shown in **Table 1**.

Clinical presentation and diagnosis of basidiobolomycosis, conidiobolomycosis, and mucormycosis

Basidiobolomycosis

Clinically, the patient may present with cutaneous, subcutaneous, intestinal, and/or disseminated infection. Initial clinical symptoms are usually non-specific, making it difficult for patients to seek medical attention (de Leon-Bojorge et al., 1988). The cutaneous or subcutaneous form is common in male pediatric patients (Shaikh et al., 2016). The lower limbs and gluteal region are frequently affected. However, several anatomic sites of infection have been reported (de Leon-Bojorge et al., 1988; Thotan et al., 2009; al Jarie et al., 2011; Kumar Verma et al., 2012; Mendoza et al., 2015). The cutaneous lesions are painless and have a mass appearance with skin color changes. They are well demarcated, have smooth margins, and no lymphatic involvement (de Leon-Bojorge et al., 1988). This infection is usually slow and progressive. Involvement of bone and muscle is unlikely. This clinical picture may resemble other pathologies such as lupus vulgaris, soft tissue tumors, Burkitt's lymphoma, synovial sarcoma, and fibrosing panniculitis. Infectious differential diagnosis may consider *Actinomyces* spp. or *Nocardia* spp., cutaneous pythiosis, chromoblastomycosis, cutaneous atypical Mycobacterium species, primary subcutaneous mucormycosis, sporotrichosis, etc., (de Leon-Bojorge et al., 1988; al Jarie et al., 2011).

Food contamination with *Basidiobolus* spp. may lead to gastrointestinal infections (Khan et al., 2001). Clinical symptoms include abdominal pain, intermittent low-grade fever, vomiting, bloody mucosal diarrhea, intestinal bleeding, gastric or intestinal ulceration, abdominal distension, obstructive symptoms, etc., (Costa, 2012; Mendoza et al., 2015; Elzein et al., 2018; Omar Takrouni et al., 2019). In the context of non-specific symptomatology, the differential diagnosis can be complex. Takrouni et al. described the case of an 18-year-old patient with an obstructing cecal mass in whom a malignant tumor was initially suspected. Because of the obstructive symptoms, surgical resolution of the case was required. During laparotomy, tissue samples were obtained from the intestine. The biopsies showed fungal hyphae that had invaded the intestinal wall and exhibited Splendore-Hoeppli phenomenon (Omar Takrouni et al., 2019). This case shows us the importance of laboratory techniques support in any form of disease. Indeed, the diagnosis in these patients can be confirmed by culture, histopathology, and detection of the microorganism by polymerase chain reaction (PCR).

Conidiobolomycosis

Infections with *C. coronatus* are common in male adults without a history of immunosuppression (Martinson and Clark, 1967). They usually involve the subcutaneous tissues of the nasal and facial regions (Martinson and Clark, 1967; Clark, 1968; Fischer et al., 2008; Isa-Isa et al., 2012). This trophism has led to the term rhinoconidiobolomycosis (Clark, 1968). In any case, it is important to note that *Basidiobolus* spp. can also infect the face (Goyal et al., 2010). Nasal nodules grow slowly during the infection. Initially, nasal obstruction occurs, followed by erythematous infiltration and thickening of the nasal skin. Eventually, the facial structures begin to deform

TABLE 1 Virulence factors associated with basidiobolomycosis, conidiobolomycosis, and mucormycosis.

A) Virulence in basidiobolomycosis

Name	Mechanism	References
Elastase	Enzyme from the class of proteases (peptidases), which degrade proteins. Probably associated with tissue invasion.	Shaikh et al., 2016
Esterase	Enzyme breaks down esters into an acid and an alcohol during a process known as hydrolysis. Probably associated with tissue invasion.	Shaikh et al., 2016
Collagenase	A group of proteolytic enzymes that break down collagen and gelatin. Probably associated with tissue invasion.	Shaikh et al., 2016
Lipase	Enzymes that catalyzes the hydrolysis of fats. Probably associated with tissue invasion.	Shaikh et al., 2016

B) Virulence in conidiobolomycosis

Name	Mechanism	References
Lipase	Enzymes that catalyzes the hydrolysis of fats. Probably associated with tissue invasion.	Shaikh et al., 2016
Proteasease	Protein degrading enzymes. Probably associated with tissue invasion.	Shaikh et al., 2016
EST (BQ622285)	Iron storage. Ferritin-like protein.	Freimoser et al., 2003
Coronatin-2	Coronatin-2 kills larvae of G. mellonella by damaging hemocytes	Boguś et al., 2017
Reactive Oxygen Species	The autophagic pathway is activated in Galleria mellonella that has C. coronatus infection as a result of stress and reactive oxygen species.	Kazek et al., 2020

C) Proven virulence in mucormycosis

Name	Mechanism	References
High-affinity iron permease	Iron uptake and transport in *Mucorales* fungi especially during the lack of iron in the environment	Stanford et al., 2021
Spore coat protein	Invasins in the pathogenesis of mucormycosis	Gebremariam et al., 2014
Alkaline *Rhizopus* protease enzyme	Enhancing the coagulation process in patients suffering from mucormycosis	Spreer et al., 2006
ADP-ribosylation factor	Fungal dimorphism and virulence in *M. circinelloides*	Patiño-Medina et al., 2018
Dihydrolipoyl dehydrogenase	Most abundant antigen in the serum of patients suffered from FLD compared to healthy donors	Rognon et al., 2016
Calcineurin	Tangible role in the transition from yeast to hyphae in *M. circinelloides*	Lee et al., 2013
Serine and aspartate proteases	Most common candidates of the secreted proteases in the genome of *L. corymbifera*	Schwartze et al., 2014
	In *Rhizopus* act as secrete lytic enzymes	
Heme oxygenase	Obtain iron from host hemoglobin and might explain the angioinvasive nature	Stanford and Voigt, 2020
Rhizoferrin	Obtain iron through a receptor-mediated, energy-dependent process	Thieken and Winkelmann, 1992
RNAi-mediated resistance to fluconazole	*M. circineloides* hides the fluconazole target by RNAi	Calo et al., 2014
Intracellular macrophage survival	Inhibition of phagolysosome formation by melanin on the spore surface of *R. arrhizus*.	Andrianaki et al., 2018
Mucoricin	The mucoricin of *R. delemar* causes cell damage, promotes inflammation and recruitment of polymorphonuclear cells, increases vascular permeability, and protects hyphae from phagocytosis in necrotic tissue.	Soliman et al., 2021
Rhizoxin	An antimitotic macrocyclic polyketide metabolite.	Ibrahim et al., 2012
RNA interference pathways	A way of negatively regulating gene expression through small non-coding RNAs or short RNAs (sRNAs)	Calo et al., 2014

Description of virulence factors and mechanisms in: (A) basidiobolomycosis, (B) conidiobolomycosis, (C) proven virulence factors in mucormycosis, and (D) unproven virulence factors in mucormycosis. In the right column the references are indicated to the bibliographical source.

and show a marked increase in volume (Clark, 1968; Fischer et al., 2008; Goyal et al., 2010; Isa-Isa et al., 2012). Chronic infection (in some cases several months to years), if untreated, leads to indolent facial deformities. Based on these findings, a classification was proposed using early, intermediate and late progression of the disease (Blumentrath et al., 2015); (1) early: the patient has been infected for less than 11 months and has no nodules in the nostril or invasion of the soft tissues of the face, (2) intermediate: the patient has been infected between 11 and 12 months and has a nodule in the nostril or invasion of the facial soft tissues, (3) late: patients with a development time of more than 12 months, (4) atypical: severe pain, necrosis, orbital involvement, or systemic spread occurs within the first 12 months of disease).

Invasive and disseminated disease in patients with conidiobolomycosis is rare but possible. Isolation of *Conidiobolus lamprauges* in a 61-year-old patient with recurrent mantle cell lymphoma and postmortem diagnosis revealed growth of *C. lamprauges* in the tracheal mucosa, lungs, kidneys, and spleen (Kimura et al., 2011). Moreover, invasive lung disease with *C. pachyzygosporus* in a 71-year-old patient with myeloid leukemia and histopathologic diagnosis of lung biopsy showed large and tortuous hyphal elements with occasional septa (Stavropoulou et al., 2020). Therefore, 18S rDNA pan-fungal polymerase chain reaction (PCR) was positive for a *Conidiobolus* spp. at 1,582 copies/ml. Finally, PCR was performed for the 28S ribosomal DNA (rDNA), which showed good discrimination for 100% identity with *C. pachyzygosporus* (Stavropoulou et al., 2020). This demonstrates that invasive and disseminated forms of conidiobolomycosis can be included in the differential diagnosis in patients with severe immunosuppression.

Mucormycosis

Mucorales infection can cause rhino-orbital-cerebral, pulmonary, cutaneous, gastrointestinal, disseminated, and uncommon forms of mucormycosis (Cornely et al., 2019). A systematic review and meta-analysis of case reports revealed rhino-orbital-cerebral mucormycosis as the most common form and affected 34% of the population (Jeong et al., 2019). Cutaneous mucormycosis accounted for 22% and pulmonary mucormycosis occurred in 20% of the cases studied. After initial infection (rhino-orbital, pulmonary, cutaneous) spread is possible in 22% of cases. Unfortunately, patients with disseminated mucormycosis have a mortality of up to 94%. Although gastrointestinal mucormycosis occurred in 8% of case reports, mortality can be as high as 85%. Pulmonary mucormycosis ranks third in mortality with 76% of patients analyzed in case reports. It is interesting to note that in the pediatric population there is a greater association between the cutaneous and gastrointestinal forms of mucormycosis (Jeong et al., 2019).

A special section deserves COVID -19 associated mucormycosis (CAM) highlighted since early 2021. This superinfection has been reported in at least 18 countries. The country with the most reports was India, followed by the United States of America (Hoenigl et al., 2022). Most affected were severely ill patients with COVID -19, diabetics, and patients taking corticosteroids (Hoenigl et al., 2022). CAM Patients were particularly susceptible to a combination of risk factors. These included an elevated ferritin, islet cell damage, cytokine storm (interleukin 6), endothelitis, ketoacidosis, use of antifungal drugs, uncontrolled corticosteroid, etc., (Gupta and Ahuja, 2021; Mathew et al., 2021; Pal et al., 2021; Hoenigl et al., 2022). In the various studies, rhino-orbital-cerebral and pulmonary mucormycosis is shown as the main presentation (Cornely et al., 2019; Gupta and Ahuja, 2021).

Rhino-orbital-cerebral mucormycosis is caused by nasal invasion of *Mucorales* sporangiospore in susceptible patients. Once the infection spreads, surrounding tissues also become infected. Depending on growth, the fungi may invade the palate from below, infect the upper part of the sphenoid sinus, invade the cavernous sinus laterally, and infect the orbits. Fungi invade the skull through the orbital apex or cribriform plate of the ethmoid bone or by hematogenous dissemination. Invasion of cerebral vessels with or without mycotic aneurysms is possible. Initially, the patient reports fever and pain in the midface or retroocular region (Hosseini and Borghei, 2005; Orguc et al., 2005). Nasal congestion, rhinorrhea, nasal hypoesthesia, and epistaxis may also occur. If the eye is affected, diplopia, blurred vision, proptosis, conjunctival chemosis, nygtasms, and amaurosis may occur (Ribes et al., 2000). If the brain is affected, the patient may show dizziness, gait disturbances, altered mental status, and even seizures. Physical examination reveals nasal and orbital cellulitis. This rapidly progresses to necrosis of the affected areas in association with fungal angioinvasion (Ribes et al., 2000; Ganesan and Sivanandam, 2022).

When *Mucorales* spores reach the lower respiratory tract. They can cause endotracheal and/or bronchoalveolar infections in patients with risk factors. Endotracheal infection can lead to airway and lung collapse (Collins et al., 1999). Invasion into the hilar region with local spread to the mediastinum, pericardium, and thoracic cavity has also been described (Connor et al., 1979; Seifert et al., 2020). Patients may initially present with fever and non-specific chest pain. These manifestations may be confused with other etiologies. For example, bacterial infections, pulmonary candidiasis, or aspergillosis (Connor et al., 1979; Passamonte and Dix, 1985). Qu et al. (2021) analyzed 59 patients with proven pulmonary mucormycosis in western China. The three most common clinical manifestations were cough (93%), fever (52%), hemoptysis (45%), dyspnea (30%),

and chest pain (15%). Because of clinical non-specificity, imaging studies are useful in these patients. In the early stages, a peribronchial opacity may be observed. In advanced stages, the disease progresses to consolidations, nodules, or masses. The reverse halo sign is associated with pulmonary mucormycosis rather than invasive pulmonary aspergillosis (Agrawal et al., 2020).

Mucorales can infect the skin directly by trauma. It has been associated with inoculation due to minor trauma in patients with comorbidities such as leukemia (Maleitzke et al., 2019). Cases have also been observed in severe trauma due to motor vehicle accidents (Tyll et al., 2016). Or associated with biomedical devices (Roden et al., 2005; Rammaert et al., 2012; Perz et al., 2020). Secondarily and rarely, the skin may be affected by the spread of pulmonary or gastrointestinal foci (Iyengar et al., 2017). Cutaneous mucormycosis can be classified as superficial and deep. It is superficial when it affects the skin or subcutaneous cellular tissue. On the other hand, if tendons, muscles and/or bones are affected, it is classified as deep (Roden et al., 2005; Spellberg et al., 2005). Initially, cutaneous mucormycosis usually has a cellulitis-like appearance. As it progresses, there is blackish skin discoloration, scabs, abscesses, ulcers, small nodules, indurated masses, etc., (Johnson et al., 1987; Eaton et al., 1994; Baradkar and Kumar, 2009; García-Pajares et al., 2012; Hsieh et al., 2013; Perz et al., 2020). This leads to an inflammatory periphery, a central black eschar surrounded by necrotic tissue (Baradkar and Kumar, 2009; García-Pajares et al., 2012; Rammaert et al., 2012; Perz et al., 2020). Although the bibliography highlights the black eschar. The analysis of 623 patients with cutaneous mucormycosis found that only 55% of the cases were associated with the term's "necrosis," "necrotic," or "necrotizing" (Skiada et al., 2022). An individualized, holistic analysis of the patient's risk factors, potential mechanisms of infection, and clinic is essential.

In gastrointestinal mucormycosis, the stomach, colon, and ileum are most affected (Michalak et al., 1980; Spellberg, 2012). In addition, patients may be mistaken for cancer due to masses in the cecum, ileum, or appendix (Busbait et al., 2020). The most important risk factors are corticosteroid use and diabetes (Michalak et al., 1980; Spellberg, 2012). In a systematic review of 80 cases with gastrointestinal mucormycosis, abdominal pain occurred in 50%, fever in 37%, and perforation in 27% (Skiada et al., 2022). This study also included neonates with abdominal pain and vomiting (40%) and gastrointestinal perforation (30%). In addition, fever, obstruction, and abdominal distension occurred in 20% of the neonates studied (Skiada et al., 2022). Imaging findings may contribute to the diagnosis. Computed tomography findings can range from gastric pneumatosis and reduced gastric wall enhancement to mass like thickening (Ghuman et al., 2021). Because of the wide range of differential diagnoses, culture, histopathology, and PCR of biopsies of the infected tissue are required for confirmation in all cases (Cornely et al., 2019). Over time, the disease may spread to many organs. After angioinvasion, *Mucorales* can migrate to any organ. This leads to rare forms such as endocarditis, osteomyelitis, peritonitis, and pyelonephritis (Echols et al., 1979; Virmani et al., 1982; Aymoré et al., 2005; Ram et al., 2007).

Within these disseminated forms, renal mucormycosis attracts our special attention. This mucoralean infection of the kidney is rare, usually unilateral, and potentially fatal. It has been observed in both immunocompromised and immunocompetent patients. Clinically, patients present with the triad of fever, low back pain, and sepsis. Assessment of general condition is important as it is associated with acute kidney injurie. Imaging studies are important as they reveal areas of lower density, focal vascular loss, and infarction. Urine microscopy and culture can be used to diagnose renal mucormycosis (Gupta and Gupta, 2012; Kuy et al., 2013; Bhadauria et al., 2018; Devana et al., 2019).

Diagnosis

Diagnosis of these fungal infections should include analysis of risk and demographic factors, clinical presentation, site of infection, clinical evolution, and imaging studies. If suspicion exists, it must be confirmed by examination of biopsy fragments from infected tissue for fungal cultures, direct visualization with potassium hydroxide (KOH), histopathology, and detection of microorganisms by polymerase chain reaction (PCR).

Direct observation from tissue and fungal cultures

It is commonly recommended to investigate several biopsy fragments of the affected tissue by microbiological and histopathological analyses. Specimens must be transported in a sterile container with a closed lid to avoid contamination by microorganisms from the environment. They should not be transported under refrigeration as this inhibits the growth of *B. ranarum* (Davis et al., 1994). Potassium hydroxide is a strong base. When it encounters tissues, it softens, digests, and cleans the tissues. Fungi are not attacked by KOH because of its composition. Therefore, it digests the cells of the tissue under study and allows observation of fungal structures (Cheesbrough, 2005; Tille, 2021). Small 2 mm^2 blocks of biopsied tissue should be placed into a glass slide with 1 drop of 10% KOH (10 g KOH, 10 ml glycerol, 80 ml distilled water) (Cheesbrough, 2005; Tille, 2021). Which will allow shows under light microscopy

broad ribbon like hyaline pauciseptate or aseptate hyphae (Cheesbrough, 2005; Tille, 2021). The importance of KOH smears in diagnosis cannot be overstated. Using a fluorescent microscope to examine Blankophor (fluorescent dye) wet-mount preparation of tissue biopsy increases diagnostic sensitivity (Kumar Verma et al., 2012).

Among the disadvantages of wet preparation with KOH is the large and variable sensitivity in detecting fungal pathogens (Afshar et al., 2018). Biopsies may have low or no microbial load at the site of collection. The site from which the biopsy was taken is not representative site of infection (Cheesbrough, 2005; Tille, 2021). It is important that personnel trained in mycology process and analyze the specimen. The detection of pauciseptate or aseptate hyphae confirms the etiology of the fungi. However, they do not allow differentiation between basidiobolomycosis, conidiobolomycosis, and mucormycosis because the findings are similar (Tille, 2021). For this reason, culture or molecular diagnosis is important for specific identification of the microorganism. Although 2% Sabouraud dextrose agar (SDA) is generally recommended as a culture medium for fungi. Some fungal pathogens may have difficulty growing. Therefore, potato dextrose agar (PDA) and even brain heart infusion agar (BHI) are much more enriched and facilitate the growth of microorganisms (Procop et al., 2016; Tille, 2021).

If the biopsy is from a tissue with a large saprophytic bacterial microbiota will be processed, the use of SDA or BHI supplemented with chloramphenicol is very helpful (Procop et al., 2016; Tille, 2021). The incubation of the cultures with the samples should be carried out at 37°C and at room temperature. *Basidiobolus ranarum* grows with a creamy rugose colony of on 2% Sabouraud dextrose agar. When genetic material is exchanged, zygospores develop with their characteristic beaks as a result. Primary culture in SDA shows a yellow-white colony of *Conidiobolus coronatus* after 48 h. Some aerial hyphae and satellite colonies can be seen on the lid of the SDA plate due to the ejection of conidia from the sporangiophores (Vilela and Mendoza, 2018). The name coronatus comes from the development into fully formed corona secondary conidia (Vilela and Mendoza, 2018). In just 5 days, *Rhizopus* and *Mucor* can growth cottony-like appearance in a Petri dish. As the sporangiospores mature within the sporangium, the growth usually turns grayish-brown with aging. The reverse remains a pale white. *Rhizopus* and *Mucor* are fast-growing fungi that can fill a Petri dish with fluffy, cotton candy-like growth in less than 5 days. The growth in PDA is usually whitish and may turn grayish brown with age due to maturation of sporangiospores in the sporangium. The reverse side remains pale white (Lass-Flör, 2009). Under the microscope, *Mucor* sp. has no apophysis, a spherical sporangium, and no rhizoids. *Rhizopus* sp., on the other hand, has not prominent apophysis, a spherical sporangium, and abundant rhizoids (often well developed) (Ribes et al., 2000; Lass-Flör, 2009).

Histopathological findings

Histopathologic examination may provide clues to distinguish mucormycosis from basidiobolomycosis or conidiobolomycosis. If fungal disease is suspected, it is recommended that a Grocott (methenamine) silver stain (GMS) or Periodic acid-Schiff stain (PAS) be ordered. The morphology of the tissue around the fungus can be better identified with PAS. Meanwhile, the fungal structures are highlighted by GMS (Denning et al., 2003; Procop et al., 2016; Tille, 2021). A commonly used stain is hematoxylin and eosin (H&E). In conidiobolomycosis and basidiobolomycosis, H&E-stained tissues that are infected show extensive fibrosis and an inflammatory reaction (Denning et al., 2003; Procop et al., 2016; Tille, 2021). This inflammatory reaction is usually characterized by demonstrated as eosinophilic cuff surrounding the coenocytic hyphae that may include eosinophils (Splendore–Hoeppli phenomenon) (Denning et al., 2003; Gopinath, 2018). Although patients with entomophthoromycosis generally do not have angioinvasion (Denning et al., 2003). Rare cases of gastrointestinal conidiobolomycosis with angioinvasion have been reported (Elzein et al., 2018).

A *Mucorales* hyphae varies in width (6–25 μm), and it is usually non-septate or sparsely septate (coenocytic hyphae). In mucormycosis, angioinvasion by coenocytic hyphae occurs with subsequent necrosis or hemorrhage of the surrounding tissue. When inflammation is present, it is often purulent, less commonly granulomatous, and neutrophils may be observed. In the study by Ben-Ami et al. (2009) in cancer patients with pulmonary mucormycosis, it is described that inflammation is limited but there is extensive angioinvasion into the wall or lumen of blood vessels. Interestingly, *Mucorales* hyphae stain poorly in GMS. For this reason, mucormycosis should be suspected if staining of hyphae with GMS is poor (Denning et al., 2003).

Molecular diagnosis

Detection of sequences of genetic material of pathogens can reduce the waiting time for results. Hata et al. reported the development of a real-time PCR assay for the detection of *Lichtheimia* (formerly *Absidia*), *Apophysomyces*, *Cunninghamella*, *Mucor*, *Rhizopus*, and *Saksenaea* in culture and tissue samples (Hata et al., 2008). The analytical sensitivity of this real-time PCR for the detection of *Mucorales* was 10 targets/μl and showed no cross-reactivity for bacteria and fungi (Hata et al., 2008). For samples from cultures, clinical sensitivity was 100% (39/39) and specificity was 92% (59/64). Meanwhile, for biopsies, unfortunately, only 2 samples were processed with a sensitivity and specificity

of 100%. It is clearly advisable to increase the number of biopsies from patients with confirmed mucormycosis in order to calculate sensitivity and specificity correctly (Hata et al., 2008).

Another method used in patients with mucormycosis is the detection of circulating DNA in blood and cerebrospinal fluid (Shigemura et al., 2014; Millon et al., 2016; Hiramoto et al., 2020). In these studies, sequences of oligonucleotide primers and markers for detecting circulating DNA of *Mucor/Rhizopus*, *Lichtheimia*, or *Rhizomucor* with detection limit of 1.0×10^3 copies/ml (Shigemura et al., 2014; Hiramoto et al., 2020). The advantage is that the detection of circulating DNA from the first day of infection (Hiramoto et al., 2020). In any case, more cases need to be studied and compared with other methods to determine the sensitivity and specificity of this technique. On the other hand, Alanio et al. evaluated the detection of *Mucorales* in 19 tissue samples by PCR/electrospray ionization mass spectrometry (PCR/ESI- MS) with a detection time of 6 h (Alanio et al., 2015). PCR/ESI-MS was more effective than other molecular methods in identifying *Mucorales* to species level. This methodology could not correctly identify samples with *Cunninghamella* spp. or *Saksenaea vasiformis*, PCR/ESI-MS databases need to be improved to improve the identification (Alanio et al., 2015).

Few studies have reported PCR primers for the identification of *Entomophthoromycotina* fungi in infected tissues in patients with conidiobolomycosis or basidiobolomycosis. Taxon-specific primer pairs were designed to amplify a 28S rDNA fragment for *Entomophthoromycotina* detection. For *B. haptosporus/ranarum* Ba1 (AAAATCTGTAAGGTTCAACCTTG) and Ba2 (TGCAGGAGAAGTACATCCGC) (Voigt et al., 1999). For the detection of *C. coronatus* was designed Cc1 (TCTCTTAACTTGCTTCTATGCC) and Cc2 (CTTTAATTAAGCTAATCAACATG) (Voigt et al., 1999). El-Shabrawi et al. detected *B. ranarum* after 6 months in paraffin-embedded tissue (FFPE). This result was consistent with morphological, physiological, and molecular evidence of basidiobolomycosis in a patient with a fungal mass (El-Shabrawi et al., 2014). While this is a promising result. Validation in other clinical cases is needed to determine sensitivity, and specificity in clinical samples.

The next-generation metagenomic sequencing (mNGS) technique analyses nucleic acids from a wide range of microorganisms at the same time. Studies and case reports have shown that this strategy can identify the causative pathogen when other strategies have failed. There are some examples in the scientific literature. Sitterle et al. detected *B. meristosporus* in a biopsy of a colonic mass (Sitterlé et al., 2017). Falces-Romero et al. reported colonization by *Conidiobolus* sp. in a respiratory specimen from a patient with chronic obstructive pulmonary disease (Falces-Romero et al., 2017). Sun et al. detected *Rhizopus microsporus* in skin and lung biopsies from a patient with clinical symptoms suggestive of disseminated mucormycosis (Sun et al., 2021). In any case, contamination can lead to false-positive results, and detailed analysis of the clinical case to validate the results by experts in clinical microbiology and infectious diseases is recommended.

Conclusion

The class Zygomycetes has been rejected because of the phylogenetic differences of the microorganisms that compose it. Currently, the classes *Mucoromycota*, which includes the order *Mucorales*, and *Zoopagomycota*, which includes the orders *Entomophthorales* (*Conidiobolus* spp.) and *Basidiobolales* (*Basidiobolus* spp.), are used. The term zygomycosis should not be used because it can lead to confusion. Instead, infection with *Mucorales* is referred to as mucormycosis, *Basidiobolus* spp. as basidiobolomycosis, and infection with *Conidiobolus* spp. as conidiobolomycosis. On the other hand, entomophthoromycosis is a broad term that includes infection with *Conidiobolus* spp. or *Basidiobolus* spp. Mucormycosis is an infection that occurs primarily in patients with impaired cellular immunity. In contrast, basidiobolomycosis and conidiobolomycosis usually occur in immunocompetent patients. However, the coenocytic hyphomycosis is proposed as a new term to summarize fungal infections caused by coenocytic (non-septate), terrestrial and basal lineage filamentous fungi which were formerly subscribed to the "zygomycetes" in a colloquial sense.

The clinical picture of patients infected with *Mucorales* shows a rapid evolution with angioinvasion and necrosis, ranging from the rhino-orbital-cerebral form to the disseminated form. Clinically, patients with *Basidiobolus* spp. show a slow course lasting many months with mass-like infection of the skin. *Conidiobolus* spp. is characterized by nasal infection with the appearance of a mass and a slow course over months that may become obstructive. The differential diagnosis based on epidemiologic, clinical, imaging, histopathologic, microbiologic, and molecular criteria must be critically analyzed in each patient. Although histopathology can be helpful in differentiating between mucormycosis and entomophthoromycosis based on angioinvasion, necrosis, and the Splendore-Hoeppli phenomenon. The confirmatory diagnosis is based on the report of a mycological culture and molecular biology tests. Confirmation by culture or molecular biology helps in the choice of antifungal agent based on the susceptibility profile of each microorganism.

In the context of the limitations of this study, it is important to note that the heat maps show prevalence based on case reports. This does not necessarily reflect the real global burden of conidiobolomycosis, basidiobolomycosis, and mucormycosis. For this reason, an international surveillance network with trained personnel and a global perspective is the solution we need and has not yet been adequately implemented.

Author contributions

JA-E and KV drafted and revised the manuscript. Both authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1035100/full#supplementary-material

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Why vary what's working? Phase variation and biofilm formation in *Francisella tularensis*

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The notoriety of high-consequence human pathogens has increased in recent years and, rightfully, research efforts have focused on understanding host-pathogen interactions. *Francisella tularensis* has been detected in an impressively broad range of vertebrate hosts as well as numerous arthropod vectors and single-celled organisms. Two clinically important subspecies, *F. tularensis* subsp. *tularensis* (Type A) and *F. tularensis* subsp. *holarctica* (Type B), are responsible for the majority of tularemia cases in humans. The success of this bacterium in mammalian hosts can be at least partly attributed to a unique LPS molecule that allows the bacterium to avoid detection by the host immune system. Curiously, phase variation of the O-antigen incorporated into LPS has been documented in these subspecies of *F. tularensis,* and these variants often display some level of attenuation in infection models. While the role of phase variation in *F. tularensis* biology is unclear, it has been suggested that this phenomenon can aid in environmental survival and persistence. Biofilms have been established as the predominant lifestyle of many bacteria in the environment, though, it was previously thought that Type A and B isolates of *F. tularensis* typically form poor biofilms. Recent studies question this ideology as it was shown that alteration of the O-antigen allows robust biofilm formation in both Type A and B isolates. This review aims to explore the link between phase variation of the O-antigen, biofilm formation, and environmental persistence with an emphasis on clinically relevant subspecies and how understanding these poorly studied mechanisms could lead to new medical countermeasures to combat tularemia.

KEYWORDS

***Francisella tularensis*, phase variation, biofilm, O-antigen, host adaptation, environmental survival, vectors and bacteria, VBNC (viable, but non-culturable)**

Introduction

Found ubiquitously across the Northern Hemisphere, *Francisella tularensis* is the etiological agent of tularemia, or more casually, "rabbit fever"(Ellis et al., 2002). In humans, illness presents in several forms with the most common being ulceroglandular, often from the bite of an insect carrying *F. tularensis* or contact with an infected rabbit. From a biodefense perspective, tularemia could also manifest as the more severe form of the

disease-causing pneumonic or typhoidal tularemia (Ellis et al., 2002). *F. tularensis* is comprised of two subspecies that cause human disease: *F. tularensis* subsp. *tularensis* (Type A) and *F. tularensis* subsp. *holarctica* (Type B). A third closely related subspecies, *F. tularensis* subsp. *novicida*, is only associated with brackish water or soil and rarely causes disease in humans. *F. novicida* is commonly used as a laboratory surrogate since it has a high degree of genetic similarity to *F. tularensis*, is able to infect macrophages *in vitro* and cause disease in mice, and can be handled under BSL-2 conditions (Kingry and Petersen, 2014). Throughout this manuscript, *F. novicida* will be used to note this subspecies exclusively while *F. tularensis* will be used to describe Type A and B isolates specifically.

Francisella tularensis is classified as a Tier 1 Select Agent in the United States (Dennis et al., 2001) and currently no approved Food and Drug Administration (FDA) vaccine is available. While a Live Vaccine Strain (LVS) exists and has been used for laboratory workers at risk in the past under Investigational New Drug (IND) status, the basis of attenuation is poorly characterized and the ancestral strain is unknown(Eigelsbach and Downs, 1961; Saslaw et al., 1961; Hornick and Eigelsbach, 1966). Complicating the approval of LVS is spontaneous variation of colony phenotypes, which is known to adversely affect immunization (Eigelsbach et al., 1951; Eigelsbach and Downs, 1961). Early studies determined that the frequency of colony variation, referred to as phase or blue/gray variation, could be as high as 10^{-3} to 10^{-4} depending on culture conditions and, paradoxically, variant colony phenotypes dramatically differed in terms of virulence when tested in a murine model (Eigelsbach et al., 1951). With this in mind, it is curious that the mechanisms responsible for this variation were not naturally selected against given the consequence for attenuation of an intracellular pathogen. Furthermore, understanding how this variation occurs in *F. tularensis* could lead to ways to prevent phase variation from occurring and allow for a more stable and efficacious live vaccine.

Biofilm refers to an adhered community of cells encased by an extracellular matrix (ECM) and typically involves distinct changes in bacterial behavior, gene expression, and metabolism that are not observed in the planktonic state (Hall-Stoodley et al., 2004). In pathogens, biofilm is often regarded as a virulence determinant as it enables bacteria to cope with the host environment by thwarting innate immunity, phagocytosis, and antibiotic treatment (Hall-Stoodley and Stoodley, 2009). Francisella species have been shown to form biofilm [reviewed by van Hoek (2013)], though clinically important subspecies of *F. tularensis* tend to form a less defined biofilm with sparse cell density than *F. novicida* or other Francisella species(Margolis et al., 2010; Mahajan et al., 2011). Recent studies demonstrate that O-antigen (O-Ag) of the LPS can influence the biofilm-forming capacity of Type A and B isolates, changing this perception of biofilm formation in *F. tularensis* (Champion et al., 2019; Mlynek et al., 2021). This review aims to explore where phase variation and biofilm may play a role in the survival and pathogenesis of *F. tularensis* which could lead to new therapies to prevent disease.

Comparing apples to oranges: Distinct genetic differences between *Francisella novicida* and *Francisella tularensis* complicate understanding biofilm formation in clinically relevant isolates

While *F. novicida* shares a high degree of genetic similarity with *F. tularensis* for which it has been utilized as a surrogate strain, important genetic differences do exist. First, *F. tularensis* lacks a functional cyclic-di-GMP system (cd-GMP) which is present in *F. novicida*. Secondly, the *wbt* locus of *F. tularensis* (involved in O-Ag synthesis) contains additional genes that are not present in *F. novicida* (McLendon et al., 2006; Sjodin et al., 2012; Kingry and Petersen, 2014). These differences between *F. novicida* and the virulent *F. tularensis* strains highlight the need to focus on the latter pathogenic strains to gain a true understanding of biofilm and variation.

cd-GMP has been shown to be an important second messenger that impacts multiple aspects of bacterial behavior, often influencing genes responsible for the transition between the environment and hosts in pathogenic species (Tamayo et al., 2007; Jenal et al., 2017). The model that has emerged is that elevated levels of cd-GMP inhibit motility and stimulate biofilm formation by downregulation of the genes encoding proteins required for flagella and/or pili and upregulation of genes encoding extracellular polysaccharide (Simm et al., 2004; Hickman et al., 2005). The intracellular concentrations of cd-GMP are tightly controlled by diguanylate cyclase (DGC; for synthesis) and phosphodiesterase enzymes (PDE; for degradation). The activity of these enzymes depends on a myriad of input signals, often through the interaction with other proteins, such as two-component systems (Galperin et al., 2001). The gene cluster responsible for modulating the levels of cd-GMP in *F. novicida* (FTN_ 0451 to FTN_0456) was noted to be absent in both LVS and Schu S4 (Rohmer et al., 2007) and appears to be absent or incomplete in all *F. tularensis* genomes published to date. While it is unclear if other genes encode DGC/PDE enzymes capable of modulating cd-GMP levels in *F. tularensis*, cd-GMP appears to control virulence and biofilm in *F. novicida* in a similar manner to what has been reported in other bacterial pathogens. A study by Zogaj et al., demonstrated that high cd-GMP levels tempered virulence of *F. novicida* by inhibiting intracellular replication and promoted biofilm formation (Zogaj et al., 2012). While Francisella genomes have a limited repertoire of *bona fide* two-component systems, it was also found that the orphaned response regulator QseB influenced DGC activity, which is consistent with other reports suggesting a role for QseB in *F. novicida* biofilm formation (Durham-Colleran et al., 2010; Zogaj et al., 2012). However, mechanisms responsible for the regulation of biofilm formation in Type A and B isolates are largely unknown.

LPS is considered a major virulence factor in *F. tularensis* as it allows the bacterial cell to evade the host immune response (Sandstrom et al., 1992; Gunn and Ernst, 2007; Wang et al., 2007; Weiss et al., 2007) and deleterious mutations within genes involved

in LPS or O-Ag synthesis often lead to attenuation (Raynaud et al., 2007; Apicella et al., 2010; Kim et al., 2012; Jones et al., 2014; Rasmussen et al., 2014, 2015; Chance et al., 2017). While the structure of *F. tularensis* LPS contains notable and unique features for lipid A, core, and O-Ag [reviewed by Gunn and Ernst, 2007], recent studies suggest the O-Ag is a driving determinant of biofilm-forming capacity (Champion et al., 2019; Mlynek et al., 2021). As the case with a wide range of other Gram-negative bacteria, O-Ag has been demonstrated to play a role in biofilm formation (Nakao et al., 2006, 2012; Murphy et al., 2014; Hathroubi et al., 2016).

Francisella tularensis isolates share an O-Ag repeat of Qui4NFm-GalNAcAN-GalNAcAN-GuiNac (Vinogradov et al., 1991, 2002; Prior et al., 2003). In contrast, the terminal saccharides differ in *F. novicida* with the QuiNAc4NAc at the reducing residue and a third GalNAcAN at the non-reducing residue (Vinogradov et al., 2004; Thomas et al., 2007). The most likely cause for this difference is that *F. tularensis* contains five additional genes (*wbtI, wbtJ, wbtK, wbtL,* and *wbtM*) within the *wbt* locus (McLendon et al., 2006; Rohmer et al., 2007; Sjodin et al., 2012). Each additional gene is thought to encode for an enzyme involved in the biosynthesis of Qui4NFm (Prior et al., 2003; Li et al., 2007; Twine et al., 2012; Zimmer et al., 2014). Furthermore, mutations made to genes within the *wbt* operon can result in an altered O-Ag phenotype consistent with "phase" or "gray" variants (Bandara et al., 2011). Interestingly, growth environment can influence the relative amount of O-Ag displayed on the cell surface, either by LPS chain length or incorporation into a capsule, suggesting that this feature may be important in host or environmental adaptation (Zarrella et al., 2011; Holland et al., 2017). However, transcriptional regulation of the O-Ag has not been associated with phase variation as this phenomenon is attributed to physical alterations of this molecule (Cowley et al., 1996; Hartley et al., 2006; Soni et al., 2010).

O-antigen influences the biofilm-forming capacity of *Francisella tularensis* isolates

Early researchers noted a distinct reduction in virulence when *F. tularensis* was cultured on artificial medium (Foshay, 1932; Ransmeier, 1943; Eigelsbach et al., 1951). A study by Eigelsbach in 1951 aimed to systematically correlate colony morphology to virulence by identifying smooth and non-smooth colonies and then further classifying these colonies using oblique lighting. It was found that medium pH, inoculum size, and culture duration influenced the rate at which spontaneous changes in colony morphology occurred. Most importantly, this study determined that colonies which appeared gray under oblique lighting were highly attenuated compared to those that appeared blue. Eigelsbach also noted that some of these morphologies were unstable, and reversion between blue and gray variant forms was possible. It would be 45 years before these findings were linked to an antigenic shift of the LPS O-Ag displayed on the cell surface (Cowley et al., 1996).

A study by Champion et al. identified O-Ag and glycosylation of the capsule-like complex (CLC) as factors that can influence biofilm formation in *F. tularensis* isolates (Champion et al., 2019). While *F. novicida* can form a robust biofilm in 2–3 days, *F. tularensis* isolates typically required 10 days to form a comparable biofilm. O-Ag deficient mutants of LVS and TI0902 (virulent Type A) developed biofilms within 5 days that were 2-5x more robust than parental strains (Champion et al., 2019). Further, surface attachment and biofilm formation were enhanced in a double mutant deficient in O-Ag and CLC glycosylation, suggesting that there is an inverse relationship between cell surface carbohydrates and biofilm formation. It is notable that *F. tularensis* can produce an electron transparent CLC as well as an electron-dense capsule, with the latter being chiefly composed of O-Ag [Hood, 1977; Cherwonogrodzky et al., 1994; Apicella et al., 2010; reviewed by Freudenberger Catanzaro and Inzana, 2020]. Highlighting the differences in biofilm formation among *F. tularensis* subspecies, O-Ag mutants in *F. novicida* form biofilms that are equal or less than the parental wild-type (Champion et al., 2019).

Mlynek et al. identified a link between variation of the O-Ag and biofilm formation in *F. tularensis* (Mlynek et al., 2021). In agreement with the Champion findings, biofilm formation typically required at least 7 days to occur. However, it was noted that robust biofilm formation occurred stochastically at earlier time points and increased in frequency as culture duration increased. It was subsequently determined that biofilm formation was associated with a distinct population of gray variants that emerged within the culture. While the exact alteration of the LPS in these variants was unknown, western blotting with α-LPS or α-capsule mAbs against O-Ag yielded no reactivity (Mlynek et al., 2021). The studies Champion et al. and Mlynek et al. found that external conditions, such as culture medium and pH, impacted biofilm development, which suggests additional gene regulation and/or environmental checkpoints beyond antigenic variation of O-Ag may factor into *F. tularensis* biofilm formation.

Is variety the spice of life for *Francisella tularensis*?

F. tularensis has been identified in a diverse collection of hosts ranging from amoeba to humans. Further, it has been well established that *F. tularensis* can be harbored within arthropod vectors as well as detected in water sources. While biofilm is typically thought to be the predominant lifestyle of bacteria in the environment, it is unclear what role, if any, biofilm plays in these diverse environments and how phase variation, or more properly, antigenic variation factors into biofilm formation by *F. tularensis* in this context (Figure 1).

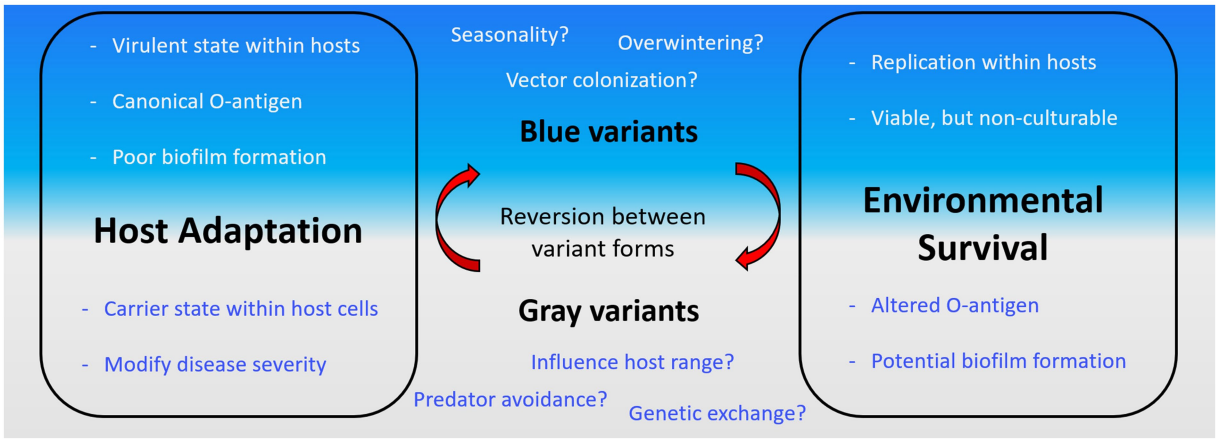

FIGURE 1
Traits of *Francisella tularensis* antigenic variation and outstanding questions. *F. tularensis* is known to transition between a "blue" or "gray" state by altering the O-antigen. The properties of these states are outlined in each box (Host Adaptation, left; Environmental Survival, right) grouped by where trait likely aids the bacterial cell. The traits are further separated within these boxes based on the associated state of the cell (blue, top; gray, bottom). Outstanding aspects of *F. tularensis* biology where phase variation is potentially important are displayed in blue or gray text.

Vertebrates and hosts cells

Given the pathogenicity of this bacterium for humans, research efforts have focused on the mammalian host cell response and the role of LPS in avoiding immune detection. While structural modifications to lipid A and core of the LPS have been attributed to phase variation (Soni et al., 2010), the most common cause of phase variation is arguably alterations to the O-Ag (Cowley et al., 1996; Hartley et al., 2006). In Francisella, it has been well established that O-Ag is important for the subversion of mammalian innate immune defenses, namely complement (Sandstrom et al., 1988; Sorokin et al., 1996; Ben Nasr and Klimpel, 2008; Clay et al., 2008) and, along these lines, serum increases the uptake of gray variants by macrophages (Hartley et al., 2006). Additionally, poor intracellular replication of gray variants within macrophages has been reported (Thomas et al., 2007; Lindemann et al., 2011). However, this begs the question why is phase variation frequently observed in *F. tularensis* if immune evasion and intracellular replication are decreased in variants?

One hypothesis is that *F. tularensis* gray variants (GVs) enable persistence inside host macrophages by stimulating bacteriostatic levels of the nitric oxide response to set up a carrier state (Cowley et al., 1996). Recently, biofilm-like structures, termed intracellular bacterial communities (IBC), have been identified for many bacteria persisting intracellularly within host cells [reviewed by Mirzaei et al., 2020]. IBCs consist of small aggregates of cells encased within a matrix, often formed from extracellular polysaccharides and/or pili. It is currently unclear if GVs produce biofilm when grown intracellularly, though conceivably, IBCs would contribute to persistence within the host. While *F. tularensis* is typically studied as an intracellular pathogen, bacteria can be detected extracellularly in host blood (Forestal et al., 2007; Yu et al., 2008). In this environment, biofilm could play a more traditional role in pathogenesis and armor variants during the extracellular phase of the infection. Outer membrane vesicles (OMV) have been shown to stimulate biofilm in *F. tularensis* (Siebert et al., 2019) and facilitate early interactions with macrophages (McCaig et al., 2013; Pavkova et al., 2021); however, OMVs were not detected when the bacterium is intracellular (Pavkova et al., 2021).

Alternatively, if GVs and by extension biofilm formation are important for *F. tularensis* pathogenesis, phase variation may impede the ability to survive within certain hosts which has implications for host range. Supporting this hypothesis, many mammalian animal models are highly susceptible to *F. tularensis*, especially at low doses (<10 CFU), while only mice typically succumb to *F. novicida* at this dose [reviewed in Kingry and Petersen, 2014]. The LPS structure is likely a contributing factor to differences in host susceptibility as *F. tularensis* growth was suppressed in rat macrophages by the nitric oxide response when co-infected with *F. novicida* (Cowley et al., 1997). While *F. tularensis* infects a plethora of vertebrate hosts (including, amphibian, fish, birds, and mammals; Burroughs et al., 1945; Hopla, 1974), it is largely unknown how the immune response of individual species reacts to *F. tularensis* LPS and phase variant O-Ag. It is possible that the LPS presented by GVs favors the survival of *F. tularensis* in vertebrate host other than routinely studied laboratory models. Further studies are needed to address the *in vivo* role of biofilm formation during vertebrate infections in *F. tularensis*.

Arthropod vectors

Arthropod transmission of *F. tularensis* to humans has been noted nearly since the first reports of tularemia in the early 1900s (Francis, 1919; Parker et al., 1924), and still today, the glandular and ulceroglandular forms are by far the most common

manifestation of tularemia in humans (Rosenberg et al., 2018). Association with a vector can increase survivorship and biological transmission of a bacterial population while decreasing the risk of "dead-end" infections in pathogens associated with a high mortality. Biofilm has been found to aid in the transmission of bacterial pathogens closely associated with arthropod vectors to mammalian hosts with the most notable example being *Yersinia pestis* in fleas (Jarrett et al., 2004; Hinnebusch and Erickson, 2008). While *F. tularensis* is transmissible through multiple vectors, tularemia is mostly associated with bites from deer flies, mosquitoes, and hard ticks [reviewed in Telford and Goethert (2020)].

Type A and B isolates tend to have different vector ecology. Classically, Type A isolates have been associated with ticks in arid environments, while Type B isolates have more often been associated with mosquitos near aquatic systems (Parker et al., 1951; Jellison, 1974; Eliasson et al., 2006; Tully and Huntley, 2020). Transstadial transmission has been reported for both vectors (Lundstrom et al., 2011; Backman et al., 2015; Coburn et al., 2015; Mani et al., 2015), with ticks maintaining bacteria capable of infection at each stage (Coburn et al., 2015; Mani et al., 2015) and the American dog tick (*Dermacentor variabilis*) was identified as a major factor for perpetuation in an environmental setting (Goethert et al., 2004; Goethert and Telford, 2009). While a fair amount of literature has been published on the ticks and tularemia (thorough reviews by the Huntley group; Zellner and Huntley, 2019; Tully and Huntley, 2020), little is known about the role, if any, of *F. tularensis* biofilm formation in tick vectors.

Mosquito larvae have been shown to feed on both planktonic and biofilm cultures of *F. tularensis* in an aquatic laboratory setting (Mahajan et al., 2011; Backman et al., 2015). In these models, *F. tularensis* localized intracellularly within the mosquito, suggesting that bacterial persistence in this vector is not solely due to ingestion. In this context, the seasonality of tularemia in mammals could be indicative of an overwintering state within arthropod vectors; however, long-term exposure to *F. tularensis* biofilm lowered the overall fitness of mosquito larvae and fecundity of adults (Mahajan et al., 2011).

An important aspect to note is that invertebrate immunology remains largely unknown in these vectors, making it difficult to interpret if phase variation or biofilm is advantageous for survival within arthropods. Drosophila have been shown to detect LPS through C-type lectins and β-glucan recognition proteins (βGRPs; Kim et al., 2000; Xia et al., 2018). A careful examination of the role biofilm formation plays in arthropod vectors, if any, would greatly advance the understanding of both biofilm and LPS variation.

Protozoan hosts and aquatic survival

The presence of *F. tularensis* in aquatic systems places the bacterium in proximity of single-celled organisms, such as amoeba (Hopla, 1974; Abd et al., 2003), as well as an environment that provides the potential for multi-species biofilms. It is possible that both phase variation and biofilm could offer a competitive advantage. For instance, it has been shown that protozoan preying upon *Salmonella* display feeding preference when presented multiple serovars and can distinguish prey based solely upon the O-Ag (Wildschutte et al., 2004). Along these lines, phase variation may enable predator avoidance as the edibility of *F. tularensis* has previously been shown to differ depending on subspecies, even isolate (Thelaus et al., 2009). However, Abd et al. demonstrated that *F. tularensis* can utilize *Acanthamoeba castellanii* as a host, following an infection cycle that is similar to murine macrophages once bacteria were engulfed (Anthony et al., 1991; Abd et al., 2003; El-Etr et al., 2009). Further work is needed to determine if protozoans display preference for a particular *F. tularensis* O-Ag variant as well as the mechanisms enabling invasion.

Francisella tularensis has been found to remain viable as determined by CFU counts in conditions mimicking natural water for at least 3 weeks (Golovliov et al., 2021). Under these conditions, wild-type *F. tularensis* strains did not form biofilms; however, a *wbtI* mutant (O-Ag deficient) formed biofilms that were maintained throughout the study at both 20°C and 4°C (Golovliov et al., 2021). Differences in virulence were observed after 24 weeks at 4°C, as mice infected with Schu S4 (Type A) displayed no symptoms of disease, while FSC200 (Type B) remained virulent (Golovliov et al., 2021). Along these lines, LVS was found to persist in a viable, but non-culturable state (VBNC) for at least 140 days at 8°C in tap water, but at the cost of virulence as mice were symptomless when challenged with VBNC cells (Forsman et al., 2000). A connection between biofilm-forming variants and a VBNC state was found by Mlynek et al. (2021), but it is unclear how *F. tularensis* prioritizes the convergence of mechanisms that could impact environmental survival. External signals such as nutrient availability or pH have been found to factor into both phase variation and biofilm formation (Eigelsbach et al., 1951; Champion et al., 2019; Mlynek et al., 2021).

Additional considerations

Biofilm formation is considered a virulence determinant in many bacteria as it facilitates the establishment of chronic infections (Costerton et al., 1999; James et al., 2008). A contributing factor to the persistence of these infections is the ability of cells within the biofilm to withstand antibiotic treatment. In *F. novicida* and LVS, biofilms have been shown to decrease the susceptibility of embedded bacteria to ciprofloxacin (Siebert et al., 2019, 2020), a first-line drug for post-exposure prophylaxis (Dennis et al., 2001). However, it was determined that ciprofloxacin-exposed biofilms readily entered a VBNC state (Siebert et al., 2020), providing yet an additional link between biofilm formation and a VBNC phenotype. Interestingly, Chung and colleagues demonstrated that biofilms formed by a *F. novicida chiA* mutant can be re-sensitized to an antibiotic it was previously resistant to if the ECM was enzymatically degraded (Chung et al., 2014). These studies highlight the potential health implications to consider if *F. tularensis* biofilm formation plays a role in human pathogenesis.

Conclusion and future directions

Recent studies have demonstrated a link between phase variation and biofilm formation in *F. tularensis*. While the field appears to agree that both these phenotypes likely aid in environmental survival, many unanswered questions remain that would significantly advance our understanding of the mechanisms enabling survival and persistence, and even potentially help identify emerging threats in this genus of bacteria. Lastly, the focus of this review was on phase variation and biofilm formation; however, it is possible that multiple mechanisms and genetic pathways exist to control biofilm formation in *F. tularensis*. Understanding what role, if any, variation and biofilm plays in *F. tularensis* could allow for insights to develop better vaccines and therapeutics to prevent tularemia.

Author contributions

KM and JB contributed to the conception and writing of this manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Peptidoglycan enzymes of *Francisella*: Roles in cell morphology and pathogenesis, and potential as therapeutic targets

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Peptidoglycan, found within the cell wall of bacteria, is a structure critical for maintaining cell morphology and providing a protective barrier in diverse environments. Peptidoglycan is a remarkably dynamic structure that is constantly remodeled during cell growth and division by various peptidoglycan enzymes. Numerous peptidoglycan enzymes have been characterized from diverse bacteria and are highly sought after as targets for therapeutics. However, very little is known about these enzymes within the biothreat agent *Francisella tularensis*. As the causative agent of tularemia, *F. tularensis* is classified as a category A biothreat pathogen, in part due to its low infectious dose and lack of FDA-approved vaccine. Many bacterial species encode multiple peptidoglycan enzymes with redundant functions that allow for compensation if one of the enzymes are inactivated. In contrast, *F. tularensis* appears to lack this redundancy, indicating peptidoglycan enzymes may be completely essential for growth and could be exploited as targets for medical countermeasures. Indeed, several peptidoglycan enzymes in *F. tularensis* have been shown to play important roles in cell division, cell morphology, virulence, and modulation of host response. The aim of this review is to summarize findings from the current literature on peptidoglycan enzymes present in *Francisella* and discuss areas where future research efforts might be directed. We conclude that *Francisella* harbors a distinct set of peptidoglycan enzymes important for cell growth and virulence and represent potentially valuable targets for the development of novel therapeutics.

KEYWORDS

***Francisella*, peptidoglycan, tularemia, antibiotics, therapeutics, lytic transglycosylase, carboxypeptidase, penicillin-binding protein**

Introduction

The vast majority of bacteria contain a cell wall consisting of peptidoglycan (PG), a structure critical for maintaining the cell morphology and membrane integrity, and providing a protective barrier against osmotic pressure, antibacterial compounds, and other environmental stresses. PG is composed of alternating *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac) residues connected by β-(1–4) linkages. These glycan strands are further crosslinked by peptide chains, a feature which contributes to the cell wall stability and resistance to changes in osmotic pressure. In Gram-negative bacteria, these peptide chains are typically composed of L-Ala-γ-D-Glu-diaminopimelate (meso-DAP)-D-ala-D-ala, with the L-ala residue attaching the peptide chain to the C3 position of the Mur*N*ac residue (Vollmer et al., 2008; Sobhanifar et al., 2013). While these features are characteristic of most Gram-negative bacteria, some level of variation in the glycan strands or peptide chains exists across bacterial species (Schleifer and Kandler, 1972). Glycan strands are then further linked by short peptide chains to form a thin meshwork that surrounds the inner membrane. The number of peptidoglycan layers has been estimated to be between 1 and 3 in *Escherichia coli*, depending on the strain and growth conditions (Yao et al., 1999; Matias et al., 2003).

The bacterial cell wall is a dynamic structure that undergoes constant remodeling to accommodate cell division, response to environmental signals, and incorporation of membrane proteins. While the PG layer offers structural stability to the cell wall, it must also be flexible to accomplish these tasks. PG remodeling relies on the coordinated activities of a series of modifying enzymes, which target the glycan strands and the peptide cross-links. Extensive knowledge has been obtained from studying the array of PG remodeling proteins in *E. coli* and other bacteria. However, very few studies have investigated the PG remodeling enzymes in *Francisella* spp., and no characterization of the PG structure or composition in this organism has been performed. *Francisella tularensis*, the causative agent of tularemia, is a Gram-negative facultative intracellular bacterium. Within *F. tularensis*, there are two subspecies that cause disease in humans, subsp. *tularensis* and subsp. *holarctica*. A third subspecies, *F. novicida*, is commonly used as a laboratory surrogate for the fully virulent strains. *F. tularensis* poses a significant biothreat risk due its low infectious dose, ease of aerosolization, and lack of approved vaccine (McLendon et al., 2006). Moreover, the potential for antibiotic resistant strains makes finding novel antibiotics and therapeutics critical (Gestin et al., 2010; Sutera et al., 2014; Jaing et al., 2016; Biot et al., 2020). As a facultative intracellular pathogen, *F. tularensis* is able to replicate within multiple host cells, primarily macrophages, which is necessary for pathogenesis. Once taken up by a macrophage, *F. tularensis* resides within a phagosome. The phagosomal membrane is then disrupted, and the bacterium released to replicate freely within the cytosol. More detailed reviews on the intracellular life cycle of *F. tularensis* are available (Clemens and Horwitz, 2007; Chong and Celli, 2010; Santic et al., 2010). Exploring the PG enzymes of *Francisella* is an exciting area of study, given the importance of PG enzymes in cell division, virulence, antibiotic resistance, and their potential as therapeutic targets. The focus of this review is to summarize the current knowledge of PG enzymes in *Francisella* and highlight gaps in our understanding that require further investigation. We hope this work becomes part of the larger effort to reveal potential targets to combat tularemia.

Overview of peptidoglycan enzymes

The initial steps of PG biosynthesis occur in the cytosol *via* the Mur pathway that results in the formation of the lipid II PG precursor, consisting of a single disaccharide, pentapeptide unit attached to the lipid carrier (Egan et al., 2020). This precursor is then flipped across the inner membrane to allow for subsequent polymerization of the PG, which is carried out primarily by penicillin-binding proteins (PBPs). PBPs are a large group of enzymes broadly divided into two classes, high molecular mass (HMM) and low molecular mass (LMM) PBPs (Sauvage et al., 2008). The HMM PBPs are involved in the first steps of PG synthesis in the periplasm, catalyzing glycosyltransferase and transpeptidase reactions, involving transfer of the growing PG chain to the PG precursor and cross-linking of the glycan chains, respectively. The LMM PBPs are involved in cell separation and PG recycling (Sauvage et al., 2008). For the purpose of simplicity and therapeutic applications of the PG enzymes, this review will focus on the final steps of PG synthesis and recycling that occur in the periplasmic space.

The LMM PBPs consist of both carboxypeptidases, which cleave the terminal D-alanine from the stem pentapeptide creating a tetrapeptide, and endopeptidases, which cleave within the peptide crosslinks (Aguilera Rossi et al., 2016). Cell wall hydrolases, also referred to as autolysins, consist of three enzyme types, amidases, peptidases, and glycosidases, all of which are involved in cell wall remodeling during cell division. These enzymes are distinct from PBPs, though share overlapping functions (Vermassen et al., 2019). The amidases cleave the bond between the peptide stem and glycan chain, peptidases cleave the peptide stem, and glycosidases act upon the glycan chains. The lytic transglycosylases (LTs), structurally related to the glycosidases, are a unique family of enzymes that cleave the beta-glycosidic bond between the N-acetylglucosamine and N-acetylmuramic acid residues of the glycan strands, and catalyze the formation of 1,6-anhydro-N-acetyl-β-D-muramoyl residue (Dik et al., 2017). The schematic in Figure 1 shows where in the PG each type of enzyme cleaves, along with the known PG enzymes in *Francisella* spp. Currently, there are at least five carboxypeptidases and two lytic transglycosylases known to be present in *Francisella*, with LdcA harboring both carboxypeptidase and endopeptidase activity, and no known amidases or glucosaminidases reported in the literature. Known

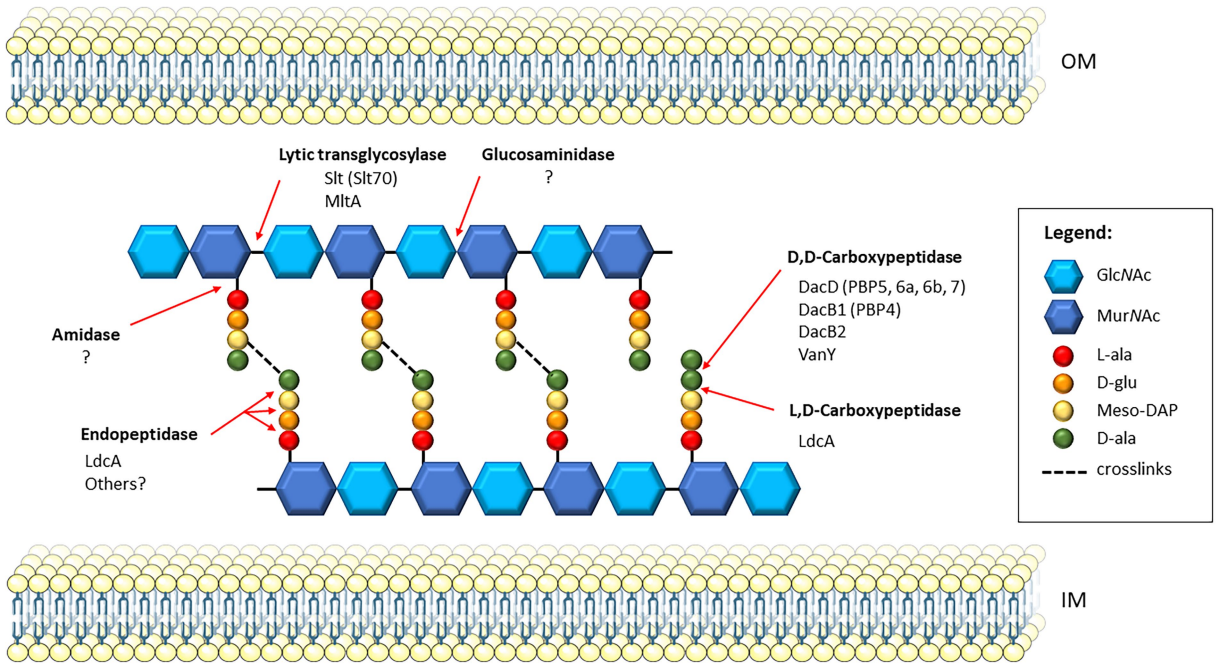

FIGURE 1
Schematic of PG and PG recycling enzymes in *Francisella*. A section of cross-linked PG is shown between the inner membrane (IM) and outer membrane (OM) of the bacterial cell. Since *Francisella* PG has not yet been characterized, this model shows the predicted PG composition based on that of *Escherichia coli*. The glycan strands composed of alternating disaccharide residues, *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac), are crosslinked *via* the stem peptide. The stem peptide is composed of L-Ala-γ-D-Glu-diaminopimelate (meso-DAP)-D-ala-D-ala. Transpeptidases catalyze the formation of crosslinks in a two-step process, first removing the terminal D-ala of the stem peptide, then linking the D-ala of the resulting tetrapeptide on donor strand to the meso-DAP residue on the acceptor strand. Red arrows indicate the cleavage sites of specific PG enzymes, and those known to be present in *Francisella*; the homologous enzymes in *E. coli*, if applicable, are indicated in parentheses. *Francisella* harbors two known lytic transglycosylases, Slt and MltA, which cleave the β-1,4 glycosidic bond between Mur*N*Ac and Glc*N*Ac residues. Currently, no glucosaminidases have been identified in *Francisella*, which also act upon glycan strands but cleave the non-reducing end of the Glc*N*Ac residues. Five D, D-carboxypeptidases are present in *Francisella*, including DacD, DacB1, DacB2, and VanY. The LdcA enzyme is an L,D-carboxypeptidase that also exhibits activity as an endopeptidase, though no others have been yet identified. Lastly, amidases, which cleave the bond between the Mur*N*ac residues and the stem peptide, have not been identified in *Francisella*.

and putative peptidoglycan enzymes and their locus tags in three representative *Francisella* strains are listed in Table 1. Both LT enzymes from *Francisella* and two of the carboxypeptidases, DacD and LdcA, have been investigated and will be discussed herein.

Carboxypeptidases

The D-alanyl-D-alanine carboxypeptidases, or DD-carboxypeptidases, are the most abundant PBPs within *E. coli*, though mutation of one or more genes does not cause lethality, owing to the highly redundant nature of these enzymes (Denome et al., 1999). The major DD-carboxypeptidase in *Francisella*, DacD, has been characterized in two separate studies. In the first study, *dacD* of the *F. holarctica* strain FSC200 was inactivated *via* insertional mutagenesis and shown to have a significant replication defect in murine bone-marrow derived macrophages (Spidlova et al., 2018). The *dacD* mutant was also attenuated in BALB/c mice after subcutaneous infection, with decreased spread to major organs in comparison to the wild-type strain. Transmission electron microscopy (TEM) revealed significantly increased cell size and breaks within the plasma membrane of the *dacD* mutant. The latter study generated an insertional *dacD* mutant in the fully virulent *F. tularensis* Schu S4 strain and showed a pH-dependent growth defect of the mutant relative to wild-type, that was restored by complementation and partially restored for growth at neutral pH (Kijek et al., 2019). Moreover, the *dacD* mutant had significant morphological defects and increased cell size as evidenced by scanning electron microscopy (SEM). Finally, during intranasal infection of BALB/c mice, the *dacD* mutant was significantly attenuated for virulence and protective against challenge with the parent Schu S4 strain, showing its potential as a live vaccine strain.

Recently, the L, D-carboxypeptidase, LdcA, of *F. tularensis* was described, which cleaves pentapeptide stems to tripeptide stems, as opposed to the D, D-carboxypeptidases which cleave the terminal D-alanine to form tetrapeptide stems (Zellner et al., 2021). Using a recombinant LdcA protein derived from the live vaccine strain (LVS), the authors demonstrated both L, D-carboxypeptidase and L, D-endopeptidase activities that were dependent on at least two residues of the Ser-Glu-His catalytic triad. Isogenic deletion of *ldcA* in LVS produced cells that were

TABLE 1 PG enzymes and corresponding locus tags in the three *Francisella* strains of interest.

Gene	*Francisella tularensis* Schu S4	*Francisella holarctica* live vaccine strain (LVS)	*Francisella novicida* U112	Protein product
ldcA[1]	FTT_0101	FTL_1678	FTN_1613	L,D-carboxypeptidase
dacD[2]	FTT_1029	FTL_1060	FTN_0907	D,D-carboxypeptidase
slt[2]	FTT_0400	FTL_0466	FTN_0496	Soluble lytic murein transglycosylase
mltA[2]	FTT_1271	FTL_1189	FTN_1286	Membrane-bound lytic murein transglycosylase A
dacB1[3]	FTT_1039	FTL_1046	FTN_0917	D,D-carboxypeptidase
dacB2[3]	FTT_0724c	FTL_1509–1,508	FTN_0635	D,D-carboxypeptidase
vanY[3]	FTT_0549	FTL_1004	FTN_0966	D,D-carboxypeptidase

[1]Enzymatic activity has been demonstrated (Zellner et al., 2021). [2]Described and characterized, discussed herein, but the specific enzymatic activity not shown. [3]Putative PG enzymes based on homology.

smaller and rounder than wild-type cells, with significantly thicker outer membranes and septum defects indicated by the presence of bacterial chains. The virulence of this mutant was completely attenuated during intranasal infection of C3H/HeN mice as compared to wild-type and complement strains, and further protected against challenge with the fully virulent Schu S4 strain.

Additional DD-carboxypeptidases, including DacB1 (FTL_1046), DacB2 (FTL_1509) and VanY (FTL_1004), have been annotated in *Francisella* spp. (Table 1), though no studies have yet been published on the characterization and confirmation of these putative enzymes. It is possible that additional PG enzymes are present in *Francisella* though not annotated; indeed, lack of consistent sequence annotation is a challenge when analyzing available genome data for *Francisella*. Homologs may be identified *via* BLAST search of known PG enzyme sequences, although *Francisella* harbors many hypothetical proteins and proteins which bear no homology to existing proteins, even if they exhibit similar functions. Interestingly, a previous study identified FTT_0924 as an essential gene in *F. tularensis*, encoding an unknown but highly conserved protein that was necessary for maintaining PG stability and replication in macrophages. This protein could represent one of the PG enzymes employed by *Francisella* spp., to maintain proper cell division (Brunton et al., 2015). Roughly 30% of the genes identified in *Francisella* are hypothetical proteins of unknown function (Titball and Petrosino, 2007).

Lytic transglycosylases

Francisella tularensis harbors only two known lytic transglycosylases, the soluble lytic transglycosylase (Slt), and the murein lytic transglycosylase A (MltA). The role of the Slt enzyme in cell morphology and virulence was previously examined using a transposon mutant of *F. novicida* (Bachert et al., 2019). The *slt* mutant showed significant growth defects and loss of viability during growth in low pH conditions. SEM further showed morphological defects including aberrant cell shapes and sizes, and extensive clumping and fusion of cells which were restored *via* complementation. The *F. novicida slt* mutant was also significantly attenuated during intranasal infection of BALB/c mice, with limited dissemination to major organs, and showed decreased replication in J774 murine macrophage-like cells (Bachert et al., 2019). The *slt* gene was also identified in a cytotoxicity study, in which a transposon mutant of *slt* was found to be less cytotoxic to THP-1 human monocytes than the parental *F. novicida* strain (Nakamura et al., 2019). The authors further constructed an in-frame deletion of *slt* in *F. novicida* and showed reduced growth of the mutant in both THP-1 cells and J774 cells. Although the mutant was still able to escape the phagosome, mutant bacteria were degraded by autophagy during the later stages of infection. Furthermore, the mutant elicited high levels of pro-inflammatory cytokines TNF-α, IL-6, and IL-1β from the cells, indicating a role for Slt in immune suppression (Nakamura et al., 2019).

More recently, Nakamura et al. (2020) identified the related MltA enzyme as necessary for the survival of *F. novicida* in silkworms. Virulence in the silkworm model was significantly attenuated using an in-frame deletion mutant of *mltA* and restored by complementation. MltA was shown to suppress immune responses in this model as evidenced by increased induction of cecropin B expression in the *mltA* mutant. Lastly, *F. novicida mltA* failed to replicate in THP-1 cells and was less cytotoxic as assessed by LDH release (Nakamura et al., 2020).

Discussion

Lack of peptidoglycan enzymes redundancy in *Francisella*

Peptidoglycan modifying enzymes are reported to be highly redundant in a variety of bacteria. For example, *E. coli* harbors at least 35 PG hydrolases, including 7 carboxypeptidases and 8 lytic transglycosylases (van Heijenoort, 2011). *Pseudomonas aeruginosa* expresses 11 LTs (Lee et al., 2017). However, *Francisella* species appear to lack the redundancy of PG enzymes observed in many bacteria. A previous search for *E. coli* PBP

homologs revealed only three PBPs in *F. tularensis*, in contrast to 12 PBPs in *E. coli*. The *F. tularensis* homologs are DacD, FtsI, and DacB1 with DacD representing the major DD-carboxypeptidase homologous to PBP5, 6a, 6b, and 7 from *E. coli* (Kijek et al., 2019). Furthermore, *F. tularensis* has been found to harbor only two LT enzymes, MltA (Nakamura et al., 2020) and Slt (Bachert et al., 2019; Nakamura et al., 2019). Moreover, *dacD*, *dacB1*, *ftsI*, *slt*, and *mltA* have all been identified as essential for *in vitro* growth of *F. tularensis* using a transposon library (Ireland et al., 2019). This finding does not rule out potential polar effects of a transposon insertion. However, multiple attempts by our laboratory to generate in-frame deletions of *slt* in LVS and the Schu S4 strain of *F. tularensis* were unsuccessful and suggest *slt* is essential for *F. tularensis* (Bachert et al., 2019). Such essential genes would be ideal targets for medical countermeasures against tularemia. The lack of gene redundancy observed is not unique to *F. tularensis*. It has been shown that host-evolved bacteria with reduced genomes, including *Mycobacterium leprae* and *Rickettsia prowazekii*, lost their genetic redundancy in favor of maintaining diverse protein families, in contrast to free living bacteria (Mendonça et al., 2011). Conversely, for pathogens with large genomes and frequent gene duplication, including duplication of PG enzymes, the effectiveness of potential PG enzymes inhibitors could be limited.

Impact of peptidoglycan enzymes on *Francisella* cell morphology

The L, D- and D, D-carboxypeptidases of *Francisella* appear to have important but very distinct roles in cell morphology, although they both act upon the peptide cross-links of the cell wall. For instance, inactivation of *dacD* resulted in a pH-dependent growth defect, but the *ldcA* mutant showed no replication defect *in vitro* (Kijek et al., 2019; Zellner et al., 2021). Moreover, some of the morphological defects observed for LVS *ldcA* were opposite those of the *dacD* mutant, including smaller rather than larger cell size and a thicker outer membrane compared to a discontinuous membrane (Spidlova et al., 2018; Zellner et al., 2021). These mutations have complex effects on the bacterial cell surface and its sensitivity to various stressors. For instance, *ldcA* mutant had increased sensitivity to cell-wall targeted antibiotics, such as vancomycin and ampicillin, but increased resistance to cytosol-targeted antibiotics such as gentamicin and ciprofloxacin (Zellner et al., 2021). Notably, increased sensitivity to sodium dodecyl sulfate was observed for both *dacD* and *ldcA* mutants (Spidlova et al., 2018; Kijek et al., 2019; Zellner et al., 2021). Interestingly, the *ldcA* mutant was reported to form chains of cells for ~10% of the bacteria (Zellner et al., 2021). We saw a similar phenotype with the *slt* mutant, although with clumping of cells rather than chains; Slt also acts upon the glycan strands rather than the cross-links (Bachert et al., 2019).

The overall phenotypes of *slt* and *dacD* mutants were highly similar, both showing increased cell size and altered membrane structure. Both mutants also exhibited pH-dependent phenotypes, with membrane defects apparent in low pH conditions that were partially restored by growth in neutral pH conditions (Bachert et al., 2019; Kijek et al., 2019). This pH-dependency could point to a role for Slt and DacD enzymes in maintaining bacterial cell morphology in the intracellular environment of the macrophage during host infection. The *slt* and *dacD* mutants were characterized using SEM, which allowed for the visualization of surface structures and observation of ruffled surface appearance, while the *ldcA* mutant was characterized using TEM; SEM analysis of the *ldcA* mutant could potentially reveal similar defects. It is also worth noting that mutation of the genes encoding for these enzymes may have different effects depending on the strain or subspecies. For example, mutation of *dacD* in the *F. tularensis* Schu S4 strain resulted in cells roughly two times larger than wild-type (Kijek et al., 2019), whereas mutation of *dacD* in the *F. holarctica* FSC200 strain resulted in cells 10 times larger than wild-type (Spidlova et al., 2018). No morphological defects have yet been reported for *mltA* mutants of *Francisella*, although the attenuated growth and virulence of *F. novicida mltA* would suggest such a phenotype.

Roles of peptidoglycan enzymes in immunomodulation

Interestingly, both Slt and MltA were implicated in immunomodulation, although discovered in different models of infection, i.e., THP-1 cells for Slt and silkworm for MltA (Nakamura et al., 2019, 2020). It is possible that aberrations in the cell wall resulting from mutation of the genes encoding for the LT enzymes alters the recognition of the PG by the TLR2 receptor. It would be interesting to test *Francisella mltA* and *slt* mutants in parallel using a mouse model and obtaining cytokine profiles of the host challenged with the mutants compared to wild-type infected mice. Additionally, since the LT enzymes have been associated with assembly of the type VI secretion system and incorporation into the cell envelope of *E. coli* (Santin and Cascales, 2017), the immunosuppressive effects observed with some of these mutants may be due in part to alterations in the type VI secretion system. However, the contribution of these enzymes to type VI secretion system assembly or stability in *Francisella* spp., are currently unknown.

Modulation of the immune response by PG fragments released during cell wall recycling of bacteria has been reported in the literature. This phenomenon is especially well-characterized in *Neisseria gonorrhoeae* and *Neisseria meningitidis*, where toxic PG fragments, released from the activity of LTs during host infection, contribute to inflammation and cytotoxicity (Chan and Dillard, 2017). This is in contrast to the LTs of *Francisella spp.*, which have been shown to dampen immune responses as described above. In general, *Francisella* is a stealth pathogen, replicating within macrophages without triggering host defenses or causing overt damage (Sjöstedt, 2006). The modified LPS structure expressed by *Francisella* is known to bypass typical TLR4 recognition, contributing to its ability to replicate unseen within

the host (Okan and Kasper, 2013). However, the structure of the PG within *Francisella* has not yet been characterized and may harbor modifications that allow the bacterium to suppress or evade host immune responses.

NOD-like receptors within host cells are known to recognize the presence of intracellular PG in host cells and trigger the inflammasome, leading to caspase-1 activation and the production of IL-1B and IL-18 leading to cell death. Similar to other intracellular pathogens, *Francisella* has been shown to activate the inflammasome and caspase-1 responses (Weiss et al., 2007; Broz and Monack, 2011). However, the mechanisms described rely on AIM2 sensing of dsDNA and NLRP3 sensing of LPS (Man et al., 2015; Mitra et al., 2018). Whether *Francisella* PG within host cells triggers a similar immune response is not currently known.

Implications in targeting

Targeting the lytic transglycosylases, specifically in combination with beta-lactam antibiotics, may be useful for the design of novel therapeutics. Bulgecin A, an O-sulfonated glycopeptide, is an LT enzyme inhibitor that was first demonstrated to be active against the Slt70 enzyme of *E. coli*, and the Slt70-Bulgecin A complex was crystallized to determine the structure (Thunnissen et al., 1995). While Bulgecin A harbors no antibiotic activity itself, it works synergistically with beta-lactams to increase their efficacy. The combination treatment of Bulgecin A and beta-lactams has been demonstrated effective against multiple pathogens, including *E. coli*, *P. aeruginosa*, *Helicobacter pylori*, and *Acinetobacter baumannii* (Templin et al., 1992; Thunnissen et al., 1995; Bonis et al., 2012; Skalweit and Li, 2016). Bulgecin A has not yet been tested against Francisella, either alone or in combination with beta-lactams. Bulgecin A harbors great potential for improving efficacy of existing drugs when used as a combinational therapy.

Conclusion

Peptidoglycan enzymes have long been considered as potential targets for antibiotics, given that it is unique to bacteria and the active sites of these enzymes have been well characterized in common pathogens, such as *E. coli*. While very little is known about how PG remodeling occurs in *Francisella* and the specific enzymes involved, the few studies discussed above highlight a significant role of PG enzymes in the survival and virulence of *Francisella*. Importantly, the LT enzymes are essential for survival in fully virulent *F. tularensis* and when mutated in *F. novicida*, have a significant impact on cell morphology and virulence. This is in contrast to *E. coli* and other species, in which mutation of multiple PG enzymes result in no detected phenotype. These observations suggest antibiotics targeting the essential LT enzymes of *Francisella* may be especially potent against tularemia. Moreover, the threat of antibiotic resistance heightens the biothreat concern of *Francisella*, making the development of novel antibiotics and inhibitors essential. The active sites for LTs and carboxypeptidases seem to be conserved between *Francisella* and *E. coli*, so existing antibiotics may be applied or redesigned for use against *Francisella*. Combination therapies targeting both LT and PBP enzymes are currently being evaluated for other pathogens, such as *P. aeruginosa*, and could prove to be effective against *F. tularensis* (Dik et al., 2019). Characterization of the PG enzymes in *Francisella* and their functions is an underdeveloped (but very promising) area of study. Research efforts in this field will likely uncover strategies to develop more effective antibiotics and inhibitors against tularemia.

Author contributions

BB and JB contributed to the conceptualization, writing, and editing. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Mechanisms of host manipulation by *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae (also known as gonococcus) has been causing gonorrhoea in humans since ancient Egyptian times. Today, global gonorrhoea infections are rising at an alarming rate, in concert with an increasing number of antimicrobial-resistant strains. The gonococcus has concurrently evolved several intricate mechanisms that promote pathogenesis by evading both host immunity and defeating common therapeutic interventions. Central to these adaptations is the ability of the gonococcus to manipulate various host microenvironments upon infection. For example, the gonococcus can survive within neutrophils through direct regulation of both the oxidative burst response and maturation of the phagosome; a concerning trait given the important role neutrophils have in defending against invading pathogens. Hence, a detailed understanding of how *N. gonorrhoeae* exploits the human host to establish and maintain infection is crucial for combating this pathogen. This review summarizes the mechanisms behind host manipulation, with a central focus on the exploitation of host epithelial cell signaling to promote colonization and invasion of the epithelial lining, the modulation of the host immune response to evade both innate and adaptive defenses, and the manipulation of host cell death pathways to both assist colonization and combat antimicrobial activities of innate immune cells. Collectively, these pathways act in concert to enable *N. gonorrhoeae* to colonize and invade a wide array of host tissues, both establishing and disseminating gonococcal infection.

KEYWORDS

gonorrhoea (*Neisseria gonorrhoeae*), apoptosis, cell signaling and infection, gonorrhoea pathogenesis, immunology and infectious disease

1. Introduction

Gonorrhoea is one of the most ancient human diseases, with written accounts dating back as far as 1550 BC (Nickel, 2005). This sexually transmitted infection has since propagated into a global public health crisis today. Strains of the causative agent, *Neisseria gonorrhoeae* (also known as gonococcus), have acquired resistance to every antibiotic used to effectively treat the disease since the late 1930s (Unemo and Shafer, 2011); and in 2018, the first treatment failure of front-line antibiotics was reported in the United Kingdom (Eyre et al., 2018). Alongside this threat of antibiotic resistance, is the continued rise in global case numbers (Kirkcaldy et al., 2019; Unemo et al., 2019; Whelan et al., 2021) and the lack of an effective vaccine against gonococcal infection (McIntosh, 2020). Together, this highlights the pressing need for gonococcal-focused research to prevent the emergence of untreatable gonorrhoea in the near future.

N. gonorrhoeae is an obligate human pathogen, and predominantly infects the mucosal epithelium of the human urogenital tract, but is also capable of infecting other exposed mucosal sites, such as the rectum, pharynx, and conjunctiva (Quillin and Seifert, 2018). Gonococcal infection can present in both women and men as symptomatic or asymptomatic, with higher rates of

asymptomatic infection reported in women (Unemo et al., 2019); although, asymptomatic infection rates in men have been estimated to be as high as 40% (Martín-Sánchez et al., 2020). The significant proportion of asymptomatic infections caused by the gonococcus, generally leaves the individual unaware of the infection they harbor, increasing the chances of both transmission and dissemination. Symptomatic infection usually presents as urethritis in males and as ectocervicitis or endocervicitis in females. Although disseminated infection to other anatomic locations occurs only in a minority of cases, extensive localized infection can cause severe complications, including pelvic inflammatory disease, infertility, ectopic pregnancy, or neonatal blindness resulting from transmission during birth. Moreover, gonococcal infection also increases the chances of the individual contracting additional sexually transmitted infections, such as HIV (Masi and Eisenstein, 1981; Sandström, 1987; Little, 2006; Quillin and Seifert, 2018). There is little information regarding the outcome of asymptomatic infections as individuals do not seek treatment. However, anecdotal evidence suggests asymptomatic infection generally self-resolves within several months, although, in extreme cases the infection may persist for many years (Russell and Hook, 2009). This parallels the observation that symptomatic infections also self-resolve in cases of treatment failures or in the absence of treatment, although the infection is thought to persist for at least 14 days (Liu et al., 2014; Lovett and Duncan, 2019).

Given the wide array of host mucosa that *N. gonorrhoeae* can infect, it is not surprising that the bacterium possesses an array of surface molecules to allow entry and invasion of these various types of tissue (Quillin and Seifert, 2018). In addition, the gonococcus possesses an extraordinary ability to undergo both phase and antigenic variation of these primary surface antigens with extremely high turn-over rates (Virji, 2009). In part, this feature of the gonococcus explains why this bacterium has remained such a prominent human pathogen as it is exceedingly difficult for the human immune system to develop immunological memory. *N. gonorrhoeae* expresses three primary outer membrane proteins, the porin ion channel protein (PorB), the colony opacity-associated (Opa) proteins, and the reduction modifiable protein (Rmp); in addition to two key pathogen-associated molecular patterns (PAMPs), lipooligosaccharides (LOS) and the type IV pili (Edwards and Apicella, 2004). The majority of these virulence factors have the ability to undergo phase and/or antigenic variation (Stern et al., 1986; Burch et al., 1997; Criss et al., 2005), with the exception of Rmp (Judd, 1982) and the abundant outer membrane protein, PorB, where all gonococcal strains express one of the PorB isotypes – $PorB_{IA}$ or $PorB_{IB}$ (Derrick et al., 1999). Despite the antigenic-stable nature of PorB within a given strain, allelic variation between gonococcal strains is common among the PorB isotypes, and occurs in the surface-exposed loops of the porin molecules (Fudyk et al., 1999; Garvin et al., 2008). In terms of antigenic variation, a single strain of *N. gonorrhoeae* can encode up to 11 distinct *opa* genes, allowing for the constitutive expression of several distinct Opa proteins (Stern and Meyer, 1987; Bhat et al., 1991). Furthermore, a pentameric pyrimidine motif (CTCTT) is located in the 5′ region of all gonococcal *opa* loci, with varying numbers of the repeating unit per locus. This motif is susceptible to slip-strand misrepair, causing either the deletion or insertion of this repeating unit, potentially leading to out-of-frame transcripts and subsequently no Opa expression (Stern et al., 1986). Antigenic variation of the type IV pili system stems from homologous recombination of the single *pilE* locus, with portions of the 19 promoterless (or "silent") variations of this gene (the *pilS* loci), which lie upstream of *pilE* (Hamrick et al., 2001; Sechman et al., 2005). Variation of the LOS molecules occurs indirectly through phase variation of the cytosolic glycosyltransferases, which enzymatically determine the glycan profile of the LOS molecules (Danaher et al., 1995; Burch et al., 1997).

In addition to constant surface variation, an additional key trait underpinning the success of the gonococcus is its ability to manipulate host cell signaling events in favor of its pathogenesis. Again, an unsurprising feat given the longevity of the relationship this bacterium has had with its human host. Inevitably, the primary mechanisms by which the gonococcus exerts this manipulation is through its key outer membrane antigens. Obtaining both a detailed and holistic understanding of these intricately evolved host manipulation events will greatly advance our fight against this high priority pathogen.

Here, we summarize the literature surrounding the host epithelial signaling pathways the gonococcus takes advantage of to both colonize and penetrate the epithelial lining of the urogenital tract to establish infection. Following this, we highlight the primary mechanisms used by the gonococcus to subsequently alter the host immune and cell death responses in a manner which favors pathogenesis. Notably, there appears to be a general consensus throughout the research community that both up- and down-regulation of the host immune and cell death pathways can promote gonococcal infection, likely dependent on the anatomical site and time-period of the infection. While the underlying detail behind these mechanisms remains largely uncharacterized, the induction of anti-inflammatory immune states and the establishment of intracellular niches by preventing cell death are ultimately essential for facilitating transmission. In contrast, an upregulation of proinflammatory responses during infection may allow the gonococcus to overcome epithelial barriers through tissue damage, gaining access to nutrients and deeper tissue, thus enabling further dissemination of the pathogen.

2. Exploitation of host epithelial cell signaling pathways to establish infection

Gonococci establish infection at the mucosal epithelia of the human genital tract using three interrelated events: colonization of the epithelia, invasion of epithelial cells, and trafficking into the subepithelial tissue. The mechanisms for each of these events differ between males and females, and within females at different anatomic locations. However, all involve close interactions with host cells and alteration of host cell signaling pathways, generally leading to decreased epithelial cell exfoliation to promote colonization or invasion into the epithelial layer.

2.1. Gonococcal interactions with male urethral epithelial cells

Gonococcal infection in men most often occurs as acute urethritis resulting from the inflammatory response to infecting gonococci (Ramsey et al., 1995). Generally, the hallmark of symptomatic gonorrhoea in men is the presence of a purulent discharge, associated with the influx of neutrophils and the shedding of urethral epithelial cells. Human studies have identified an incubation period of up to 40 h extending from the moment of infection to the onset of clinical symptoms, where gonococci cannot be cultured from the male urethra (Schneider et al., 1995). This suggests that the gonococci enter the protective environment of epithelial cells early in disease, enabling both

survival and replication (Edwards and Apicella, 2004). While pili mediate initial cell-to-cell contact events essential for colonization, and Opa proteins promote gonococcal adherence, the concurrent binding of both pili and Opa proteins further promotes colonization and infection. The exact mechanism and signaling pathways affected upon binding to male urethral epithelial cells have only partially been deciphered. Although pili expression is a known requirement for invasion, the receptor which the pili engages with during male infection has not been identified *in vivo*. Known pili receptors include the complement receptors, CD46 and CR3, and other I-domain-containing integrins, such as the α1β1 and α2β1 integrins. CR3 expression is absent within the male urogenital epithelium, leaving CD46 and other I-domain-containing integrins as likely candidates. Although binding of pili to the complement regulatory protein, CD46, initiated infection in various cell types *in vitro* (Källström et al., 1997; Gill et al., 2003; Gill and Atkinson, 2004), and CD46 expression has been demonstrated in female genital tissue (Edwards et al., 2002), to date no pilus-CD46 interaction has been shown *in vivo* in male exudates (Edwards and Apicella, 2005). In support of this, the first interactions of the gonococcal pili with male urethral epithelial cells *in vitro*, demonstrated an early preference for binding the I-domain-containing integrins, α1β1 and α2β1, over CD46 to initiate gonococcal adherence and colonization (Edwards and Apicella, 2005). Gonococcal invasion of male urethral epithelial cells is facilitated by interactions of the asialoglycoprotein receptor (ASGP-R) with LOS on the gonococcal cell membrane, resulting in the formation of an actin pedestal underneath the bacterium (Apicella et al., 1996; Harvey et al., 1997). Following pedestal formation, ASGP-R-mediated endocytosis of the bacterium ensues, in both an actin- (Giardina et al., 1998) and clathrin-dependent (Harvey et al., 1997) manner, subsequently mediating cytoskeletal rearrangements to further promote gonococcal invasion and transcytosis.

2.2. Gonococcal interactions with epithelial cells of the female reproductive tract

In contrast to the male urogenital tract, the female reproductive tract presents a variety of epithelial cell types, from the vagina and ectocervix of the lower reproductive tract, to the endocervix, uterus, and fallopian tubes of the upper reproductive tract. The gonococci manipulate different host pathways by binding to differentially expressed receptors on the surface of different cervical epithelial cells to promote either colonization or cell invasion. Cells in the female reproductive tract transition from non-polarized squamous epithelial cells in the ectocervix, to single-layer, polarized columnar cells in the endocervix, with the area of cellular transition known as the transition zone (Prendiville and Sankaranarayanan, 2017). The monolayer epithelium of the endocervix differs in that it is held together by apical junctions compared to adherent junctions in the squamous epithelium of the ectocervix, which act to prevent pathogen entry and maintain polarity of the endocervical layer. Clinical observations and microscopy studies by Harkness in the 1940s demonstrated that during natural infection, *N. gonorrhoeae* was incapable of invading the ectocervix, but colonization of the endocervix led to transmigration into the subepithelium or lymphatic vessels (Harkness, 1948). Transmigration into the subepithelium has also been shown to occur within the transition zone (Yu et al., 2019). Furthermore, recent research using a human cervical tissue explant model demonstrated that expression of gonococcal Opa proteins promotes colonization in the ectocervical region of the female genital tract by preventing epithelial cell exfoliation. Early studies using organ cultures of human fallopian tubes demonstrated piliated gonococci result in sloughing of ciliated epithelial cells compared to non-piliated gonococci, highlighting the importance of pili in infection (McGee et al., 1981). This damage was not observed for non-ciliated epithelial cells, with gonococci attaching to these cells, leading to cell invasion and trafficking to the subepithelial tissue (see (Lenz and Dillard, 2018) for a review on *N. gonorrhoeae* pathogenesis of the human fallopian tubes). Similar to that within the male urethral epithelium, binding of pili, Opa, and PorB also activates host cell signaling pathways to promote colonization and infection within the female reproductive tract.

2.2.1. Binding of the type IV pilus activates host epithelial cell signaling pathways to promote colonization and infection of the female reproductive tract

Attachment of the gonococcus to female epithelial cells is initiated by the type IV pilus. The pilus binds to the complement receptors, CD46 and CR3, and I-domain containing integrin receptors. CR3 is an I-domain containing receptor, where pilus binding activates the CR3 I-domain, leading to membrane ruffling, subsequently mediating cell invasion (Edwards et al., 2001). Together with membrane ruffling, this I-domain activation of CR3 modulates additional downstream signaling events to further facilitate infection of endocervical cells which have high expression of the CR3 receptor (Jennings et al., 2011). Binding of the pilus to CD46 does not occur at the early stages of infection, but CD46 clustering and colocalization with the gonococcus is observed at later time points in infection (>6 h; Källström et al., 1997; Gill et al., 2003; Gill and Atkinson, 2004). Binding to CD46 triggers phosphorylation of tyrosines on the CD46 cytosolic tail (Lee et al., 2002), and the subsequent release of intracellular Ca^{2+} (Ayala et al., 2005). Calcium release promotes intracellular survival by redirecting the core lysosomal membrane protein, Lamp1, to the plasma membrane where it is cleaved by the immunoglobulin A1 (IgA1) protease secreted by adherent gonococci, reducing the number of lysosomes in infected cells (Källström et al., 1997; Lin et al., 1997; Hopper et al., 2000; Ayala et al., 2001). To further promote infection, binding of pili to epithelial cells results in remodeling of the cytoskeleton and modulation of phagocytosis by activation of the phosphoinositide-3 (PI-3) kinase/Akt signaling pathway (Lee et al., 2005). Activation of this cascade triggers the epithelial cell to produce the lipid secondary messenger, [PI(3,4,5) P3], which activates Akt and the PI-3 kinase to be translocated to the outer face of the infected cell membrane. The subsequent close proximity of these kinases to the attached microcolony has been postulated to enable the gonococcus access to the lipid secondary messenger, which is known to affect bacterial motility and behavior, thereby enhancing colonization and possibly host cell invasion (Lee et al., 2005). In addition, the force generated from pilus retraction on host cells activates expression of infection-regulated genes known to be expressed upon mechanical stress, through activation of mitogen-activated protein kinase (MAPK), which influences the ability of the cell to survive apoptosis and is discussed later in this review (Howie et al., 2005). Retraction of the pilus upon binding to epithelial cells also plays an important role in the activation of NF-κB, a global transcription factor that controls transcription, cytokine production, and cell survival. The microcolony is critical for NF-κB activation during gonococcal infection, presumably by generating an increased retraction force on the epithelial cell membrane leading to increased NF-κB activation (Dietrich

et al., 2011). Through regulation of this global transcription factor, gonococcal microcolonies have the ability to regulate a range of host cell processes, including apoptosis and the innate immune response, influencing how infection proceeds.

2.2.2. Opa proteins interact with host cells to promote gonococcal pathogenesis by manipulation of cell signaling pathways

Pilus retraction by the gonococcus triggers a tight association between Opa outer membrane proteins on the gonococcus and host cell receptors (Merz et al., 1996, 2000). The importance of Opa proteins in infection is highlighted by the findings that Opa expression increases gonococcal fitness in the female reproductive tract of mice (Cole et al., 2010), and the presence of Opa antibodies in the blood of infected women correlates with a decreased risk of gonococcal salpingitis (Plummer et al., 1994). Additionally, *N. gonorrhoeae* isolates from naturally infected men and women are Opa^{+}, as are isolates obtained post-infection of men with Opa^{-} colonies (James and Swanson, 1978; Jerse et al., 1994; Isbey et al., 1997). The majority of Opa proteins bind the carcinoembryonic antigen cell adhesion molecules (CEACAMs; Gray-Owen et al., 1997a; Sadarangani et al., 2011; Virji et al., 1996), however, the Opa50 isoform is also able to bind heparin sulfate proteoglycan (HSPG) receptors (van Putten and Paul, 1995; Freissler et al., 2000). Although, expression of the Opa50 variant has no significant effect on infection with any cervical cell types in tissue explant models (Yu et al., 2019). This could be due to HSPG receptors not being exposed on the apical surface of some epithelial tissues, therefore Opa interactions with HSPG do not mediate gonococcal attachment to the apical membrane (Lin et al., 1997), given HSPG binding does allow effective gonococcal uptake into non-polarized epithelial cells (Chen et al., 1995; van Putten and Paul, 1995; Freissler et al., 2000). The interaction of Opa proteins with HSPG also activates phosphatidylcholine-specific phospholipase C (PLC) and the acid sphingomyelinase, resulting in the release of diacylglycerol and ceramide, initiating signaling pathways to promote gonococcal invasion of epithelial cells (Grassmé et al., 1997).

Four different CEACAM receptors (CEACAM1, CEACAM3, CEACAM5, and CEACAM6) on various cell types can independently associate with gonococcal Opa proteins (for a review of gonococcal Opa proteins see Sadarangani et al., 2011). CEACAM1, CEACAM5, and CEACAM6 are expressed on the apical membrane of various epithelial cells, whereas CEACAM3 is expressed exclusively by neutrophils. CEACAMs are differentially recognized by Opa variants, and as such the expression and distribution of CEACAM receptors can influence gonococcal interactions with host cells. For example, epithelial cells of the female reproductive tract differentially express CEACAMs, with high expression in ecto- and endo-cervical cells and low expression in cells found in the transition zone. To date, there has been no study investigating CEACAM expression within epithelial cells of the male urethra, but a mouse model expressing human CEACAMs shows expression of CEACAM5 on the male urethral cell surface (Chan and Stanners, 2004).

Exfoliation of infected epithelial cells is often a first line defense mechanism of the innate immune system to prevent bacterial colonization. The interactions between CEACAM and Opa promote colonization of ecto- and endo-cervical cells by suppressing the exfoliation of mucosal cells (Figure 1). A high level of CEACAM expression in the lumen of the ectocervix drives strong colonization of Opa-expressing gonococci (Yu et al., 2019). Downstream CEACAM signaling increases integrin-β1 activity (Muenzner et al., 2010; Yu et al., 2019), which decreases epithelial cell shedding, promoting colonization, while also inhibiting β-catenin phosphorylation to stabilize cell-to-cell junctions (Figure 1) leading to reduced cell layer penetration (Yu et al., 2019). Binding of gonococci to CEACAM receptors also triggers the *de novo* expression of CD105, a member of the transforming growth factor-β1 receptor family, to promote integrin activity and enhance binding to extracellular matrix proteins leading to decreased exfoliation of epithelial cells, increasing gonococcal colonization (Muenzner et al., 2005, 2010; Figure 1).

It is well established that gonococcus binding to CEACAMs modulates cell signaling (Huber et al., 1999; Poy et al., 2002; Nagaishi et al., 2006; Leung et al., 2008). But how CEACAM clustering and binding at the cell membrane leads to altered gene expression was unknown until a recent study by Muenzner and Hauck (2020) identified an unexpected role for the soluble, diffusible gas nitric oxide (NO) in CD105 expression (Muenzner and Hauck, 2020). NO is produced by gonococci during anaerobic metabolism, and this membrane permeable gas initiates a conserved eukaryotic cell signaling pathway involving soluble guanylate cyclase (sGC), protein kinase G, and the transcription factor, CREB, to upregulate expression of CD105, causing increased cell-matrix adhesion and decreased epithelial cell exfoliation (Figure 1; Muenzner and Hauck, 2020). CEACAM clustering or CEACAM engagement by metabolically inactive bacteria does not lead to the downstream expression of CD105, highlighting the importance of metabolically active and anaerobic pre-conditioning of the gonococci to produce NO. The close interaction between gonococci and epithelial cells resulting from Opa-CEACAM interactions is a requirement to allow sufficient amounts of NO to reach the host cell cytoplasm, as although NO can transverse freely it can only act across short distances. The Opa-CEACAM interaction leads to a tight embedding of the bacteria into the host membrane or endocytic uptake into the host cell (Wang et al., 1998; Schmitter et al., 2007), allowing bacterial produced NO to reach the host cell cytoplasm in sufficient quantities to initiate NO-based signaling pathways. The production of NO in gonococci is dependent on the nitrate reductase protein, AniA, which is used in the switch to anaerobic respiration. AniA is one of the major proteins expressed under low oxygen conditions, such as those in the female genital tract. An interesting aside as to the importance of AniA indirectly leading to decreased epithelial cell exfoliation is the emergence of a novel group of *Neisseria meningitidis* that are capable of colonizing the urogenital tract and can transmit by sexual contact (Bazan et al., 2016). This ability to colonize the urogenital tract has been accompanied by gene loss, but also by the acquisition of a large gonococcal genomic fragment containing the AniA gene (Tzeng et al., 2017).

Low CEACAM expression in cells of the transition zone and endocervix abolish the effects of Opa binding, enabling gonococci to penetrate host cells regardless of which Opa variant they express. In a 2019 study utilizing female reproductive tissue explants, binding of Opa50 to HSPG receptors was found to induce phosphorylation of the cell junction associated protein, β-catenin, and reduce integrin expression to promote gonococci penetration of the epithelial layer (Figure 1; Yu et al., 2019). β-catenin is an adapter protein for the apical junction protein E-cadherin, and upon phosphorylation, induces the disassembly of this apical junction. Phosphorylated β-catenin is subsequently redistributed from the apical junction to the cytoplasm and gonococcal adherence sites to facilitate internalization (Edwards et al., 2013), while E-cadherin is also redistributed to the cytoplasm (Rodríguez-Tirado et al., 2012). These cell junction protein

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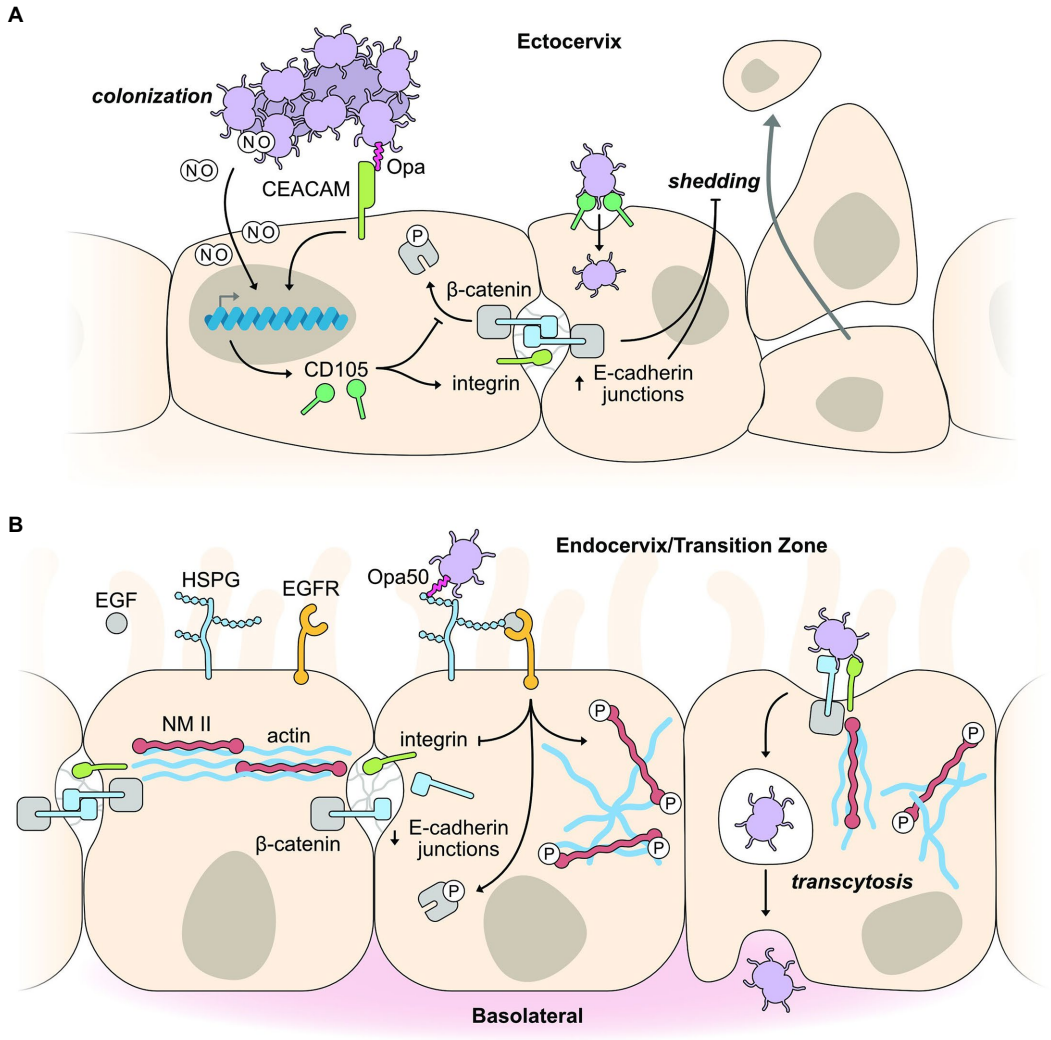

FIGURE 1
Exploitation of host epithelial cell signaling pathways for colonization and infection. **(A)** Binding of Opa-expressing gonococci to CEACAM receptors on squamous epithelial cells of the ectocervix promotes colonization by inhibiting epithelial cell shedding. Opa binding by CEACAM receptors triggers changes in host gene expression, including the expression of CD105, which activates integrin and decreases phosphorylation of the cell junction protein, β-catenin. Collectively, this strengthens and maintains the E-cadherin junction between epithelial cells leading to decreased epithelial cell shedding in response to gonococcal infection. Opa-CEACAM interactions result in a tight association between the gonococcus and host cell, allowing nitric oxide (NO) produced by the gonococci (under anaerobic respiration) to diffuse into the host cell, activating expression of genes, such as CD105, to decrease cell shedding as outlined above. **(B)** Binding of gonococci to endocervical cells of the transition zone initiates cell invasion by cytoskeletal rearrangements and weakening of cell junctions. CEACAM expression is reduced in endocervical cells of the transition zone. Therefore, gonococci interact with HSPG receptors leading to activation of the EGFR receptor. Activation of the associated signaling pathways results in decreased integrin expression, increased phosphorylation of β-catenin, and movement of E-cadherin junction proteins to the membrane under the gonococcus, acting in concert to weaken cell junctions. Phosphorylation of NMII results in its rearrangement alongside actin filaments to facilitate cell invasion.

redistributions do not lead to a significant increase in the permeability of the epithelial layer, yet weakens the lateral mobility between the apical and basolateral membranes. The gonococcal-induced phosphorylation of β-catenin and its redistribution depends on the kinase activity of the epidermal growth factor receptor (EGFR; Edwards et al., 2013). EGFR activation appears not to depend on a specific surface molecule on the gonococcus but on a variety of different surface molecules which interact with different host receptors to induce the production of EGFR ligands for EGFR activation (Swanson et al., 2011). Although only a small percent of adherent gonococci invade and cross the epithelial cell layer, these gonococci can cause both local and disseminated infections. Gonococci cross the epithelial barrier primarily *via* an intracellular rather than an intercellular route, although they may gain access to deeper tissues *via* an intercellular route (McGee et al., 1981; Apicella et al., 1996; Mosleh et al., 1997).

Interactions of gonococcal surface molecules with the epithelial cell surface also activates host cell signaling cascades that trigger the reorganization of the actin cytoskeleton, which is required for host cell entry and transmigration across the epithelial layer. EGFR, as discussed above, is activated by gonococci binding and is a common surface receptor that is essential for epithelial cell survival and proliferation through the activation of several signaling cascades (PI3K, PLCγ, calcium flux, and PKC and MAP kinases), all of which can lead to actin rearrangement (Swanson et al., 2011; Figure 1). Gonococci induce disassembly of the actomyosin ring, which

supports cell junction complexes by inducing calcium-dependent phosphorylation of nonmuscle myosin II (NMII), leading to activation and reorganization of NMII from cell junctions to gonococcal adherence sites (Wang et al., 2017). The reorganization of NMII to gonococcal adherence sites is part of the substantial rearrangements seen in the host cell directly beneath the points of bacterial attachment. Early studies demonstrated membrane perturbations resulting in membrane ruffles upon gonococci infection of primary ecto- and endo-cervical cells (Edwards et al., 2000). Actin-associated proteins, such as ezrin and vinculin, accumulate in response to gonococcal adhesion, further promoting the actin-dependent entry of primary epithelial cells (Edwards et al., 2000). Although, differences in the involvement of the actin cytoskeleton have been observed depending on which receptor is used for internalization.

2.2.3. PorB interacts with host cell membranes, influencing host cell signaling to promote intracellular survival

PorB is the most abundant protein in the gonococcus and is essential for its viability (Britigan et al., 1985). The porin complex is a trimeric arrangement spanning the membrane, forming an anion selective channel. Gonococcal strains expressing the $PorB_{IA}$ protein are more invasive (van Putten et al., 1998), and are often isolated from patients with disseminated gonococcal infection, while strains expressing $PorB_{IB}$ tend to be associated with pelvic inflammatory disease, and are isolated from patients with localized gonococcal infection (Morello and Bohnhoff, 1989). Notably, the majority of gonococcal PorB research appears to be focused on the $PorB_{IB}$ isoform; given the implications the $PorB_{IA}$ isoform clearly has on disseminating infection, research efforts should be extended to include this more potent PorB protein. Like the pilus, PorB induces calcium transients in the host cell, leading to a reduction in the Lamp1 glycoprotein *via* gonococcal IgA1 protease cleavage, and a general reduction of lysosome numbers in infected cells (Lin et al., 1997; Hopper et al., 2000). These effects ultimately increase gonococcal intracellular survival and growth. The pilus and porin act in concert to induce calcium fluctuations in the host cell; an important secondary messenger that can influence cell growth, differentiation, motility, apoptosis, and necrosis (Ayala et al., 2005).

3. Modulation of the host immune response

Throughout infection, the gonococcus has also evolved several intricate mechanisms to mitigate the impact of the host immune response (Figure 2). These evasion mechanisms limit the generation of a host protective immune response, leaving the host defenseless against reinfection and proving vaccine development difficult. Understanding in detail these intricate immune evasion mechanisms will greatly advance vaccine design, and also enable insight into the causes of asymptomatic infection. Throughout this section, we highlight the immune evasion mechanisms of the gonococcus in both cervical epithelial cells and immune cells. Lenz and Dillard have recently reviewed the interactions between the gonococcus and the immune system in the fallopian tubes (Lenz and Dillard, 2018), demonstrating both similarities and differences to the lower genital tract.

3.1. The innate immune response

3.1.1. *Neisseria gonorrhoeae* expresses surface-exposed lysozyme inhibitors

The host innate immune system acts quickly upon the detection of an invading pathogen, and begins attacking components of the bacterial cell wall (Ragland and Criss, 2017). Peptidoglycan is targeted by lysozyme, which hydrolyzes the bonds holding the peptidoglycan subunits together. Various isoforms of lysozyme are distributed throughout the human genome (Irwin et al., 2011); consequently, bacterial pathogens have evolved to express several potent lysozyme inhibitors (Callewaert et al., 2012). Upon infection, gonococci are likely to encounter the human c-type lysozyme found on mucosal surfaces and secretions, and within the degradative granules of innate immune cells (Ragland et al., 2020). *N. gonorrhoeae* encodes two surface-exposed inhibitors of human c-type lysozyme (Figure 2A) – the adhesin complex protein (ACP; Hung et al., 2013; Humbert et al., 2017) and the surface-exposed lysozyme inhibitor of c-type lysozyme (SilC; Zielke et al., 2018). In the absence of SilC, gonococcal colonization of the mucosa of the murine female genital tract is significantly reduced (Zielke et al., 2018).

3.1.2. Engagement of pattern recognition receptors can promote pathogenesis

Pattern recognition receptors (PRRs) are expressed by the host on both epithelial and innate immune cells, and are an additional first line defense against invading pathogens. These PRRs recognize evolutionarily conserved molecules produced by pathogens, commonly referred to as PAMPs, stimulating a cascade of signaling events to upregulate innate immune mechanisms. It is well established that gonococci are recognized by several PRRs upon infection, including toll-like receptors (TLR), nucleotide-binding oligomerization domain-like receptors (NOD), and stimulator of interferon genes (STING; Fichorova et al., 2001; Fisette et al., 2003; Kaparakis et al., 2010; Liu et al., 2010; Woodhams et al., 2013; Mavrogiorgos et al., 2014; Zhu et al., 2018). Activation of these receptors typically culminates in early NF-κB-dependent inflammatory responses, frequently seen in symptomatic infections. Although pro-inflammatory responses can enhance gonococcal pathogenesis (Fisette et al., 2003; Kaparakis et al., 2010; Mavrogiorgos et al., 2014; Lenz and Dillard, 2018; Quillin and Seifert, 2018; Lovett and Duncan, 2019), the direct activation of these PRRs and the subsequent effects on gonococcal pathogenesis have been seldom studied.

In one notable example, Andrade et al., demonstrated an upregulation of the anti-inflammatory cytokine, type 1 interferon-β (IFN-β), upon gonococcal invasion of both human and murine cells, which occurred *via* both STING- and TLR-4-dependent engagement (Andrade et al., 2016). Elevated levels of IFN-β suppressed both neutrophil and macrophage killing of gonococci. TLR-4 engagement general TLR-4 signaling is known to upregulate two distinct pro- and anti-inflammatory pathways. The anti-inflammatory pathway causes the upregulation of IFN-β (Figure 2A) *via* the IRF3 transcriptional regulator, which is activated upon TRAM/TRIF signaling. In contrast, several pro-inflammatory cytokines can be upregulated by two central transcription factors, NF-κB and/or activator protein-1, upon stimulation of MyD88 signaling by TLR-4 engagement (Molteni et al., 2016). Consequently, despite the activation of TLR-4 *via* the same ligand (LOS), it is unknown how gonococci-TLR-4 interactions appear to sway TLR-4 signaling

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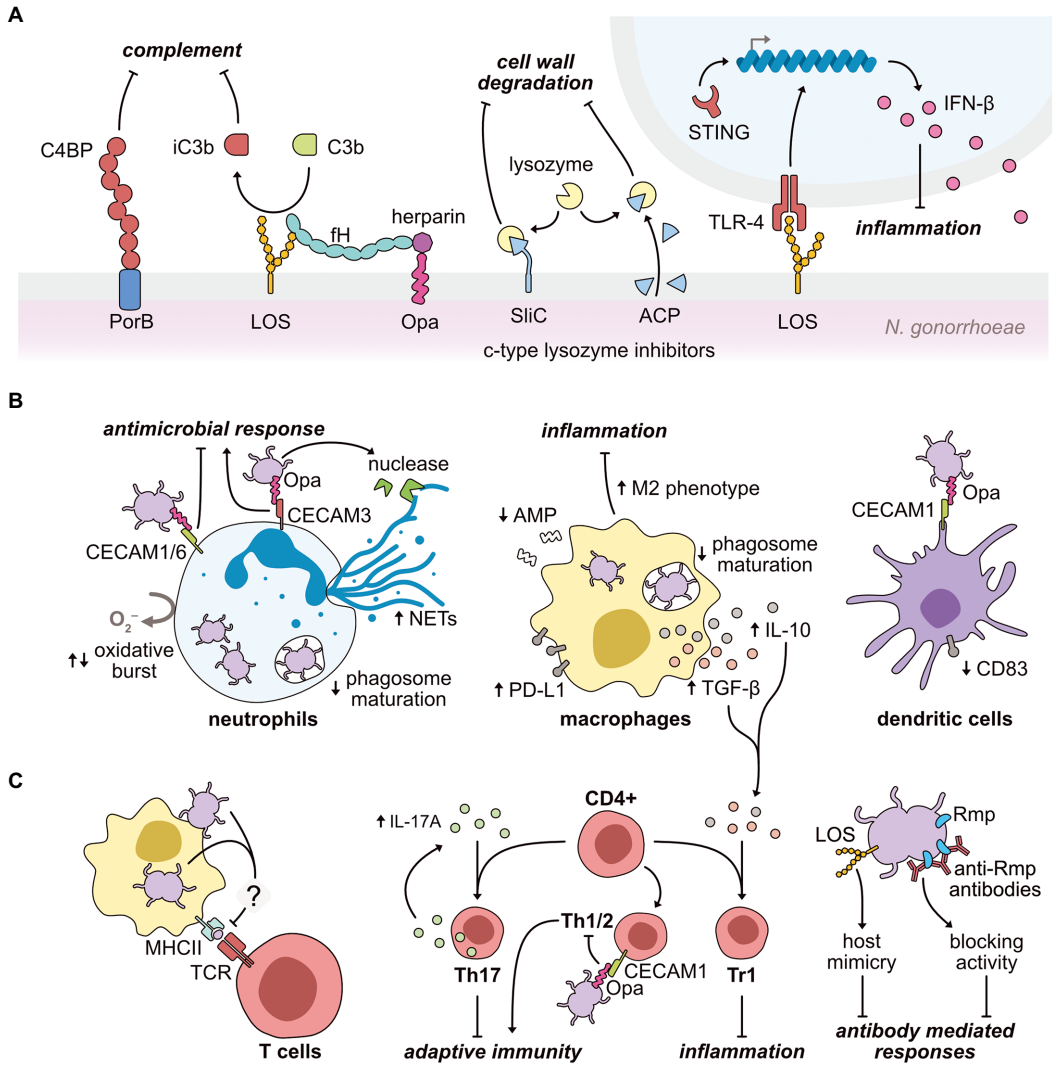

FIGURE 2
Modulation mechanisms exerted by *N. gonorrhoeae* on the host immune response during infection. **(A)** Gonococci use their primary outer membrane antigens to limit the efficacy of host lysozyme, complement, and pattern recognition systems. PorB and sialylated LOS interact with the soluble complement inhibitory proteins, C4BP and factor H (fH), respectively. LOS also binds to the soluble complement protein, C3b, inducing its inactivation (iC3b). Gonococcal Opa proteins further interact with host heparin to promote C3b inactivation. The gonococcus also expresses two surface-exposed lysozyme inhibitors (SilC and ACP), preventing the bactericidal activity of host lysozyme upon infection. In parallel, LOS interaction with the pattern recognition receptor, TLR-4, upregulates the expression of the anti-inflammatory cytokine, IFN-β. Gonococci-induced upregulation of IFN-β also occurs *via* activation of the cytosolic pattern recognition receptor, STING. **(B)** Gonococci repress antimicrobial functions of key innate effector cells to promote infection. The gonococcus engages with CEACAM receptors on neutrophils *via* its Opa proteins to repress (Opa-CEACAM1 and Opa-CEACAM6) or activate (Opa-CEACAM3) inflammatory responses, such as the oxidative burst. Neutrophil extracellular traps (NETs) are upregulated by gonococcal infection, although the secretion of a gonococcal nuclease may aid gonococci in resisting NET action. In both neutrophils and macrophages, gonococci are adept at delaying phagosome maturation to prolong intracellular survival. To specifically limit macrophage function, gonococci downregulates antimicrobial peptide (AMP) expression, while simultaneously upregulating immunosuppressive molecules to promote type 1 regulatory T (Tr1) cell proliferation. As part of this reprogramming, macrophages are polarized toward an anti-inflammatory M2 phenotype. Finally, Opa-CEACAM1 interactions on dendritic cells cause a downregulation of the CD83 maturation marker. **(C)** Gonococci act both directly and indirectly on the host adaptive immune system to minimize production of immunological memory. Gonococcal-challenged macrophages fail to increase major histocompatibility complex class II (MHCII) expression, which limits efficient antigen-presentation to cognate T cell receptors (TCRs). Instead, gonococcal infection upregulates the cytokine, IL-17A, directing $CD4^+$ T cells toward a Th17 phenotype, less capable of inducing a protective response. Opa-CEACAM1 interactions on Th1/2 cells further reduces the efficacy of the adaptive immune response. Alongside the blunting of T cell-mediated immunity, B cell-mediated immunity is also minimized during gonococcal infection. Antibodies generated against the gonococcal Rmp protein block bactericidal effects of other circulating antibodies. Furthermore, upon sialylation *in situ*, the gonococcal LOS molecule mimics host structures, thus presenting as "self" to the immune system.

toward an anti-inflammatory path. However, the subcellular localization of TLR-4 during activation may explain how these opposing responses are induced. MyD88 signaling becomes activated at the plasma membrane, in contrast, the anti-inflammatory pathway is initiated upon TLR-4 engagement within the endosome (Molteni et al., 2016).

3.1.3. *Neisseria gonorrhoeae* exploits complement regulatory proteins for silent epithelial entry and immune masking

Working in concert with innate pattern recognition, are host proteins of the complement system: an enzymatic cascade that circulates in blood and permeates tissues to rapidly clear invading pathogens

(Merle et al., 2015). The gonococcus has evolved to effectively counter various host complement proteins and significantly reduce complement activity (Figure 2A). Specifically, the gonococcal PorB protein binds both soluble C4b-binding protein (C4BP; Ram et al., 2001) and factor H (fH; Ram et al., 1998a), with sialylated LOS also capable of interaction with fH (Ram et al., 1998b). Both C4BP and fH are inhibitory components of the complement system, which prevent the recognition of host cells as foreign. Therefore, recruitment of these proteins enables the gonococcus to mask itself from complement-mediated attack. Further prevention of complement activity is enabled by the interaction of LOS with the soluble complement component, C3b, causing its inactivation (iC3b), again presenting the gonococci as "self" to the immune system (Edwards and Apicella, 2002). A secondary effect of the deposition of iC3b on the gonococcal surface is the promotion of host cell invasion *via* the host iC3b receptor, CR3. Engagement of this receptor by gonococci triggers increased CR3 expression within cervical epithelial cells, promoting further invasion of the host cell, without triggering a host inflammatory response given the immune silent-nature of this receptor (Edwards et al., 2001, 2002). Notably, CR3 is absent from male, but not female, urogenital epithelia, suggesting a potential link between the higher degree of asymptomatic infection in females compared to males. CR3 is also expressed by several immune cells, including neutrophils, macrophages, and T cells (Lamers et al., 2021), however, whether the gonococcus silently enters these immune cells *via* this receptor is unknown.

Furthermore, the gonococcus has evolved to take advantage of the host compound, heparin, to aid complement system evasion through interaction with its Opa proteins (Chen et al., 1995). Mucosal surfaces contain reasonably high levels of extracellular glycosaminoglycans, including heparin (Jackson et al., 1991), therefore it is likely that gonococci have access to these compounds upon infection (Chen et al., 1995). Extracellular heparin directly binds fH, increasing the affinity of the gonococcal surface for C3b (Figure 2A), further promoting the deposition of iC3b on the gonococcal surface (Meri and Pangburn, 1990). This ability to associate with heparin-like compounds is unique to *Neisseria* species. In addition, the Opa-heparin interaction acts as a bridge for further interaction with the human glycoprotein, vitronectin (Duensing and van Putten, 1998; Singh et al., 2010). Vitronectin is used by the host as a mechanism to prevent lysis by membrane attack complexes, and therefore provides another layer of immune avoidance when co-opted by the gonococcus.

3.1.4. *Neisseria gonorrhoeae* establishes intracellular niches within neutrophils during infection

A well-established trait of gonococcal symptomatic infection is the rapid influx of high numbers of neutrophils to the site of infection, forming gonococci-neutrophil-rich exudates (Johnson and Criss, 2011; Palmer and Criss, 2018; Quillin and Seifert, 2018). Upon the detection of inflammatory signaling, neutrophils rapidly transmigrate from circulation into various layers of host tissue, and ultimately the mucosae (Rosales, 2020). Problematically for host immunity, *N. gonorrhoeae* can not only survive within neutrophils but also persist and replicate (Johnson and Criss, 2011; Zughaier et al., 2014; Gunderson and Seifert, 2015; Quillin and Seifert, 2018), clearly demonstrating an ability to effectively dampen intracellular bactericidal effects exerted by neutrophils (Figure 2B).

Generally, the first interaction between neutrophils and the gonococcus occurs between the CEACAM family of receptors (CEACAM1, CEACAM3, or CEACAM6) and the gonococcal Opa protein (69102). While CEACAM1 and CEACAM3, respectively, inhibit or activate neutrophil functions *via* cytoplasmic immunoreceptor tyrosine-based signaling motifs, CEACAM6 lacks a cytoplasmic domain (McCaw et al., 2004; Muenzner et al., 2010). Nonetheless, engagement of CEACAM6 induces inhibitory signaling similar to CEACAM1 (Schmitter et al., 2007). Consequently, gonococcal engulfment as a result of CEACAM1 and CEACAM6 engagement by Opa inhibits pro-inflammatory neutrophil effector functions (Sarantis and Gray-Owen, 2012). Conversely, CEACAM3 binding stimulates neutrophil effector functions (Schmitter et al., 2004), including degranulation and the oxidative burst response (Booth et al., 2003; Sarantis and Gray-Owen, 2012), as well as the stimulation of neutrophil influx (Sintsova et al., 2014). Thus, the nature of the Opa-CEACAM interaction during infection has been proposed as a key contributor to either the presence or absence of symptoms, particularly given that the presentation of symptoms in gonococcal infection is largely a result of tissue damage caused by neutrophil influx (Sintsova et al., 2014). Furthermore, Sintsova et al., demonstrated the requirement of CEACAM3 activation in the stimulation of these neutrophil antimicrobial actions (Sintsova et al., 2014). Despite the potential for inflammatory responses to aid pathogenesis, this potent inflammatory response caused by CEACAM3 engagement is thought not to be the case. Instead, the Opa-CEACAM3 interaction has been proposed as a consequence of human evolution to combat invading pathogens, which utilize non-stimulating CEACAM receptors to infect host cells (Bonsignore et al., 2020). Ultimately, it remains whether CEACAM3-dependent phagocytosis of gonococci into neutrophils allows the gonococcal cells to persist within these potent immune cells, or whether the gonococcus can only establish intracellular niches within neutrophils when engulfed *via* CEACAM1 or CEACAM6. Notably, several gonococcal isolates from disseminated infections did not possess binding-capacity for CEACAM3, unlike isolates that primarily cause localized infections (Roth et al., 2013). This suggests that CEACAM3 engagement is not utilized by the gonococcus to promote pathogenesis.

In addition to the Opa-CEACAM3 interaction promoting an oxidative burst response within neutrophils, the gonococcal PorB protein may induce a similar effect when integrated into the neutrophil cell membrane (Gray-Owen et al., 1997b; Lorenzen et al., 2000). The oxidative burst response culminates in the production of reactive oxygen species, which are produced either in the extracellular milieu *via* NADPH oxidase activity, or within the intracellular environment *via* myeloperoxidase. Notably, infection experiments with Opa-deficient gonococci failed to stimulate a neutrophil oxidative burst response (Rest et al., 1982; Virji and Heckels, 1986; Fischer and Rest, 1988; Criss and Seifert, 2008; Johnson and Criss, 2011), suggesting that PorB likely acts to enhance this Opa-dependent effect. Again, this finding may provide a link between the tendencies for asymptomatic infection to present more often in women compared to men, given that Opaless gonococci are primarily isolated from female infections, whereas, Opa-expressing gonococci are primarily isolated from male infections (Swanson et al., 1988; Jerse et al., 1994; Jerse, 1999; Johnson and Criss, 2011). Nevertheless, Gunderson et al., demonstrated a situational dependency of this gonococcal effect on neutrophil function (Gunderson and Seifert, 2015). At a high multiplicity of infection (MOI; >20), which is thought to better emulate infection, oxidative burst responses were suppressed, whereas the opposite response was seen at low MOIs (<20). Despite an anti-inflammatory state seen at

high MOIs, the production of neutrophil extracellular traps (NETs) were still observed. However, gonococci appeared to be resistant to these NETs, which is thought to be a result of the gonococcal nuclease secretion, which has been shown to degrade the NET matrix (Juneau et al., 2015). In addition to the effects on the oxidative defense mechanism of neutrophils, following internalization, the gonococcus slows the maturation of the phagosome *via* its pilus and PorB proteins (Bjerknes et al., 1995; Lorenzen et al., 2000; Johnson and Criss, 2011, 2013; Palmer and Criss, 2018). Phagosomes are termed "mature" upon fusion with cytoplasmic granules containing potent antimicrobial compounds. Therefore, avoiding these components greatly enhances gonococcal intracellular survival within neutrophils.

3.1.5. Macrophages are reprogrammed by invading gonococci

The ability of the gonococcus to survive within the hostile intracellular environment of neutrophils is certainly unique, but the infiltration of the longer-lived antigen-presenting cells of the immune system significantly aids gonococcal pathogenesis. The relationship between the gonococcus and antigen-presenting cells provides a critical link between gonococci infection and the adaptive immune system. The specific manipulation of both macrophages and dendritic cells (DCs) by the gonococcus serves to dampen the establishment of protective immune memory (Figure 2B). As a result, reinfection is common given the lack of population adaptive immunity (Zhu et al., 2011). Similar to neutrophils, upon invasion of macrophages, gonococci can also delay the maturation of the phagosome, subsequently promoting intracellular survival. Mosleh et al., demonstrated profound differences in various Rab GTPases (Rab4, Rab5, and Rab7) within human macrophage phagosomes treated with and without the gonococcal PorB protein (Mosleh et al., 1998). These Rab GTPases are known regulators of phagosomal maturation (Gorvel et al., 1991; Bucci et al., 1992), and consequently, phagosomes treated with PorB took longer to acquire late endocytic markers (Rab7 and cathepsin D) compared to untreated phagosomes, demonstrating the PorB-induced delay in phagosome maturation.

Upon establishment of macrophage infection, the gonococcus can actively suppress expression of numerous antimicrobial peptides (AMPs), including LL-37, HBD-1, and SLPI, where this suppression is correlated to epigenetic modification of the macrophage genome (Zughaier et al., 2020). The gonococcus constitutively expresses a histone deacetylase (HDAC)-like protein, at a four-fold higher rate under anaerobic conditions compared to aerobic, suggesting a primary role for this protein during host infection. Counterintuitively, this HDAC-like protein appears not to directly modify host DNA, and therefore acts *via* a yet to be identified mechanism. A second avenue by which the gonococcus directly modifies immune cell gene expression is by targeting the regulatory elements of iron availability in macrophages to combat the host iron-limiting defense (Zughaier et al., 2014). This innate immune defense carried out by macrophages can occur *via* both intra- and extra-cellular mechanisms of action. One key pathway is the expression of hepcidin, a hormone which limits iron uptake within the gut, while maintaining intracellular iron stores within macrophages. Macrophages can also generate their own siderophores to minimize access to free iron, and this process is highly dependent on the production of enterobactin by the enzyme BDH2. Gonococcal infection of both a murine (RAW264) and human (THP-1) macrophage cell line caused the overexpression of hepcidin and the downregulation of BDH2 expression, while also manipulating the expression of several other genes involved in the iron-limiting response of macrophages. Iron is essential for gonococcal survival (Zughaier et al., 2014), and therefore, the ability of the gonococcus to circumvent the iron-limiting defense of macrophages is critical to a successful infection.

Once the gonococcus has evaded the primary defense mechanisms exerted by macrophages, immunomodulatory mechanisms to alter macrophage-induced adaptive responses are initiated. The gonococcus suppresses critical T-cell responses through the induction of macrophage anti-inflammatory signaling (Ortiz et al., 2015; Château and Seifert, 2016; Escobar et al., 2018). However, conflicting reports have emerged that suggest gonococcal infection can induce both pro- and anti-inflammatory signaling in macrophages (Makepeace et al., 2001; Duncan et al., 2009; Feinen et al., 2010; Packiam et al., 2010; Zughaier et al., 2014; Ortiz et al., 2015). In part, these discrepancies are linked to differences in experimental cell models and require the development of more representative macrophage models of infection to resolve. Nevertheless, an interesting correlation noted in many bacterial infections is the link between chronic and persistent infection and an ability to reprogramme macrophages toward an M2 phenotype (Ortiz et al., 2015). The M2 phenotype of macrophages is strongly anti-inflammatory and stimulates tissue repair, whereas the opposing M1 phenotype favors an inflammatory response, characterized by the proliferation of T helper 1 (Th1) cells. Notably, a shift toward an M2 phenotype has been noted for gonococcal infection (Escobar et al., 2013; Ortiz et al., 2015). Elevated levels of immunosuppressive IL-10, TGF-β1, and PD-L1 were noted upon gonococcal challenge of both murine and human macrophages, despite increases in inflammatory IL-6. This cytokine profile is indicative of a T helper 17 (Th17) cell response, which is generally less capable of targeting intracellular pathogens. In addition, static levels of the cell surface co-stimulatory molecule, CD86, and the major histocompatibility complex class II, were noted within gonococci-infected macrophages, which are both involved in stimulating an adaptive immune response (Magee et al., 2012; Wieczorek et al., 2017). Taken together, these data suggest a reduced ability of gonococci-infected macrophages to stimulate a T cell response (Escobar et al., 2013; Ortiz et al., 2015). Likewise, a failure to raise expression levels of the antibody receptor, CD64, was also demonstrated upon gonococcal macrophage infection, and is proposed to weaken the antibody-dependent cellular cytotoxicity response against the gonococcus (Ortiz et al., 2015).

3.1.6. Gonococcal antigens induce an anti-inflammatory phenotype in dendritic cells

Typically, dendritic cell responses to foreign antigens are pro-inflammatory in nature and promote both T cell development and maturation (Cabeza-Cabrerizo et al., 2021). However, in both human and murine DCs, gonococcal antigens induce an anti-inflammatory response (characterized by IL-10 and PD-L1 production), which results in reduced production of $CD4^+$ T cells (Zhu et al., 2012; Yu et al., 2013). One mechanism by which *N. gonorrhoeae* is hypothesized to elicit this DC response is *via* interactions of the gonococcal Opa protein with CEACAM1 on DCs. In support of this, Yu et al., correlated the Opa-CEACAM1 interaction on DCs with a significant downregulation in the DC maturation marker, CD83 (Yu et al., 2013). Combined with the gonococcal-macrophage research, it is clearly evident that the gonococcus exerts very similar effects on the antigen-presenting cells of

the innate immune system, with an overall reduced development of an adaptive T cell response.

3.1.7. Pro- and anti-inflammatory cytokine responses are induced during gonococcal infection

Cytokines are master regulatory molecules, which coordinate immune defense upon infection. *N. gonorrhoeae* manipulates host cytokine signaling pathways, again creating a balance between pro- and anti-inflammatory states. The greatest perturbations are generally observed in the tumor necrosis factor (TNF), interferon (IFN), and interleukin (IL) cytokine signaling pathways, which generally culminate in the upregulation of an inflammatory state; however the induction of anti-inflammatory cytokines has also been noted (Fichorova et al., 2001; Kaparakis et al., 2010; Mavrogiorgos et al., 2014; Andrade et al., 2016; Château and Seifert, 2016; Płaczkiewicz et al., 2022). Although the majority of these studies have not provided direct links as to whether these inflammatory states promote gonococcal pathogenesis, a smaller number of studies demonstrated direct manipulation events on host cells by the gonococcus, ultimately influencing inflammation to promote infection.

An example unique to the gonococcus is the secretion of heptose-1,7-bisphosphate (HBP; Gaudet et al., 2015). During the synthesis of gonococcal LOS, HBP is secreted from the cell as a by-product and penetrates the host cytosol *via* endocytosis. Gonococcal HBP triggers inflammatory responses in many mammalian cell lines, including epithelial and immune cells. Importantly, this activation does not induce infected host cell death (Malott et al., 2013; Gaudet et al., 2015), suggesting that an HBP-induced inflammatory response promotes pathogenesis. An additional mechanism used by the gonococcus to directly influence inflammation is the upregulation of host long non-coding RNAs (lncRNAs). Płaczkiewicz et al., first demonstrated the upregulation of the lncRNA, MALATI, in epithelial cells in response to gonococcal challenge (Płaczkiewicz et al., 2022). This lncRNA reduces the expression of pro-inflammatory cytokines through the NF-κB pathway in macrophages (Wu et al., 2015; Zhao et al., 2016; Mao et al., 2017). In the same study, the lncRNA, RP11-510N19.5, was also upregulated in epithelial cells upon gonococcal challenge and can influence the expression of the chemokine, CCL20, which attracts lymphocytes *via* chemotaxis. Lastly, the lncRNA, ERICD, was also overexpressed, and is thought to be indirectly involved in the upregulation of the inflammatory TNFα molecule through RNA-mediated gene silencing.

Less well understood are the recent findings by Landig et al. that the gonococcus can interact with host Siglec receptors expressed by immune cells (Landig et al., 2019). Siglec receptors are one of several mechanisms utilized by the immune system to differentiate "self" from "foreign." These receptors recognize sialic acids presented on host cells, and upon binding induce anti-inflammatory signaling pathways within the immune cell to prevent immune attack. Therefore, by interacting with these receptors, gonococci can present as "self" to the host immune system. Humans also possess Siglec receptors that culminate in pro-inflammatory responses upon engagement, and the gonococcus has demonstrated binding to both pro- and anti-inflammatory Siglecs (Landig et al., 2019). Although at a glance it appears contradicting, it is hypothesized that humans have evolved these inflammatory Siglecs as a mechanism to combat invading pathogens that were utilizing anti-inflammatory Siglecs to hide from the immune system. Whether this interaction of the gonococcus with the human pro-inflammatory Siglecs is a product of gonococcal evolution remains to be elucidated.

3.2. The adaptive immune response

Protective immunity against gonococcal infection is generally reported to be minimal or absent, even in symptomatic infections, and gonococcal re-infection is common in both women and men (Kasper et al., 1977; Buchanan et al., 1980; Hook et al., 1984; Plummer et al., 1989; Hedges et al., 1998b, 1999; Fox et al., 1999; Schmidt et al., 2001). Although the poor adaptive immune response is a consequence of innate immune evasion strategies, the gonococcus has evolved several additional strategies to specifically hinder the development of both B and T cell immunity (Figure 2C).

3.2.1. *Neisseria gonorrhoeae* hinders antibody production and function

Several studies have reported antibody titers against the gonococcus both during and following infection, however, it has been proposed that the specificity and/or levels of these antibodies are insufficient for robust protection against re-infection (Kasper et al., 1977; Buchanan et al., 1980; Hook et al., 1984; Plummer et al., 1989; Hedges et al., 1998b, 1999; Fox et al., 1999; Schmidt et al., 2001). Antibodies generated against the gonococcal outer membrane protein, Rmp, during infection increase the likelihood of both ascension of the infection and re-infection (Plummer et al., 1993). A fraction of these anti-Rmp antibodies prevent the function of various bactericidal antibodies (Figure 2C; Joiner et al., 1985; Rice et al., 1986; Virji et al., 1987; Lovett and Duncan, 2019). Supporting this, sera from patients with disseminated gonococcal infection hinder the bactericidal effects of uninfected sera against the gonococcus *in vitro* (Allen Mccutchan et al., 1978; Rice and Kasper, 1982).

Molecular mimicry exhibited by gonococcal LOS upon sialylation *in situ* contributes to the reduced production and/or efficacy of anti-gonococcal antibodies (Figure 2C; Mandrell et al., 1988; Moran et al., 1996; Quillin and Seifert, 2018). Sialylated LOS moieties share significant structural similarities with glycosphingolipids predominantly found on human erythrocytes (Moran et al., 1996), allowing the pathogen to present as "self" to the immune system (Tan et al., 1986; Wetzler et al., 1992; Moran et al., 1996). The substrate(s) used by the gonococcus for enzymatic sialylation are not only found within the genital tract, but also the bloodstream and neutrophils (Apicella and Mandrell, 1989), allowing the gonococcus to evade humoral immune responses throughout different stages of the infection.

In addition to the role the gonococcal IgA1 protease has in promoting intracellular survival, this protease can also further reduce mucosal antibody levels by cleaving the hinge region of secretory IgA1 (Blake and Swanson, 1978; Johnson and Criss, 2011). However, despite extensive studies of the equivalent protein from meningococci (Brooks et al., 1992; Parsons et al., 2004; Tommassen and Arenas, 2017; Spoerry et al., 2021; Zhigis et al., 2021; Kilian et al., 2022), the role of the gonococcal IgA1 protease in antibody cleavage during infection has not been extensively studied. Although the IgA1 protease from vaginal washings in women with gonorrhoea demonstrated antibody cleavage activity *in vitro* (Blake et al., 1979), no activity has been noted *in vivo* to date (Hedges et al., 1998a).

3.2.2. *Neisseria gonorrhoeae* induces a Th17 polarization in T cells

Another well-conserved immunomodulatory mechanism of the gonococcus is the promotion of a Th17 cell response at the expense of a Th1/Th2 cell response (Figure 2C) – a critical contributor to the lack of lasting adaptive immunity (Feinen et al., 2010; Liu et al., 2013, 2014). Th17 cells are a subset of $CD4^+$ T cells, and primarily function to invoke innate immune defenses, which the gonococcus is clearly well armed to defend. In contrast, Th1/Th2 cells are primarily involved in stimulating an adaptive immune response (Feinen et al., 2010). Specifically, gonococcal infection induces the cytokine IL-17A (Gagliardi et al., 2011; Masson et al., 2014), a stimulator of Th17 cell differentiation, and not IL-12, which is required for Th1 cell differentiation (Ramsey et al., 1995; Naumann et al., 1997; Simpson et al., 1999). Intriguingly, the absence of TLR-4 *in vitro* minimizes the degree of Th17 polarization, suggesting that a TLR-4-LOS interaction may be responsible for the induction of IL-17A expression (Feinen et al., 2010). Moreover, IL-17A is expressed stably throughout the murine infection period, suggesting that gonococci may maintain this polarization throughout infection. IL-17A also increases the secretion of neutrophil-attracting chemokines, LIX and MIP-2α, from murine genital cells. Accordingly, removal of the IL-17A receptor prolonged infection and reduced neutrophil recruitment within the murine infection model.

Th17 polarization is further biased through the inhibitory Opa-CEACAM1 interaction on Th1/Th2 cells (Boulton and Gray-Owen, 2002; Pantelic et al., 2005; Feinen et al., 2010; Packiam et al., 2010; Liu et al., 2012). Interestingly, CEACAM1 engagement by gonococci on T cells, in contrast to all other CEACAM1-expressing cells, does not result in phagocytosis of the bacterial cells, but instead increases the level of CEACAM1 expression, allowing for stable Opa-CEACAM1 binding and prolonged inhibition (Lee et al., 2008). This difference may reflect the active recruitment of downstream effector molecules, SHP-1 and SHP-2, by gonococcal CEACAM1 interactions with T cells. In contrast, SHP-1 activity is suppressed upon gonococcal interactions with CEACAM1 in both macrophages and neutrophils (Hauck et al., 1999). Notably, the inhibitory Opa-CEACAM1 interaction also occurs with human B cells, although ultimately results in cell death (Pantelic et al., 2005) – an effect that has not been demonstrated for T cells. One consequence of this inhibitory signaling within T cells is a reduction in T cell proliferation, where CEACAM1 inhibition alone is able to exert these suppression effects, without any additional contributing factors (Boulton and Gray-Owen, 2002).

Alongside the Th17 response, an upregulation of the anti-inflammatory cytokines, IL-10 and TGF-β, by gonococcal infection stimulates the proliferation of immunosuppressive type 1 regulatory T (Tr1) cells (Imarai et al., 2008; Liu et al., 2012, 2013, 2014, 2017). Dendritic cells are the likely source of IL-10 overexpression upon interactions with the gonococcus, as discussed above (Zhu et al., 2012). Notably, when the action of TGF-β and IL-10 are inhibited, a protective immune response is generated in a gonococcal mouse model (Liu et al., 2012, 2014). Although, when IL-10 function alone is prevented, the Th17 response is not affected, demonstrating a TGF-β requirement for Th17 cell production. Separate infection experiments with an Opa^- gonococcal strain in murine cells *in vitro*, and TLR-4 deficient murine lymph node cells with wild-type gonococci, demonstrated reduced levels of IL-10 expression, suggesting a likely role for Opa and the LOS-TLR-4 interaction in this response; although these findings remain to be confirmed *in vivo*.

4. Manipulation of host cell death pathways

In addition to the host immune response, regulated cell death pathways are a crucial mechanism used by the host to alleviate infection. At the highest level, there are three main overarching categories of regulated cell death, defined by the morphological features associated with the death phenotype: apoptosis, autophagy, and necroptosis. These pathways are tightly controlled by complex interactions between pro-death and pro-survival signals, which coordinate to orchestrate cell fate, and, in some cases, elicit an immune response. The manipulation of these pathways by *N. gonorrhoeae* is an important virulence mechanism used to both establish and promote infection.

4.1. Apoptosis

Apoptosis is the most well-characterized programmed cell death pathway, and is renowned for its seemingly immuno-silent nature given it elicits a minimal inflammatory response. Mammals possess two distinct, yet overlapping pathways, which lead to apoptotic cell death: the intrinsic (or mitochondrial-mediated) and extrinsic (or receptor-mediated) apoptotic pathways. These pathways are defined by a series of signaling events preceding the activation of the effector caspases (−3 and −7), which commit the cell to apoptosis by stochastically degrading cellular proteins. *N. gonorrhoeae* infection elicits both pro- and anti-apoptotic responses (Figure 3) in a variety of cell types, including epithelial cells of the urethra, cervix, and fallopian tubes, in addition to immune cells, such as macrophages and neutrophils (Muller, 1999, 2000, 2002; Beck and Meyer, 2000; Binnicker et al., 2003, 2004; Simons et al., 2005, 2006; Morales et al., 2006; Kepp et al., 2007, 2009; Reyes et al., 2007; Howie et al., 2008; Follows et al., 2009; Kozjak-Pavlovic et al., 2009; Chen and Seifert, 2011; Château and Seifert, 2016; Deo et al., 2018; Cho et al., 2020).

4.1.1. *Neisseria gonorrhoeae* manipulates early events in the mitochondrial-mediated apoptotic pathway to both prevent and stimulate cell death

The defining event of the mitochondrial-mediated apoptotic pathway is the permeabilization of the mitochondrial membrane. This event is tightly regulated by members of the Bcl-2 family of proteins, comprising of three interacting protein groups: the anti-apoptotic proteins (Bcl-2), the pro-apoptotic proteins (BH3-only), and the pro-apoptotic effector proteins (Bax and Bak). Typically, cellular stress caused by bacterial infection results in the upregulation of the BH3-only proteins, which function to activate the pro-apoptotic effector proteins. This causes the membrane to permeabilize and subsequently releases cytochrome *c* into the cytoplasm, activating the caspase cascade. The gonococcus both subverts and accelerates this pathway *via* two main modes of manipulation — the secretion of PorB and the activation of host cell signaling pathways, ultimately affecting the balance of pro- and anti-apoptotic proteins (Muller, 1999, 2000, 2002; Binnicker et al., 2003, 2004; Simons et al., 2005, 2006; Kepp et al., 2007, 2009; Howie et al., 2008; Follows et al., 2009; Kozjak-Pavlovic et al., 2009; Chen and Seifert, 2011; Château and Seifert, 2016; Deo et al., 2018; Cho et al., 2020).

Prevention of mitochondrial-mediated apoptosis, specifically the early mitochondrial membrane permeabilization event, is observed in epithelial cells, neutrophils, and macrophages infected with high doses (MOI > 10) of *N. gonorrhoeae* (Binnicker et al., 2003; Simons et al., 2006;

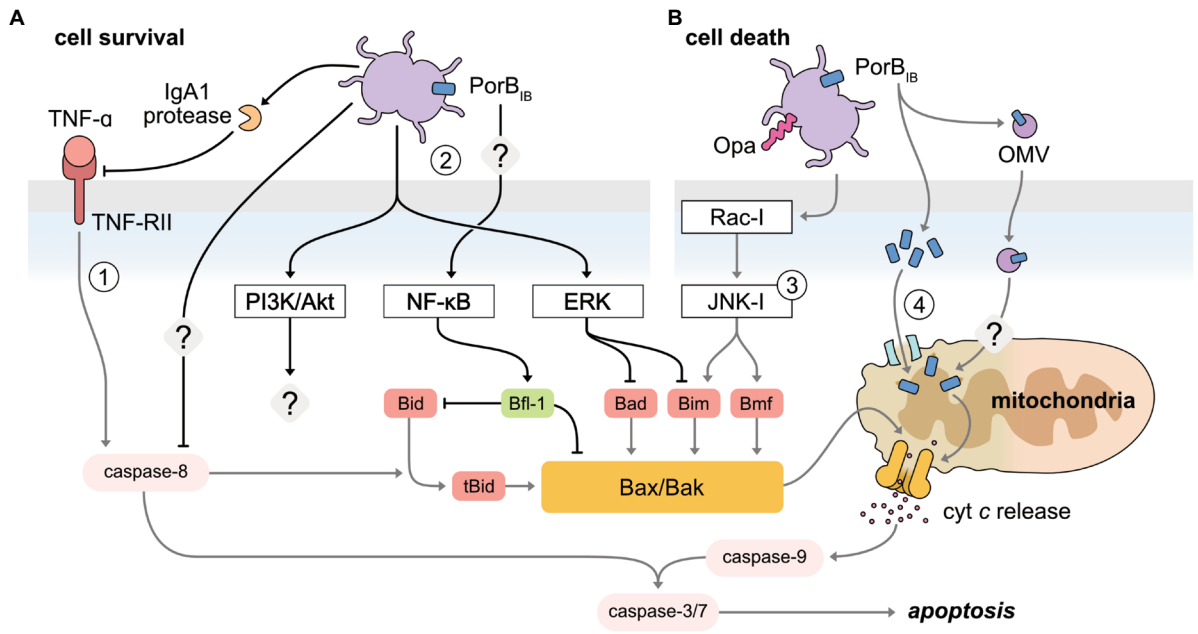

FIGURE 3
Manipulation of apoptosis pathways by *N. gonorrhoeae* to both inhibit and induce host cell death. **(A)** The receptor-mediated and mitochondrial-mediated apoptosis pathways are inhibited by gonococci to promote cell survival. (1) Secreted IgA1 protease has been shown to specifically cleave the TNFα conjugate receptor, TNF-RII, inhibiting receptor-mediated apoptosis. Caspase-8 is also inhibited, though the mechanism is undefined. (2) Host pro-survival pathways are activated upon gonococcal infection of host cells. NF-κB, activated through PorB$_{IB}$ in an unidentified mechanism, increases the pro-survival protein Bfl-1, which inhibits the pro-apoptotic proteins, Bim and Bad. PI3K/Akt and ERK pathway activation is mediated by pili-expressing gonococci. The downstream impact of gonococcal-activated PI3K/Akt has not been investigated to date. Gonococcal-mediated activation of ERK downregulates the BH3-only proteins, Bim and Bad. **(B)** Gonococci promote apoptotic cell death by disrupting the integrity of the mitochondrial membrane. (3) Opa-expressing gonococci activate JNK-,1 through Rac-1, releasing BH3-only proteins, Bim and Bmf, sensitizing the mitochondria to permeabilization. (4) PorB is targeted to the inner mitochondrial membrane, disrupting the membrane potential to induce apoptosis. PorB can also be translocated to the mitochondria *via* delivery from outer membrane vesicles (OMV).

Chen and Seifert, 2011; Château and Seifert, 2016; Cho et al., 2020). While it is unknown how the MOI used in experimental settings emulates the different stages of infection, *N. gonorrhoeae*-mediated protection against apoptosis is thought to benefit pathogenesis by preserving replicative niches to facilitate both intra- and extra-cellular colonization and dissemination. In addition, prevention of apoptosis is yet another tactic employed by the gonococcus to overcome the antibacterial properties of innate immune cells.

N. gonorrhoeae infection of epithelial cells can activate several host pro-survival signaling pathways aimed at preserving the mitochondrial membrane potential. For example, the extracellular signal-regulated kinase (ERK) pathway and the PI3K/Akt pathway are activated upon infection of pili-expressing gonococci, in a manner that is enhanced by the physical force of the pili retraction motor (Lee et al., 2005; Howie et al., 2008). Although PI3K/Akt is a known negative regulator of mitochondrial-mediated apoptosis (Song et al., 2005), the downstream impact on apoptosis as a result of gonococcal-mediated PI3K/Akt activation has not been specifically investigated. Nevertheless, *N. gonorrhoeae*-mediated ERK activation results in the differential downregulation of the BH3-only proteins, Bim and Bad, *via* proteasome degradation and protein inactivation, respectively, in human T84 colonic epidermoid cells (Howie et al., 2008). Notably, inhibition of ERK in gonococcal-infected cells only partially restores the apoptosis phenotype of uninfected cells, suggesting that additional ERK-independent mechanisms are required for the full anti-apoptotic response *N. gonorrhoeae* elicits (Howie et al., 2008). In a separate instance, intracellular gonococci reduced ERK activation in transduced human urethral epithelial cells, which was correlated to a reduction in apoptosis. However, how this ERK-reduction leads to differences concerning the mitochondrial-mediated pathway was not investigated (Liu et al., 2015).

Alongside the ERK pathway, the gonococcus can also upregulate several pro-survival genes through the activation of NF-κB, including *bfl-1*, *cIAP-2*, *cox-2*, *mcl-1*, and *cFLIP* (Binnicker et al., 2003, 2004; Follows et al., 2009). However, only *bfl-1* and *cIAP-2* upregulation correspond to increases at the protein level in urethral and endocervical epithelial cells, respectively, suggesting a non-essential role for the remaining genes in *N. gonorrhoeae*-mediated apoptosis prevention (Binnicker et al., 2004; Follows et al., 2009). Bfl-1 is a member of the Bcl-2 pro-survival proteins, tasked with protecting mitochondrial membrane integrity by inhibiting the BH3-only protein, Bid, and effector protein, Bax (Zhang et al., 2000; Werner et al., 2002). In contrast, cIAP-2 belongs to the inhibitor of apoptosis proteins (IAPs), which were initially thought to only function as direct inhibitors of effector caspases. While this holds true for some IAP members, such as XIAP, more recent insight suggests other members, such as cIAP-2, offer additional functions, such as targeting components of the TNFα signaling pathway for ubiquitin degradation (Park et al., 2004). Intriguingly, the *N. gonorrhoeae*-stimulated increase of intracellular cIAP-2 was later determined to provide protection against a caspase-independent necroptosis-like cell death, as opposed to apoptosis, as caspase inhibition was observed to the same extent when cIAP-2 was experimentally deactivated (Nudel et al., 2015). This suggests that gonococcus-mediated

activation of NF-κB may simultaneously modulate multiple cell death pathways, which is unsurprising given the wide array of signaling networks regulated by NF-κB. Furthermore, prolonged exposure of endocervical epithelial cells with live gonococci stimulated the production of exosomes for the extracellular exportation of cIAP-2, potentially as a mechanism to induce cell death at later time points in infection (Nudel et al., 2015).

In contrast to epithelial cell studies, the trend between gonococcal infection dose and the apoptotic outcome in neutrophils presents inconsistently. For example, increasing infection doses (such as MOIs between 1 and 100) have shown to both reduce and increase apoptosis inhibition, where the latter is in agreement with observations in epithelial cells (Simons et al., 2006; Chen and Seifert, 2011; Cho et al., 2020). Although the reasons for these discrepancies are unclear, previous studies highlight the importance of cell line choice in apoptotic outcome, suggesting that conflicting reports are likely valid (Binnicker et al., 2003; Château and Seifert, 2016). Nonetheless, the gonococcus is clearly able to subvert mitochondrial-mediated apoptosis in neutrophils, which constitutes an important antimicrobial mechanism used by these potent immune cells (Allen and Criss, 2019). Whether this mechanism of defense occurs in a similar manner to epithelial cells remains to be elucidated; although increased gene expression of the anti-apoptotic, *XIAP* and *cIAP-2*, is observed in gonococci-infected neutrophils, in parallel to depressed caspase activity (Simons et al., 2006), it has not been confirmed if this corresponds to increased protein levels, nor would this explain how the upstream event, mitochondrial membrane permeabilization, is prevented.

There are conflicting reports as to whether circumventing the mitochondrial-mediated pathway requires viable gonococci. Nonetheless, several studies have demonstrated partial apoptosis resistance after treatment of heat- or gentamicin-killed gonococci, leading researchers to hypothesize that gonococcal surface components are involved in apoptosis modulation (Binnicker et al., 2004; Simons et al., 2006; Château and Seifert, 2016). Binnicker et al., crucially demonstrated that purified $PorB_{IB}$ at high doses (MOI equivalent of >10), but not other outer membrane components, such as pili, Opa proteins or LOS, was sufficient to upregulate pro-survival genes under the control of NF-κB activation in urethral epithelial cells. However, this was insufficient to confer the significant apoptosis-resistance observed for whole gonococci (Binnicker et al., 2004). This suggests that continued production of $PorB_{IB}$ by viable gonococci could be important in maintaining resistance to apoptosis through an as yet undefined mechanism. Accordingly, treatment with PorB offers partial protection against apoptosis in endocervical epithelial cells but is dependent upon viable gonococci (Follows et al., 2009).

In striking contrast to infection at high doses, gonococcal infection of macrophages and epithelial cells at low doses (MOI of 1) induces apoptosis through similar manipulation strategies (Muller, 1999, 2000, 2002; Kozjak-Pavlovic et al., 2009; Deo et al., 2018). *N. gonorrhoeae*-induced apoptosis is postulated to promote infection by facilitating dissemination from initial sites of infection or as a means to evade death by executing macrophages. Integral to the mechanism of apoptosis-induction is the secretion of PorB; reliant on close contact with the host cell, $PorB_{IB}$ translocates to the inner mitochondrial membrane, facilitated by existing mitochondrial import machinery (Kozjak-Pavlovic et al., 2009). Dependent upon ATP-binding, PorB subsequently lodges into the inner mitochondrial membrane, leading to a breakdown of mitochondrial membrane potential — a requirement for apoptosis induction (Kozjak-Pavlovic et al., 2009). Activation of host cell signaling pathways also plays a role in *N. gonorrhoeae*-induced apoptosis. Specifically, Opa-expressing *N. gonorrhoeae* mediate activation of the Rho family GTPase, Rac-1, stimulating a proliferation of activation events within the Jun-N-terminal Kinase-1 (JNK-1) signaling pathway, ultimately inducing the BH3-only proteins, Bim and Bmf, enabling the pro-apoptotic effector functions of Bak and Bax (Kepp et al., 2009). This, in combination with PorB sensitization, has been shown to stimulate gonococcal-induced apoptosis in epithelial cells (Kepp et al., 2007, 2009; Kozjak-Pavlovic et al., 2009). In macrophages, secreted outer membrane vesicles (OMVs) containing PorB have been found to facilitate PorB-targeting to the mitochondria, a mechanism sufficient to cause membrane permeabilization, cytochrome *c* release, and the downstream activation of effector caspases, all independent of the activation of host signaling pathways (Deo et al., 2018). Whether PorB dissociates from the OMV for transportation to the inner mitochondrial membrane *via* existing import machinery, as seen in epithelial cells, or if the OMV directly associates with the membrane to deliver PorB is not currently known. Although the latter explanation is currently favored given the observed "clustering" of PorB when associated with the mitochondria. Additionally, import through existing machinery requires unfolded protein, while PorB associated with OMVs adopts a similar native confirmation to that found in the outer membrane (Deo et al., 2018). It is interesting to note that OMV-facilitated delivery of PorB may be specific to macrophages based on the difference in the cell type responses to OMVs. In epithelial cells, OMVs are targeted to lysosomes for degradation (Bielaszewska et al., 2013), yet there is evidence OMVs may escape endosomes in macrophages and are detectable in the cytosol (Kozjak-Pavlovic et al., 2009; Vanaja et al., 2016), enabling the delivery of PorB. Intriguingly, the localization of PorB to the mitochondria appears to be a pathogenic-specific function of the *Neisserial* porin, as for commensal species, such as *Neisseria mucosa,* porin remains localized in the cytoplasm, further implicating the importance of PorB in pathogenesis (Muller, 2002).

4.1.2. *Neisseria gonorrhoeae* manipulates early events in the receptor-mediated apoptotic pathway to both prevent and stimulate cell death

The receptor-mediated apoptosis pathway requires binding of death ligands to the corresponding death receptors, initiating caspase-8, and subsequently the effector caspases. Caspase-8 may also cross-communicate with the mitochondrial-mediated pathway through the cleavage of the BH3-only protein, Bid, where truncated Bid activates both Bax and Bak. In addition to its function as an immune inflammatory cytokine, the death signaling ligand, TNFα, initiates important signaling events within host cells that encounter *N. gonorrhoeae*, culminating in apoptosis to prevent colonization (Maisey et al., 2003; Morales et al., 2006; Yang et al., 2020). Epithelial cells of the fallopian tubes infected with gonococci at low doses (MOI of 1), induces TNFα-mediated apoptosis in adjacent cells (not associated with the bacteria), potentially as a means to disseminate from the initial sites of colonization and gain access to deeper tissue (Morales et al., 2006). However, at higher doses of gonococcal infection (MOI 10 and 100), the bacterium is able to circumvent TNFα-mediated apoptosis in cells of which it has direct association with, to colonize the fallopian tube epithelium, despite the presence of high TNFα concentrations. Based on these findings, Morales et al., postulate that an increase of an unidentified gonococcal product is responsible for protection against TNFα-induced apoptosis (Morales et al., 2006). Secretion of the IgA1 protease by the gonococcus upon

infection of the monocytic cell line, U937, specifically cleaves the TNFα conjugate receptor, TRF-RII, but not TRF-RI, to inhibit apoptosis (Beck and Meyer, 2000). Given that TRF-RII is constitutively expressed in epithelial cells of the fallopian tube (Maisey et al., 2003; Reyes et al., 2007), it would be interesting to examine if the IgA1 protease contributes to apoptosis-inhibition in a similar manner to U937 cells. The hypothesis that the gonococcus acts early to inhibit the receptor-mediated apoptosis pathway is further supported by a study in U937 differentiated macrophages, where caspase-8 and subsequent Bid cleavage is inhibited in response to TNFα stimulated apoptosis (Château and Seifert, 2016), advantageously ceasing the communication between the two pathways for effective inhibition.

In addition to TNFα, the TNF-related apoptosis-inducing ligand (TRAIL) also functions as a death signaling ligand to initiate the receptor-mediated pathway. Inhibition of TRAIL-induced apoptosis has been demonstrated in neutrophils infected with gonococci. However, as apoptosis inhibition was determined by monitoring effector caspase-3 activation compared to uninfected cells, it is unclear if caspase inhibition was the mechanism of manipulation, or if the gonococcal targets lie upstream of this event, as has been postulated in macrophages (Chen and Seifert, 2011).

4.2. Autophagy

Autophagy is a complex, highly regulated, cytoprotective mechanism to control cellular homeostasis and adapt to metabolic stress to the benefit of the organism. To control infection upon intracellular pathogen invasion, autophagy mechanisms can be used by the host, by directly targeting the pathogen (here termed xenophagy), or by limiting their ability to survive through the elimination of critical replication factors. Regarding the former, autophagosomes target the invading pathogen and subsequently fuse with lysosomes for degradation, known as autophagy flux.

4.2.1. *Neisseria gonorrhoeae* escapes xenophagy in epithelial cells and macrophages

Transcytosis is a prerequisite for disseminating gonococcal infection, during which intracellular gonococci are targeted by the autophagy pathway in epithelial cells through CD46-cyt1/GOPC signaling however a subpopulation of gonococci escape autophagy clearance (Lu et al., 2018). A recent mini-review published by Mendes et al., details current research on how *N. gonorrhoeae* escapes autophagy-mediated killing in epithelial cells (Mendes et al., 2020). In summary, the gonococcus possesses the ability to dampen CD46-cyt1/GOPC intracellular signals used by infected cells to initiate autophagy over time, and inhibit the maturation of autophagosomes and autophagy flux through the canonical pathway of activating an autophagy repressor complex, rapamycin complex 1 (Lu et al., 2018; Kim W. J. et al., 2019). A reduction of autophagic flux is also observed in murine macrophage and human macrophage-like cell lines, partially dependent upon phosphoethanolamine (PEA)-decorated lipid A (PEA-lipid-A)-expressing gonococci (Zughaier et al., 2015). While PEA-lipid-A is necessary for gonococcal survival when associated with phagocytes (Zughaier et al., 2015), how PEA-lipid-A enhances autophagy delay requires further investigation. Despite the conservation of autophagy machinery in neutrophils, and their involvement in antimicrobial activities (Shrestha et al., 2020), it has yet to be explored how the autophagic response in neutrophils is influenced during infection with *N. gonorrhoeae*.

4.3. Necroptosis-like cell death

Necrotic cell death pathways are distinctively characterized by rupture of the cell membrane. Originally thought to be a completely uncontrolled process, it is now known that necrosis has specific, programmed signaling events, referred to as necroptosis. Pyroptosis and pyronecrosis are two forms of necroptosis-like cell death, with similar inflammatory responses that are frequently directed against intracellular pathogens.

Pyroptosis is executed by the pore-forming protein, gasdermin D, following cleavage by caspase-1 or caspase-4 proteases. Caspase-1 is specifically activated by NOD-like receptor (NLR) inflammasome complexes, which forms in the cytosol upon the recognition of pathogenic stimuli (canonical pathway), or by caspase-4 which becomes activated following the direct binding of bacterial lipopolysaccharides (non-canonical pathway). Strong inflammatory responses are further mediated by caspase-1 maturation of IL-1β and IL-18 cytokines, which are released into the surroundings upon rupture. Pyronecrosis is predominantly activated through the NLR family pyrin domain containing 3 (NLRP3), forming an NLRP3-inflammasome, leading to cell death and the release of IL-1β and the pro-inflammatory factor, HMGB1, independent of caspase activation.

4.3.1. *Neisseria gonorrhoeae* eliminates macrophages and propagates inflammatory responses by activating pyroptosis and pyronecrosis pathways

N. gonorrhoeae is a potent inducer of pyroptosis in monocyte-derived macrophages *via* both canonical and non-canonical pathways (Ritter and Genco, 2018). Nonetheless, the number of viable gonococci do not appear to be significantly impacted by pyroptosis, and it is hypothesized that the elimination of these phagocytic cells blunts the clearance of infection (Ritter and Genco, 2018). More recently, Li et al., determined induction of pyroptosis required viable gonococci rather than heat- or freeze/thaw-killed gonococci in a TLR-2-dependent manner (Li et al., 2019). At a mechanistic level, *N. gonorrhoeae* was found to prime NLRP3-inflammasome formation through the activation of NF-κB- and MAPK-dependent pathways, causing the increase in NLRP3 and proIL-1β transcription (Li et al., 2019). Various molecular signals that have been proposed to act upstream of NLRP3 activation and inflammasome formation are observed during gonococcal infection leading to caspase-1 activation (Li et al., 2019). In the absence of caspase activation, *N. gonorrhoeae* maintains the capacity to propagate an inflammatory response and cause cell death in monocytic THP-1 cells *via* pyronecrosis, dependent on cathepsin B activation. However, in either case the underlying gonococcal agonist has not yet been discovered (Duncan et al., 2009; Li et al., 2019).

5. Conclusion

The ability of the gonococcus to effectively colonize both the male and female urogenital tracts, with a diverse array of cellular structures requires a diversity of sophisticated infection strategies. Consequently,

the gonococcus has evolved extensive capabilities to manipulate a wide range of host cell signaling pathways, dependent on both the site and progression of the infection. This review highlights that these manipulation events can favor opposing signaling cascades, such as either promoting or inhibiting the host inflammatory response or cell death pathways. Yet the balance between these opposing responses during infection remains largely unclear, in particular how these infection strategies may differentiate between both male and female infection, and symptomatic versus asymptomatic infections.

A common limitation highlighted throughout this review, and an unfortunate requirement to elucidate the aforementioned infection strategies, is the inability of current model systems to accurately reflect both the pathobiology and time-length of gonococcal infection. Animal models exclude human-specific components, immortalized cell lines do not truly mirror host expression profiles, and both organoids and primary cell culture lack extracellular influences (Edwards and Butler, 2011). Clearly, human volunteer studies are the optimal infection model to study, however, these studies are only able to be conducted in males, given the high risk of complications associated with female infection. Furthermore, male volunteer studies can only examine the early stages of infection, as ethically, treatment must be delivered upon the onset of symptoms.

The extensive ability of the gonococcus to elicit anti-inflammatory responses during infection is highlighted throughout this review. Interestingly, the acquisition of these responses is suggested as a defining event in the evolution of the commensals, *Neisseria lactamica* and *Neisseria polysaccharea*. These commensals are hypothesized to have evolved from the pathogenic *Neisseria* species, suggesting an evolutionary route from commensalism, to pathogenicity, and back to commensalism (Priniski and Seifert, 2018).

Overall, this review highlights the immense host manipulation capabilities the gonococcus possesses to promote pathogenesis, with additional mechanisms not covered in this review surrounding the direct manipulation of the host cell growth cycle (Jones et al., 2007; Vielfort et al., 2013; Weyler et al., 2014). Nevertheless, progress toward elucidating these infection strategies *in situ* will greatly support advancements in both prevention and treatment of this long-lasting human disease.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Bacterial effector kinases and strategies to identify their target host substrates

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Post-translational modifications (PTMs) are critical in regulating protein function by altering chemical characteristics of proteins. Phosphorylation is an integral PTM, catalyzed by kinases and reversibly removed by phosphatases, that modulates many cellular processes in response to stimuli in all living organisms. Consequently, bacterial pathogens have evolved to secrete effectors capable of manipulating host phosphorylation pathways as a common infection strategy. Given the importance of protein phosphorylation in infection, recent advances in sequence and structural homology search have significantly expanded the discovery of a multitude of bacterial effectors with kinase activity in pathogenic bacteria. Although challenges exist due to complexity of phosphorylation networks in host cells and transient interactions between kinases and substrates, approaches are continuously being developed and applied to identify bacterial effector kinases and their host substrates. In this review, we illustrate the importance of exploiting phosphorylation in host cells by bacterial pathogens *via* the action of effector kinases and how these effector kinases contribute to virulence through the manipulation of diverse host signaling pathways. We also highlight recent developments in the identification of bacterial effector kinases and a variety of techniques to characterize kinase-substrate interactions in host cells. Identification of host substrates provides new insights for regulation of host signaling during microbial infection and may serve as foundation for developing interventions to treat infection by blocking the activity of secreted effector kinases.

KEYWORDS

post-translational modifications, phosphorylation, secretion systems, signal transduction, host-pathogen interactions, phospho-proteome, bacterial pathogenesis

Introduction

The infection process of bacterial pathogens is a fascinating display of how microbes utilize their arsenal to subvert host defenses. On the defensive side, host cells need to detect and promptly signal the presence of invading pathogens in order to generate proper responses against infection. To evade host responses, gram negative bacterial pathogens use specialized secretion machinery such as type III or type IV secretion systems (T3SS/T4SS), to translocate effector proteins across the bacterial envelope and plasma membrane of targeted hosts (Costa et al., 2015; Bienvenu et al., 2021; Horna and Ruiz, 2021; Grishin et al., 2022; Sanchez-Garrido et al., 2022). After being deployed into the host cytoplasm, these bacterial effectors can target different host proteins and consequently alter signaling pathways to modulate host responses, which is critical for establishing successful infection by the pathogen (Alto and Orth, 2012; Macho and Zipfel, 2015; Ratner et al., 2017; Pinaud et al., 2018).

Post-translational modifications (PTMs) play key roles in regulating protein functions and signal transduction. Protein phosphorylation is one of the most commonly used PTMs

for signal transduction in organisms ranging from bacteria to humans (Ubersax and Ferrell, 2007). Protein phosphorylation involves transferring the γ-phosphate of adenosine triphosphate (ATP) onto tyrosine, serine, or threonine residues of a substrate protein. This process is catalyzed by kinases and the phosphate group covalently conjugated on the substrate can be reversibly removed by phosphatases. Phosphorylation is critical in a multitude of signaling pathways that are crucial for the cells, for example, cell cycle initiation through phosphorylation of PP-1, insulin signaling through phosphorylation of AKT, and organ development through phosphorylation of YAP/TAZ (Kwon et al., 1997; Van Weeren et al., 1998; Chen et al., 2020). It is not surprising that host innate immunity is also highly regulated by protein phosphorylation and kinase targeting is a promising field for therapeutics (Gaestel et al., 2009). Thus, many bacterial pathogens have evolved to utilize protein phosphorylation to hijack host signaling pathways and gain control of their host cellular processes for replication and survival (Grishin et al., 2015; Tegtmeyer et al., 2017; Park et al., 2019). Despite the versatility of kinases in signaling networks and diversity of their substrates, many eukaryotic kinases share amino acid sequence homology in their kinase domains, particularly with highly conserved functional motifs participating in phosphate transfer. Structurally, kinases also share high degrees of similarity. Eukaryotic kinase domains, typically consisting of 12 subdomains, display structural similarity by forming a catalytic cleft surrounded by an N-terminal lobe and C-terminal lobe (Hanks and Hunter, 1995; Canova and Molle, 2014). These similarities allow pathogens to mimic a wide range of host kinases and phosphorylate target substrates during infection (Grishin et al., 2015).

Genome and amino acid sequence comparisons have discovered that many bacterial pathogens, including *Salmonella*, *Shigella*, *Yersinia*, and *Legionella*, encode effectors with primary sequence homology to eukaryotic kinase domains (Table 1; orange shade). In addition, with the increasing number of solved kinase structures and the development of structural prediction tools, several bacterial effectors with high structural similarity but limited primary sequence homology to eukaryotic kinases have been recently identified as a new class of effector kinases, including LegK7, XopC2, HopBF1, and VopG (Table 1; blue shade). Advancements in identifying effector proteins of bacterial secretion systems *via in silico* analyses have also allowed more effector kinases to be discovered. For example, a bioinformatic search of effector repertoires in sequenced genomes from *Legionella* species discovered protein kinase activity as the second largest functional domain predicted in the effectors and hundreds of putative effector kinases were identified (Gomez-Valero et al., 2019).

One of the key steps in understanding the role of effector kinases in microbial pathogenesis is identification of their host substrates. This identification allows for the characterization of effector kinase function at a molecular level and provides a foundation for developing interventions to treat infection by interfering with the actions of effector kinases. However, it is particularly challenging due to the transient nature of kinase-substrate interactions and the complexity of phosphorylation networks since endogenous host kinases also catalyze phosphorylation. In this article, we document approaches including genetic, biochemical, proteomic, and high-throughput screening techniques that have been developed and applied to identify host substrates of bacterial effector kinases. Like eukaryotic kinases, activity and substrate specificity of effector kinases can be regulated by their interacting partners, adding another layer of research interest in studying molecular mechanisms by which effector kinases target host proteins. Thus, we also discuss how effector kinases are regulated by their trans-kingdom interacting partners in host cells.

TABLE 1 Bacterial effector kinases, currently identified targeted host pathway, and substrate identification methods.

Effector *Organism*	Interacting Protein	Targeted Host Pathways	Host Substrates	Methods	References
YpkA (YopO) *Y. pseudotuberculosis*	Actin	Cytoskeleton Rearrangement	Gαq, Otubain-1, gelsolin (+others)	Affected pathway, chemical genetics, SILAC	Juris et al. (2000, 2006); Navarro et al. (2007)
SteC *S. enterica*		Cytoskeleton Rearrangement	MEK	Affected pathway	Poh et al. (2008)
LegK1 *L. pneumophila*		NFκB	IκB	Affected pathway	Ge et al. (2009)
LegK2 *L. pneumophila*		Cytoskeleton Rearrangement	ARPC1B, ARP3	Protein–protein interaction	Michard et al. (2015)
LegK4 *L. pneumophila*		Host translation	HSP70	Chemical genetics	Moss et al. (2019)
PknG *M. tuberculosis*	RAB14	Lysosome trafficking; Autophagy	TBC1D4	Affected pathway	Ge et al. (2022)
OspG *S. flexneri*	UbcH5b, Ubiquitin	NFκB	?	?	Kim et al. (2005), Pruneda et al. (2014)
NleH1/2 *E. coli*		NFκB, Micropillus formation	CRKL, EPS8L2	Proteomic microarray screen; Phosphoproteome analysis	Pham et al. (2013), Pollock et al. (2022)
Lem28 *L. pneumophila*	IP6	LCV?	?	?	Anderson et al. (2015), Sreelatha et al. (2020)
LegK7 *L. pneumophila*	MOB1	Hippo pathway	MOB1	Proteomic microarray screen	Lee et al. (2017)
XopC2 *X. oryzae*		Stomatal Closure	OSK1	Affected pathway	Wang S. et al. (2021)
HopBF1 *P. syringae*		Immune sensing	HSP90	Protein–protein interaction	Lopez et al. (2019)
VopG *V. parahaemolyticus*	?	NFκB?	?	?	Plaza et al. (2021)

?, currently unidentified.

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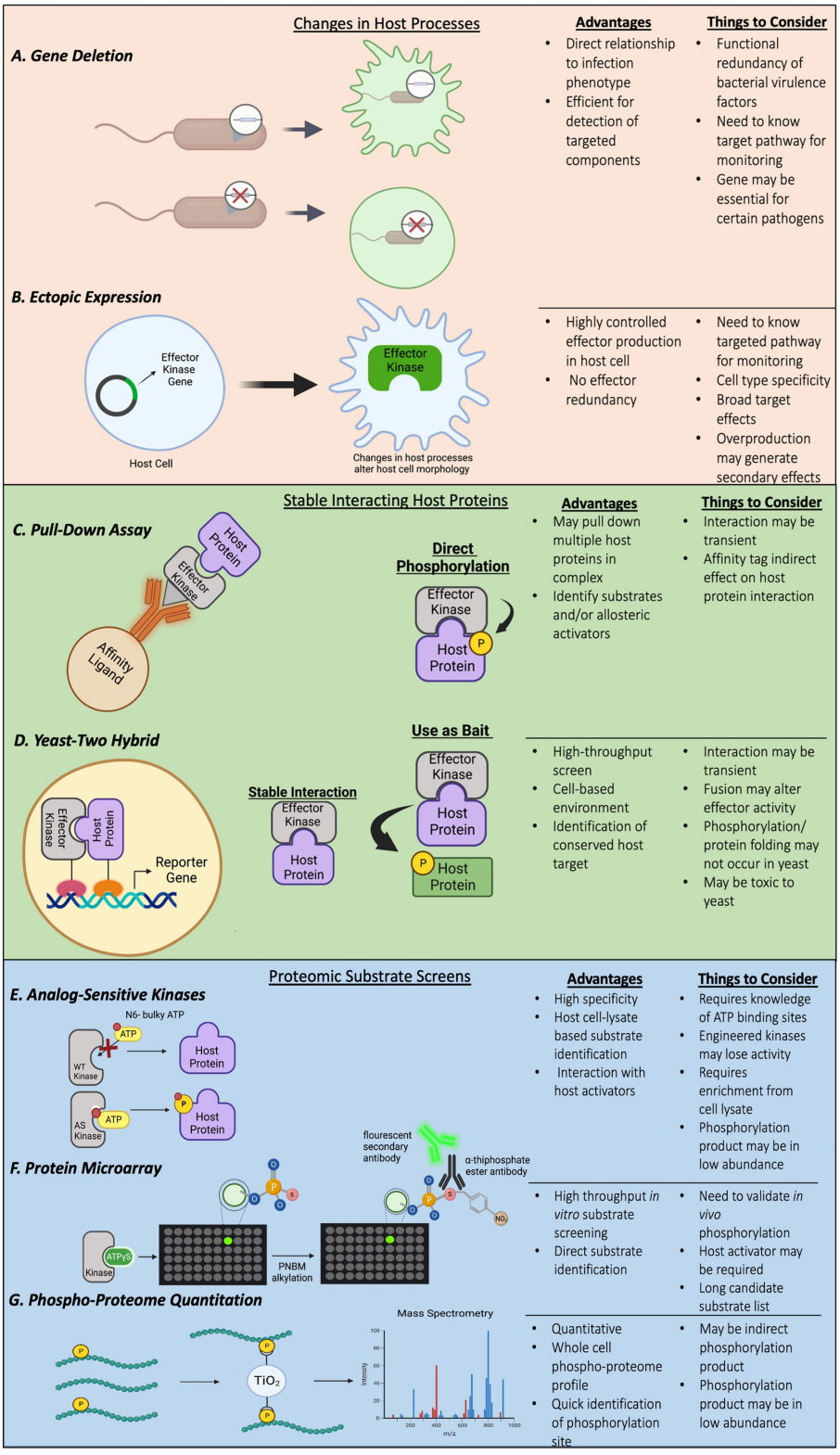

FIGURE 1
(A–G) Examples of methods and techniques used to identify bacterial effector kinases and their host substrates.

Substrate identification based on affected host pathways

Bacterial pathogens use effectors to subvert host defense, including phagocytosis and inflammatory gene expression (Alto and Orth, 2012). Characterizing functions of effector proteins in bacterial pathogenesis can be achieved by comparing responses in host cells infected by a wild-type bacterium or a mutant with the effector gene of interest deleted (Figure 1A). Alterations in targeted cellular processes, such as cytoskeleton rearrangement, maturation of pathogen-containing vacuoles, and

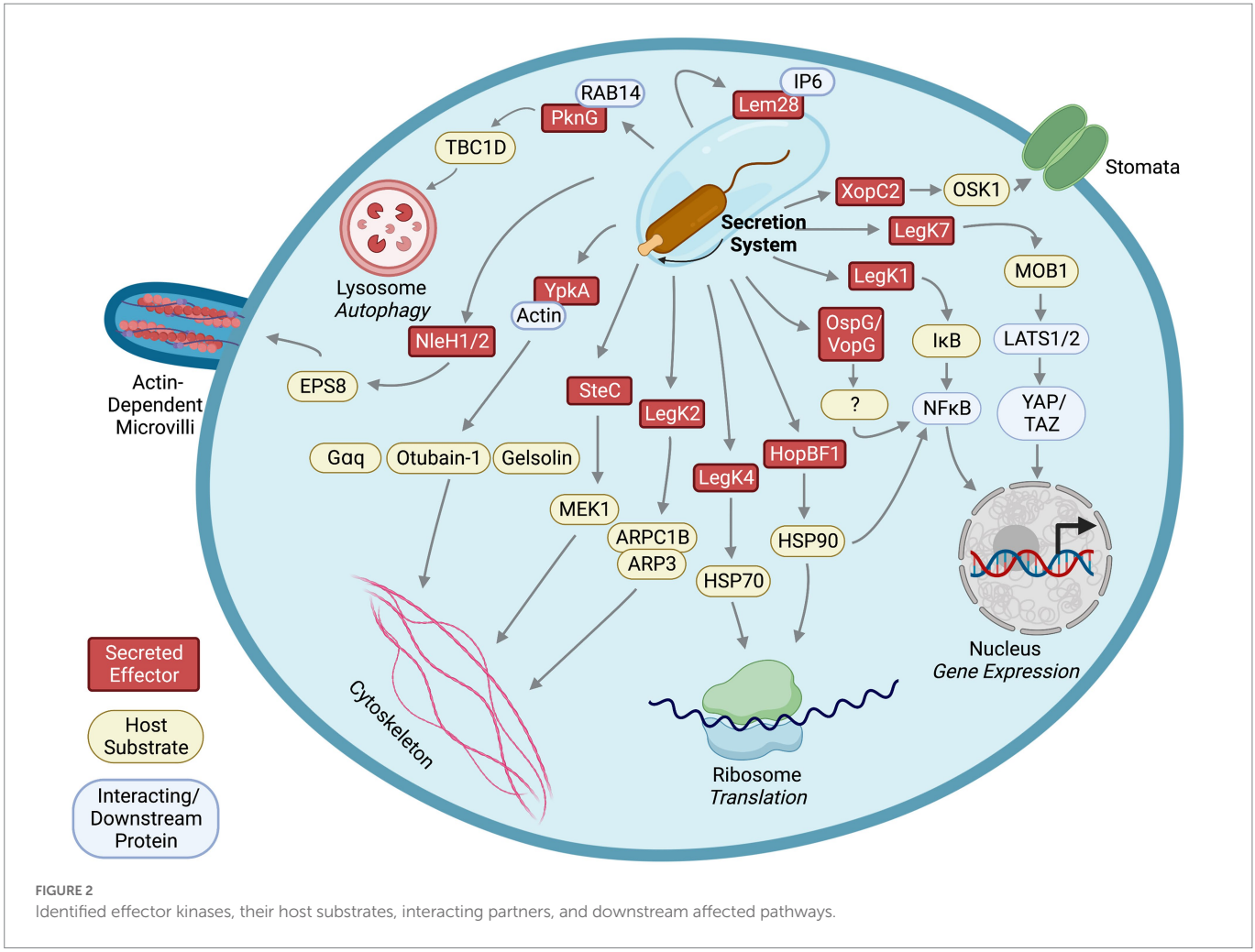

FIGURE 2
Identified effector kinases, their host substrates, interacting partners, and downstream affected pathways.

activation of inflammatory signaling are commonly monitored. As an alternative approach to simplify the complexity of infection process and potential redundancy of virulence factors produced by the pathogens, ectopically expressing bacterial effectors in host cells may generate similar effects as observed in infected host cells (Figure 1B). Both approaches have been used to characterize bacterial effector kinases and identify specific host protein substrates. Once the host responses or pathways affected are identified, it would suggest the components in the affected pathway are likely targeted by the effector, which allows researchers to narrow down the search for candidate host substrates of effector kinases.

Yersinia YpkA phosphorylates host Gαq to impair phagocytosis

Yersinia spp., including *Y. pestis, Y. pseudotuberculosis and Y. enterocolitica*, use a T3SS to translocate effector proteins into host cells (Cornelis, 2002). YpkA, also called YopO in *Y. pseudotuberculosis*, is a T3SS effector that is required for the virulence of *Yersinia spp.* (Galyov et al., 1993). The N-terminus of YpkA exhibits primary sequence homology and structural similarity to eukaryotic serine/threonine kinases, such as the family of RhoA-binding kinases, and the C-terminus contains a Rho-GTPase binding domain that interacts with host small GTPases RhoA and Rac, important for cytoskeletal organization (Dukuzumuremyi et al., 2000). The kinase activity of YpkA is critical for immediate *Yersinia pseudotuberculosis* survival in a cell-based infection model and virulence in a mouse model (Wiley et al., 2009). YpkA induces morphological changes in infected host cells and production of YpkA in mammalian cells disrupts arrangement of actin filaments, a phenomenon that requires both the kinase activity and C-terminus of YpkA (Juris et al., 2000). This destabilization likely contributes to an impairment of phagocytic ability of the host and is dependent on the interaction between YpkA and host monomeric Rho-GTPases (Grosdent et al., 2002). These observations suggest that YpkA targets host proteins involved in actin cytoskeleton regulation.

Interestingly, although YpkA binds to both GTP-and GDP-bound RhoA, a constitutively active RhoA mutant which mimics the GTP-bound status rescues the alternation of actin stress fibers by YpkA (Navarro et al., 2007), suggesting that the host target of YpkA acts upstream of RhoA and that YpkA manipulates this host target to generate suppressive effects on RhoA. Because G heterotrimeric proteins, Gα12/13 and Gαq, are regulators for RhoA activity in G-protein-coupled receptor (GPCR)-mediated actin rearrangement, screening GPCR agonists in human cells ectopically expressing YpkA revealed that YpkA inhibits activity of Gαq to suppress RhoA-mediated stress fibers formation induced by the GPCR agonists (Navarro et al., 2007). This inhibition effect is due to YpkA's ability to phosphorylate and interact with Gαq. Specifically, YpkA phosphorylates the serine-47 residue on Gαq to prevent the Gαq-GTP interaction, thereby inhibiting Gαq activation and altering actin filament structure in the host cells (Figure 2). Interfering with actin rearrangement by YpkA leads to a disruption of phagocytosis of *Y. psuedotuberculosis* by host cells (Navarro et al., 2007). In addition to affected host pathway analysis identifying

Gαq as a YpkA substrate involved in cytoskeleton rearrangement, other approaches, such as stable isotope labeling of amino acids in cell culture (SILAC; Lee et al., 2015) and chemical engineering of YpkA (Juris et al., 2006) also identified additional cytoskeleton regulators as substrates of this effector kinase, which we will discuss in later sections.

Salmonella SteC phosphorylates host MAP kinase MEK1 to promote SCV formation

When invading host cells, *Salmonella enterica* Typhimurium forms a *Salmonella*-containing vacuole (SCV) for replication. Two T3SS, named *Salmonella* pathogenicity island I and II (SPI-1, −2), are essential for *S. enterica* infection (Chakravortty et al., 2005; Jennings et al., 2017; Galán, 2021). Formation of the SCV requires effectors secreted by the *Salmonella* T3SS and is associated with an F-actin meshwork surrounding the SCV. *Salmonella* SteC is a T3SS SPI-2 effector that shares sequence homology with human kinase Raf-1 (Poh et al., 2008). Formation of F-actin meshwork during *Salmonella* infection depends on SteC and its kinase activity. Consistently, ectopic expression of SteC in mammalian cells induces F-actin rearrangement similar to the effects caused by overproduction of host Rho-associated protein kinase (ROCK), suggesting that SteC likely affects host pathways regulated by ROCK to alter actin filament structure and conformation (Poh et al., 2008).

In mammalian cells, ROCK and myosin light chain kinase (MLCK) phosphorylate myosin light chain (MLC) to activate Myosin II, a key regulator of actin organization and cross-linking (Vicente-Manzanares et al., 2009). It was shown that phosphorylated Myosin IIB is recruited to the SCV and F-actin meshwork in an SteC-dependent manner during *Salmonella* infection (Odendall et al., 2012). Furthermore, siRNA-mediated knockdown experiments showed that MLCK is required for SteC-induced F-actin rearrangement, indicating that SteC activates MLCK to phosphorylate Myosin IIB. Since extracellular signal-regulated kinases (ERKs) regulate MLCK, and SteC shares sequence similarity to host Raf which participates in the Raf/MEK (MAPK/ERK kinases)/ERK pathway, further investigation into this signaling pathway revealed that SteC directly phosphorylates MEK1 *in vitro* at serine-200. Phosphorylation of this residue contributes to MEK1 activation by promoting autophosphorylation (Odendall et al., 2012). Thus, *Salmonella* SteC phosphorylation of host MEK1 activates the MEK/ERK/MLCK pathway to modulate Myosin IIB-mediated F-actin rearrangement (Figure 2). Interestingly, an *S. enterica steC* mutant replicates more efficiently than a wildtype strain (Odendall et al., 2012), suggesting that SteC may modulate intracellular replication of the bacterium.

Legionella LegK1 phosphorylates Host IκB to activate NFκB signaling

The NFκB pathway plays a central role in controlling inflammatory gene expression and host cell survival during microbial infection (Rahman and McFadden, 2011). *Legionella pneumophila* induces nuclear accumulation of the NFκB transcription factors and expression of NFκB-regulated genes in a T4SS-dependent manner (Losick and Isberg, 2006; Abu-Zant et al., 2007). Blocking NFκB nuclear translocation causes host cell death and prevents replication of *L. pneumophila* within host cells (Losick and Isberg, 2006). These results suggest that *Legionella* T4SS effector (s) manipulate host NFκB expression and translocation to promote intracellular replication of the bacterium. Because *L. pneumophila* encodes ~300 T4SS effectors that share functional redundancy (Ensminger and Isberg, 2009; Ensminger, 2016), it is challenging to use genetic knockout approaches to identify *L. pneumophila* effectors that affect specific host cell pathways. Thus, NFκB-luciferase reporter assays combined with ectopic expression of *Legionella* effector libraries in human embryonic kidney cells (HEK cells) were used (Ge et al., 2009; Losick et al., 2010). Two *L. pneumophila* effectors, LnaB and LegK1, highly activate NFκB-controlled luciferase expression and nuclear translocation of NFκB upon production in the human cells (Ge et al., 2009; Losick et al., 2010), suggesting that these effectors target the NFκB pathway.

LegK1 is one of the four *L. pneumophila* effectors that have primary sequence homology to eukaryotic serine/threonine kinases (Hervet et al., 2011). Expression of a LegK1 catalytically inactive mutant fails to stimulate NFκB-driven luciferase expression and nuclear translocation in HEK cells (Losick et al., 2010). Therefore, LegK1 likely modulates the NFκB pathway *via* phosphorylation of host proteins. The NFκB transcription factor consists of two protein subunits, p50 and RelA, and is sequestered in the cytoplasm by binding to inhibitors of κB (IκB). Phosphorylation of IκB by IκB kinases (IKKs) results in the ubiquitination and proteasomal degradation of IκB, allowing translocation of NFκB to the nucleus (Rahman and McFadden, 2011). Subsequently, expression of LegK1 in HEK cells also increases IκBα phosphorylation and the addition of purified LegK1 into host cell lysate results in IκBα phosphorylation and processing independent of cellular IKKs (Ge et al., 2009). Finally, *in vitro* kinase assays revealed that LegK1 directly phosphorylates IκBα and other members of the IκB family, such as IκBβ, IκBε, and p100 (Ge et al., 2009), indicating that *L. pneumophila* LegK1 may phosphorylate a multitude of IκB proteins to regulate the host NFκB pathway (Figure 2). Mutants with individual *legK1* or *lnaB* genes deleted replicated within host cells as efficiently as wildtype *L. pneumophila* did, a common phenomenon due to functional redundancy of the large effector repertoire (Ge et al., 2009; Losick et al., 2010). Interestingly, a *legK1/lnaB* double mutant did not exhibit defects in pathogenesis within host cells (Losick et al., 2010), suggesting that other *L. pneumophila* effectors might compensate for the loss of LegK1 and LnaB.

Xanthomonas XopC2 phosphorylates host OSK1 to prevent stomatal closure in plants

Similar to bacteria that use effector kinases to infect their animal hosts, bacterial pathogens also interfere with plant hosts by using secretion systems to deliver effectors (Feng and Zhou, 2012), including kinase effectors. XopC2 of *Xanthomonas oryzae pv. oryzicola* has been characterized as a T3SS effector kinase that is capable of countering plant defense (Wang S. et al., 2021). Traditional primary sequence or structural homology search tools, such as Pfam and Phyre, did not identify kinase motifs or kinase folds in XopC2. However, HHPred, used for detecting remote protein homology, predicted a putative kinase motif within XopC2. Subsequent *in vitro* kinase assays demonstrated that XopC2 possesses kinase activity (Wang S. et al., 2021), suggesting that XopC2 is an atypical kinase. Importantly, transgenic rice plants expressing XopC2 are more susceptible to *X. oryzae pv. oryzicola* with more severe disease lesions, indicating a role of this effector in suppressing host defense. Moreover, kinase activity of XopC2 is required

for this suppression effect (Wang S. et al., 2021). Therefore, XopC2 phosphorylates a host protein to counteract anti-bacterial activities in infected plants.

In plants, stomata are pores for gas exchange and are major targets for pathogens as entrances to invade plant tissues. Plants close the stomal pores as a defense against invading pathogens. Defects in stomal closures were observed in rice plants expressing XopC2 upon infection, indicating regulation of stomal closures could be targeted by XopC2 (Wang S. et al., 2021). Stomal defense is negatively controlled by jasmonic acid (JA) signaling, and activation of JA signaling is mediated by degradation of a family of inhibitory proteins, JAZs. Investigation of JAZ degradation revealed enhanced JAZ9 ubiquitination by XopC2 (Wang S. et al., 2021). The SCF (SKP, cullin, and F-box E3 ubiquitin-protein ligase complex) is majorly involved in the regulation of stomatal closure *via* a ubiquitination-mediated proteasomal degradation pathway (Howe et al., 2018). Under normal conditions, OSK1, the SCF adaptor protein, is loosely associated with COI1b, promoting low levels of JAZ ubiquitination, degradation, and JA signaling. Basal or low JA signaling allows for maintenance of stomatal closure and consequent prevention of bacterial entry and infection. Further investigations into the effect of XopC2 on SCF ubiquitination complex unveiled that XopC2 directly phosphorylates OSK1 (Wang S. et al., 2021; Figure 2). XopC2-dependent OSK1 phosphorylation at serine-53 enhances its binding to the jasmonate receptor COI1b, thereby promoting the ubiquitination and proteasomal degradation of JAZ (Wang S. et al., 2021). Complete degradation of JAZ activates JA-signaling, suppressing stomatal closure, allowing bacterial entry and promoting *Xanthomonas* infection and disease susceptibility in rice plants. Like YpkA interacting with its host substrate Gαq, pulldown assays showed that XopC2 also interacts with OSK1 (Wang S. et al., 2021), demonstrating that bacterial effector kinases utilize stable interactions to phosphorylate host substrates.

Mycobacterium PknG phosphorylates host TBC1D4 to prevent autophagosome maturation

Mycobacterium tuberculosis is an intracellular pathogen that is generally confined to macrophage phagosomes upon infection. As an anti-microbial defense, the phagosomes undergo a maturation process and fuse with lysosomes to eliminate the pathogen. As observed with the pathogen-host molecular arms race, *M. tuberculosis* is adept at escaping these maturing phagosomes to enter the cytosol, simultaneously allowing their recapture by host autophagosomes in the cytosol (Russell et al., 1997; Simeone et al., 2012; Hu et al., 2020). Like other bacterial pathogens, *M. tuberculosis* have evolved to secrete effector proteins, including an effector kinase, PknG, to counteract host defense and promote infection (Walburger et al., 2004; Nicholson and Champion, 2022). The kinase activity of PknG is essential to *M. tuberculosis* pathogenicity (Walburger et al., 2004) and has therefore been a potential antibiotic target for tuberculosis treatments (Gil et al., 2013; Swain et al., 2021). Studies in PknG's role in pathogenesis show that this effector contains versatile activities, such as E3-ubiquitin ligase activity for host protein ubiquitination (Wang J. et al., 2021; Shariq et al., 2022) and regulatory activity for metabolism, virulence, and stress response within *M. tuberculosis* (Khan et al., 2017; Rieck et al., 2017; Lima et al., 2021). In this section, we focus on a newly identified host protein that is phosphorylated by PknG and approaches used to identify this host substrate.

The kinase activity of PknG is required to suppress fusion of lysosomes to phagosomes and autophagosomes, where a catalytically inactive mutant loses the ability (Ge et al., 2022). Interestingly, PknG promotes formation of autophagosome as suggested by increased LC3 puncta, a key scaffold protein and marker for autophagosomes, in host cells infected with *M. tuberculosis* (Ge et al., 2022). Similar to preventing lysosomal fusion, PknG kinase activity is required for promoting autophagosome formation, suggesting that a host protein involved in autophagosome formation is targeted by PknG. A yeast two-hybrid screen in mouse cDNA library identified host AKT interacts with PknG (Ge et al., 2022). Although PknG inhibits AKT activation by this interaction to promote autophagosome formation, phosphorylation of AKT by PknG is not shown. Instead, another host protein interacting with PknG, RAB14, emerged from the yeast two-hybrid screen, serving as a lead to identification of host substrates for PknG (Ge et al., 2022). RAB14, a small GTPase that is known to regulate vesicle trafficking and formation of *M. tuberculosis*-containing phagosomes (Kyei et al., 2006), is initially targeted and bound by PknG to maintain its GTP-bound state in order to prevent lysosomal fusion. This process is dependent on kinase activity of PknG, but like AKT, RAB14 is not phosphorylated by PknG (Ge et al., 2022). To keep RAB14 in GTP-bound state, PknG phosphorylates host TBC1D4, a GAP (GTPase-activating protein) for RAB14 (Ge et al., 2022). Phosphorylation of threonine-642 in TBC1D4 by PknG prevents its ability to activate GTP hydrolyzation in RAB14 (Ge et al., 2022). Thus, by interacting with RAB14, PknG directly phosphorylates TBC1D4, interfering with autophagosome maturation processes in the host cells (Figure 2). This illustrates another example of identifying host targets of bacterial effector kinases and their underlying molecular mechanisms through investigating affected pathways.

Host substrates identified by screening for interacting partners of effector kinases

Typically, the interaction between an enzyme and substrate is transient. Nevertheless, some kinases and their substrates can have stable interactions, with the close substrate proximity facilitating phosphorylation, for example Gαq and OSK1 for *Yersinia* YpkA and *Xanthomonas* XopC2, respectively. With feasibility of large-scale proteomic screens for protein interacting partners such as yeast two-hybrid analyses, and advances in mass spectrometry to identify proteins co-purified with bacterial effectors by pulldown assays (Figures 1C,D), host substrates that form stable complexes with effector kinases were identified.

Legionella LegK2 interacts with and phosphorylates host ARP2/3 complex for ER recruitment

As an intracellular pathogen, *L. pneumophila* enters the host cells through phagocytosis and forms a *Legionella*-containing vacuole (LCV) where the bacterium replicates within host cells. The *L. pneumophila* T4SS effectors are essential for LCV formation by manipulating many host processes, such as phagocytosis, cytoskeleton rearrangement, and vesicle trafficking (Mondino et al., 2020). In addition to LegK1, LegK2

is one of the four *L. pneumophila* T4SS effectors that share primary sequence homology to eukaryotic serine/threonine kinases (Hervet et al., 2011). Notably, it has been shown that LegK2 and its kinase activity are required for optimal intracellular replication of *L. pneumophila* within amoeba hosts and recruitment of host ER to the LCV (Hervet et al., 2011).

To identify host factors interacting with LegK2, a yeast two-hybrid screen was utilized and subsequently revealed that host ARPC1B and ARP3 interact with LegK2 (Michard et al., 2015). An *in vitro* kinase assay showed that recombinant LegK2 phosphorylates ARPC1B/ARP3 proteins and immunoblotting demonstrated phosphorylated ARPC1B and ARP3 when co-expressed with LegK2 in HEK cells (Michard et al., 2015). ARPC1B and ARP3 are components of the actin nucleator ARP2/3 complex, and phosphorylation of ARPC1B is required for activation of actin nucleation (LeClaire et al., 2008; Michard et al., 2015), suggesting that LegK2 may interfere with host actin polymerization by phosphorylating ARPC1B/ARP3 (Figure 2). In addition, ectopically expressed LegK2 in human HeLa cells disrupts formation of actin filaments, and LegK2 inhibits actin polymerization around the LCV in amoeba (Michard et al., 2015). Despite detection of ARPC1B and ARP3 phosphorylation by LegK2 using immunoblotting, mass spectrometry failed to detect phosphorylation of ARPC1B or ARP3 (Michard et al., 2015), indicating that phosphorylation level of ARPC1B and ARP3 by LegK2 is relatively low in the cells. Therefore, further analysis is necessary to dissect whether LegK2 disrupts host actin filaments through phosphorylation or interaction with the host ARPC1B/ARP3 and to determine whether the impact of LegK2 on host cytoskeleton is mediated entirely by modulating ARP2/3 complex or interactions with other host factors. Nevertheless, through yeast two-hybrid screen for LegK2 interacting partners, the ARP2/3 components are identified as host targets of the kinase effector, which may contribute to manipulation of host cytoskeleton rearrangement.

Pseudomonas HopBF1 phosphorylates host Hsp90 to prevent immune sensor activation

HopBF1 is a T3SS effector encoded by the plant pathogen *Pseudomonas syringae* and its homologs can be found in other plant and animal pathogens, such as *Ewingella americana* and *Burkholderia* spp. (Lopez et al., 2019). Despite limited primary sequence homology to eukaryotic kinases, HopBF1 exhibits predicted folding similar to protein kinases and aminoglycoside phosphotransferase. Crystal structure analyses with an *E. americana* HopBF1 homolog revealed that HopBF1 exhibits a minimal and atypical protein kinase folds (Lopez et al., 2019). Ectopic production of *P. syringae* HopBF1, but not its catalytic inactive variant, induces necrosis and collapse of leaf tissue in tobacco plants and inhibits cell growth in a yeast strain (Lopez et al., 2019), suggesting that HopBF1 targets a conserved host pathway in eukaryotic cells.

To identify host substrates of HopBF1, purified HopBF1 was incubated with yeast cell lysate and a phosphorylated protein product between 80-90kD was characterized (Lopez et al., 2019). Pulldown assays followed by mass spectrometry using yeast cell lysate containing inactive HopBF1 effector kinase revealed that yeast HSP82/HSC82, orthologs of human HSP90, co-precipitated with HopBF1 (Lopez et al., 2019), suggesting that HopBF1 interacts with eukaryotic HSP90. Consistent with the lysate-based phosphorylation experiment, *in vitro* kinase assays using purified proteins confirmed that HopBF1 directly phosphorylates serine-99 on yeast HSP90 (Lopez et al., 2019). Eukaryotic HSP90 functions as a molecular chaperone and plays an important role in plant immune responses against pathogens. Phosphorylation of host HSP90 by HopBF1 inhibits HSP90 ATPase and chaperone activity, leading to inactivation of plant nucleotide binding domain, leucine rich repeat containing proteins (NB-LRRs) that are important immune sensors against pathogens and interactors with plant HSP90 (Schulze-Lefert, 2004; Jones et al., 2016; Lopez et al., 2019; Figure 2). Because catalytically inactive HopBF1 also interacts with yeast HSP90 and many protein kinases are known clients of HSP90, it is likely that the interaction with host HSP90 stabilizes HopBF1 and HopBF1 mimics a host HSP90 client to interfere with the normal function of HSP90. Here, a technique of using a catalytic inactive effector kinase to co-precipitate host substrates, potentially through retaining the interactions with its substrates, lead to the identification of conserved HSP90 as a substrate of HopBF1. Together, this example illustrates the use of inactive enzymes to lock the transient enzyme-substrate interactions to facilitate substrate identification.

Yersinia YpkA phosphorylates actin-interacting proteins to cripple host phagocytosis

Early studies reported that host actin interacts with YpkA, strongly activating the kinase activity of YpkA, indicating that actin is an allosteric activator for YpkA (Juris et al., 2000). Crystal structure analysis of a YpkA/actin complex revealed that YpkA binds to actin monomers through the kinase domain and C-terminal GDI binding domain (Lee et al., 2015, 2017). YpkA prevents actin polymerization by binding at actin subdomain 4 to sequester actin monomers (Lee et al., 2015). However, the region between actin subdomains 1 and 3 that normally mediates actin regulator protein binding remains unoccupied in the YpkA/actin complex, which may allow actin regulatory proteins to be incorporated into the complex.

To identify additional host proteins in the YpkA/actin complex, SILAC (Stable Isotope Labeling of Amino acids in Cell culture) was utilized to label mouse RAW264.7 cell lysate (Lee et al., 2015). The C^{13} heavy isotope labeled cell lysate was incubated with YpkA. After affinity purification of His-tagged YpkA from the labeled lysate, mass spectrometry was used to identify host proteins interacting with YpkA by measuring the enrichment ratio of proteins co-purified with His-tagged YpkA from heavy isotope labeled lysate compared to protein purified from bead-only, non-labeled lysate. This approach detected known interacting partners, such as actin and the Rho GTPase Rac2, and several additional actin-binding proteins, including profilin, EVL, VASP, mDia1, INF2, WASP, WIP, gelsolin, and cofilin1 (Lee et al., 2015), suggesting that YpkA, actin, and these actin-binding proteins form a ternary complex. It is possible that YpkA may use host actin as a "bait" to recruit these proteins. To test this hypothesis, *in vitro* kinase assays were used with purified YpkA, actin, and identified actin-binding proteins. The *in vitro* assays showed that YpkA phosphorylated VASP, EVL, mDia1, WASP, and gelsolin in an actin-dependent manner (Lee et al., 2015). Adding gelsolin domain 1 (G1) as a competitor for the binding region between actin subdomain 1 and 3 decreased phosphorylation of the actin-binding proteins (Lee et al., 2015), which supports that YpkA uses actin as an activator for its kinase activity as well as a bait to recruit host actin-associated proteins into the YpkA/actin kinase complex for further phosphorylation. *In vitro* polymerization assays revealed that phosphorylation of VASP by YpkA

disrupts formation of actin filaments (Lee et al., 2015). In addition to phosphorylating host Gαq, these findings suggest an alternative mechanism by which YpkA interferes with host cytoskeleton rearrangement (Figure 2). This study demonstrates an approach of combining pulldown assays and SILAC to identify unknown substrates of the kinase effector from host cell lysate.

Phosphorylation proteomic screens for identifying host substrates of effector kinases

Substrate identification by testing components of host pathways affected by bacterial effector kinases may provide direct links between substrates and affected pathways, but may, however, be limited by the knowledge of components participating in the pathways and complicated by indirect effects from other pathways. Yeast two-hybrid screens and pulldown assays mainly identify host proteins that stably interact with the effector kinases but are likely to miss host substrates that have a transient interaction with the effector kinases. To achieve more comprehensive, non-biased screening, ATP analog-sensitive chemical genetic engineering and high-density protein microarrays combined with radioisotope ATP or non-radioactive ATPγS labeling have been developed and applied to identification of host substrates for bacterial effector kinases (Figure 1).

Chemical genetic engineering: Identifying Otubain 1 as a substrate of *Yersinia* YpkA and HSP70 as a substrate of *Legionella* LegK4

Identification of substrates with the use of purified effector kinases and radioactive ATP in cell lysate is challenging because of the high level of background phosphorylation by host kinases. To overcome this challenge, a chemical genetic technique was successfully developed by modifying the gatekeeper residues in the ATP-binding pocket of kinases of interest, which allows the modified kinases to accept synthetic ATP analogs with bulky groups attached to the N^6 position of the nucleotide (Shah et al., 1997). Because the bulky ATP analogs can only be used by the engineered analog sensitive (AS) kinase, but not wildtype kinases or endogenous kinases, only substrates of the AS kinase will be phosphorylated in the cell lysate upon co-incubation of the AS kinase and the bulky ATP analog (Figure 1E).

Based on this method, researchers mutated the methionine-211 residue in the kinase subdomain V of YpkA to glycine, which confers the $YpkA^{M211G}$ capable of using bulky ATP analogs (Juris et al., 2006). Co-incubation of the AS YpkA, radiolabeled N^6 ATP, and mouse J774 cell lysate revealed a ~36kD band was specifically phosphorylated by the AS YpkA (Juris et al., 2006). Gel-filtration and SDS-PAGE were used to isolate and separate the cytosolic fraction containing the 36 kD protein that was identified as otubain 1 (OTUB1), a deubiquitinating enzyme, by mass spectrometry (Juris et al., 2006). This phosphorylation event was further confirmed using *in vitro* kinase assays. Phosphorylation of OTUB1 by YpkA is actin-dependent, which is consistent to the model that YpkA uses actin as an allosteric activator (Figure 2). Interestingly, like other YpkA substrates, pulldown assays showed that OTUB1 also interacts with YpkA (Juris et al., 2006; Edelmann et al., 2010). It is likely that this phosphorylation modulates bacterial entry and uptake within the cell due to actin dependence and YpkA/OTUB1 complexing with the small GTPase RhoA (Edelmann et al., 2010).

Using radioactive bulky ATP analogs provides specificity and sensitivity to detect protein phosphorylation by AS kinases. However, it is still difficult to isolate and identify phosphorylated substrates from the cell lysate since the substrate is labeled by phosphates with radioisotopes that do not have significant chemical differences for affinity purification. To facilitate isolation of labeled substrates, a method that uses bulky ATPγS as phosphate donors was developed (Allen et al., 2007). The γ-thiophosphate of the bulky ATP analogs can be transferred to protein substrates by majority of kinases, and the sulfur atom on the γ-thiophosphate can serve as a molecular handle for chemical modifications, such as alkylation by *p*-nitrobenzylmesylate (PNBM). Furthermore, generation of an antibody that specifically recognizes the thiophosphate ester moiety conjugated on thiophosphorylated serine/threonine/tyrosine residues after PNBM alkylation allows for detection and purification of thiophosphorylated substrates by immunoblotting and immunoprecipitation, respectively (Allen et al., 2007).

Using the bulky ATPγS labeling technique, a host substrate of *L. pneumophila* effector kinase, LegK4, was recently identified. LegK4 contains primary sequence homology to eukaryotic serine/threonine kinases (Hervet et al., 2011) and is one of the *L. pneumophila* effectors that inhibit host protein translation when ectopically produced in HEK cells (Barry et al., 2013; Moss et al., 2019). Surprisingly, LegK4 can use N^6 benzyl ATPγS as a phosphate donor without the need to engineer the gatekeeper residues in its ATP binding pocket. Co-incubation of purified LegK4, N^6-benzyl ATPγS, and HEK cell lysate revealed two thiophosphorylated protein bands with molecular weights between 70-80kD (Moss et al., 2019). After immunoprecipitation of the thiophosphorylated proteins and mass spectrometry, it was discovered that LegK4 directly phosphorylates the host HSP70 family proteins, HSC70, HSP72, and Bip, on the threonine-495 of HSC70/HSP70 (threonine-518 of Bip; Moss et al., 2019; Figure 2). HSP70 family proteins contain ATPase activity and function as chaperones that regulate protein folding and homeostasis. The phosphorylation of HSC70 by LegK4 results in reduced ATPase activity and protein folding activity *in vitro,* and ectopic expression of LegK4 in mammalian cells causes suppression of the unfolded protein response and protein translation (Moss et al., 2019), phenomena that likely result from phosphorylation of HSC70 by LegK4.

Legionella LegK7 phosphorylates MOB1, the scaffold protein in the host hippo pathway

L. pneumophila LegK7 is a T4SS effector kinase that is conserved in many sequenced *Legionella* genomes. Unlike other previously known *L. pneumophila* effector kinases, LegK1 to LegK4, LegK7 has limited primary sequence homology to eukaryotic serine/threonine kinases but a high degree of protein folding similarity to eukaryotic kinases based on HHPred prediction (Lee and Machner, 2018). To identify host substrates of *L. pneumophila* effector kinases, a high throughput screen platform that combines the ATPγS labeling technique and a high-density protein microarray was developed (Lee and Machner, 2018). Purified LegK7 proteins were co-incubated with ATPγS on a protein microarray containing ~10,000 purified human proteins, and thiophosphorylated protein spots were then alkylated by *p*-nitrobenzylmesylate (PNBM; Lee and Machner, 2018; Figure 1F). This modification can be detected by the specific antibody that recognizes the thiophosphate ester moiety as mentioned in the earlier section followed by a fluorescence conjugated secondary antibody. This platform provides

fast screening of thousands of human proteins for potential direct substrates *in vitro* with advantages of high sensitivity based on antibody-fluorescence detection and use of non-radioactive ATP analogs without engineering the kinase. Screens using this platform revealed that LegK7 directly phosphorylates host MOB1 (Mps one binder kinase activator-like 1), a key scaffold protein of the conserved Hippo pathway, to alter host gene expression to promote *L. pneumophila* infection (Lee and Machner, 2018; Figure 2).

The eukaryotic Hippo pathway plays important roles in tissue development and cancers by regulating cell growth and proliferation (Harvey et al., 2013). The scaffold protein, MOB1, is phosphorylated by the Hippo kinase MST1 in humans, on threonine-12 and-35, which allows phosphorylated MOB1 to interact with the LATS/NDR kinases and activate their kinase activity (Praskova et al., 2008). Like host MST1, *L. pneumophila* LegK7 phosphorylates threonine-12 and-35 of MOB1 (Lee and Machner, 2018). Unexpectedly, kinase activity of LegK7 was elevated upon co-incubation with MOB1 in an *in vitro* kinase assay, suggesting that MOB1 is not only a substrate, but also an activator of LegK7 (Lee et al., 2020). Structural analyses of a LegK7/MOB1 complex confirmed that MOB1 interacts with the N-terminal non-kinase domain and the kinase domain of LegK7 at an interface opposite the catalytic cleft and that disruption of the interaction abolished upregulation of LegK7 kinase activity (Lee et al., 2020). Furthermore, the LegK7/MOB1 complex utilizes the intrinsic activity of MOB1 N-terminal extension to recruit downstream substrates, such as the host transcription factor YAP1, into the kinase complex for phosphorylation (Lee et al., 2020). Thus, *L. pneumophila* LegK7 is a functional chimera of two core kinases, MST1 and LATS/NDR, of the host hippo pathway. Like *Yersinia* YkpA exploits host actin, LegK7 uses its MOB1 substrate as an allosteric activator and recruiter for downstream host substrates.

Escherichia coli NleH1/NleH2 phosphorylate host EPS8 to modulate formation of intestinal microvilli

Enteropathogenic *E. coli* encode two T3SS effectors, NleH1 and NleH2, that are on different genomic loci, but highly similar with 84% amino acid sequence identity (Gao et al., 2009). NleH1/NleH2 contain kinase domains that are similar to T3SS effector kinases in other enteropathogenic bacteria, including *Shigella flexneri* OspG (Tobe et al., 2006), *Citrobacter rodentium* NleH (García-Angulo et al., 2008), and *Vibrio parahaemolyticus* VopG (Plaza et al., 2021). NleH1/NleH2 can prevent host apoptosis *via* their ability to interact with Bax inhibitor-1 (BI-1), an anti-apoptotic protein in host cells. However, the anti-apoptotic effect does not require the kinase activity of the NleH effectors (Hemrajani et al., 2010), suggesting that BI-1 or other host proteins controlling apoptosis are not phosphorylated by the NleH effectors. Studies have shown that another host pathway affected by the NleH effectors is the NFκB signaling pathway (Gao et al., 2009; Wan et al., 2011; Pham et al., 2013). NleH1 suppresses phosphorylation of ribosomal protein S3 (RPS3), a component of the NFκB complex, by altering substrate specificity of IκB kinase β (IKKβ; Wan et al., 2011). Moreover, NleH1 interacts with RPS3 (Gao et al., 2009). Interestingly, NleH1 kinase activity is essential for altering substrate specificity of IKKβ and suppressing RPS3 phosphorylation (Wan et al., 2011); however, both IKKβ and RPS3 are not substrates of NleH1.

A screen for host substrates of NleH1 was performed by co-incubation of purified NleH1 with radioactive γ-^{33}P ATP on a high-density human protein microarray (Pham et al., 2013). This technique is the same in principle as the LegK7 substrate screen discussed in the previous section, and the main difference is the detection methods for phosphorylated host proteins on the microarrays. The NleH1 screen identified v-Crk sarcoma virus CT10 oncogene-like protein (CRKL), EGF receptor kinase pathway substrate 8-like protein 2 (EPS8L2), and microtubule-associated protein RP/EB family member 1 (MAPRE1) are NleH1 candidate substrates (Pham et al., 2013). Separate *in vitro* kinase assays confirmed that NleH1 directly phosphorylates CRKL, and pulldown assays revealed that these two proteins stably interact (Pham et al., 2013). While a catalytic inactive NleH1 effector kinase failed to interact with CRKL, it is unclear whether this interaction is mediated by CRKL phosphorylation or autophosphorylation of NleH1. Nevertheless, knocking down CRKL expression by siRNA reduces impacts of NleH1 on RPS3, which is possibly due to that CRKL can interact with IKKβ (Pham et al., 2013).

Recently, another attempt to identify host substrates for NleH1 and NleH2 was conducted using label-free phosphoproteome analyses (Pollock et al., 2022) in infected host cells (Figure 1G). Human colon cancer cells, HT-29, were infected with wildtype enteropathogenic *E. coli* or mutants with the NleH effector genes deleted. Phosphorylated peptides were isolated by TiO2-beads from the infected cell lysate, and comparative mass spectrometry was used to quantitate phospho-peptides that were enriched in the host cells infected with wildtype bacteria. This assay revealed increased phosphorylation in multiple host proteins. Surprisingly, EGF receptor kinase pathway substrate 8 (EPS8) family proteins, including EPS8, EPS8L1 and EPS8L2, had the highest levels of phosphorylation (Pollock et al., 2022). EPS8L2 was previously identified as a putative substrate of NleH1 in the screen using human protein microarrays (Pham et al., 2013), suggesting that the EPS8 family proteins are likely genuine substrates of NleH1. Serine-775 of EPS8 was confirmed as a specific phosphorylation site by NleH1 and NleH2, and phosphorylation of this residue affected EPS8 bundling activity and cellular localization in host cells (Pollock et al., 2022). Moreover, the NleH effectors interact with EPS8, and this interaction, together with the kinase activity, are required for suppressing EPS8 bundling (Pollock et al., 2022; Figure 2).

Effector kinases with unknown host targets

Thus far, a plethora of techniques to identify host substrates of bacterial effector kinases, such as host pathway characterization, protein–protein interactions, and ATP analog-based proteomic screen techniques, have proven to be applicable. Bacteria are constantly evolving to target new host pathways and avoid host defenses. These bacterial effector kinases possess intriguing abilities to phosphorylate versatile host substrates and utilize host factors as allosteric activators. However, while there exists other bacterial effector kinases with no known host substrates, recent advances may allow for the identification of previously challenging kinase substrates and their effects in host cell signaling.

Shigella OspG and host NFκB pathway

Shigella flexneri T3SS effector OspG shares primary sequence homology with eukaryotic kinases and *E. coli* NleH1/NleH2 (Zhou

et al., 2013), as discussed in the previous section. Using a yeast two-hybrid screen, host E2 ubiquitin conjugating enzymes, such as UbcH5c and UbcH5b, were revealed to interact with OspG, and *in vitro* co-purification assays showed that OspG specifically interacts with ubiquitin-conjugated UbcH5b (Kim et al., 2005). Ubiquitin-conjugated UbcH5c stimulated autophosphorylation of OspG, and structural analysis of OspG/UbcH5c ~ ubiquitin complex revealed that OspG contacts both UbcH5c and ubiquitin (Pruneda et al., 2014). Consequently, binding with UbcH5c ~ ubiquitin stabilizes OspG in an active conformation, showing that UbcH5c ~ ubiquitin complex is an allosteric activator for OspG (Pruneda et al., 2014). However, no UbcH5c phosphorylation was observed in an *in vitro* kinase assay, indicating that UbcH5c is not a substrate of OspG.

UbcH5c is one of the components of the SCF complex, which mediates ubiquitination of phosphorylated IκB for subsequent proteasomal degradation. Ectopic expression of wild type OspG, but not catalytically inactive OspG, prevents degradation of IκBα and suppresses expression of an NFκB-driven luciferase reporter in TNFα-treated HEK cells (Kim et al., 2005), suggesting that OspG inhibits the host NFκB pathway. Consistently, human HeLa cells infected with an *S. flexneri ospG* mutant had faster IκBα degradation than cells infected with wild type *S. flexneri*, and rabbits infected with *ospG* knockout *S. flexneri* displayed more severe inflammation in the intestinal ligated ileal loop model (Kim et al., 2005). While it is clear that OspG affects the NFκB pathway and modulates host inflammatory responses during infection, host substrates of OspG remain unidentified.

Legionella Lem28 effector kinase is activated by host inositol Hexakisphosphate

L. pneumophila effector, Lem28, is encoded by the gene *lpg2603*, and its homologs can be found in other *Legionella* species. Functional motif searches suggest that Lem28 contains a phosphatidylinositol-4-phosphate (PI4P) binding domain in the C-terminus and a putative tyrosine kinase domain in the N-terminus (Burstein et al., 2016). Like other *L. pneumophila* PI4P-binding domain containing effectors, SidM and Lpg1101, the PI4P binding activity is required for localizing Lem28 to the host plasma membrane. In infected host cells, Lem28 is secreted by the T4SS and associated with the *Legionella*-containing vacuole (LCV); however, Lem28 without PI4P-binding activity still localizes on the LCV. When ectopically produced in yeast, Lem28 suppresses yeast growth and requires the PI4P binding domain (Hubber et al., 2014), suggesting that this effector disrupts a conserved eukaryotic pathway. Testing recombinant Lem28 kinase activity using *in vitro* assays did not detect autophosphorylation of the effector kinase or phosphorylation of a pseudo-substrate, myelin basic protein (MBP). Because many bacterial effectors use host factors to activate their enzymatic activities, the addition of host factors into the kinase assays lead to the interesting discovery that Lem28 co-incubated with inositol hexakisphosphate (IP6) phosphorylates MBP (Sreelatha et al., 2020). Biochemical and structural analyses also confirmed that IP6 interacts with Lem28 and that this interaction stabilizes Lem28 and facilitates ATP-binding. These assays demonstrate that IP6 is an allosteric activator for Lem28 kinase activity, which is an additional example of a bacterial effector being activated by a trans-kingdom activator from the host cells (Anderson et al., 2015). Although substrate identification *via* isolating host proteins interacting with the effector was attempted (Anderson et al., 2015), host proteins phosphorylated by Lem28 are still unknown. Thus, substrate screening from host cell lysate using phosphoproteomic mass spectrometry or a high-throughput microarray with Lem28 co-incubated with IP6 are potential options to address the transient interactions between this effector kinase and its host substrates.

Vibrio VopG contains key catalytic residues similar to NleH effector kinases

Vibrio parahaemolyticus uses a T3SS to secrete nine effector proteins into host cells during infection to target actin cytoskeleton and inflammatory responses, although more effectors are continuously being identified (Plaza et al., 2021). The newly identified *V. parahaemolyticus* VopG is a homolog of *Vibrio cholerae* VopG, and exists in many *Vibrio* species (Plaza et al., 2021). Using HHPred and pGenTHREADER to analyze *V. parahaemolyticus* VopG, sequence and structural similarity to the NleH family of T3SS effector kinases in *E. coli* and *S. flexneri* was found (Plaza et al., 2021), suggesting a potential role for VopG in phosphorylation of host substrates. NleH proteins display functional similarity in their serine/threonine kinase domain to eukaryotic kinases and promote intracellular replication by targeting NFκB signaling (Hodgson and Wan, 2016), preventing programmed cell death and defensive immune responses. Recently, VopG's sequence was analyzed and discovered to contain key catalytic residues and motifs required for kinase activity and shares specific residues with the NleH family of proteins involved in phosphorylation (Plaza et al., 2021). However, dissimilar to NleH proteins, VopG did not appear to suppress apoptosis or IL-8 production, suggesting a novel, but unidentified role for VopG in host cells (Plaza et al., 2021). While VopG likely acts as an effector kinase based its sequence and structural similarity, its activity and host substrate are both uncharacterized, lending to the importance of multiple approaches for substrate identification and pathway interactions.

New approaches for effector kinases and host substrate identification

Thus far, we have described a multitude of techniques that have been used to identify bacterial effector kinases and their host substrates. There are, however, other approaches that may also be applicable identification methods for effector kinases and host substrates. For example, similar to the ATPγS protein microarray used to screen the substrates of LegK7 as previously described, peptide microarrays may also be utilized for identification of peptide sequences or motifs that are preferred by bacterial effector kinases. This method relies on the phosphorylation screening of a microarray chip containing short amino acid motifs co-incubated with the kinase of interest (Xue and Tao, 2013; Figure 3). This approach has been used to identify host substrates of a kinase from Kaposi's sarcoma-associated herpesvirus (KSHV), vPK (Bhatt et al., 2016). The screening revealed that vPK preferentially phosphorylates peptide motifs overlapping with the motifs phosphorylated by the host kinase S6KB1, suggesting that vPK might also target the same substrates. Further analysis demonstrated that vPK functionally mimics S6KB1 to phosphorylate ribosomal S6 and promote protein translation in host cells (Bhatt et al., 2016). While this method is relatively less expensive than full protein microarrays, it mainly provides information related to the preferred substrate sequence motifs of a kinase and does not identify a direct substrate interaction. Any proteins that contain the sequence motifs could be potential substrates and will require additional validation. However, the recent construction of a kinome database of mammalian kinase substrate sequences may provide further aid in

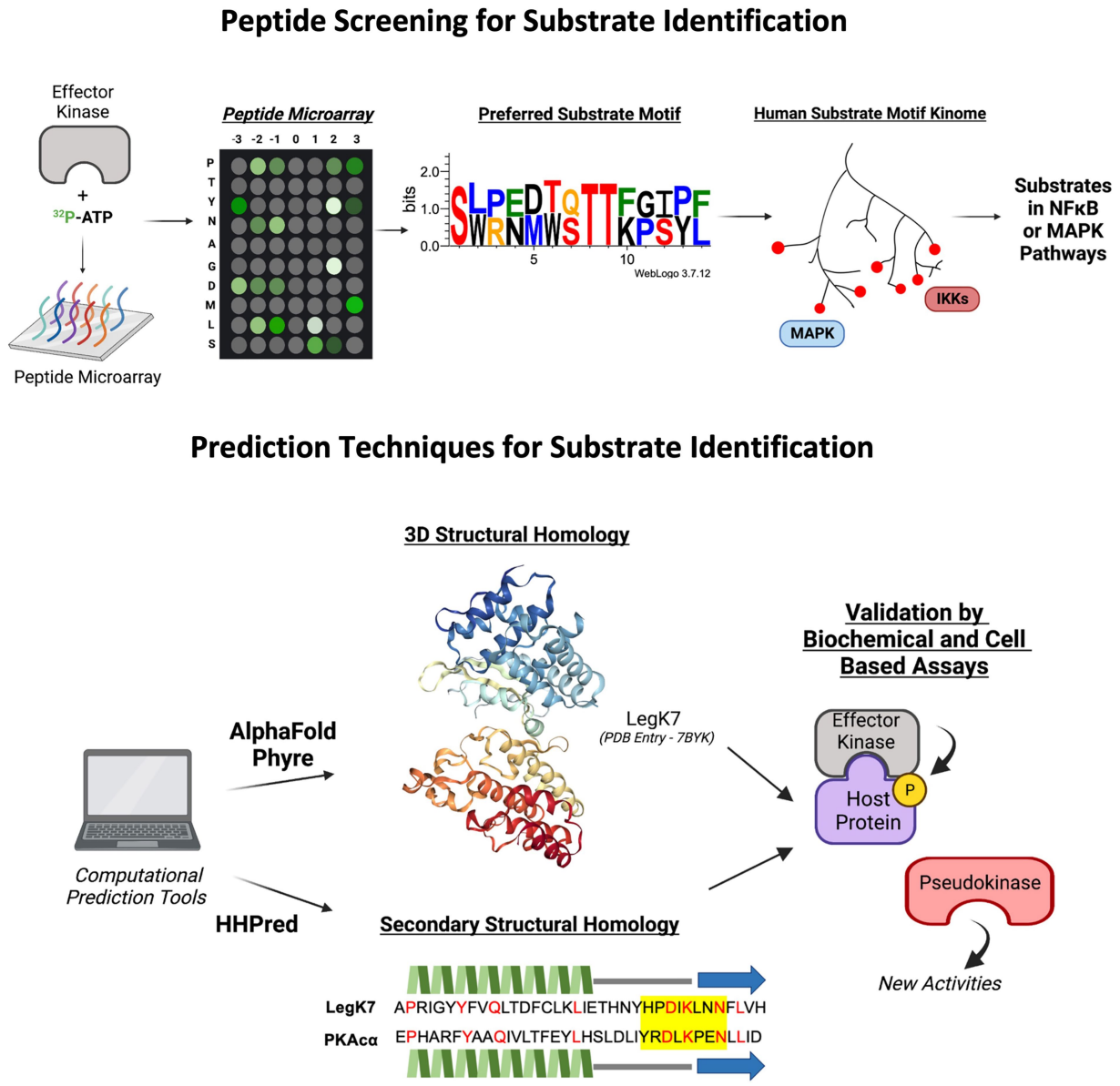

FIGURE 3
Novel predictive tools and applications, such as peptide screens and computational structure and folding algorithms in identifying putative effector kinases and their host substrates. LegK7 crystal structure from Protein Database (PDB Entry Number: 7BYK). Protein motif sequence generated through WebLogo3. The predicted secondary structure alignment of PKAcα and LegK7 is shown. Beta strands are represented by blue arrows, helices are represented by green jagged lines, loops are represented as straight gray lines, catalytic motif is highlight yellow, and identical residues are indicated by red color.

narrowing the search to specific substrate families or pathways that may be targeted by the effector kinase through shared preference in substrate motifs (Johnson et al., 2023; Figure 3).

With the advancements in artificial intelligence (AI)- and neural network-based algorithms to compute complex biological interactions, predictive computational tools may prove beneficial in the identification of effector kinases based on structural and folding homology to known kinases (Figure 3). For example, AlphaFold, HHPred, and Phyre2 are complex *in silico* applications capable of predicting three-dimensional protein structure and folding probabilities based on the protein's amino acid sequence, and in the case of AlphaFold, to near atomic accuracy even without any homologous structure (Hildebrand et al., 2009; Kelley et al., 2015; Jumper et al., 2021). Applications such as AlphaFold have recently been used to characterize the conformational properties of eukaryotic kinases such as diacylglycerol kinases (DGKs; Aulakh et al., 2022) and may aid in the identification of bacterial effector kinases through structural and folding pattern predictions. *In silico* techniques are constantly evolving, such as the AlphaFold partner algorithm, AlphaFill, which uses sequence and structural information to fill in missing small molecules such as ATP (Hekkelman et al., 2022). These new technologies may further advance the ability to predict putative effector kinases for following experimental validation and functional studies.

Conclusion

Kinases play a central role in signal transduction in living cells. Many bacterial pathogens deliver effector kinases into their host cells to interfere with signaling and subvert immune responses. Studying bacterial effector kinases has revealed how host responses, such as

phagocytosis and inflammatory gene expression are manipulated. Moreover, investigation of bacterial kinases has led to the discovery of new cellular components and pathways, including heat shock chaperone proteins and the Hippo pathway, which serve as targets for bacterial effector kinases and play new roles in microbial pathogenesis. With the increasing number of sequenced bacterial genomes, more effector kinases are expected to be discovered. For example, a genomic analysis that included 58 *Legionella* genomes showed that these *Legionella* species encode more than 18,000 T4SS effectors and eukaryotic protein kinase motifs are the second most abundant functional motifs found in these effectors based on primary sequence homology (Gomez-Valero et al., 2019). In addition, like *Pseudomonas* HopBF1 and *Legionella* LegK7, effector proteins that have limited sequence homology but high folding similarity to eukaryotic kinases have been discovered with advances in structural prediction tools, which opens a new class of effector kinases that remain to be explored. More interestingly, a *Legionella* effector SidJ, which also shares structural similarity and borderline sequence homology to eukaryotic kinases, has recently been shown to be a "pseudokinase" with novel enzyme activity capable of polyglutamylating the *Legionella* SidE family of effectors (Black et al., 2019). The discovery of pseudokinases presents another layer of complexity in identifying effector kinases and their substrates, due to these effectors being characterized as catalytically inactive in protein phosphorylation, but with structural or sequence homology to known kinases (Poh et al., 2008). For example, *Pseudomonas syringae* SelO shares structural and folding homology with eukaryotic kinases, but has been shown to catalyze AMPylation (Sreelatha et al., 2018; Pon et al., 2023). SidJ and SelO, among other microbial proteins, demonstrate that sequence or structural homology is not sufficient to categorize effectors as kinases, and their biochemical activity must be experimentally tested. Previously, SidJ was shown to modify another *L. pneumophila* effector, SdeA, and mass spectrometry analysis revealed glutamylation of sdeA as a new activity of SidJ (Black et al., 2019). *In vitro* assays using α-P32 labeled ATP instead of γ-P^{32} ATP for protein phosphorylation uncovered self-AMPylation of SelO, and further mass spectrometry analysis detected its AMPylation activity (Sreelatha et al., 2018). Some effectors with putative kinase domains, such as *V. parahaemolyticus* VopG, have not been experimentally validated for their biochemical activity. Thus, it is important to examine whether VopG may possess another activity other than protein phosphorylation. While new biochemical activities of effectors that share structural and sequential homology to kinases are exciting, challenges arise in predicting and determining their new activities. These discoveries highlight the diverse and novel functions of the effector kinase superfamily in bacterial pathogenesis.

Throughout this review, we have described multiple techniques to identify many novel bacterial effector kinase substrates (Figure 1). Each of these methods presents inherent advantages and limitations that require additional validation, and different approaches may lead to the discovery of different sets of kinase substrates, highlighting the versatile impact of effector kinases on host signaling (Figure 2). It is evident that many biological processes in host cells are highly regulated by protein phosphorylation. Although pathogens secrete phylogenetically distinct effector kinases and target different host substrate proteins, many of them converge on the same pathway with similar outcomes in the host cell. For example, SteC from *S. enterica* and LegK2 from *L. pneumophila* phosphorylate different host proteins but both result in cytoskeletal rearrangement. Conversely, effectors from different organisms that share a high degree of sequence similarity, such as NleH1/2 and OspG, target different host pathways and result in different cellular outcomes. These examples highlight the importance of protein phosphorylation in the host and the diversity in substrate targeting by effector kinases during infection. Ultimately, advancements in the discovery and characterization of effector kinases and their function in subverting host defenses to promote bacterial survival and replication allows for novel antibiotic targets and therapeutic treatments.

Author contributions

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Conflict of interest

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Co-infection of the respiratory epithelium, scene of complex functional interactions between viral, bacterial, and human neuraminidases

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The activity of sialic acids, known to play critical roles in biology and many pathological processes, is finely regulated by a class of enzymes called sialidases, also known as neuraminidases. These are present in mammals and many other biological systems, such as viruses and bacteria. This review focuses on the very particular situation of co-infections of the respiratory epithelium, the scene of complex functional interactions between viral, bacterial, and human neuraminidases. This intrinsically multidisciplinary topic combining structural biology, biochemistry, physiology, and the study of host-pathogen interactions, opens up exciting research perspectives that could lead to a better understanding of the mechanisms underlying virus-bacteria co-infections and their contribution to the aggravation of respiratory pathology, notably in the context of pre-existing pathological contexts. Strategies that mimic or inhibit the activity of the neuraminidases could constitute interesting treatment options for viral and bacterial infections.

KEYWORDS

influenza viruses, host-pathogen interactions, bacterial superinfections, co-infection, respiratory epithelium, neuraminidase (NA), sialic acids, paramyxoviruses

Introduction

Sialic acids and their importance in the respiratory epithelium

Sialic acids (SA) are typically found on the extremities of glycan chains in nearly all cell types. These acidic sugars with a nine-carbon backbone are present at all cell surfaces and most secreted proteins and lipids of vertebrates and large invertebrates, mediating or modulating various normal and pathological processes (Varki, 2008; Lewis and Lewis, 2012; Schauer and Kamerling, 2018). SA are highly expressed on the surface of the epithelial cells that constitute the airways and are also major components of the secreted mucins in the respiratory tract. SA are a main source of negative charge and

hydrophilicity that contribute to the rheological properties of mucus (Varki, 2008). The mucus is a complex secretion including mucins, mainly composed of carbohydrate chains. It can absorb excess water, which confers viscoelasticity and maintains hydration of the respiratory tract and mucociliary clearance (Lewis and Lewis, 2012; Zanin et al., 2016). In the airways, sialylated glycoproteins and glycolipids are strategically positioned on the plasma membranes of epithelia to regulate receptor-ligand, cell-cell, and host-pathogen interactions at the molecular level.

The structure of SA is related to their chemical link to the glycan (in particular, O-, or N-glycan), and this structure determines their biological properties. The mother molecule of the SA family is the neuraminic acid (Neu); this molecule does not occur as a free form in nature as it is immediately transformed by cyclization as an internal Schiff base. Other important SA are the N-acetylneuraminic acid (Neu5Ac), the N-glycolylneuraminic acid (Neu5Gc), and the keto-deoxy-nononic acid (Kdn) (Schauer and Kamerling, 2018). Neu5Gc is absent in humans, ferrets, seals, dogs, and most avian species (because of the absence of the enzyme cytidine monophosphate N-acetylneuraminic hydroxylase, but this SA is present in equine, bovine, and swine species). The N- and O-glycan composition of the human respiratory tract, including the nasopharynx, bronchus, and lungs, was studied by mass spectrometry, highlighting the complexity and broad spectrum of SA α2-3 and α2-6 glycans both present in the bronchus and lung tissue (Walther et al., 2013). Recently, new studies of the composition of the lung glycome revealed greater complexity with large complex N-glycans with linear poly-N-acetyllactosamine (PL) extensions terminated with α2-3 linked SA, smaller N-glycans terminated with α2-6 linked SA, and large glycosphingolipids (GSLs) glycans containing linear PL terminated with α2-3 linked SA (Jia et al., 2020).

The biology of SA is finely regulated by a class of enzymes called sialidases, also known as neuraminidases (NAs EC 3.2.1.18), that cleave the glycosidic bonds of terminal SA form carbohydrates, glycolipids, or glycoproteins (sialo-conjugates). NAs are present in a wide range of biological systems, such as viruses, bacteria, fungi, protozoa, and Mammalia (von Grafenstein et al., 2015). In this minireview, we will focus on viral, bacterial, and human neuraminidases and their functional interactions in the context of respiratory co-infections.

Three types of Neuraminidases and their respective roles

The Carbohydrate Active Enzymes database classifies NAs into glycoside hydrolase (GH) families 33, 34, 58, and 83 [1](Drula et al., 2022). The GH33 family comprises non-viral NAs, including human hydrolytic NAs (NEU1, NEU2, NEU3, and NEU4) and bacterial hydrolytic NAs, *trans*-sialidases, and anhydro-sialidases. The influenza virus NAs are classified in the GH34 family, and the *Paramyxoviridae* hemagglutinin-neuraminidases (HN) are classified in the GH83 family (**Table 1**). GH58 family comprises endo-N-acetyl neuraminidases (also termed endo-sialidases) found in bacteriophage K1F, for example (Stummeyer et al., 2005).

1 http://www.cazy.org

The common structure of NAs is conserved up to the tertiary level with a common six-blade beta-propeller fold around a catalytic site, with several residues at similar positions in members of both families, as determined by X-ray crystallography (Taylor, 1996). Interestingly, their quaternary structures are distinct. Indeed, viral NAs are homotetramers by the assembly of the catalytic domain and need to be tetramerized to be catalytically active, while most other NAs are monomers or associate with oligomers via adjacent protein domains (Air, 2012).

Influenza virus neuraminidases

Discovered more than 80 years ago by Hirst (1942), the NA of influenza viruses is undoubtedly the one that has been most characterized in terms of structure, activity, and biological functions. Each monomer of the influenza virus NA tetramer contains around 470 amino-acids (aa) (MW 50–60 KDa) (depending on the type of NA) and is folded from the N-terminal to the C-terminal end by a cytoplasmic tail, a transmembrane region, a stalk, and a catalytic head (Colman et al., 1983; McAuley et al., 2019). The tetrameric form allows an optimal enzymatic activity of the NA. The N2 NA is the primary reference model for numbering the residues involved in NA functionality. The catalytic site, in direct contact with the sialic acid substrate, is formed by eight highly conserved residues: three conserved Arginine residues (Arg118, Arg292, and Arg371) interact with the carboxylate group of the SA, Arg152 binds to the acetamido group on the sugar ring, and the Glu276 interacts with the 8- and 9- hydroxyl groups; the Tyr406/Glu276 is the nucleophile pair, and the Asp151 is responsible for the acid/base catalysis reaction. The catalytic site is surrounded by framework residues playing a structural role (Colman et al., 1993; McAuley et al., 2019).

The neuraminidase (NA) also has non-cleavage binding activity, mediated by a second sialic acid-binding site (2SBS) distinct from the NA catalytic site. Oseltamivir carboxylate preferentially binds to NA catalytic site, and 2SBS can attach to α2-3 sialyllactose in presence of oseltamivir carboxylate. The 2SBS site is a highly conserved feature in most avian NA (except for N3) and seems to help recruit SA substrates due to its proximity to the catalytic neuraminidase site. The 2SBS is modified or lost after adaptation from avian to another host species (the lack of conservation of the 2SBS in N1 may represent a marker of human adaptation) (McAuley et al., 2019; Du et al., 2021). Moreover, the catalytic site of the NA may also play a role in the binding to SA, as it is observed for recent human A(H3N2) viruses (Lin et al., 2010).

The different subtypes of NA are phylogenetically classified within group 1 (N1, N4, N5, and N8) and group 2 NAs (N2, N3, N6, N7, and N9); the NA of influenza B viruses form a separate group (Russell et al., 2006). The crystal structure of the different NA heads was now established for at least one representative of each subtype (Sun et al., 2014). The neuraminidase-like glycoproteins from H17N10 and H18N11 viruses isolated from bats do not contain the conserved residues involved in sialic acid binding and cleavage (García-Sastre, 2012; Li et al., 2012; Zhu et al., 2012).

Avian IAV hemagglutinin (HA) typically recognizes SA α2-3, whereas human IAV HA recognizes SA α2-6 linked to glycans. These different SA linkages have different structural conformations.

TABLE 1 Classification and main characteristics of viral, bacterial, and human neuraminidase.

Glycoside hydrolase family (GH)	Organism	Enzyme Name	Enzymatic activity	Structure
GH33	Human NA	NEU1 (lysosomes) NEU2 (cytosol) NEU3 (plasma membrane) NEU4 (lysosomes or mitochondria and endoplasmic reticulum)	Hydrolytic neuraminidases	monomer or oligomer
	Bacterial NA	*Streptococcus pneumoniae* (Nan A, Nan B, Nan C) Vibrio cholerae (NanH)	Hydrolytic neuraminidases (NanA, NanH) Intramolecular trans-sialidases (anhydrosialidases) (NanB)Trans-sialidases (NanC)	
GH34	Viral NA	Influenza A Virus NA (N1 to N9 Influenza B Virus NA	Hydrolytic neuraminidases	homotetramer
GH83	Viral NA	*Paramyxoviridae* family HN	Hydrolytic neuraminidases	

NA, neuraminidases; HN, hemagglutinin-neuraminidase.

In a human airway epithelium model, avian IAVs preferentially infect ciliated cells. In contrast, human IAVs infect secretory non-ciliated cells: this particular cellular tropism correlates with the predominant localization of SA α2-3 or α2-6 receptors, respectively (Matrosovich et al., 2004). In human airway organoids that had similar characteristics to human airways epithelium, H1N1pdm09, H7N9, and H5N6 influenza viruses were all able to infect both ciliated cells and non-ciliated goblet cells, but not basal cells (Hui et al., 2018). When an avian IAV adapts to humans, its HA specificity evolves from α2-3 to α2-6 specificity improving human transmission; the NA specificity may evolve in the same way; however, the NA maintains a capacity for cleaving α2-3 linkages to allow virus movement toward the mucus barrier rich in α2-3 mucins (McAuley et al., 2019). Influenza strains vary in their specificity regarding the type of linkage (α2-3 or α2-6) and the type of SA residue Neu5Ac, Neu5Gc, and 9-O-Ac-Neu5Ac (Villar and Barroso, 2006).

The biological functions of NA in influenza viruses are closely related to those of the other surface glycoprotein, the HA. Both NA and HA interact with SA, and an optimal viral infection needs a balance between NA and HA activities both at the entry phase of the virus, including entry in the respiratory tract, attachment to the cell surface, and at the stage of exit after budding of viral particles (Dou et al., 2018; McAuley et al., 2019; de Vries et al., 2020).

The NA has a role in the first steps of infection (Matrosovich et al., 2004). When viral particles contained in aerosols or mucosal secretions are deposited on the mucosal surface, NA activity enables the cleavage of SA α2-3 in mucus and facilitates the access of virions to epithelial cells. At the surface of the epithelial cells, virions move to reach the base of cilia, where endocytosis is possible. By cleaving SA, NA facilitates the movement of virions on the cell surface: the NA cuts the SA and prevents the virions from rolling back. The balance of NA and HA activity drives the motility of viral particles to reach cellular sites allowing receptor-mediated endocytosis (Sakai et al., 2017; de Vries et al., 2020). Using fluorescence labeling and super-resolution microscopy, it was shown that NA glycoproteins are present in clusters at a pole of filamentous viral particles and visualized that the alternate between binding and cleaving activity causes virus directional motility away from their rich NA pole (Vahey and Fletcher, 2019).

In recent years, the entry phases of influenza viruses were better described. The different attachment routes were recently reviewed (Karakus et al., 2020; Sempere Borau and Stertz, 2021), suggesting a multivalent binding mode of virions at the cell surface and redundant ways for viral entry. The attachment of viral particles is mediated by HA and NA on sialoglycans (O-glycans, N-glycans, and Glycosphingolipid (GSL)-glycans present on cellular glycoproteins and glycolipids) and also on non-sialylated structures on phosphorylated glycans (Byrd-Leotis et al., 2019; Jia et al., 2020). The attachment and entry are also mediated by cellular co-receptors that do not bear sialoglycans: the link is rendered possible by the glycans present on the HA (Hillaire et al., 2013). After the attachment of viral particles on cells, internalization is mediated by clathrin-dependent or independent endocytosis and macropinocytosis (Karakus et al., 2020). Finally, NAs play a role in the late phases of the viral cycle enabling the efficient release of new virions by cleaving SA, which retain virions in cells and prevents the aggregation of virions between them by cleaving SA from the HAs of the virions (Air, 2012; McAuley et al., 2019).

Parainfluenza neuraminidases

Human parainfluenza viruses (hPIV) are responsible for acute respiratory tract infections. These viruses are classified among the *Paramyxoviridae* family in two genera: hPIV1 and hPIV3 are Respirovirus, and hPIV2 and hPIV4 are Rubulavirus. These viruses bear different surface glycoproteins: the hemagglutinin-neuraminidase (HN) and the fusion protein (F). Following receptor recognition and endocytosis, the conformational change in HN activates the F protein mediating the fusion between viral and cellular membranes resulting in viral entry into the cell (Henrickson, 2003).

The global structure of HN is similar to the Influenza virus NA with a homotetramer conformation. The head domain of each monomer contains a receptor binding and a cleaving activity. Neu5Ac was identified as a receptor of HN. The role of HN was identified at the end of the viral multiplication cycle to promote the release of virions from the cells after budding. Conversely to influenza virus bearing distinct HA and NA glycoproteins, for hPIV HN both SA binding and cleaving functions reside in the same domain (Villar and Barroso, 2006).

The sequence alignment of hPIV HN and influenza NA has revealed the conservation of specific amino-acids, but framework residues are not conserved between IAV NA and hPIV HN (Colman et al., 1993). The catalytic site is formed by seven conserved aa residues (Arg192, Arg424, Arg502, Tyr530, Glu409, Glu549, and Asp216). The three Arg residues in positions 192, 424, and 502 interact with the carboxyl group of the sialic acid. The Glu409 and Glu549 interact and stabilize the Arg192 and Tyr530. The Tyr530/Glu409 is the nucleophilic pair playing a role in the catalytic reaction performed by the catalytic Asp216 residue (Chibanga et al., 2019). The crystal structure was obtained for the head domain of hPIV3 HN (Xu et al., 2013) but not yet for hPIV1 HN. The hPIV HN N-glycosylation is important in host-receptor interactions, thus, in the hemagglutinin function of HN. The presence of a 2SBS is still controversial for hPIV HN (Chibanga et al., 2019). Regarding substrate specificity, hPIV1 and hPIV3 HN preferentially recognize Neu5Ac bound to Galactose with an α2-3 linkage (Neu5Acα2-3Gal), hPIV3 also recognize Neu5Acα2-6Gal and Neu5GAcα2-3Gal (Villar and Barroso, 2006).

Bacterial neuraminidases

Many pathogenic bacteria produce neuraminidases, most commonly associated with mucosal tissues where SA are abundant in the gut (*Vibrio cholerae*) and the respiratory epithelium (*Streptococcus pneumoniae*; *Haemophilus influenzae*; *Pseudomonas aeruginosa*). In most cases, the primary role of these bacterial NAs is to exploit SA as a carbon source or to "cap" bacterial markers such as LPS and contribute to biofilm formation (Vimr and Lichtensteiger, 2002).

One of the best-characterized examples of bacterial NAs is *S. pneumoniae*, a widespread colonizer of the nasopharynx and a major human pathogen responsible for respiratory tract infection (Shak et al., 2013). The *S. pneumoniae* genome encodes up to three distinct neuraminidases NAs (NanA, NanB, and NanC). NanA and NanB are found in most clinical isolates and are considered as pneumococcal virulence factors. NanA is present on the bacteria's outer membrane surface and can hydrolyze α2,3- or α2,6-sialyllactoses, thus releasing α-Neu5Ac (Xu et al., 2011). NanB has a slightly different substrate specificity and acts as an intramolecular *trans*-sialidase with specificity for α2,3-sialoconjugates (Gut et al., 2008; Xu et al., 2011). NanC, found less frequently in clinical strains and the least characterized, hydrolyzes sialo-conjugated α2,3 to Neu5Ac2en (2-deoxy-2,3-dehydro-N-acetylneuraminic acid, also called DANA). This sialidase inhibitor presumably plays a regulatory role concerning the sialidase activity of NanA and NanB (Xu et al., 2011; Owen et al., 2015). Several studies suggest that the contribution of NanA to colonization and pathogenesis, observed in different *in vivo* models (Tong et al., 2000; Manco et al., 2006), relies on the role of NanA in "scavenging" host receptors to promote bacterial adhesion, the release of sialic acid as a carbon source, but also in allowing escape from the host response or altering the surface SA of other bacteria present in the same niche (Vimr and Lichtensteiger, 2002). NanB also appears to play a similar role in pathogenesis (Manco et al., 2006). Recently, *S. pneumoniae* NanB was shown to regulate NanA expression, this regulation possibly playing a role in mucus binding and mucociliary clearance (Hammond et al., 2021).

The role of NanA in the adherence and invasion of endothelial cells was demonstrated using wild type or deleted NanA mutant *S. pneumoniae* in an *in vitro* model of human brain microvascular endothelial cells and a murine model, suggesting that NanA plays an important role in the invasion of the blood-brain barrier (Uchiyama et al., 2009; Banerjee et al., 2010). The adhesin function of the N-terminal lectin part of the NanA plays a critical role in the adherence of the bacteria to endothelial cells (Uchiyama et al., 2009). The sialidase function may modify host cell receptors to reveal higher affinity ligands and increase the invasion of the endothelial cells (Uchiyama et al., 2009; Limoli et al., 2011). NanA also plays a crucial role in biofilm formation (Parker et al., 2009). The mechanism underlying this phenomenon may come from the catalytic domain able to increase sialic acids concentrations that may be used as sugars and stimulate *S. pneumoniae* growth. In addition, bacterial neuraminidases, including NanA, are also known to modulate the function of host immune factors by removing glycans which alter their stability and function [reviewed in Mathew et al. (2023)].

Similarly, to the non-enzymatic second binding site present on viral neuraminidases, a Carbohydrate Binding Module (CBM) is also present on bacterial neuraminidases, but its exact role remains to be clarified (Cantarel et al., 2009; Drula et al., 2022). This lectin domain is located in the N-terminal part, upstream of the catalytic domain of the *S. pneumoniae* NanA (Yang et al., 2015). The reconstitution of NanA structure with bioinformatics tools suggested that the lectin and catalytic domains were separated with a 16 amino-acid long flexible linker, whereas these two domains for NanB and NanC formed rigid globules stabilized by multiple interdomain interactions (Sharapova et al., 2018).

The opportunistic pathogenic bacterium *P. aeruginosa*, one of the primary etiological agents of pulmonary infections in cystic fibrosis patients (CF), is a particular case, as this bacterium does not use SA as a carbon source and does not possess sialic acid conjugated LPS (YuA et al., 1988). The comparative infection study in a mouse model of wild-type strains versus a mutant strain of *P. aeruginosa* deleted from its NA locus (D2794) showed the role of bacterial NA in biofilm formation, highlighting its role in virulence (Soong et al., 2006). Neuraminidase appears to play a role in the pathogenesis of *P. aeruginosa* in the context of cystic fibrosis, with NanA expression being increased under hyperosmolarity conditions, under the control of the gene involved in alginate expression and in the mucoid phenotype (Cacalano et al., 1992), which would further increase bacterial adhesion.

Mammalian neuraminidases

Four NAs have been identified in mammals (NEU1, 2, 3, and 4), exhibiting different localizations and functions (Monti et al., 2010; Miyagi and Yamaguchi, 2012). NEU1 is localized in lysosomes; its lysosomal activity is closely associated with cathepsin A and beta-Galactosidase. Mutations in the human NEU1 locus led to the lysosomal storage disease called sialidosis. NEU2 is mainly localized in the cytosol, but like NEU1, it can also be translocated to the membrane via mechanisms that are still poorly characterized to play other roles, particularly in the immune response (van der Wal et al., 2020). NEU3 is located in the plasma membrane, particularly on the surface of certain epithelia, and NEU4 is located

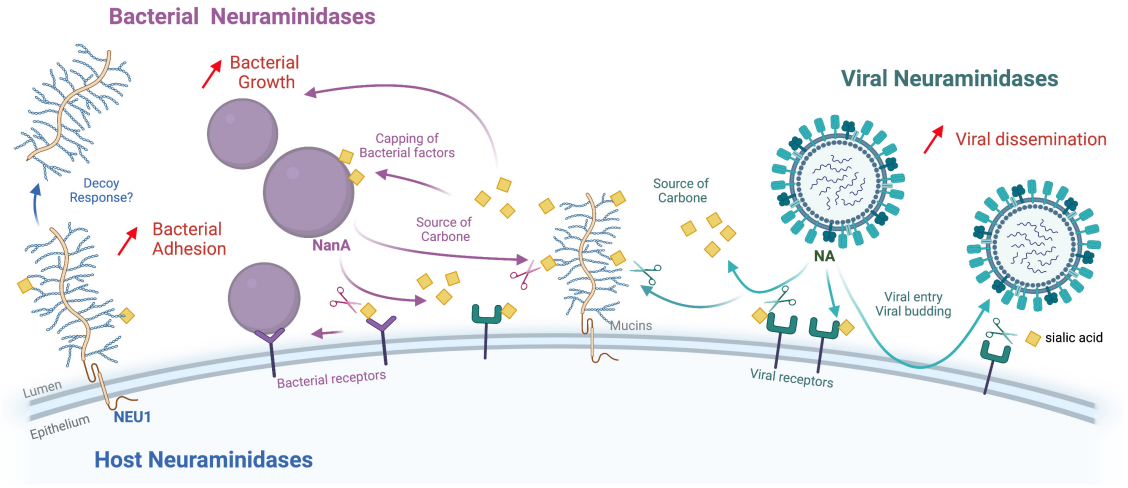

FIGURE 1
Functional interactions between viral, bacterial, and human neuraminidases. This diagram summarizes the known functions of the different neuraminidases (NA) in the context of coinfections (Viral, bacterial and host neuraminidases are pictured in green, purple, and blue, respectively. Sialic acids are represented by yellow squares). While viral NA (NA/HN) play a crucial role in virus entry and budding mechanisms, bacterial NA (NanA, NanB, and NanC) allow the exploitation of sialic acids as a carbon source or to "cap" bacterial markers such as LPS, and/or promote biofilm formation. The role of host neuraminidases at the epithelial level remains relatively uncharacterized, but their action on glycoproteins and gangliosides certainly plays an important role in regulating different types of cellular responses. Viral NA can promote bacterial infections by "stripping" SA that mask bacterial receptors on the surface of the epithelium. Another mechanism that may explain the frequent superinfection by bacteria after influenza virus infection is that influenza NA increases the availability of sialic acid as a nutrient for bacteria (carbon source). Influenza NA and bacterial NA play synergistic roles in upper respiratory tract superinfections. Host neuraminidase NEU1 activity reduces EGFR stimulation by EGF and increases *P. aeruginosa* adhesion through increased MUC1 binding to bacterial flagellin. Interestingly, other work has confirmed that *P. aeruginosa* mobilizes NEU1 via its flagellin to enhance its pathogenicity. In response, the cell releases the MUC1 ectodomain into the airway lumen as a hyperadhesive decoy receptor. Figure created with Biorender.com.

in mitochondria. Some functions of mammalian NAs seem to be in opposition or complementary, e.g., NEU3 and NEU4 oppositely regulate neuronal differentiation (Miyagi et al., 2018), and NEU1 and NEU3 target glycoproteins and gangliosides, respectively, Monti et al. (2010), Miyagi and Yamaguchi (2012). Interestingly, relevant to the focus of this review, these NAs, most notably NEU1 and NEU3, have been shown to have activity at the respiratory epithelium, contributing to 70 and 30% of the detected sialidase activity, respectively (Lillehoj et al., 2014, 2015).

Functional interactions between the different neuraminidases in the context of respiratory epithelial viral and bacterial infections

Within the complexity of the respiratory epithelial tract, the interactions between viral and bacterial pathogens may lead to more severe diseases than those caused by viruses or bacteria alone. The mechanisms involved in viral and bacterial co-infections or superinfections were reviewed recently (Oliva and Terrier, 2021). Lesions of the respiratory epithelium, by direct viral cytopathic effect or inflammatory response, expose the basement membrane and favor bacterial adherence to epithelial cells; lesions also decrease mucociliary clearance. The viral infection may also induce immunosuppression and leukopenia that favor bacterial superinfections. In this review, we will focus only on data regarding mechanisms implying the different NAs, summarized in **Figure 1**.

Viral NAs and bacterial infections

Viral NA can promote bacterial infections by "stripping" SA that masks receptors on the epithelium's surface. Experimental studies have shown that Influenza virus NA or hPIV HN can remove SA on host cells and expose *S. pneumoniae* receptors (Peltola and McCullers, 2004). The mechanisms by which Influenza NA increases bacterial adherence are not fully understood, but different hypotheses are suggested. Influenza NA, through the removal of SA from latent TGF-β, was demonstrated to activate TGF-β expression (Carlson et al., 2010), which in turn up-regulates host adhesion molecules, fibronectin, and α5-integrin, increasing the bacterial binding of group A *Streptococcus* (Li et al., 2015). Epidemiological studies have shown an association between influenza and *Neisseria meningitidis* infections; one hypothesis is that influenza infection contributes to a higher adhesion of *N. meningitidis* to respiratory epithelial cells, thus permitting entry through the epithelial barrier to the blood and meningeal spaces. Indeed, a recombinant influenza NA increased the adhesion of *N. meningitidis* strains serogroups B, C, and W135 to Hec-1-B human epithelial cells. The influenza NA permits the cleavage of sialic acid on capsular polysaccharides; this could unmask subcapsular bacterial factors that could interact with cell membrane receptors (Rameix-Welti et al., 2009).

The mechanism that may explain the frequent superinfection by *S. pneumoniae* after Influenza virus infection is that influenza NA will increase sialic acid availability as nutrient for *S. pneumoniae* promoting its proliferation. Consequently, suppressing mucins or using mucolytic treatment limits influenza-promoted

S. pneumoniae replication (Siegel et al., 2014). In addition to influenza virus, there is also a direct link between hPIV infection and *S. pneumoniae* infections in young children (Grijalva et al., 2014). Neuraminidase inhibitors have an impact on the reduction of influenza-related complications and their use may reduce the use of antibiotics. This impact may be due to the fact that blocking influenza NA will limit sialic acid availability and limit bacterial superinfections (McCullers, 2014). A similar impact of the use of hPIV HN inhibitors may limit *S. pneumoniae* superinfections (Alymova et al., 2005, 2009).

The NA of each pathogen may also mediate pathogen interactions, and interactions may be possible via the ubiquitous SA. Visualized by confocal and super-resolution microscopy, the direct interaction between IAV and different bacteria (*S. pneumoniae*, *S. aureus*, and *H. influenzae*) may promote bacterial adherence (Rowe et al., 2019). More recently, a study has shown that hPIV3 can recognize the α2-3 linked SA on capsular polysaccharides of Group B *streptococci*. Experiments of co-infections delayed hPIV3 infection but increased Group B *streptococci* adherence to virus-infected Hep-2 cells (Tong et al., 2018).

Impact of bacterial NAs on viral infection

In the Human airway epithelial model (HAE), NAs with different SA cleavage specificity were used to better understand the physiopathology of viral infections. Non-ciliated cells present a higher proportion of α2-6-linked SA, while ciliated cells possess both α2-3 and α2-6-linked SA. Experimental studies on HAE have shown that avian IAV and hPIV3 mainly infect ciliated cells, whereas human IAV mainly infects non-ciliated cells (Matrosovich et al., 2004; Zhang et al., 2005; Thompson et al., 2006). Treatment of HAE with the *Vibrio cholerae* NA, which cleaves SA residue with α2-3, α2-6, or α2-8 linkages, did not significantly impact human IAV infection but abolished hPIV3 infection. These results suggest that SA are more important for hPIV3 infection than for human IAV infection of HAE (Thompson et al., 2006). The use of NAs with different cleavage specificity provided evidence that hPIV3 utilizes α2-6-linked SA on ciliated cells to initiate infection (Zhang et al., 2005). The IAV NA and bacterial NA play synergistic roles in upper respiratory tract superinfections. The role of the bacterial NA was recently studied using different models. In a mouse model, NanA deficient *S. pneumoniae* were impaired in their ability to induce nasal and middle ear infections. IAV and NanA synergize to influence bacterial pathogenesis (Wren et al., 2017).

Interactions between mammalian NEUs and pathogens

Mammalian NAs and their interactions with pathogens have been relatively less studied than their counterparts in bacteria and viruses. Nevertheless, several studies have highlighted the role played by NEU1 in the adhesion and pathogenicity mechanisms of certain bacteria. Indeed, both in the A549 cell line and in primary respiratory epithelial cells, it has been demonstrated that two receptors present on the surface of respiratory epithelia, Epithelial Growth Factor Receptor (EGFR) and mucin 1 (MUC1), are substrates of NEU1 *in vivo*. NEU1 regulates the activation of signaling pathways associated with these receptors, in particular, the ERK1/2 (extracellular signal-regulated kinase) pathway. NEU1 activity reduces EGFR stimulation by EGF and increases *P. aeruginosa* adhesion via increased binding of MUC1 to bacterial flagellin (Lillehoj et al., 2012). Interestingly, other work has confirmed that *P. aeruginosa*, mobilizes NEU1 via its flagellin to increase its pathogenicity. Still, in response, the cell releases the ectodomain of MUC1 into the airway lumen as a hyper-adhesive decoy receptor (Lillehoj et al., 2015). Because of its localization to the surface of epithelia and ciliated brush borders, and its ability to desialylate surface gangliosides, NEU3 could play a role similar to that of NEU1, but this has yet to be demonstrated.

Treatment options and research avenues inhibiting or using neuraminidases in the context of viral and bacterial infections

Sialic acids and NAs are ubiquitous molecules that mediate cell infections by viral and bacterial pathogens and contribute to increasing their pathogenicity. Therefore, there may be an interest in inhibiting viral and bacterial NAs to prevent viral and bacterial propagation or cleaving SA to prevent viral infections.

Neuraminidase inhibitors with anti-influenza activity

The discovery of the Neu5Ac2en (DANA) initiated the discovery of neuraminidase inhibitors (NAIs). Knowledge of the structure of the active site of NA interacting with SA or DANA has led to the discovery of NAIs with antiviral activity (Colman et al., 1983; Varghese et al., 1983). Zanamivir, very close to DANA (4-guanidino-Neu5Ac2en), is only active by inhalation. Oseltamivir carboxylate contains a cyclohexene base and a bulky hydrophobic group (6-pentyl ether chain): it can bind to the active site of the viral NA after reorientation of the E276 residue allowing the formation of a hydrophobic pocket (Varghese et al., 1998). Peramivir bears a cyclopentane base, a 4-guanidino group, and a hydrophobic side chain (Babu et al., 2000); due to low oral bioavailability, it is used parenterally for severe infections in adults in the USA, Japan, and Korea (McLaughlin et al., 2015). Laninamivir is a long-acting NAI that contains a 4-guanidino group and a 7-methoxy group. The prodrug laninamivir octanoate used by inhalation is converted to laninamivir in the lungs with a prolonged duration of action; it is approved for use in Japan in uncomplicated forms (Watanabe et al., 2010). The trials concerning the efficacy of oseltamivir in the curative treatment of influenza show a limited clinical efficacy (Hayden et al., 1999; Nicholson et al., 2000; Treanor et al., 2000). Meta-analyses of randomized clinical trials have demonstrated that oral oseltamivir, in the context of uncomplicated influenza, decreases the duration of clinical signs by 1 day and increases the risk of nausea (Jefferson et al., 2014; Dobson et al., 2015). Treatment with oseltamivir reduces respiratory complications

requiring antibiotics and hospitalizations (Dobson et al., 2015). Meta-analyses of individual data (compilation of 78 observational studies on 29,234 patients of all ages between 2009 and 2011) show a 50% reduction in mortality when taking NAIs less than 48 h after the onset of signs clinics (Muthuri et al., 2014). The addition of oseltamivir carboxylate at 1 μM on HAE culture and to IAV suspensions used for infection decreased significantly (20 to 500-fold fewer cells) the number of infected cells compared to untreated cultures. These results indicate that inhibition of the IAV NA suppresses the initiation of the IAV infection (Matrosovich et al., 2004). The potential inhibition by these NAIs of NAs other than IAV NAs represent one research avenue to discover other molecules.

Neuraminidase inhibitors with anti-hPIV HN activity

The roles of hPIV HN in the viral multiplication cycle at early steps (attachment to SA and activation of the F protein for fusion) and later stages (release of new viral particles by the neuraminidase catalytic site) make it an ideal target for antiviral agents. Competitive inhibitors derived from DANA were studied as possible hPIV HN inhibitors. The *in vitro* zanamivir IC_{50} against hPIV HN was very high (evaluated at 0,25mM). Using the crystal structure of Newcastle Disease virus (NDV) HN, BCX 2798 and BCX 2855 inhibitors were developed. The hPIV inhibitor BCX-2798 was the most studied compound presenting prophylactic efficacy in a mouse model of hPIV1 infection (Alymova et al., 2009). Therapeutic options for inhibition of hPIV HN with DANA-derived analogs were reviewed recently, and targeting the receptor binding activity appears more efficient than blocking the neuraminidase activity alone (Chibanga et al., 2019).

Neuraminidase inhibitors to elucidate mammalian NAs activity

Studies using recombinant purified mammalian NAs (NEU) and the MUNANA (4'-methylumbelliferyl α-d-N-acetylneuraminic acid) substrate revealed a low inhibitory activity of DANA, mainly against NEU3 and NEU4, and of zanamivir against NEU2 and NEU3 (Hata et al., 2008; Richards et al., 2018). Recent studies reported the discovery of NEU1, NEU2, and NEU3-specific inhibitors derived from DANA (Guo et al., 2018a,b). These specific inhibitor molecules are interesting tools to elucidate mammalian NAs roles, and a recent study has shown that NEU1 and NEU3 promote leukocyte infiltration, whereas NEU4 was anti-inflammatory (Howlader et al., 2022).

Neuraminidase inhibitors and *S. pneumoniae* NanA and NanB

Crystal structures of *S. pneumoniae* NanA in complex with zanamivir or oseltamivir carboxylate revealed a weak to medium NanA competitive inhibition, respectively (Gut et al., 2011). Other molecules katsumadain A and artocarpin, can inhibit recombinant NanA (rNanA) at low micromolar concentrations in different NA inhibition assays; however, artocarpin was the only one able to inhibit *S. pneumoniae* adherence to A549 cells, reduce biofilm formation and bacterial growth (Walther et al., 2015). The study of S. *pneumoniae* NanA has revealed the evolutionary diversity of this enzyme and the different inhibitory efficiency of oseltamivir and DANA on these enzymes (Xu et al., 2016). The potential role of *S. pneumoniae* NanA and NanB in post-influenza virus infection complications was studied in A549 and MDCK cells. The addition of low dilutions (less than 1:1,000 and 1:100) of rNanA or rNanB hampered the virus spread in A549 cells, whereas higher dilutions (more than 1:10,000 and 1:1,000) promoted virus spread. These results suggest that high levels of rNanA or rNanB remove viral receptors and prevent cells from IAV infection, whereas at higher concentrations, rNanA, and B enhance the cleavage function for viral release (Walther et al., 2016). In this co-infection *in vitro* model, the presence of zanamivir at 1 μM inhibited the IAV NA, but zanamivir was inactive on rNanA, which could promote the viral release and spread. Oseltamivir at 1 μM inhibited both IAV NA and rNanA, and viral spread was limited (Walther et al., 2016). These results suggest that the discovery of molecules able to inhibit both viral NAs and bacterial NAs may prevent complications linked to viral and bacterial co-infections.

Use of neuraminidases to cleave sialic acids and prevent viral infections

The DAS181 is a recombinant fusion protein composed of *Actinomyces viscosus* sialidase catalytic domain fused to an epithelium anchorage heparin-binding domain (Malakhov et al., 2006). The DAS181 removes α2-6 or α2-3 linked to terminal galactose SA from respiratory epithelium cells, preventing the infection by viruses using sialic acid as a receptor (Malakhov et al., 2006). Influenza and hPIV infections can be controlled *in vitro* and *in vivo* by the DAS181 (Fludase®) used topically as an inhaled treatment. Studies performed in a mouse model confirmed that DAS181 was efficient for preventing and treating influenza A(H5N1) and oseltamivir-sensitive or resistant A(H7N9) infection in a mouse model (Belser et al., 2007; Marjuki et al., 2014). A phase II clinical trial performed in healthy adult participants has shown that the DAS181 allows a significant decrease in influenza viral load. However, no impact was detected on alleviating clinical symptoms, possibly due to the healthy patient recruitment (Moss et al., 2012). A phase II clinical trial (NCT04298060) is registered to study the DAS181 in patients with severe influenza virus infections. DAS181 was also used as a compassionate treatment in immunocompromised patients infected with hPIV. The results of phase 2 clinical trial NCT01644877 studying the clinical impact of DAS181 in immunocompromised patients suffering from a low respiratory infection with hPIV suggest that DAS181 improves oxygenation in hPIV infected immunocompromised patients not requiring mechanical ventilation (Chemaly et al., 2021). Albeit these encouraging data, the therapeutic use of DAS181 has some limitations. The DAS181 is well tolerated, although some possible hepatic disturbances (Moss et al., 2012). Antibodies directed against DAS181 develop in treated patients; this could preclude a prolonged or repeated use of this molecule (Zenilman et al., 2015).

SA and mucins function as decoys for pathogens and are essential to protect epithelial cells. The desialylation of the epithelium may expose host receptors and increase bacterial adherence and superinfection (Zhang, 2008). However, bacterial adherence needs exposure of basal membranes after epithelial necrosis mediated by viral infection (Nicholls et al., 2013). DAS181 treatment alone did not result in any cytopathic HAE effect and did not increase the adhesion of *S. pneumoniae* (Nicholls et al., 2008).

The Carbohydrate Binding Module (CBM) from *S. pneumoniae* has been used as a preventive strategy, as a single intranasal administration 7 days before challenge, to mask sialic acids and protect mice from a lethal challenge by A(H1N1)pdm09 influenza virus (Connaris et al., 2014). The idea of diverting the natural biological properties of some pathogens to mask host receptors and prevent infection by another pathogen is an is an interesting avenue for development.

Conclusion

Today, we have a relatively good understanding of the central role of sialic acids and neuraminidases in pathogen biology. In comparison, our knowledge of the role of different neuraminidases in the mutual interactions between viruses, bacteria, and host cells is still limited and deserves further exploration in the future. In the context of respiratory co-infections between viruses and bacteria, the respective contribution of host neuraminidases, virus(es), and bacteria to the evolution of respiratory pathology remains to be explored and better understood. This will require the development of new biologically relevant experimental models that allow a comprehensive approach to the study of host-pathogen interactions, requiring a combination of techniques from different disciplines. These different sialidases with similar enzymatic activities could constitute a common target of interest for the future development of specific treatments for bacterial superinfections, for example.

Author contributions

VE and OT wrote the first draft of the manuscript and wrote the sections of the manuscript. Both authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Functional diversity of staphylococcal surface proteins at the host-microbe interface

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Surface proteins of Gram-positive pathogens are key determinants of virulence that substantially shape host-microbe interactions. Specifically, these proteins mediate host invasion and pathogen transmission, drive the acquisition of heme-iron from hemoproteins, and subvert innate and adaptive immune cell responses to push bacterial survival and pathogenesis in a hostile environment. Herein, we briefly review and highlight the multi-facetted roles of cell wall-anchored proteins of multidrug-resistant *Staphylococcus aureus*, a common etiological agent of purulent skin and soft tissue infections as well as severe systemic diseases in humans. In particular, we focus on the functional diversity of staphylococcal surface proteins and discuss their impact on the variety of clinical manifestations of *S. aureus* infections. We also describe mechanistic and underlying principles of staphylococcal surface protein-mediated immune evasion and coupled strategies *S. aureus* utilizes to paralyze patrolling neutrophils, macrophages, and other immune cells. Ultimately, we provide a systematic overview of novel therapeutic concepts and anti-infective strategies that aim at neutralizing *S. aureus* surface proteins or sortases, the molecular catalysts of protein anchoring in Gram-positive bacteria.

KEYWORDS

***Staphylococcus aureus*, surface proteins, sortase A, abscess, vaccine**

Introduction

Staphylococcus aureus is a notorious pathogen that causes fatal diseases in the human population (Lowy, 1998; Lee et al., 2018). This microbe is a leading causative agent of skin and soft tissue infections (SSTIs), pneumonia, endocarditis, septic arthritis, osteomyelitis, bacteremia, and sepsis (Lowy, 1998; Kuehnert et al., 2006; Klevens et al., 2007). Of note, a global survey indicates that this pathogen accounts for more than 1 million deaths annually (GBD 2019 Antimicrobial Resistance Collaborators, 2022), an alarming death count which undoubtedly correlates with multidrug resistance (Chambers and Deleo, 2009; Lee et al., 2018), genetic flexibility and adaptive evolution (Chambers and Deleo, 2009; Malachowa and DeLeo, 2010; Smith et al., 2022; Howden et al., 2023), as well as refined immuno-evasive maneuvers this microbe evolved to overcome host immunity (Spaan et al., 2013; Thammavongsa et al., 2015a). Specifically, *S. aureus* secretes an extraordinary repertoire of virulence factors into the environment in order to establish acute and persistent infections in mammalian hosts (Foster, 2005; Thammavongsa et al., 2015a). Examples involve pore-forming and cytolytic toxins, superantigens, and multiple immuno-modulatory exoenzymes, which harbor an N-terminal

signal peptide required for a Sec-machinery-dependent translocation across the cytoplasmic membrane (Foster, 2005; Spaan et al., 2013; Thammavongsa et al., 2015a; Tam and Torres, 2019). Moreover, *S. aureus* expresses up to 24 signal peptide-bearing and pathogenicity-associated cell surface proteins that are characterized by diverse functional domains and flexible host ligand binding properties, as well as by a short C-terminal sorting sequence (Foster, 2019; Schneewind and Missiakas, 2019). This sequence, typically an LPXTG motif (Schneewind et al., 1992; Schneewind and Missiakas, 2019), is sensed and cleaved by sortase A (SrtA), a type II membrane protein and transpeptidase that catalyzes anchoring of cell surface proteins to the peptidoglycan of *S. aureus* and other Gram-positive bacteria (Mazmanian et al., 1999; Ton-That et al., 1999; Schneewind and Missiakas, 2019). Remarkably, *S. aureus srtA* mutants largely fail to colonize the host and are strongly attenuated in animal models of infectious disease (Mazmanian et al., 2000; Jonsson et al., 2003; Schaffer et al., 2006; Bubeck Wardenburg et al., 2007; Weidenmaier et al., 2008; Cheng et al., 2009; Chen et al., 2014; Misawa et al., 2015), a striking phenotype that inspired the staphylococcal research community to examine the individual roles of cell surface-displayed proteins at the host-microbe interface.

Herein, we briefly summarize the multi-facetted and sometimes redundant functions of cell surface proteins during local and invasive *S. aureus* infections. We also discuss how these proteins affect staphylococcal immune evasion and interaction with professional and non-professional phagocytes. Ultimately, we highlight the potential role of staphylococcal surface proteins in the design of vaccines, unique anti-infective agents, and novel therapeutic intervention strategies.

Role of staphylococcal surface proteins during colonization and establishment of skin and soft tissue infections

S. aureus is a very frequent cause of SSTIs which include cellulitis, inflamed hair follicles (folliculitis), furuncles and deep-seated abscesses, and surgical site infections (Lowy, 1998; Lee et al., 2018). During the establishment of these infections, cell surface proteins play a substantial role and largely contribute to initial adhesion and invasion of host cells (Foster et al., 2014; Lee et al., 2018; Foster, 2019). For example, several staphylococcal surface proteins including clumping factor B (ClfB), fibronectin-binding protein B (FnBPB), and iron-regulated surface determinant protein A (IsdA) mediate binding to human loricrin (Clarke et al., 2009; Mulcahy et al., 2012; da Costa et al., 2022), the most abundant protein of the cornified cell envelope and terminally differentiated corneocytes (Candi et al., 2005). Thus, it is not unexpected that some surface proteins influence staphylococcal colonization of the nasal cavity which is the natural niche of *S. aureus* (Figure 1; O'Brien et al., 2002; Clarke et al., 2006; Wertheim et al., 2008; Mulcahy et al., 2012; Weidenmaier et al., 2012; Sun et al., 2018). Specifically, ClfB- and IsdA-mediated binding to loricrin has been shown to affect interaction with squamous nasal epithelial cells thereby facilitating stable colonization of rodent or human nares (Clarke et al., 2006, 2009; Wertheim et al., 2008; Mulcahy et al., 2012). This process is further strengthened by IsdA-mediated interaction with involucrin and cytokeratin-10 as well as other staphylococcal surface proteins such as serine aspartate repeat containing protein D (SdrD) and *S. aureus* surface protein G (SasG) which also confer attachment to desquamated epithelial cells (Clarke et al., 2009; Corrigan et al., 2009; Askarian et al., 2016; Mills et al., 2022). Nonetheless, colonization and initial binding to upper skin layers not necessarily correlate with establishment of purulent infections of the skin. Albeit colonization of the host is generally accepted to be a risk factor for acquiring local and invasive staphylococcal diseases (von Eiff et al., 2001; Wertheim et al., 2005), establishment of these infections often requires skin lesions, wounds, or other medical conditions that favor pathogen entry (Cheng et al., 2011; Tong et al., 2015). For example, patients with atopic dermatitis, a chronic inflammatory skin disease associated with an IgE-mediated allergic response (Bieber, 2008; Werfel, 2009), are at elevated risk to be colonized with *S. aureus* and therefore often suffer from local infections of the skin (Geoghegan et al., 2018; Ogonowska et al., 2020). During atopic dermatitis, ClfB and particularly fibronectin-binding proteins (FnBPs) not only mediate binding to skin cells but also react with IgE antibodies thereby triggering specific inflammatory and allergic immune responses (Cho et al., 2001; Reginald et al., 2011; Fleury et al., 2017; Farag et al., 2022). In that regard, we further note that ClfB contributes to SSTIs and early stages of abscess formation in experimental skin infection models (Figure 1; Lacey et al., 2019). Mice subcutaneously infected with a *clfB* mutant of the *S. aureus* MRSA isolate USA300 developed smaller skin lesions over the course of the infection as compared to animals infected with the parental strain (Lacey et al., 2019). This phenomenon is associated with loricrin, which was found to be a component of the abscess wall and major host factor required for the development of skin lesions in mammals (Lacey et al., 2019). Likewise, bacterial mutants lacking FnBPs exhibited attenuated virulence in skin abscess models (Kwiecinski et al., 2014), probably also as a result of impaired host cell invasion and altered interaction with loricrin or extracellular matrix components (Fowler et al., 2000; Edwards et al., 2011; da Costa et al., 2022). Reduced bacterial loads in these models may further be explained by FnBPB-mediated neutralization of histones (Pietrocola et al., 2019), an antimicrobial component of neutrophil extracellular traps (NETs) which are formed in response to *S. aureus* during infection of the skin or other body parts (Brinkmann et al., 2004; Yipp et al., 2012; von Kockritz-Blickwede and Winstel, 2022). With this in mind, it is also worth noting that various other *S. aureus*-derived surface proteins assist in protecting staphylococci against professional phagocytes thereby essentially contributing to the development of abscesses and SSTIs (Foster, 2019; Schneewind and Missiakas, 2019). In particular, staphylococcal protein A (SpA) is a chief factor required for proper abscess formation in the skin as staphylococcal mutants lacking this determinant display virulence defects and reduced abscess volume in experimental murine models of skin infection (Patel et al., 1987; Kwiecinski et al., 2014). Moreover, clumping factor A (ClfA) has been linked to skin infections inasmuch as subcutaneous abscesses from rabbits infected with *clfA*-deficient staphylococci differed in size and had only weak evidence of vasculitis and thrombosis when compared to lesions formed by the parental *S. aureus* isolate (Malachowa et al., 2016). Thus, SpA and ClfA influence the pathogenesis of skin abscesses and associated SSTIs, presumably due to their anti-phagocytic properties which are known to promote staphylococcal evasion from neutrophil-mediated killing (Dossett

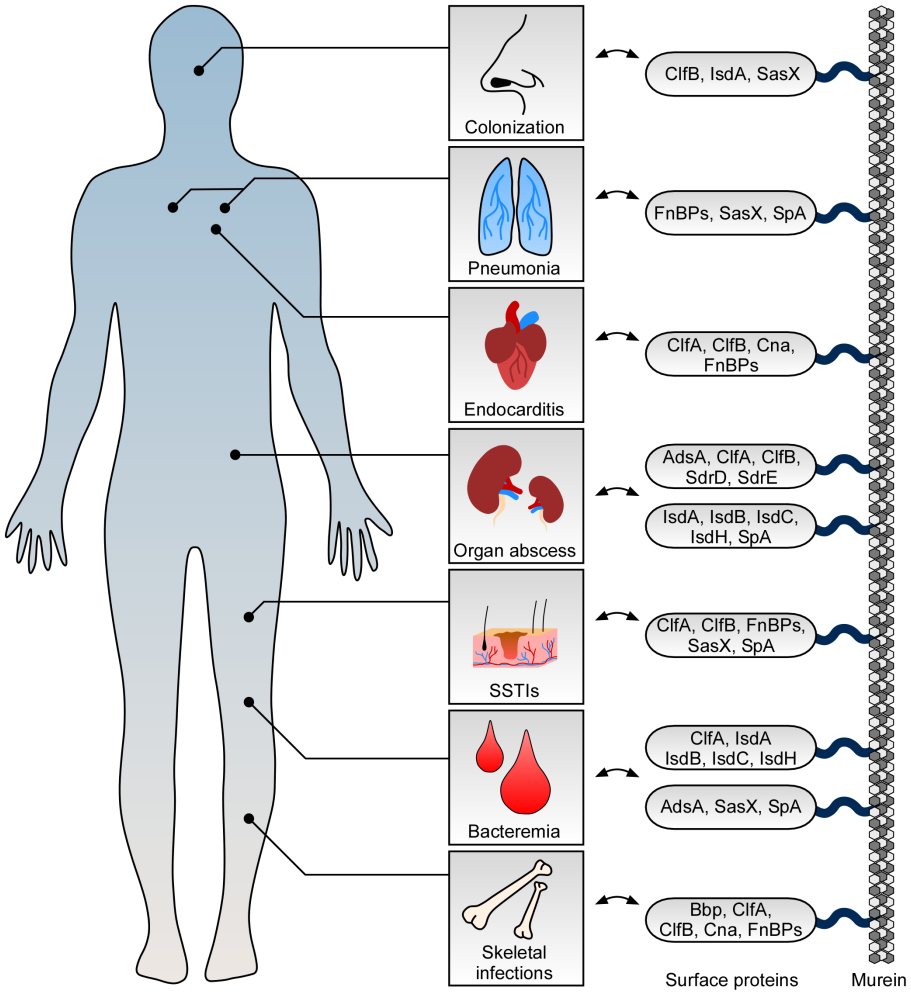

FIGURE 1
Role of staphylococcal surface proteins at the host-microbe interface. *Staphylococcus aureus* deploys surface proteins to promote interaction with mammalian hosts. While some surface proteins affect *S. aureus* nasal colonization, others contribute to skin and soft tissue infections (SSTIs) and fatal invasive diseases. Key surface proteins including adenosine synthase A (AdsA), bone sialoprotein (Bbp), clumping factor A and B (ClfA and ClfB), collagen adhesin (Cna), fibronectin-binding proteins (FnBPs), iron-regulated surface determinant proteins (IsdABCH), *S. aureus* surface protein X (SasX), serine aspartate repeat containing proteins D and E (SdrD and SdrE), and staphylococcal protein A (SpA) along with their proposed function during colonization and infection of human hosts are highlighted.

et al., 1969; Higgins et al., 2006). However, protein A was also found to affect infections of the skin by modulating inflammatory signaling cascades and cell death modalities in neutrophils and epithelial cells, highlighting the multi-facetted functions of staphylococcal cell surface proteins during establishment of SSTIs (Classen et al., 2011; Soong et al., 2012; Gonzalez et al., 2019; Ledo et al., 2020). Lastly, we note that not all cell surface proteins impacting SSTIs are part of the staphylococcal core genome. Specific MRSA clones with the sequence type ST239, for instance, carry a large ΦSPβ-like prophage in their genome that encodes a unique cell wall-anchored protein termed SasX (Li et al., 2012). Of note, mutant bacteria lacking *sasX* failed to colonize the nares of mice and were attenuated during experimental skin infection, a pioneering observation that has been linked to MRSA spread in China and other Asian countries (Li et al., 2012; Liu et al., 2015). Together, this comprehensive work underscores the relevance and importance of cell surface-displayed proteins during *S. aureus* colonization of host tissues and infections of the skin.

Impact of cell surface proteins on *Staphylococcus aureus* bacteremia and intra-organ abscess formation

S. aureus is also a frequently encountered agent of invasive and life-threatening diseases (Lowy, 1998; Lee et al., 2018). Bacteremia, for example, is a serious medical condition associated with high morbidity and mortality rates that often occurs upon staphylococcal entry into the bloodstream (Thomer et al., 2016; Bai et al., 2022). But how does *S. aureus* manage to survive within this hostile environment? Earlier studies and particularly transcriptional profiling of *S. aureus* in human blood identified multiple staphylococcal virulence determinants that became highly expressed under bacteremia-mimicking conditions (Malachowa et al., 2011). Among these factors are secreted toxins and various cell surface proteins including IsdA, IsdB, and IsdC, all elements of the iron-regulated surface determinant system (Isd; Figure 1; Malachowa et al., 2011). This system is required for iron and heme uptake in staphylococci and thus helps *S. aureus* to overcome

iron limitation in the host (Mazmanian et al., 2003; Hammer and Skaar, 2011). Accordingly, *S. aureus* mutants lacking IsdA, IsdB, IsdC, as well as IsdH exhibit decreased survival in blood and virulence defects in mouse models of bloodstream infection (Cheng et al., 2009; Visai et al., 2009; Kim et al., 2010b). This also holds true for staphylococcal variants that fail to express protein A, SasX, or adenosine synthase A (AdsA; Palmqvist et al., 2002; Thammavongsa et al., 2009; Li et al., 2012; Falugi et al., 2013). While protein A and SasX predominantly aid in preventing phagocytic clearance of *S. aureus* by either capturing immunoglobulins or promoting intercellular aggregation (Forsgren and Sjoquist, 1966; Dossett et al., 1969; Forsgren and Quie, 1974; Li et al., 2012), AdsA along with its 5′–3′-nucleotidase activity dampens neutrophil responses and coupled killing of *S. aureus* during acute bloodstream infection by converting host-derived adenosine monophosphate into immuno-suppressive adenosine (Thammavongsa et al., 2009). Nevertheless, entry and survival of *S. aureus* in blood causes organism-wide dissemination and formation of new replicative niches that often manifest as abscesses (Thomer et al., 2016). Establishment of these lesions can occur in almost all organs and requires, *inter alia*, the activity of specific cell surface proteins (Cheng et al., 2011; Thomer et al., 2016). For example, elements of the Isd machinery, ClfA and ClfB, as well as protein A significantly contribute to intra-organ abscess formation and priming of persistent infections (Cheng et al., 2009). Moreover, lack of SdrD, a cell wall-anchored protein that is only prevalent in approximately 60% of all *S. aureus* isolates (Sabat et al., 2006), dramatically lowered abscess formation and bacterial loads in organ tissues in murine models of systemic infection (Cheng et al., 2009; Askarian et al., 2017). Likewise, genetic ablation of *adsA* decreased the staphylococcal burden in renal tissues following intravenous challenge and concurrently ameliorated overall disease outcomes in mice (Thammavongsa et al., 2009). However, this phenomenon not only correlated with the failure of *adsA*-deficient staphylococci to synthesize adenosine during the initial phase of a bloodstream infection. Previous work showed that AdsA, together with the help of a secreted nuclease, converts NETs and host-derived DNA molecules into phagocyte-eliminating deoxyadenosine and deoxyguanosine, two purine effector-deoxyribonucleosides that promote killing of immune cells by targeting the purine salvage pathway-apoptosis axis (Thammavongsa et al., 2013; Winstel et al., 2018, 2019; Tantawy et al., 2022). Following this strategy, phagocyte entry into purulent cavities of deep-seated abscesses is efficiently suppressed thereby enhancing staphylococcal survival and establishment of persistent infections in organ tissues (Thammavongsa et al., 2013; Winstel et al., 2019). Thus, staphylococcal surface proteins essentially contribute to *S. aureus* bloodstream infection and intra-organ abscess development.

Contribution of staphylococcal surface proteins to skeletal infections, endocarditis, and pneumonia

Not all of the aforementioned cell surface proteins exclusively affect abscess formation upon bloodstream infection and staphylococcal dissemination in the host (Figure 1; Foster, 2019). ClfA and protein A, for instance, play a key role during septic arthritis (Josefsson et al., 2001; Palmqvist et al., 2002), a dangerous joint disease which is characterized by fever, joint pain and swelling, as well as redness of affected body regions (Shirtliff and Mader, 2002; Mathews et al., 2010). Development of septic arthritis is also linked to the expression of staphylococcal collagen adhesin (Cna), a protein and member of the MSCRAMM (microbial surface component recognizing adhesive matrix molecule) family that mediates binding to collagen and cartilage (Patti et al., 1994; Xu et al., 2004). Moreover, fibrinogen-and fibronectin-binding proteins (i.e., ClfA, ClfB, FnBPA, and FnBPB) promote bacterial aggregation in human synovial fluid, a biofilm-like state that protects *S. aureus* from antibiotics and phagocytes within the joint cavity (Dastgheyb et al., 2015). In this regard, we further note that some of these proteins impact staphylococcal skeletal infections and chronic bone diseases (i.e., osteomyelitis; Gimza and Cassat, 2021; Masters et al., 2022). For example, *S. aureus* Cna and bone sialoprotein (Bbp), another MSCRAMM that facilitates adhesion to fibrinogen (Vazquez et al., 2011), confer binding to the bone matrix and thus contribute to the pathogenesis of osteomyelitis (Ryden et al., 1989; Elasri et al., 2002; Campoccia et al., 2009; Persson et al., 2009). Likewise, protein A is a major modulator of this disease as binding of SpA to osteoblasts prevents cellular proliferation and stimulates apoptotic cell death in bone-synthesizing cells (Claro et al., 2011; Widaa et al., 2012). Development of osteomyelitis and establishment of replicative niches in the bone environment is further promoted by FnBPs (Ahmed et al., 2001), crucial *S. aureus* surface proteins that also impact non-osseous and fatal staphylococcal diseases of the heart (Foster, 2019). More specifically, FnBPs along with fibrinogen-and collagen-binding proteins of *S. aureus* influence the pathogenesis of infective endocarditis (Kuypers and Proctor, 1989; Moreillon et al., 1995; Hienz et al., 1996; Entenza et al., 2000; Que et al., 2005; Claes et al., 2017), a serious and life-threatening disease affecting the endocardial surface of the heart (Holland et al., 2016). Mechanistically, these proteins promote attachment of *S. aureus* to vessel walls, thrombi, and traumatized or inflamed heart tissues (Kuypers and Proctor, 1989; Moreillon et al., 1995; Entenza et al., 2000; Que et al., 2005; Claes et al., 2017). At later stages, FnBPA together with other virulence factors trigger staphylococcal invasion of the valve endothelium thereby aiding in the establishment of novel proliferative sites that provoke tissue destruction, cardiac abscess formation, and organ failure (Hamill et al., 1986; Que et al., 2005; Holland et al., 2016). Not surprisingly perhaps that FnBPs have a similar role during acute lower respiratory tract infection (pneumonia) as these factors confer binding to and uptake of *S. aureus* into airway epithelial cells (Figure 1; McElroy et al., 2002; Mongodin et al., 2002). Yet, failure to enter host cells due to missing expression of FnBPs may also boost staphylococcal pathogenicity as demonstrated in a rat model of experimental pneumonia (McElroy et al., 2002). Presumably, intracellular replication and persistence is favored by specific *S. aureus* isolates and might help to better adapt to the inflamed lung environment. This is also exemplified by the persistent lifestyle of staphylococcal small colony variants (SCVs), an auxotrophic and hard-to-treat subpopulation of *S. aureus* that often emerges during airway infections and in patients with cystic fibrosis (Proctor et al., 2006; Kahl et al., 2016). SCVs particularly aim at infiltrating host cells by upregulating FnBPs and other cell surface proteins to establish a protective niche that shields the microbe from neutrophils and alveolar macrophages (Vaudaux et al., 2002; Kahl et al., 2005; Mitchell et al., 2008; Tuchscherr et al., 2010, 2011; Kriegeskorte et al., 2014). Since SCVs as well as wildtype *S. aureus* often co-infect the lung together with other pathogens (McCullers, 2014; Oliva and Terrier, 2021), we finally appreciate that staphylococcal surface proteins may even impact

outcomes of polymicrobial infections. Most notably, recent advances suggest that *S. aureus* IsdA manipulates the Janus kinase-signal transducer and activator of transcription (JAK–STAT) signaling cascade thereby accelerating proliferation of severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) in epithelial cells (Goncheva et al., 2023). Moreover, protein A was found to protect *Pseudomonas aeruginosa* from neutrophil-mediated killing and altered the capacity of this microbe to form biofilms (Armbruster et al., 2016). This mechanism involves binding of protein A to cell surface structures of *P. aeruginosa* and the release of SpA from the staphylococcal cell wall, an earlier described phenomenon that may even be linked to binding of protein A to tumor necrosis factor receptor 1 (TNFR1) on lung epithelial cells thereby shaping staphylococcal pneumonia (Gomez et al., 2004; Becker et al., 2014; Armbruster et al., 2016). Overall, these compelling studies highlight the variable functions of cell surface proteins during *S. aureus* bone and joint infections, endocarditis, and pneumonia.

Targeting cell surface proteins and sortase A to improve *Staphylococcus aureus* infection outcomes

Due to their near-essential role during *S. aureus* pathogenesis and colonization of the host, cell surface proteins represent attractive targets for the development of new prophylactic and anti-infective intervention strategies. Earlier studies demonstrated that vaccination of laboratory animals with staphylococcal cell surface proteins together with passive immunization approaches confer protective effects against *S. aureus* disease (Table 1). For example, IsdA-or IsdB-based immunization of mice and interference with heme-iron uptake attenuated the adaptive properties and virulence potential of staphylococci in multiple *in vivo* models (Table 1; Clarke et al., 2006; Kuklin et al., 2006; Brown et al., 2009; Kim et al., 2010b; Bennett et al., 2019a,b). Likewise, immunization of mice with SpA_{KKAA}, a non-toxigenic protein A-based vaccine (Kim et al., 2010a), or safety-improved variants thereof abolished staphylococcal pathogenicity in murine and guinea pig models of bloodstream infection, and even promoted decolonization of rodent nares (Table 1; Kim et al., 2010a, 2015; Sun et al., 2018; Shi et al., 2021). Moreover, SpA-targeting monoclonal antibodies (mAbs) and derived humanized variants displayed therapeutic activity in abscess mouse models and concurrently offered protection against bacteremia and neonatal sepsis (Table 1; Kim et al., 2012; Thammavongsa et al., 2015b; Chen et al., 2019, 2020, 2022). These effects correlated with the antibody-mediated neutralization of the immunoglobulin Fcγ-binding and B-cell receptor crosslinking properties of SpA and enhanced opsonophagocytic killing of staphylococci in mouse or human blood (Kim et al., 2012; Thammavongsa et al., 2015b; Chen et al., 2019, 2020, 2022). Accelerated killing of *S. aureus* in host blood paired with ameliorated outcomes of septic arthritis or bacteremia was also observed in passively immunized animals that received ClfA-or SraP (serine-rich adhesin for platelets)-targeting antibodies (Josefsson et al., 2001; Tkaczyk et al., 2016; Yang et al., 2016; Zhou et al., 2021). Administration of an antiserum raised against staphylococcal AdsA further aided in rescuing mice from fatal bloodstream infection and peritonitis, presumably as a result of enhanced killing of staphylococci by circulating neutrophils that can no longer be suppressed by pathogen-derived adenosine (Zhang et al., 2017b). Accordingly, cell surface proteins and their immunogenic potential have often been exploited to formulate an effective vaccine against *S. aureus* (Clegg et al., 2021). Examples involve a recombinant, protein A-and IsdB-N2-containing five-antigen *S. aureus* vaccine (rFSAV) as well as SA4Ag, a multicomponent vaccine composed of capsular polysaccharide conjugates and recombinant forms of ClfA and the staphylococcal manganese transporter C (MntC; Creech et al., 2017; Frenck et al., 2017; Zeng et al., 2020). However, various clinical trials ended in failure due to adverse effects or limited efficacy in diseased patients (Clegg et al., 2021), thereby asking for improved vaccination strategies that may encompass probiotic-based immunization (Pan et al., 2023), advanced antibody engineering (Chen et al., 2020, 2022), or usage of live-attenuated vaccine platforms (Cabral et al., 2017; Moscoso et al., 2018). Alternatively, chemical interference with the transpeptidase activity of sortase A may also help to limit *S. aureus* colonization and severity of staphylococcal disease. In fact, previous work demonstrated that small molecule inhibitor-based blockade of sortase A can reduce *S. aureus* virulence in different animal model of infectious disease (Table 1). Computational drug engineering, for instance, identified 3,6-disubstituted triazolothiadiazole as a potent inhibitor of sortase A that improved infection outcomes of lethal *S. aureus* bacteremia (Table 1; Zhang et al., 2014). Further, compound library screening helped to isolate several natural products with sortase A-blocking and anti-infective properties (Table 1; Song et al., 2022a,b). Some of these agents even potentiated the efficacy of cell wall biosynthesis-targeting antibiotics during experimental pneumonia, presumably aiding in the design of poly-therapeutic approaches that may also encompass usage of allantodapsone, a prototype pan-inhibitor of staphylococcal adhesion to extracellular matrix proteins (Prencipe et al., 2022), to combat complicated MRSA infections in the future (Table 1; Song et al., 2022a,b; Wang et al., 2022b).

Concluding remarks

Cell surface proteins are key determinants of *S. aureus* virulence that largely affect host adaptation and immune evasion (Foster et al., 2014; Schneewind and Missiakas, 2019). Undoubtedly, many of these elements modulate host-microbe interaction and essentially contribute to the diverse clinical syndromes *S. aureus* may trigger in mammals (Foster et al., 2014; Schneewind and Missiakas, 2019). Staphylococcal surface proteins may even shape local outbreaks and emergence of new hyper-virulent clones (Li et al., 2012), as well as host tropism as exemplified by the biofilm-associated protein (Bap) which is prevalently expressed in *S. aureus* strains that provoke mastitis in animals (Valle et al., 2020). Notwithstanding, the antigenic variation, diversity, and functional multiplicity of cell surface proteins have hampered attempts to exploit these structures for the development of preventive therapeutics. Although active or passive immunization of laboratory animals conferred protective effects, neutralization of surface proteins may not necessarily represent a suitable approach to prevent staphylococcal infectious diseases in humans. Yet, experimental vaccines and antibody-based immunotherapies that seek to inactivate surface proteins in staphylococci may help to optimize future vaccine trials in diseased individuals. Concomitantly, resolving crystal structures of surface protein-antibody complexes, as recently implemented with ClfA and the mAb tefibazumab (Ganesh et al., 2016), along with an in-depth investigation of non-protective immune imprinting, a phenomenon that correlates with therapeutic failure of

TABLE 1 Selected mono-therapeutic approaches to attenuate *Staphylococcus aureus* pathogenicity *in vivo*.

Target	Therapeutic approach[a, b, c, d]	Effect[e]	References
AdsA	Vaccination with rAdsA or α-AdsA rabbit serum	therapeutic effect in peritonitis, survival, and skin abscess mouse models	Zhang et al. (2017b)
ClfA	Immunization of laboratory animals with rClfA or α-ClfA antibodies	reduces severity of *S. aureus*-mediated septic arthritis; protective effect in bacteremia and prosthetic-device infection models	Josefsson et al. (2001), Arrecubieta et al. (2008), Tkaczyk et al. (2016)
	Application of humanized mAb targeting ClfA	offers protection in a rabbit model of infective endocarditis	Domanski et al. (2005)
	Vaccine approach by using a ClfA-specific murine mAb	attenuates *S. aureus* virulence in a mouse sepsis model	Hall et al. (2003)
ClfB	Vaccination with UV-killed *S. aureus*, rClfB, or a ClfB-targeting antibody	abolishes nasal colonization in mice; protects against *S. aureus* skin infection	Schaffer et al. (2006), Lacey et al. (2019)
Cna	Immunization with rCna or α-Cna rat serum	protects from *S. aureus* infection and reduces mortality of mice upon lethal challenge	Nilsson et al. (1998)
FnBPA	Administration of FnBPA fusion proteins or rFnBPA for vaccination purposes	ameliorates outcomes of experimental mastitis in mice; protective effect in lethal challenge mouse model; reduced bacterial loads in organ tissues	Mamo et al. (1994), Zuo et al. (2014)
IsdA	Application of human mAb specific for IsdA	decreases bacterial loads in a murine model of systemic infection	Bennett et al. (2019a)
	Exploitation of purified and IsdA-specific rabbit antibody	lowers bacterial loads in a renal abscess mouse model; protective effect upon lethal *S. aureus* infection	Kim et al. (2010b)
	Vaccine approach with purified IsdA	diminishes nasal colonization of cotton rat nares	Clarke et al. (2006)
IsdB	Vaccination of mice with rIsdB	improves survival of mice upon lethal challenge with *S. aureus*	Kuklin et al. (2006)
	Application of probiotic-based vaccine (WXD171-IsdB)	mediates protection from *S. aureus* in skin, pneumonia, and sepsis mouse models	Pan et al. (2023)
	Immunization of mice with human mAb binding to IsdB-NEAT2	attenuates *S. aureus* virulence in a murine septic model	Bennett et al. (2019b)
	Purified rabbit antibody specific for IsdB	decreases bacterial loads in a renal abscess mouse model; protects mice from lethal *S. aureus* challenge	Kim et al. (2010b)
	Murine mAb that targets IsdB	reduces mortality in a murine intravenous challenge model	Brown et al. (2009)
SasX	Immunization with rSasX or α-SasX rabbit serum	reduces size of skin abscesses and severity of acute lung infection; reduces nasal colonization in mice	Liu et al. (2015)
SpA	Vaccine trial with purified SpA_{KKAA}, SpA_{KKE} or SpA_{KKT}	provides activity against *S. aureus* in murine and guinea pig models of bloodstream infection; reduces *S. aureus* nasal colonization in mice	Kim et al. (2010a), Kim et al. (2015), Sun et al. (2018), Shi et al. (2021)
	Rabbit polyclonal antibody targeting SpA	prevents hyper-inflammatory responses during experimental osteomyelitis	Gehrke et al. (2023)
	Immunization with recombinant or mouse hybridoma-derived SpA_{KKAA}-binding mAb	promotes decolonization of mice; therapeutic effect in a renal abscess mouse model; offers protection against neonatal sepsis in mice	Kim et al. (2012), Thammavongsa et al. (2015b), Chen et al. (2019)
	Human mAb specific for SpA	shields mice from *S. aureus* in a bacteremia model	Varshney et al. (2018)
	Humanized α-SpA mAb and Fcγ-engineered antibodies	therapeutic effect against MRSA in a renal abscess mouse model; reduces kidney abscess formation	Chen et al. (2020), Chen et al. (2022)
SraP	Immunization with murine mAb targeting SraP	reduces staphylococcal loads in the bloodstream; improves outcomes of *S. aureus*-mediated sepsis and peritonitis	Vahdani et al. (2021), Zhou et al. (2021)

(Continued)

TABLE 1 (Continued)

Target	Therapeutic approach[a, b, c, d]	Effect[e]	References
SrtA	Small molecule inhibitor-based approach (monotherapy with either orientin, punicalagin, rhodionin, scutellarin, or taxifolin)	attenuates staphylococcal virulence during experimental pneumonia	Wang et al. (2021a), Wang et al. (2021c), Song et al. (2022a), Wang et al. (2022a), Wang et al. (2022b)
	Hypodermic injection of chlorogenic acid	reduces mortality of *S. aureus*-infected mice	Wang et al. (2015)
	Infection control by using ML346	protects *Galleria mellonella* larvae from *S. aureus* infection	Guan et al. (2022)
	Acacetin-based therapeutic approach	dampens staphylococcal virulence in a renal abscess mouse model	Bi et al. (2016)
	Anti-infective therapy with either hibifolin, isovitexin, eriodictyol, cyanidin chloride, or chalcone	ameliorates outcomes of staphylococcal lung infection	Zhang et al. (2017a), Wang et al. (2021b), Song et al. (2022b), Su et al. (2022), Tian et al. (2022)
	Therapeutic administration of erianin or 3,6-disubstituted triazolothiadiazole	improves survival of mice following *S. aureus* bloodstream infection	Zhang et al. (2014), Ouyang et al. (2018)
	Administration of an oligopeptide (LPRDA)	protective effect in a mouse model of experimental mastitis	Wang et al. (2018)

[a]mAb: monoclonal antibody;
[b]WXD171-IsdB: *Limosilactobacillus reuteri* WXD171 expressing *Staphylococcus aureus* IsdB;
[c]SpA_{KKAA}, SpA_{KKA}, or SpA_{KKT}: non-toxigenic protein A vaccine variants;
[d]3-(4-pyridinyl)-6-(2-sodiumsulfonatephenyl) [1,2,4]triazolo[3,4-b][1,3,4]thiadiazole and related compounds;
[e]MRSA: methicillin-resistant *Staphylococcus aureus*.

IsdB-based immunization trials (Tsai et al., 2022), could assist in the exploitation of *S. aureus* surface proteins for the reformulation of an effective vaccine candidate or fabrication of unique prophylactic tools that foster decolonization of high-risk patients. Ultimately, the discovery of new host ligands of non-excessively studied surface proteins such as the plasmin-sensitive surface protein (Pls), an MRSA-specific cell envelope-displayed glycoprotein (Savolainen et al., 2001; Josefsson et al., 2005; Bleiziffer et al., 2017), may also aid in the design of additional anti-infective strategies and further fuels our knowledge of staphylococcal infection dynamics.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Advancements in understanding the molecular and immune mechanisms of *Bartonella* pathogenicity

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Bartonellae are considered to be emerging opportunistic pathogens. The bacteria are transmitted by blood-sucking arthropods, and their hosts are a wide range of mammals including humans. After a protective barrier breach in mammals, *Bartonella* colonizes endothelial cells (ECs), enters the bloodstream, and infects erythrocytes. Current research primarily focuses on investigating the interaction between *Bartonella* and ECs and erythrocytes, with recent attention also paid to immune-related aspects. Various molecules related to *Bartonella*'s pathogenicity have been identified. The present review aims to provide a comprehensive overview of the newly described molecular and immune responses associated with *Bartonella*'s pathogenicity.

KEYWORDS

***Bartonella*, blood-sucking arthropods, endothelial cells, erythrocytes, antibody, immune escape**

Introduction

Bartonellae are Gram-negative facultative intracellular bacteria (Brenner et al., 1993) that were first described in 1909. Since the last reclassification in 1993, the number of Bartonellae species has increased to 45 as of Okaro et al. (2017), and new species continue to be identified in recent years (Gutierrez et al., 2020; do Amaral et al., 2022a,b; Liu et al., 2022). Phylogenetic analyses have categorized the genus *Bartonella* into three phylogenetic clades, which include the honeybee symbiont *Bartonella apis*, the pathogenic *Bartonella tamiae*, and the eubartonellae, which are further separated into *Bartonella australis* and four distinct lineages (Segers et al., 2017). *Bartonella bacilliformis* and *Bartonella ancashensis*, which are human pathogens, belong to lineage 1. *Bartonella* species specific to ruminants are found in lineage 2. The most abundant *Bartonella* species are those in lineage 3 and 4, which infect a diverse range of mammalian hosts (Wagner and Dehio, 2019). Recently, two bat-associated *Bartonella* strains showed significant differences with other lineages in phylogenetic relationship analysis (Goncalves-Oliveira et al., 2023). Transmission of Bartonellae between hosts is primarily mediated by diverse blood-sucking arthropod vectors such

as fleas, body lice, ticks, sandflies, and others (Breitschwerdt et al., 2010a). These pathogens have a broad range of mammalian hosts, including, but not limited to, primates, rodents, and cats (Gundi et al., 2004; Dehio, 2005). However, each *Bartonella* species is typically adapted to a specific mammalian host (Deng et al., 2012).

In arthropods, the life cycle of most *Bartonella* species is divided into replication in the midgut of intestinal tracts and spreading through excretion (Foil et al., 1998). The pathogen is shed within arthropod feces onto mammalian skin and can be superficially inoculated into the derma by scratching or biting (Okujava et al., 2014). The derma-niched *Bartonella* might penetrate the endothelial cells (ECs) facilitated by dendritic cells (DCs) and Bartonella effector proteins (Beps) (Siamer and Dehio, 2015; Fromm and Dehio, 2021). Subsequently, the ECs-residing bacteria enter the bloodstream, invade erythrocytes, multiply inside them, and await the next round of transmission when arthropods bite the infected mammalian host again (Harms and Dehio, 2012; Pulliainen and Dehio, 2012; Fromm and Dehio, 2021).

Bartonella bacilliformis, *Bartonella quintana*, and *Bartonella henselae* are three major species that trigger pathological angiogenesis during infection in humans. These three species are the etiological agents of Carrion's disease, trench fever, and cat scratch disease (CSD), respectively (Rolain et al., 2004; Breitschwerdt et al., 2010a; Harms and Dehio, 2012; Liu et al., 2012). The symptoms and syndromes of bartonelloses are diverse, ranging from bacillary angiomatosis (BA), bacillary peliosis hepatis, chronic asymptomatic bacteremia, and infectious endocarditis, to neurological disorders. CSD, a relatively common zoonotic infection acquired from cats or cat fleas carrying *B. henselae*, is characterized by enlarged regional lymph nodes and fever, particularly in children and adolescents (Biancardi and Curi, 2014; Chang et al., 2016; Okaro et al., 2021). Atypical manifestations of CSD, such as hepatic and/or splenic lesions, discitis, granulomatous conjunctivitis, endocarditis, myocarditis, neuroretinitis, osteomyelitis, and encephalomeningitis, can mimic serious disorders. Moreover, *B. henselae* and *B. clarridgeiae* have been detected in blood samples from human donors at a Brazilian blood bank (Pitassi et al., 2015), indicating their presence in healthy humans, and highlighting the possibility of undetected cases of *Bartonella* infection in humans. *B. bacilliformis*, the pathogen of Carrion's disease, is primarily found in the Andean valleys of South America (Sanchez Clemente et al., 2012). This disease is characterized by an acute phase, known as Oroya fever, marked by fever, pallor, hemolytic anemia, myalgia, and arthralgia, followed by a chronic phase characterized by the development of vascular proliferative lesions on the skin, known as verruga peruana, which can persist for several months or even years (Maguina et al., 2009; Harms and Dehio, 2012; Sanchez Clemente et al., 2012; Gomes et al., 2016a). *B. quintana*, the etiological agent of trench fever, is naturally restricted to human hosts and louse vectors. The pathogen is now frequently identified among urban homeless and marginalized populations in the United States and Europe, and is responsible for various conditions, including endocarditis, pericardial effusion, bacillary angiomatosis-peliosis, and even asymptomatic bacteremia (Leibler et al., 2016; Sasaki et al., 2021).

This review intends to summarize recent findings about the pathogenicity of *Bartonella* and the immune response in hosts elicited by the infection, to provide valuable insights for future research.

Blood-sucking arthropods as vectors for *Bartonella* transmission

Blood-sucking arthropods are important for the transmission of *Bartonella*. Various blood-sucking arthropods have been identified as vectors capable of transmitting *Bartonella*, such as the sand flies *Lutzomyia verrucarum* for *B. bacilliformis* (Minnick et al., 2014), cat flea *Ctenocephalides felis* for *B. henselae* (Chomel et al., 2009; Harms and Dehio, 2012; Duscher et al., 2018), human body lice *Pediculus humanus corporis* for *B. quintana* (Byam and Lloyd, 1920), and the flea *Ctenophthalmus nobilis* for *Bartonella grahamii* and *Bartonella taylorii* (Bown et al., 2004). Most Bartonellae reside in the intestinal tract of arthropods, where they replicate and are eventually transmitted to mammalian hosts through vector defecation (**Figure 1B**; Chomel et al., 2009). Although *Bartonella* bacteria (such as *B. henselae*) typically survive in arthropod feces for several days (Higgins et al., 1996), *B. quintana* remains infectious for up to 1 year in louse feces (Kostrzewski, 1950; Chomel et al., 2009), making it more likely for hosts to contract an infection. Bartonellae can also infect the salivary glands of arthropods, as evidenced by the transmission of *B. henselae* through tick saliva to an artificial membrane feeding system (Cotte et al., 2008). Additionally, the transovarial transmission of *B. henselae* Marseille, *Bartonella schoenbuchensis* DSMZ 13525, and *B. grahamii* ATCC700132 from female tick *Ixodes ricinus* to their offspring via eggs may also occur, as *Bartonella* DNA has been detected in eggs laid by the three kinds of *Bartonella*-positive female ticks and in hatched larvae (**Figure 1A**; Krol et al., 2021). The spread of *B. bacilliformis* by *L. verrucarum* represents an exception of vector transmission. The current research has shown that *B. bacilliformis* can colonize the midgut of *L. verrucarum* (Battisti et al., 2015), yet the mechanism by which midgut-residing *B. bacilliformis* in sandflies enters human hosts remains unclear (Garcia-Quintanilla et al., 2019).

Climate change indirectly affects the spread of *Bartonella* by perturbing the proliferation of blood-sucking arthropods (Chamberlin et al., 2002). When the number of blood-sucking arthropods increases during warm and humid conditions such as during an El Niño, the infection rate of *Bartonella* also seems to increase correspondingly (Chamberlin et al., 2002; Clemente et al., 2016). These findings highlight the critical role of blood-sucking arthropods in the transmission of *Bartonella*.

The flea *C. felis* is the primary means of transmitting *B. henselae* from cat to cat. Studies have shown that healthy cats do not contract *Bartonella* infection or seroconversion after sharing food or playing with highly bacteremic cats (Chomel et al., 1996; Abbott et al., 1997). However, fleas that have taken a blood meal from highly bacteremic cats can effectively transmit *B. henselae* to specific-pathogen-free (SPF) cats (**Figure 1A**; Chomel et al., 1996). Although there is no evidence to prove that *B. henselae* can be spread among cats through salivation, *B. henselae* DNA has been detected in the saliva of infected cats (Oskouizadeh et al., 2010). *Bartonella* may be present in the saliva of infected mammals, but the bacteria are fastidious haemophilia (Liu and Biville, 2013), and therefore, the concentration of *Bartonella* in the blood of a highly bacteremic host is likely higher than that in its saliva. Furthermore, different mechanisms exist in certain arthropods that enable the transmission of *Bartonella* from arthropods to hosts. It

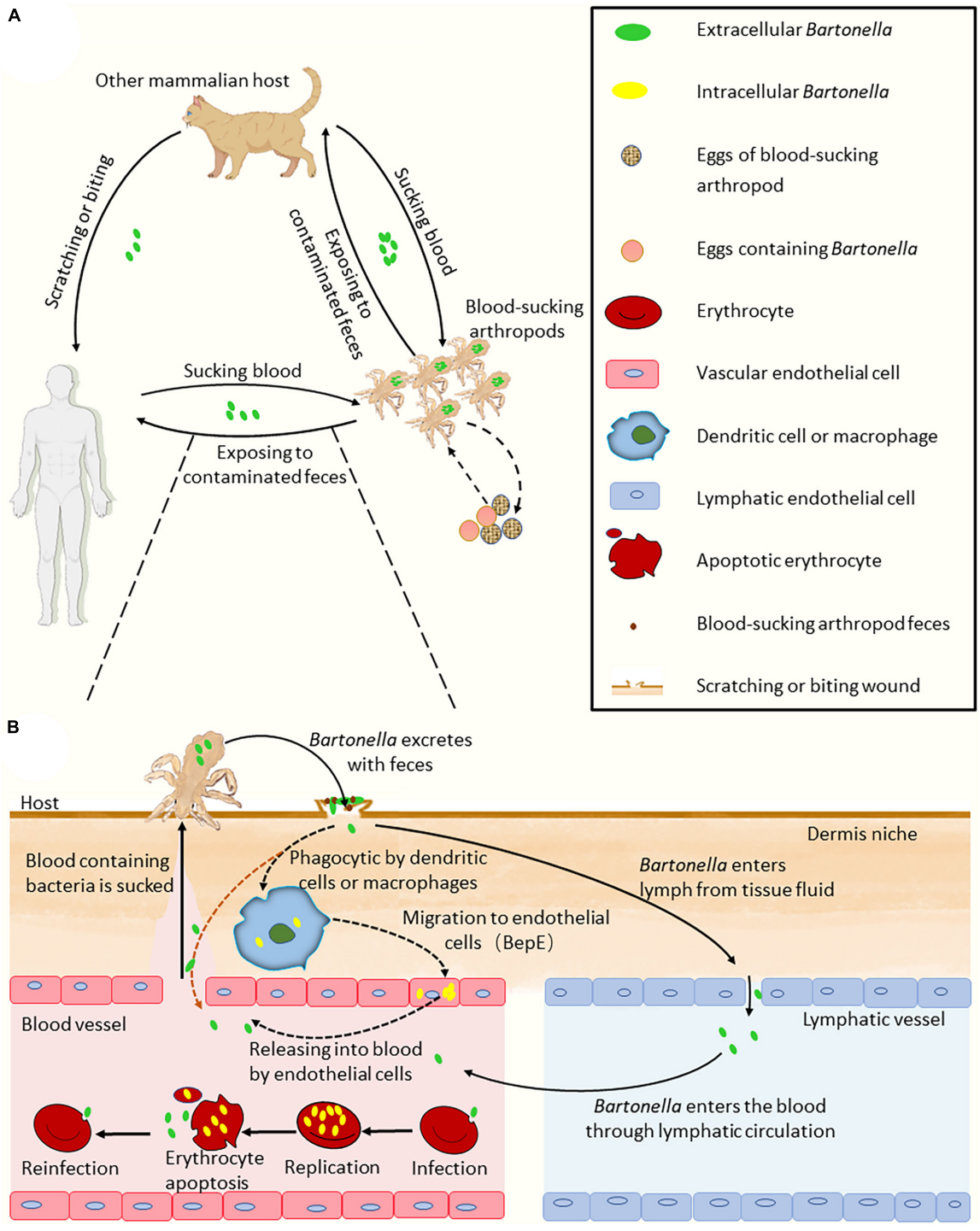

FIGURE 1
Model of *Bartonella* infection cycle. **(A)** The process of *Bartonella* transmission in the hosts. **(B)** The ways of Bartonellae enter the blood. There are two ways of Bartonellae with VirB/VirD4 T4SS enter the blood. The first way may be through the host's macrophages, endothelial cells and other migration into the blood. The second way is through lymphatic circulation into the blood. Bartonellae absent of VirB/VirD4 T4SS, such as *B. bacilliformis*, may enter the blood with feces directly through the wound bitten by sand flies (the orange dashed line arrow). In the figure, the solid line arrow indicates the confirmed process, and the dashed line arrow indicates conjecture. Some elements in the figure were drawn by Figdraw.

is important to note that the transmission of *Bartonella* from cat to human is usually achieved through cat scratches due to contact with *B. henselae*-contaminated flea feces.

The importance of some arthropod vectors for the transmission of *B. henselae* underscores the likelihood that this applies to most *Bartonella* species (Pulliainen and Dehio, 2012). After *Bartonella*-contaminated feces are inoculated to the wound of the host, the bacteria invade the dermis layer of the host and begin their journey of invasion (**Figure 1B**).

Migration and blood entry of *Bartonella* in mammalian host

Bartonella initiates its infection cycle in a mammalian host after replication in the midgut of arthropod vectors (Harms and Dehio, 2012). When these arthropods suck blood from mammals, the affected area on the host's skin experiences irritation, followed by scratching that can lead to the inoculation of *Bartonella*-containing insect feces into the dermis. Transmission of *Bartonella* can also occur through traumatic contact with infected animals (Chomel et al., 2009). After superficially inoculating the dermis, the bacteria sequentially access the dermal and blood-seeding niches. During dermal niche colonization, migrating cells such as DCs or macrophages are likely kidnapped by *Bartonella* to reach the blood-seeding niche (Fromm and Dehio, 2021). Transmission from the dermal niche to the blood-seeding niche may also occur via the lymphatic system (Hong et al., 2017). In the blood-seeding niche, the cell type is still puzzling and the bacteria most likely colonize ECs (Eicher and Dehio, 2012). It is believed that the blood-seeding niche releases bacteria into the bloodstream periodically, which invade erythrocytes and replicate until the critical limit of eight bacteria on average per erythrocyte is reached (Litwin and Johnson, 2005; Pulliainen and Dehio, 2012). After the apoptosis of infected erythrocytes, bacterial cells are released again into the bloodstream to invade new erythrocytes for replication, which may partly explain the periodic occurrence of host bacteremia after infection (Harms and Dehio, 2012). Once arthropods bite the infected mammals again, *Bartonella* enters the next round of transmission (**Figure 1A**; Harms and Dehio, 2012; Fromm and Dehio, 2021).

Bartonellae (lineage 3 and lineage 4), along with other bacteria like *Helicobacter pylori*, *Legionella pneumophila*, and *Brucella* spp. (Cascales and Christie, 2003; Harms et al., 2017), utilize the VirB/VirD4 type IV secretion system (T4SS) as a key virulence factor to infect humans and other mammals' target cells (Cascales and Christie, 2003). The pathogens translocate Bartonella effector proteins (Beps) into host cells via the VirB/VirD4 T4SS, which orchestrates multiple cellular processes in host cells, including modulating the immune response and subverting cellular functions to benefit the bacterial survival and proliferation (Okujava et al., 2014; Sorg et al., 2020). The VirB/VirD4 T4SS and the translocated Beps are the best characterized *Bartonella*-specific virulence factors. The invasion of *B. henselae* into human ECs occurs in two distinct pathways, either as a single bacterium through endocytosis or as bacterial aggregates in the form of invasomes (Truttmann et al., 2011). Induced by F-actin rearrangements and stress fiber formation, the formation of invasome relies on the functional VirB/VirD4 T4SS associated with either BepG alone or the combination of BepC and BepF (Truttmann et al., 2011). BepF is comprised of three Bep intracellular delivery domains, with two non-terminal domains triggering invasome formation in conjunction with BepC (Wagner and Dehio, 2019). BepC contributes to the formation of the invasome by modulating the F-actin cytoskeleton. Recently, it has been discovered that BepC triggers stress fiber formation by activating the RhoA GTPase signaling cascade through the recruitment of the Rho guanine nucleotide exchange factor H1 (GEF-H1) to the plasma membrane (Marlaire and Dehio, 2021).

The VirB/VirD4 T4SS system also plays a crucial role in the migration of *Bartonella* from the dermal niche to the blood-seeding niche. It is achieved by translocating BepE into host cells, which inhibits cell fragmentation caused by BepC or other Beps and ensures the migration of *Bartonella*-infected DCs to deliver the pathogen from the derma site to the blood-seeding niche (Okujava et al., 2014). The distributional pattern of VirB/VirD4 T4SS and Beps is proposed to correlate with arthropod vectors' blood-feeding behavior and the mode of bacterial transmission (Dehio and Tsolis, 2017). Bartonellae that are absent of VirB/VirD4 T4SS, such as *B. bacilliformis*, are exclusively transmitted by sandflies. However, sandflies display a violent mode of blood-feeding by damaging microvessels of the skin to allow them to access the freshly draining blood (Ribeiro, 1995; Serafim et al., 2021), which potentially provides a direct route for *Bartonella* to invade the bloodstream (Dehio and Tsolis, 2017).

Furthermore, the presence of either *B. henselae* or *B. quintana* enhances the proliferation of human ECs and prevents apoptosis. This anti-apoptotic effect is mediated by the pathogen's BepA protein, which interacts with human adenylyl cyclase 7 (AC7), followed by elevated cAMP levels (Pulliainen et al., 2012). The prolonged survival of host cells allows for an efficient time course for intracellular bacteria to have multiple replications, which is believed to be critical for a successful infection from the primary niche to the bloodstream. However, the BepA ortholog in *B. tribocorum* lacks anti-apoptotic activity (Dehio, 2008), indicating that orthologous BepA proteins may have varying functions in different bacteria.

Infection of endothelial cells (ECs)

Bartonella species have been found to inhabit various host cells, including mononuclear phagocytes, $CD34^+$ progenitor cells, and mesenchymal stromal cells (MSCs) (Mändle et al., 2005), but the vascular endothelium is considered the primary blood-seeding niche for *Bartonella* colonization in the mammalian host (Deng et al., 2012, 2019). To infect the ECs of blood vessels in a mammalian host, Bartonellae must first traverse the extracellular matrix of the cells. The degradation of extracellular matrix proteins has been confirmed to be facilitated by the interaction between fibrinolysis and several pathogen proteins (Lähteenmäki et al., 2001). *Bartonella* utilizes a metabolic enzyme known as α-enolase or phosphopyruvate hydratase, which is involved in the synthesis of pyruvate, to act as a plasminogen receptor and mediate the activation of plasmin and extracellular matrix degradation (**Figure 2**; Díaz-Ramos et al., 2012; Cappello et al., 2017). Plasmin, the proteolytically active form of plasminogen, is responsible for promoting fibrin dissolution in the extracellular matrix of host cells (Keragala and Medcalf, 2021). The sequence of α-enolase in *B. henselae* is highly homologous to that of many other *Bartonella* species, and they all possess typical plasminogen-binding modes. Accordingly, the α-enolase of *Bartonella* has been hypothesized to function as a plasminogen-binding protein, which has been recently confirmed in experiments (Deng et al., 2019). As a result, the enolase-plasminogen interaction is identified as one potential mechanism exploited by *Bartonella* to loosen and degrade the extracellular matrix of ECs before entering (Deng et al., 2019).

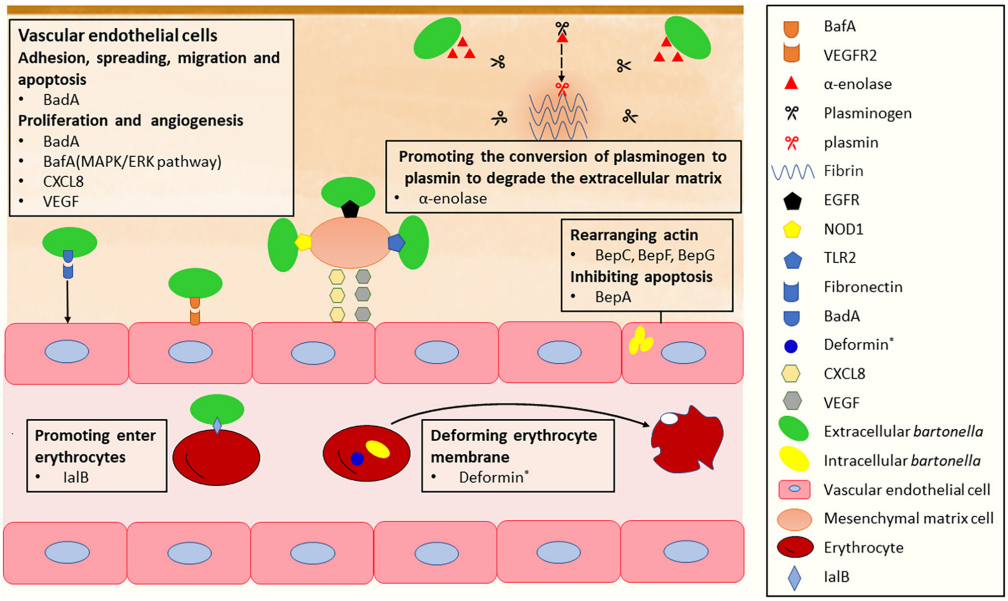

FIGURE 2
The mechanism of *Bartonella* infects vascular endothelial cells, mesenchymal stromal cells and erythrocytes. *Deformin is only found in *B. bacilliformis* and *B. henselae*.

To efficiently adhere to extracellular matrix proteins of ECs, *Bartonella* expresses surface adhesins, such as Bartonella adhesin A (BadA) in *B. henselae* (Riess et al., 2004), variably expressed outer membrane proteins (Vomps) in *B. quintana* (Zhang et al., 2004), and *Bartonella* repeat protein A (BrpA) in *Bartonella vinsonii* (Gilmore et al., 2005). These adhesins belong to the trimeric autotransporter adhesin (TAA) family (Linke et al., 2006). Among them, VompC confers the ability of *B. quintana* to bind collagen IV in extracellular matrix proteins of ECs; BadA is essential for the attachment of *B. henselae* to extracellular matrix proteins including collagen, laminin, and fibronectin, with fibronectin being particularly important (Kaiser et al., 2008; Muller et al., 2011). Fibronectin orchestrates various cellular processes of ECs, including cell adhesion, spreading, migration, proliferation, and apoptosis (Patten and Wang, 2021; Vaca et al., 2022). The binding of BadA to fibronectin occurs at repetitive motifs in the neck/stalk region of BadA and is a cumulative effect leading to rapid saturation (Thibau et al., 2022b). This interaction helps the bacteria to adhere to host cells. As another important virulence factor in *Bartonella*, BadA was described to negatively affect the Beps-translocating activity of VirB/D4 T4SS, while the function of BadA itself remain intact when both factors were co-expressed in *B. henselae* (Lu et al., 2013).

The outer membrane protein BadA is also responsible for *Bartonella*-induced vasoproliferation. It activates hypoxia-inducible factor-1 and stimulates the secretion of pro-angiogenic cytokines, such as vascular endothelial growth factor (VEGF) and C-X-C motif chemokine ligand (CXCL) 8 (Riess et al., 2004; Kempf et al., 2005; McCord et al., 2006), contributing to *Bartonella*-induced vasoproliferation. However, the observed BadA-dependent VEGF secretion was limited to certain cells and not in *B. henselae*-infected ECs (Kempf et al., 2001). Another autotransporter protein, *Bartonella* angiogenic factor A (BafA), is identified as a key *Bartonella*-derived mitogenic factor (Tsukamoto et al., 2020, 2022). BafA acts as a VEGF analog that promotes angiogenesis by binding to vascular endothelial growth factor receptor-2 (VEGFR2) on the EC surface and activating the MAPK/ERK pathway, which facilitates EC proliferation, tube formation, and subsequent angiogenesis (Tsukamoto et al., 2020). BafA family proteins are common among many *Bartonella* species, and BafA-triggered angiogenesis plays a central role in the formation of vasoproliferative lesions during *Bartonella* infection. Studies have shown that *B. quintana*, *B. henselae*, and *B. elizabethae* induce angiogenesis and proliferation by stimulating the VEGFR2 signaling pathway through the production of BafA (Tsukamoto et al., 2020, 2022; Suzuki et al., 2023). In addition, other cell types recruited to the vasoproliferative lesions, including monocytes, macrophages, and MSCs, stimulate EC proliferation through the production of VEGF and CXCL8. In particular, MSCs distributed in various tissues, including the bone marrow and the adipose tissue, play the role of a *B. henselae* reservoir and modulator of EC functions. *B. henselae*-infected MSCs release angiogenic factors, such as CXCL8, VEGF, etc., leading to the induction of a proangiogenic phenotype in ECs. Toll-like receptor 2 (TLR2), nucleotide-binding oligomerization domain-containing protein 1 (NOD1), and epidermal growth factor receptor (EGFR) are identified as the receptors involved in the recognition of *B. henselae* by MSCs (**Figure 2**; Scutera et al., 2021). Meanwhile, *B. henselae*-infected MSCs also release proinflammatory chemokines, which recruit monocytes/macrophages in the vasoproliferative lesions. The angiogenic factors produced by phagocytic cells play a central role in mediating angiogenesis (Resto-Ruiz et al., 2002; McCord et al., 2006; O'Rourke et al., 2015). The main virulence factors involved in the invasion of the ECs are summarized in **Table 1**.

TABLE 1 The main virulence factors involved in the invasion of endothelial cells and erythrocytes.

Virulence factor	Functions	Lineages	References
Trw type IV secretion system	Adhesion to erythrocyte surface	*Bartonella australis;* Lineage 4	Harms and Dehio, 2012
BadA/Vomps	Binding to extracellular matrix proteins	*B. henselae/B. quintana* (lineage 4)	Riess et al., 2004
Deformin	Damage in erythrocyte membranes	*B. bacilliformis* (lineage 1); *B. henselae* (lineage 4)	Mernaugh and Ihler, 1992; Iwaki-Egawa and Ihler, 1997
VirB/VirD4-T4SS, Bartonella effector proteins	Inhibition of apoptosis, proinflammatory activation, modulation of angiogenesis, invasome formation	*B. ancashensis* (lineage 1); Lineage 3–4	Wagner and Dehio, 2019
BafA	Angiogenesis	*B. quintana*, *B. henselae* and *B. elizabethae* (lineage 4)	Tsukamoto et al., 2020, 2022
CFA	Invasion of erythrocytes	Lineage 1–4	Siewert et al., 2022b
IalB protein	Promoting enter erythrocytes	*B. bacilliformis* (lineage 1); *B. henselae* (lineage 4)	Mitchell and Minnick, 1995; Coleman and Minnick, 2001

Infection of erythrocytes

The induction of intra-erythrocytic bacteremia is a hallmark of Bartonellae infection in mammalian hosts. The infection process of erythrocytes by *Bartonella* is divided into three main stages: adhesion, erythrocyte deformation, and invasion, with various virulence factors involved (**Table 1**).

The initial step is the adhesion of *Bartonella* to erythrocytes. In some *Bartonella* species, a type of T4SS called Trw has been identified as a key factor promoting host-specific erythrocyte infection, leading to prolonged bacteremia (Frank et al., 2005). Upon attachment, Bartonellae invade and penetrate mature erythrocytes, although the exact mechanism of this process is not fully understood. However, several necessary factors have been identified. The invasion-associated locus B genes (*ialB*) are crucial for the invasive behavior of the bacteria. IalB protein locates on the outer membrane of *B. henselae* (Coleman and Minnick, 2001; Chenoweth et al., 2004), and the inner membrane of *B. bacilliformis* (Mitchell and Minnick, 1995). The loss of *ialB* does not significantly affect bacterial adhesion, yet it leads to a ten-fold reduction in the number of bacteria residing in erythrocytes (**Figure 2**; Deng et al., 2016). Another important factor is deformin, a small molecule with protease resistance, heat resistance, and high affinity with albumin (Iwaki-Egawa and Ihler, 1997; Hendrix and Kiss, 2003). During infection, *B. bacilliformis* interacts with human erythrocytes to produce trenches, pits, conical invaginations, and internal vacuoles in the erythrocyte membrane (Mernaugh and Ihler, 1992; Xu et al., 1995), which is stimulated by deformin (**Figure 2**). Similarly, deformation activity on the erythrocyte membrane has also been reported in *B. henselae* (Iwaki-Egawa and Ihler, 1997).

A recent study showed that the CAMP-like factor autotransporter (CFA) of *B. taylorii* is crucial for infecting erythrocytes. Mice infected with a mutant strain lacking the *cfa* locus remained free of bacteremia, while the wild-type strain caused infection (Siewert et al., 2022b). CFA was first identified as an autotransporter virulence protein with potential cohemolysin activity in *B. henselae* (Litwin and Johnson, 2005). Autotransporters are a family of proteins secreted by gram-negative bacteria through the type V secretion mechanism, and they transport themselves through the outer membrane. The extracellular passenger domain at the N-terminal region of autotransporters often binds to a β-barrel folded by the C-terminal region in the bacterial outer membrane (Tame, 2011). Comparative genomics analysis has shown that the *cfa* locus is present in all Eubartonellae, with a hypervariable antigenic region (Siewert et al., 2022b). Meanwhile, CFA is one of the major targets for protective neutralizing antibodies that can prevent the attachment of pathogens to erythrocytes independent of complement or Fc receptors in hosts (Siewert et al., 2022b).

The undetectable situation of *Bartonella* after erythrocyte invasion plays a significant role in its spread, aided by the mobility and the lack of organelles in mature red blood cells, posing challenges for treatment selection. Research on *Bartonella* will provide insight into intracellular parasitology.

Host immune response after *Bartonella* invasion

Bartonella can cause a broad spectrum of diseases in humans, and the severity of symptoms is closely related to the immune status of the patients. It can be self-limited in immunocompetent individuals (Windsor, 2001; Gai et al., 2015), and it also can be very serious and even fatal for individuals with human immunodeficiency virus (HIV) or advanced immunosuppression (Mosepele et al., 2012). Bartonellae can establish long-lasting intraerythrocytic bacteremia, therefore, the intraerythrocytic cellular niche in host cells poses a daunting challenge to antibacterial immune defense. The lack of the major histocompatibility complex (MHC) on the cell surface of erythrocytes prevents antigen presentation, making MHC-dependent cytotoxicity an ineffective response. However, other immune responses are employed by the host to eliminate the pathogen.

Macrophages and DCs are innate immune cells that are vital for host's defense against *Bartonella* invasion. *In vitro*, co-incubation of murine macrophage cell line J774 with *B. henselae* led to rapid internalization of the bacterium by the macrophages. The

phagocytosis of unstimulated murine macrophages to *B. henselae* achieved full saturation within 4 h, accompanied by a significant increase in the expression of tumor necrosis factor α, interleukin (IL)-1β, and IL-6 by J774 (Musso et al., 2001). Following phagocytosis of *B. henselae*, DCs highly express the aforementioned three cytokines and upregulate the expression of CXCL8, CXCL1, and CXCL13, which recruit neutrophils and B cells to the site of infection (Vermi et al., 2006). The secretion of pro-inflammatory cytokines contributes to the formation of characteristic granuloma in CSD, which promotes the confinement of *B. henselae* to specific sites, limiting its spread within the host.

Mounting antibody responses that neutralize the pathogen is proved to be critical for clearing *Bartonella* cells in the blood of infected mice (Koesling et al., 2001). Studies have demonstrated that antibodies can prevent bacterial attachment to erythrocytes and suppress bacteremia independent of complement or Fc receptors (Pulliainen et al., 2012). The antibodies that interfere with *B. taylorii* adhesion to erythrocytes *in vitro* belong to the IgG2a and IgG3 isotypes (Siewert et al., 2022b; **Figure 3**). In this humoral immune defense process, the bacterial surface determinant CFA was recently identified as a target for protective antibodies (Siewert et al., 2022b). Furthermore, antibodies against *B. bacilliformis* could confer long-term immune protection. Individuals living in high-risk areas for *B. bacilliformis* have been found to possess significantly higher serum IgG levels specific to *B. bacilliformis* compared to those living in areas where the first outbreak of Carrion's disease occurred (Gomes et al., 2016b). During CSD infection, IgG and IgA are the main antibodies induced, with IgG1 being the major subclass of IgG (McGill et al., 1998). Specific IgM is the predominant antibody during the acute infectious phase in *B. bacilliformis*-infected patients and elevated specific IgG levels indicate a history of infection (Pons et al., 2017). However, *in vitro* experiments have shown extensive cross-reactivity between *B. henselae* to various microorganisms, such as *Treponema pallidum* and *Chlamydia* group (McGill et al., 1998). The above humoral immune processes can be seen in **Figure 3**.

The complement system, an important part of the innate immune system, plays a key role in defending against *Bartonella* before it enters erythrocytes in blood vessels and other cell types in peripheral tissues. An *in vitro* experiment showed that the alternative pathway of the complement system was primarily involved, with activation of the classical pathway also detected when human-derived non-immune serum was exposed to *B. henselae* (Rodriguez-Barradas et al., 1995; **Figure 3**).

In addition, humoral immune defense is essential in preventing and eliminating vertical transmission of *Bartonella*. Vertical transmission of *Bartonella* exists in mice and has been reported in a patient (Breitschwerdt et al., 2010b). Siewert et al. (2022a) recently found that only B cell-deficient offspring developed persistent bacteremia upon vertical transmission of *B. taylorii* in mice, whereas the corresponding wild-type offspring cleared the infection and developed protective immune memory. This result severely challenges the proposal that immunological tolerance in offspring due to vertical transmission is a mechanism of *Bartonella* persistence (Kosoy et al., 1998).

Besides, *Bartonella* infection stimulates the secretion of certain cytokines in the host. Specifically, interferon γ, which activates macrophages to destroy intracellular pathogens, is highly expressed in the peripheral blood of cats with bacteremia (Kabeya et al., 2009) and in the spleen cells of *B. henselae*-infected mice (Kabeya et al., 2007). Additionally, innate immune defense mechanisms, such as mannan-binding proteins, may also play a role in the recognition and elimination of *B. henselae* (Ezekowitz and Stahl, 1988; Rodriguez-Barradas et al., 1995). In contrast, cell-mediated immune response (CMI) is crucial for eliminating pathogens when *Bartonella*, especially *B. henselae*, enters host cells (excluding erythrocytes) (Kabeya et al., 2009).

Bartonella immune escape in mammalian host

Bartonella has also developed several strategies to evade the host's immune response. Evasion of innate immune response is a prerequisite for *Bartonella* to establish intracellular infection in the host. When *Bartonella* is inoculated at subcutaneous or intradermal sites, it is first and foremost exposed to resident DCs and macrophages. *B. tribocorum* has been reported to be resistant to phagocytosis in rats (Hong et al., 2017). The phagocytosis resistance is likely delivered by the structural mechanism of bacterial aggregates and is validated by the fact that a *badA* knock-out *Bartonella* strain is more susceptible to phagocytosis by macrophages (Riess et al., 2004). Even if engulfed by macrophages, *Bartonella* can inhibit pyroptosis and suppress the expansion of the inflammatory response (Hong et al., 2017). Alternatively, it can form a unique *Bartonella*-containing vacuole (BCV) that can delay lysosomal targeting and destruction (Kyme et al., 2005).

As gram-negative bacteria, Bartonellae possess a lipopolysaccharide (LPS) component on the outer membrane that is a well-known ligand for Toll-like receptor 4 (TLR4). However, *Bartonella* LPS is poorly recognized by TLR4, which may also contribute to the low efficiency of phagocytosis. LPS of *B. quintana* and *B. henselae* exhibits several unusual features, including a unique structure, lipid A with a long fatty acid side chain, and a lack of an O-chain polysaccharide (Zahringer et al., 2004; Popa et al., 2007; Malgorzata-Miller et al., 2016). Furthermore, the LPS of *B. quintana* is an antagonist of TLR4, inhibiting the expression of cytokines, including IL-1β, IL-6, and tumor necrosis factor α, generated by TLR4-linked pathways (Popa et al., 2007; Mosepele et al., 2012; Malgorzata-Miller et al., 2016). During *Bartonella* infection, TLR4 does not play a significant role in pathogen recognition. Instead, the host's innate immune response is triggered through TLR2 recognition (Vermi et al., 2006; Matera et al., 2008). In the case of *Candida albicans* infection, TLR2 has been shown to induce IL-10 secretion and Treg cell survival, thereby inhibiting inflammation (Netea et al., 2004). However, it is still unknown whether *Bartonella* infection triggers a similar response.

An antagonistic mechanism against the complement system has also been identified in *Bartonella*. BadA is proven to be critical for bacterial resistance to the host complement system. A *badA*-knockout *Bartonella birtlesii* mutant was susceptible to mouse serum, whereas the wild-type *B. birtlesii* expressing active BadA was resistant, which could be neutralized by anti-BadA antibodies (Deng et al., 2012).

Antigenic variation is another efficient strategy for *Bartonella* to evade the host immune response (**Figure 3**). Two important

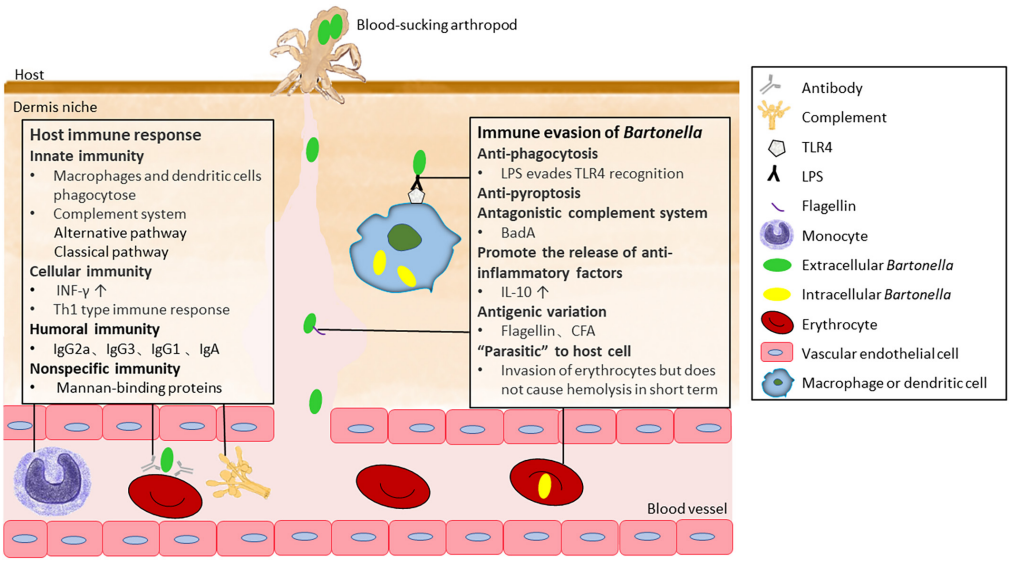

FIGURE 3
The immune response of the host to *Bartonella* and immune escape mechanism of *Bartonella*.

virulence factors, BadA in *B. henselae* (Riess et al., 2004) and the Vomp family in *B. quintana* (Zhang et al., 2004), have great potential to evade the host immune response through antigenic variation. The stem domain of *badA* and *vomp* contains modular and repetitive DNA sequences that perhaps increase the recombination frequency of the corresponding genes (Linke et al., 2006), while the internal structure of the *vomp* locus promotes recombination and deletion of the *vomp* gene (Zhang et al., 2004). The variable expression of Vomp family members was detected in both macaque animal models and *B. quintana*-infected humans (Zhang et al., 2004), and the expression of BadA seemly exhibits the characterization of phase variation (Thibau et al., 2022a). Intriguingly, *B. bacilliformis* can evade Toll-like receptor 5 by possessing unique amino acid sequences in flagellin that are different from the evolutionarily conserved ones required for microbial fitness (Andersen-Nissen et al., 2005; **Figure 3**). Bartonella CFA, an important target of protective antibodies, has a hypervariable antigenic region in both human- and mouse-hosted *Bartonella* strains (Siewert et al., 2022b). This hypervariability has the potential to allow for antibody evasion in the same mammalian host infected with different *Bartonella* strains through multiple sequential or timewise overlapping modes.

Suppression of immune response is also employed by *Bartonella* to escape the host immune response. Some *Bartonella* species, such as *B. quintana* and *B. henselae*, can promote mononuclear cells and DCs to secrete IL-10 (Papadopoulos et al., 2001; Vermi et al., 2006; Foca et al., 2012; Schmidgen et al., 2014), which suppresses inflammation response and facilitates the continuous progression of asymptomatic pathogen infection (Kabeya et al., 2007; Couper et al., 2008). The vital function of IL-10 in *Bartonella*'s immune evasion has been demonstrated in mouse models, as the pathogen *B. birtlesii* is unable to establish bacteremia in IL-10 knockout mice (Marignac et al., 2010). What's more, recent studies showed that BepD activated the STAT3 pathway and promoted the secretion of anti-inflammatory cytokine IL-10, which may play a role in the resistance of *Bartonella* to innate immune cells in the dermal niche (Fromm and Dehio, 2021). In addition, *B. vinsonii* reduces MHC-II expression on the surface of B cells in dogs, suggesting the declined B-cell antigen presentation to helper T cells (Pappalardo et al., 2001). In cats, post-infection with *B. henselae* leads to a decreased number of $CD4^+$ cells (Kabeya et al., 2009), yet the corresponding mechanism is still unknown.

The intracellular persistence of *Bartonella* is another unique strategy to avoid the host humoral immune system. *B. quintana*, for example, colonizes intracellularly without causing hemolysis of erythrocytes, which benefits the spread through body lice and cause repeated infection (Rolain et al., 2002, 2003). Recent studies have also detected *B. quintana* in human dental pulp stem cells (DPSCs), and the increase of bacterial load within the cellular niche does not affect the proliferation of DPSCs (Oumarou Hama et al., 2021).

The host immune response and *Bartonella* immune escape competes and restricts each other as shown in **Figure 3**. A strong immune response can reduce the frequency of host bacteremia, leading to recovery under the combined action of specific and non-specific immunity. However, weak immune response can result in repeated and long-term *Bartonella* infection, which can even be life-threatening. Therefore, studying the host immune response after *Bartonella* infection can help understand the causes of bacillary angiomatosis, peliosis hepatis, CSD, and other diseases, and provide new ideas for alleviating the clinical symptoms of patients after *Bartonella* infection.

Conclusion

Bartonella is primarily transmitted through the feces of blood-sucking arthropods or traumatic contact with infected animals. The invasion of Bartonellae in lineage 3 and lineage 4 is facilitated by the VirB/VirD4 T4SS system and Beps protein. The pathogen's invasion triggers the humoral and cellular immune responses of the

host. Particularly, the immune system produces various antibodies to neutralize pathogens, playing a vital role in the removal of *Bartonella* from the bloodstream.

However, *Bartonella* species have developed several mechanisms to evade or resist the host's immune response, which allow them to cause long-termed and repeated bacteremia. Some *Bartonella* species rely on special LPS structure or are hidden in host cells, while others stimulate the host to produce cytokines that weaken the immune response. Here we briefly summarized the recent findings on how *Bartonella* interacts with host ECs. Among the well-studied molecules are the trimeric family of autotransporters, α-enolase, and BafA. Additionally, *Bartonella* infection of MSCs can increase the susceptibility of ECs to *Bartonella*. The molecular involvement during erythrocyte invasion has also deepened our understanding of *Bartonella* infection. Investigating the mechanism of *Bartonella* infection on ECs and erythrocytes is crucial for comprehending *Bartonella* disease. Despite significant efforts, there remain numerous uncertain aspects that require further investigation, such as fully elucidating the functions of various types of molecules mentioned above. Future studies on *Bartonella* may require more appropriate *in vitro* and *in vivo* infection models, as well as functional genomics studies. Our efforts would be directed toward developing more effective and economical methods for detecting *Bartonella* in the population, as well as preventing and treating the diseases caused by *Bartonella*.

Author contributions

TL and JF contributed to the conception of the review and reviewed and revised the manuscript. XJ, YG, TL, JF, YX, JL, and JS collected and organized the data. XJ and YG wrote the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Corrigendum: Advancements in understanding the molecular and immune mechanisms of *Bartonella* pathogenicity

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***Bartonella*, blood-sucking arthropods, endothelial cells, erythrocytes, antibody, immune escape**

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In the published article, there was an error in the section **Infection of endothelial cells (ECs)**, paragraph 1. The citation in "*Bartonella* species have been found to inhabit various host cells, including mononuclear phagocytes, $CD34^+$ progenitor cells, and mesenchymal stromal cells (MSCs) (Scutera et al., 2021), but …" was incorrect. The correct citation is "(Mändle et al., 2005)". In the published article, this work was not included in the list of references. The reference is "Mändle, T., Einsele, H., Schaller, M., Neumann, D., Vogel, W., Autenrieth, I. B., et al. (2005). Infection of human CD34+ progenitor cells with *Bartonella henselae* results in intraerythrocytic presence of *B. henselae*. *Blood* 106, 1215–1222. doi: 10.1182/blood-2004-12-4670".

In the published article, there was an error in the section **Infection of endothelial cells (ECs)**, paragraph 1. The citation in "The degradation of extracellular matrix proteins has been confirmed to be facilitated by the interaction between fibrinolysis and several pathogen proteins (Vaca et al., 2022)." was incorrect. The correct citation is "(Lähteenmäki et al., 2001)" In the published article, this work was not included in the list of references. The reference is "Lähteenmäki, K., Kuusela, P., Korhonen, T. K. (2001). Bacterial plasminogen activators and receptors. *FEMS Microbiol. Rev.* 25, 531–552. doi: 10.1111/j.1574-6976.2001.tb00590.x".

In the published article, there was an error in the section **Infection of endothelial cells (ECs)**, paragraph 2. The citation in "These adhesins belong to the trimeric autotransporter adhesin (TAA) family (Hoiczyk et al., 2000)." was incorrect. The correct citation is "(Linke et al., 2006)". As the work is no longer cited, the reference "Hoiczyk E, Roggenkamp A, Reichenbecher M, Lupas A, Heesemann J (2000). Structure and sequence analysis of Yersinia YadA and Moraxella UspAs reveal a novel class of adhesins. *EMBO J.* 19, 5989–5899. doi: 10.1093/emboj/19.22.5989" has been removed.

In the published article, there was an error in the section **Infection of endothelial cells (ECs)**, paragraph 3. The citation in "It activates hypoxia-inducible factor-1 and stimulates the secretion of pro-angiogenic cytokines, such as vascular endothelial growth factor (VEGF) and C-X-C motif chemokine ligand (CXCL) 8 (Kaiser et al., 2008, 2012), contributing to *Bartonella*-induced vasoproliferation." was incorrect. The correct citation is "(Riess et al., 2004; Kempf et al., 2005; McCord et al., 2006)" In the published article, the following works were not included in the list of references, but have been added:

"Riess, T., Andersson, S. G., Lupas, A., Schaller, M., Schäfer, A., Kyme, P., et al. (2004). *Bartonella* adhesin A mediates a proangiogenic host cell response. *J. Exp. Med.* 200, 1267–1278. doi: 10.1084/jem.20040500"

"Kempf, V. A., Lebiedziejewski, M., Alitalo, K., Wälzlein, J. H., Ehehalt, U., Fiebig, J., et al. (2005). Activation of hypoxia-inducible factor-1 in bacillary angiomatosis: evidence for a role of hypoxia-inducible factor-1 in bacterial infections. *Circulation* 111, 1054–1062. doi: 10.1161/01.CIR.0000155608.07691.B7"

As it is no longer cited, the reference "(1) Kaiser, P. O., Linke, D., Schwarz, H., Leo, J. C., and Kempf, V. A. (2012). Analysis of the BadA stalk from Bartonella henselae reveals domain-specific and domain-overlapping functions in the host cell infection process. *Cell. Microbiol.* 14, 198–209. doi: 10.1111/j.1462-5822.2011.01711.x" has been removed.

In the published article, there was an error in the section **Blood-sucking arthropods as vectors for *Bartonella* transmission**, paragraph 1. The citation in "… human body lice *Pediculus humanus corporis* for *B. quintana* (Kloch et al., 2018)…" was incorrect. The correct citation is "(Byam and Lloyd, 1920)". In the published article, this work was not included in the list of references. The reference is "Byam, W., and Lloyd, L. (1920). Trench fever: its epidemiology and endemiology. *Proc. R. Soc. Med.* 13, 1–27." As it is no longer cited, the reference "Kloch, A., Wenzel, M. A., Laetsch, D. R., Michalski, O., Bajer, A., Behnke, J. M., et al. (2018). Signatures of balancing selection in toll-like receptor (TLRs) genes - novel insights from a free-living rodent. *Sci. Rep.* 8:8361. doi: 10.1038/s41598-018-26672-2" has been removed.

The authors apologize for these errors and state that they do not change the scientific conclusions of the article in any way. The original article has been updated.

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Novel evidence on sepsis-inducing pathogens: from laboratory to bedside

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Sepsis is a life-threatening condition and a significant cause of preventable morbidity and mortality globally. Among the leading causative agents of sepsis are bacterial pathogens *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus pyogenes*, along with fungal pathogens of the *Candida* species. Here, we focus on evidence from human studies but also include *in vitro* and *in vivo* cellular and molecular evidence, exploring how bacterial and fungal pathogens are associated with bloodstream infection and sepsis. This review presents a narrative update on pathogen epidemiology, virulence factors, host factors of susceptibility, mechanisms of immunomodulation, current therapies, antibiotic resistance, and opportunities for diagnosis, prognosis, and therapeutics, through the perspective of bloodstream infection and sepsis. A list of curated novel host and pathogen factors, diagnostic and prognostic markers, and potential therapeutical targets to tackle sepsis from the research laboratory is presented. Further, we discuss the complex nature of sepsis depending on the sepsis-inducing pathogen and host susceptibility, the more common strains associated with severe pathology and how these aspects may impact in the management of the clinical presentation of sepsis.

KEYWORDS

sepsis, inflammation, immunology, microorganisms, diagnostics, prognosis, therapy

1. Introduction

Sepsis, a life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer et al., 2016), is a significant cause of geriatric, maternal, neonatal, and child mortality. The World Health Organization (WHO) responded in 2017 with a resolution acknowledging sepsis as a major cause of preventable morbidity and mortality globally, while highlighting some of the most frequent pathogens connected etiologically to this condition, their primary site of infection, the increasing contribution of nosocomial infections, and their alarming resistance to antibiotics [World Health Organization (WHO), 2017]. Great strides toward an accurate quantification of its incidence and mortality (Rudd et al., 2020), and the pathogens accounting for its excess of mortality (GBD 2019 Antimicrobial Resistance Collaborators, 2022), have been made since that resolution. While

epidemiological findings raise awareness and aid in the shaping of public health policies, molecular discoveries contribute to the identification of host factors of susceptibility and pathogen mechanisms of immune evasion, structuring the therapeutic tools of the future.

Bacterial pathogens lead cases of bloodstream infections (BSI; Gouel-Cheron et al., 2022) with *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Streptococcus pyogenes*, as the as the pathogens with the largest number of attributable deaths (Diekema et al., 2019; GBD 2019 Antimicrobial Resistance Collaborators, 2022). Among fungal pathogens, *Candida* species are the most frequent pathogens in the critical care setting, with strong nosocomial association (Delaloye and Calandra, 2014; Gouel-Cheron et al., 2022). Viral agents are less frequently regarded as the cause of sepsis and are often deemed as facilitators to infection by secondary agents (Lin et al., 2018). Nevertheless, Herpes simplex virus, Enterovirus, Influenza, Adenovirus, Dengue virus, and recently the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), are considered as viral causes of sepsis (Shane et al., 2017; Teparrukkul et al., 2017; Iuliano et al., 2018; Alhazzani et al., 2020).

Because only a fraction of the articles included in this work regarded the comprehensive clinical continuum that sepsis represents, a pragmatic approach translating results from invasive infection and progression into the bloodstream, either by extension or by analogy, was adopted. Thus, this review summarizes recent findings in terms of pathogen epidemiology, virulence factors, host factors of susceptibility, mechanisms of immunomodulation, current therapies, antibiotic resistance, and opportunities for diagnosis, prognosis, and therapeutics, focusing on evidence from human studies but also including cellular and molecular evidence from *in vitro* and *in vivo* studies, all through the looking glass of BSI and sepsis.

2. *Escherichia coli*

Escherichia coli is a Gram-negative bacillus with remarkable phylogenetic diversity. Although mostly recognized for its pathogenic role in diarrheal diseases, and less frequently to extraintestinal illness, *E. coli* is also a part of the commensal microbiome of the host. Commensal strains (originating typically from phylogroup A) rarely cause diseases in healthy hosts, as they lack specialized virulence traits. Intestinal-pathogenic *E. coli* comprise groups diarrheagenic, enteropathogenic, enterohemorrhagic, enterotoxigenic, enteroaggregative, enteroinvasive, and diffusely adherent (originating typically from phylogroups B1 and E; Kaper et al., 2004; Bachmann et al., 2015). Extraintestinal *E. coli* (ExPEC) comprise a growing group that includes uropathogenic *E. coli* (UPEC), sepsis-associated *E. coli* (SEPEC), and neonatal meningitis *E. coli* (NMEC), among others (Desvaux et al., 2020), originating typically from phylogroups B2 and occasionally from phylogroups D, F, or G (Escobar-Páramo et al., 2004; Clermont et al., 2019). In fact, a recent prospective observational cohort study on *E. coli* bacteremia found that phylogroups most frequently associated with fatal outcome after 28 days were, in order, B2 (46%), D (17%), and B1 (15%; de Lastours et al., 2020). The main source of *E. coli* bacteremia is urinary tract infection (representing more than 50% of the cases; Bonten et al., 2020), with advanced age (>65 years) representing the greatest risk factor for asymptomatic *E. coli* bacteremia of urinary source (OR = 1.8–2.95; Bai et al., 2020). Consistently, UPEC isolated from patients with pyelonephritis exhibit much higher serum resistance (82–93%) than fecal *E. coli* isolates (57%; Coggon et al., 2018; Table 1). Although widely considered as the leading pathogen for bacteremia (Shorr et al., 2006; Al-Hasan et al., 2012), the predominance of *E. coli* depends on the timeframe, geographical location, and age of the patients included in the analysis.

TABLE 1 Summary of novel diagnostic/prognostic markers and resistance factors of interest for sepsis detailed in this review.

Organism	Marker/factor	References
Resistance factors		
E. coli	UPEC bacteremia	Coggon et al. (2018)
K. pneumoniae	Serotype O1, O2, or O3 bacteremia	Choi et al. (2020)
S. aureus	PVL$^+$ MRSA bacteremia	Zhao et al. (2022)
P. aeruginosa	Resistance genes *blaGES*, *aadB*, *gyrA* (T83I), and *parC* (S87L)	Recio et al. (2021)
S. pyogenes	*emm43.4*/PBP2x-T553K or emm93.0 bacteremia	Hayes et al. (2020) and Ron et al. (2022)
Novel diagnostic/prognostic markers		
S. aureus	Measurement of genes *COX7C*, *NDUFA4*, *ATP5J*, *NDUFB3*, and *COX7A2*	Wu H. et al. (2021)
	Measurement of m6A-SNPs	Sun et al. (2020)
	Measurement of caspase-1, IL-18, and NLRP3	Rasmussen et al. (2019)
	Measurement of C5a and IL-10	Eichenberger et al. (2020)
	Measurement of neutrophil/lymphocyte ratio	Greenberg et al. (2018)
S. pyogenes	Multidimensional scaling of leukocyte and platelet abundance	Loof et al. (2018)
Candida spp.	Machine-learning	Ripoli et al. (2020)
	Delta neutrophil index	Park et al. (2020)
	Multivariate risk score	Poissy et al. (2020)
	Composite SOFA/CCI score	Asai et al. (2021)
	Prior corticosteroid use	Kayaaslan et al. (2021)

ATP5J, ATP synthase-coupling factor 6; C5a, complement component 5a; COX7A2, cytochrome c oxidase subunit 7A2; COX7C, cytochrome c oxidase subunit 7C; IL-10, interleukin 10; IL-18, interleukin 18; m6A-SNPs, N6-methyladenosine associated single-nucleotide polymorphisms; MRSA, methicillin-resistant *S. aureus*; NDUFA4, cytochrome c oxidase subunit NDUFA4; NDUFB3, NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3; NLRP 3, NOD-like receptor family pyrin domain-containing 3; PVL, panton–valentine leukocidin; UPEC, uropathogenic *E. coli*; VEGF, vascular endothelial growth factor.

2.1. Epidemiology

The SENTRY program for global antimicrobial surveillance places *E. coli* as the number one pathogen causing BSI worldwide since 2005 (Diekema et al., 2019). However, this is not consistent in the United States, where *S. aureus* leads the ranking since 1997. In fact, a recent report on the epidemiology of hospital-acquired BSI in intensive care unit patients in Europe reveals that *Klebsiella* spp. lead the ranking of Gram-negative bacteremia, relegating *E. coli* to the second place (Tabah et al., 2023). Similarly, data from a prospective observational study conducted in Thailand, which included data from 3,806 patients, found that *E. coli* was the most frequent causative agent for BSI, and that age over 70 years carries an increased hazard ratio of 1.5 for 28-day mortality (Somayaji et al., 2021). A recent meta-analysis reported that the incidence of *E. coli* bacteremia is increasing over time in select high-income countries around the globe with pooled mortality rates ranging from 10.7 to 14.3% (Bonten et al., 2020). Also, data from the SENTRY program place *E. coli* as the leading pathogen causing BSI only in ages older than 64 years old, while for the rest of the age groups *S. aureus* leads the ranking, a fact that was ratified in a 10-year report on the epidemiology of bacteremia in a Portuguese pediatric population (Ferreira et al., 2023). Nevertheless, a recent cohort study representative of the United States, which analyzed 217,480 neonatal patients, found an overwhelming majority of *E. coli*-associated sepsis (Stoll et al., 2020). Remarkably, *E. coli* dominance in hospital onset bacteremia (Diekema et al., 2019) is significantly diluted during concomitant viral co-infection (Glass et al., 2022), especially in patients hospitalized for coronavirus disease 2019 (COVID-19; Garcia-Vidal et al., 2021).

2.2. Pathogen factors

Virulence factor diversity for *E. coli* isolated from blood comprises genes coding for proteins related to adherence (*ecp*, *hcp*, *eaeH*, *fim*, and *pap*), intracellular traffic (*upaG*, *ehaB*, and *agn43*), invasion (*tia*), iron uptake (*sitA*, *chuA*, *fyuA*, and *iuccC*), and toxins (*hlyE*/*clyA*, *usp.*, and *senB*) allowing bacteria to reach and survive in the bloodstream (Kim et al., 2022). However, ExPEC makes use of additional virulence traits, members of the serine protease autotransporters of Enterobacteriaceae (SPATE) superfamily involved in evasion from host immune defense mechanisms (Abreu et al., 2015). A recent prospective observational cohort study analyzing blood isolates from 278 patients found SPATE coding genes in 61% of the isolates, with an overwhelming presence in phylogroup B2, with *sat* and *vat* as the most prevalent of them (Freire et al., 2020). SPATE gene *sat* is a class 1-SPATE recognized as a cytotoxic factor with proteolytic activity (Freire et al., 2022) and *vat* is a class 2-SPATE cytotoxic factor (Nichols et al., 2016; Díaz et al., 2020), and both displayed a significant presence in ExPEC isolates of phylogroups B2, D, E, and F (Freire et al., 2020). *In vitro* evidence on virulence factors reveals that *E. coli* adheres to endothelial cells by a direct and necessary interaction between bacterial cell membrane protein OmpA and endothelial integrin αVβ3 which, in turn, activates endothelial cells via calcium-dependent intracellular cascades that ultimately lead to a downregulation of VE-Cadherin (Tapia et al., 2019), increased vascular permeability (Gatica et al., 2020), and endothelial apoptosis (McHale et al., 2018). Interestingly, all these readouts displayed not different to uninfected controls when using OmpA-deficient mutant strains, strongly underlining the role of OmpA in the genesis of sepsis at the cellular level. A summary of the factors detailed in this section is listed in Table 2.

TABLE 2 Summary of novel pathogen factors of interest for sepsis detailed in this review.

Organism	Activity	Pathogen factor	References
E. coli	Survival	*iss*	Fröding et al. (2020)
	Adhesion	*iha17, ecp, hcp, eaeH, fim,* and *pap*	de Lastours et al. (2020) and Kim et al. (2022)
	Intracellular traffic	*upaG*, *ehaB*, and *agn43*	
	Invasion	*tia*	
	Iron metabolism	*sitA*, *chuA*, *fyuA*, and *iuccC*	
	Toxins	*hlyE*/*clyA*, *usp,* and *senB*	
	Cytotoxicity	SPATE coding genes *sat* and *vat*	Freire et al. (2020)
	Cell infection	*ompA*	McHale et al. (2018)
K. pneumoniae	Invasion	*rmpA/2*	Cienfuegos-Gallet et al. (2022), Kochan et al. (2022), and Liao et al. (2022)
	Iron metabolism	*ybt,* iucA, and *iroB*	
	Cytotoxicity	*clb*	
	Metabolism	*peg-344*	
S. aureus	Survival	*capA*	Recker et al. (2017)
	Cytotoxicity	PVL, TSST-1, and *hly*	Ahmad et al. (2020), Monecke et al. (2020), and Sun et al. (2021)
P. aeruginosa	Survival	*mifR*	Xiong et al. (2022)
	Invasion	*hepP*	Dzvova et al. (2018)
	Iron metabolism	Hxu, Has, and Phu systems	Otero-Asman et al. (2019) and Yang F. et al. (2022)
	Cytotoxicity	*exoS*	Recio et al. (2021)
S. pyogenes	Invasion	*emm*, *speG*, *speH*, *speJ*, and *speK*	Imöhl et al. (2017) and Sánchez-Encinales et al. (2019)
	Metabolism	*spy1476*, *spy1343*	Sitkiewicz and Musser (2017) and Kant and Pancholi (2021)

PVL, panton–valentine leukocidin; SPATE, serine protease autotransporters of Enterobacteriaceae; and TSST-1, toxic shock syndrome toxin-1.

2.3. Host factors

Clinical manifestations of *E. coli* bacteremia include fever, disorientation, hypotension, and respiratory failure, and may include septic shock, which is estimated to be present in about 25% of bacteremic patients (Kang et al., 2005). In fact, a recent prospective observational cohort study found a significant association between *E. coli* bacteremia and adverse outcomes after 28 days for the following clinical presentations: cancer, chronic peripheral arteritis, sepsis, and septic shock at initial presentation, infection of the digestive tract and airway, and start of adequate antibiotic therapy after 48 h of onset bacteremia (de Lastours et al., 2020). Interestingly, bacteremias starting as urinary tract infections displayed a greater strength of association with the survivor group, representing a noteworthy point of inflection for these types of infections. The latter is not an isolated figure, as confirmed in a multivariate analysis from a recent retrospective cohort study on *E. coli* bacteremia, where this observation urinary tract infection is reported as a remarkable protective factor (OR = 0.07) for the outcome 30-day mortality (Chapelet et al., 2017). Furthermore, multivariate analysis reveals an astounding OR of 6.54 for pulmonary portal of entry as determinant of 28-day mortality, after adjustment. Other determinants included in the final multivariate model were infection with bacterial factor STc88 (OR = 3.62), expression of virulence factor iha_17 (OR = 4.41), and other comorbidities (OR = 1.14; de Lastours et al., 2020). Similarly, another prospective observational cohort study found a significant association between *E. coli* bacteremia and septic shock or death within 72 h in patients presenting hematologic cancer or history of transplantation (OR = 16.34), reduced daily living activity (OR = 3.85), and presence of virulence factor *iss* (OR = 7.71), after adjustment in a multivariate analysis (Fröding et al., 2020). The excess risk revealed for oncologic, or transplantation patients is a call for caution to practitioners attending such conditions. A summary of the factors detailed in this section is listed in Table 3.

2.4. Treatment

Empiric antimicrobial therapy follows a general Gram-negative bacteremia algorithm with prolonged delivery of broad-spectrum β-lactam antibiotics as the first indication (Evans et al., 2021). Directed therapy is aimed at restraining resistance with recommendations to switch to a single agent with the narrowest spectrum to which the organism is susceptible. A recent prospective observational cohort study found that amoxicillin/clavulanic acid, cotrimoxazole, and fluoroquinolone, had the strongest association between antibiotic resistance and 28-day mortality in *E. coli* bacteremia (de Lastours et al., 2020). Favorably, none of the non-survivors presented any carbapenem resistance, which was present in only one out of 493 survivors. Moreover, multivariate analysis reveals an increased risk for 28-day mortality by broad-spectrum β-lactam and/or third-generation cephalosporin resistant strains in patients presenting chronic alcoholism (OR = 3.04), initiating adequate antibiotic treatment after 48 h (OR = 3.04), or with a history of bacteremia (OR = 2.81), after adjustment (de Lastours et al., 2020). According to the latest global report on antimicrobial resistance by the WHO, the median resistance to third generation cephalosporins in *E. coli* bloodstream confirmed infections is 41.8% [World Health Organization (WHO), 2022a]. It is suggested that cefotaxime and ceftriaxone are the most suitable antibiotics to monitor cephalosporin resistance in *E. coli* BSI. Thus, pathogen-directed analyses are key to guide the development and implementation of strategies on age-targeted prevention, antimicrobial resistance, and new therapies.

3. *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative, encapsulated, non-motile, facultatively anaerobic bacterium. *Klebsiella* species are commonly found in soil, water, plants, and livestock (Morgado et al., 2022), and *K. pneumoniae* in human hosts is not strictly pathogenic. In fact, *K. pneumoniae* colonizes the nasal and digestive tract without causing any symptomatic disease (Chang et al., 2021), and it is proposed that gastrointestinal colonization serves as a major reservoir for transmission and infection to other sites (Martin et al., 2016). Moreover, studies show a high level of association between gastrointestinal carriage and subsequent infection by a patient's own *K. pneumoniae* strains (Martin et al., 2016; Gorrie et al., 2017). Although the exact mechanisms are unclear, *K. pneumoniae* progression from intestinal territories has been related to a disproportion in bacterial density of colonizing strains, especially when host defenses are challenged, e.g., during cancer, diabetes mellitus, and alcohol abuse (Happel and Nelson, 2005). Traditionally, serotypes of *Klebsiella* isolates have been identified and followed using typing antisera. Different O and K serotypes are produced as a result of the detection of distinctive variants of surface-exposed polysaccharides, named O-antigens and K-antigens, by certain antibodies. O-antigens constitute the outer layer of lipopolysaccharide (LPS), whereas K-antigens are a component of the bacterial capsule polysaccharide (CPS). To date, eight serotypes for O-antigens and 77 for K-antigens have been described (Follador et al., 2016).

3.1. Epidemiology

A recent multi-country collection study revealed that *K. pneumoniae* geographical diversity is dominated worldwide by antigen O1, followed by antigen O2, which displayed a larger proportion in Europe, and antigen O3, less represented in Africa (Choi et al., 2020). Additionally, a recent report on worldwide BSI identified *K. pneumoniae* as the third most prevalent pathogen consistently over the last 25 years (Diekema et al., 2019). Moreover, various publications on *K. pneumoniae* progression into the bloodstream identified pulmonary, abdominal, and urinary sites as the leading sources of infection (Togawa et al., 2015; Hyun et al., 2018; Juan et al., 2019; Huang Y. T. et al., 2020; Li M. et al., 2023). Interestingly, all these reports are successful in identifying the primary source of infection, a fact that differs from clinical findings reported before 2010, in which *K. pneumoniae* bacteremia of unknown origin reached up to 58% of cases (Tsay et al., 2002; Tumbarello et al., 2006). Among the most widely distributed *K. pneumoniae*, serotype antibiotic resistance was predominantly associated to serotypes carrying antigens O2 and O3, while serotypes carrying antigen O1 (the most frequently distributed worldwide) was associated with sensitivity to extended spectrum cephalosporins, fluoroquinolone, and carbapenems, among many other antibiotics (Choi et al., 2020;

TABLE 3 Summary of novel risk and protective factors of interest for sepsis detailed in this review.

Organism	Factor	References
Pathogen risk factors		
E. coli	Presence of phylogroups B2, D, and B1	de Lastours et al. (2020)
K. pneumoniae	Presence serotypes K1, K2, K20, K54, K57	Liao et al. (2022)
	Presence of *bla*KPC-bearing strains	Hu et al. (2021)
S. aureus	Presence of PVL^+ MRSA strains	Imauven et al. (2022)
P. aeruginosa	Presence of MLST, ST235 or O11 serotype	Recio et al. (2021)
	Strong biofilm producing strains	di Domenico et al. (2021)
	Non-motile strains	Gupte et al. (2021)
Candida spp.	Presence of BDG^+ species	Agnelli et al. (2019)
Host risk factors		
E. coli	Advanced age	Bai et al. (2020) and Somayaji et al. (2021)
	Neonate infection	Stoll et al. (2020)
	Chronic comorbidities: cancer, chronic peripheral arteritis	de Lastours et al. (2020)
	Bacteremia with pulmonary portal of entry	Chapelet et al. (2017)
K. pneumoniae	Bacteremia with pulmonary portal of entry	Chen I. R. et al. (2022)
	Presence of central venous catheter	Ang et al. (2022)
S. aureus	Advanced age, male sex	Bassetti et al. (2018) and Imam et al. (2019)
	Presence of genes *COX7C, NDUFA4, ATP5J, NDUFB3*, and *COX7A2*	Wu H. et al. (2021)
P. aeruginosa	Advanced age, male sex	Esparcia et al. (2019)
	Indwelling urinary catheter	Esparcia et al. (2019) and Tan et al. (2021)
	Long-term hospital stay	Tan et al. (2021)
	Immunocompromise	Hammer et al. (2017)
	Bacteremia with pulmonary portal of entry	
	X-linked agammaglobulinemia	Bhardwaj et al. (2017), Biscaye et al. (2017), Birlutiu et al. (2019), and Huang H. et al. (2020)
S. pyogenes	Low levels of VEGF	Lu et al. (2022)
Candida spp.	Concomitant bacteremia	Lee et al. (2020), Pieralli et al. (2021), Zhong et al. (2022), and Gebremicael et al. (2023)
	SOFA score	Bienvenu et al. (2020), Jung et al. (2020), Huang H. Y. et al. (2020), and Kutlu et al. (2022)
	CVC	Lee et al. (2020) and Huang H. Y. et al. (2020)
	Liver cirrhosis	González-Lara et al. (2017), Battistolo et al. (2021), and Meyahnwi et al. (2022)
	Kidney dysfunction	Poissy et al. (2020), Mazzanti et al. (2021), and Kutlu et al. (2022)
	Charlson Comorbidity Index ≥4	Bassetti et al. (2020), Yoo et al. (2020), and Kim et al. (2021)
	Concomitant neoplasia	Lee et al. (2020), Battistolo et al. (2021), and Vázquez-Olvera et al. (2023)
	Current azole therapy	Lee et al. (2020)
	Age ≥ 65	Meyahnwi et al. (2022)
	Concurrent antibiotic therapy	
	Neutropenia	Kim et al. (2021)
	Total parenteral nutrition	Pieralli et al. (2021) and Kutlu et al. (2022)
	Hemodialysis	Bassetti et al. (2020)
	Cardiovascular surgery	Mazzanti et al. (2021)
	IV catheter	Huang H. Y. et al. (2020)
	MODS ≥6	Chen et al. (2020) and Yoo et al. (2020)
	Concomitant severe sepsis	González-Lara et al. (2017)
	Required vasopressor therapy	Gebremicael et al. (2023)
	Liver dysfunction	
	Broad-spectrum antibiotic use before candidemia	Kutlu et al. (2022)
	Thrombocytopenia	
	Delayed treatment	Bienvenu et al. (2020)
Host protective factors		
E. coli	Bacteremia with urinary portal of entry	Chapelet et al. (2017) and de Lastours et al. (2020)
P. aeruginosa	Levels of hemoglobin in pediatrics	Kung et al. (2020)
S. pyogenes	VEGF	Lu et al. (2022)
	Endosomal TLR13 pathogen recognition	Hafner et al. (2019)
Candida spp.	CVC removal	Kutlu et al. (2022)

ATP5J, ATP synthase-coupling factor 6; BDG, (1,3)-β-D-Glucan; COX7A2, cytochrome c oxidase subunit 7A2; COX7C, cytochrome c oxidase subunit 7C; CVC, central venous catheter; GI disease, gastrointestinal disease; blaKPC, b-lactamase *K. pneumoniae*; MLST, multilocus sequence typing; MODS, multiple organ dysfunction score; MRSA, methicillin-resistant *S. aureus*; NDUFA4, cytochrome c oxidase subunit NDUFA4; NDUFB3, NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3; PVL, panton–valentine leukocidin; SOFA, sepsis organ failure assessment; TLR13, toll-like receptor 13; VEGF, vascular endothelial growth factor.

Table 1). According to the latest global report on antimicrobial resistance by the WHO, *K. pneumoniae* is the third leading agent causing bacteremia with an increasing resistance to third- and fourth-generation cephalosporin and co-trimoxazole therapy [World Health Organization (WHO), 2022a], pushing the use of carbapenems (Souli et al., 2017).

3.2. Pathogen factors

Among the most prevalent virulence factors identified in BSI in the United States are genes *ybt*, *clb*, *iucA*, *rmpA*, *rmpA2*, and *iroB*, of which, *iucA* and *rmpA/2* have been associated with carbapenem resistance (Kochan et al., 2022). Consistently, *iucA*, *rmpA*, *rmpA2*, *iroB*, and *peg-344*, were recently described as the most prevalent virulence genes in Taiwan (Liao et al., 2022), while genes *ybt*, *iuc*, and *rmp* were recently described as the most prevalent in China (Cienfuegos-Gallet et al., 2022). Co-infection of *K. pneumoniae* with other bacterial agents (i.e., *Acinetobacter baumannii*, *P. aeruginosa*, and *E. coli*, among other less frequent bacteria) has been documented and sized previously (Karakonstantis et al., 2022; Lv et al., 2022).

3.3. Host factors

Multivariate analyses report nosocomial pneumonia, high SOFA score, inappropriate treatment (Chen I. R. et al., 2022), high APACHE II score, development of septic shock (Wu X. et al., 2021), and central venous catheter (Ang et al., 2022), as significant risk factors for mortality from *K. pneumoniae* BSI. Moreover, BSI with serotypes K1, K2, K20, K54, and K57, has also been found associated to increased mortality distributions (Liao et al., 2022). Furthermore, infection with *K. pneumoniae* β-lactamase (bla_{KPC})-harboring strains has been found as an independent risk factor for mortality (Hu et al., 2021). Co-infection of *K. pneumoniae* with other viral agents, particularly with SARS-CoV-2, has been well described (Damico et al., 2022; Said et al., 2022), reviewed (Santos et al., 2022), and sized (Lansbury et al., 2020; Kariyawasam et al., 2022; Santos et al., 2022) elsewhere. Of note, the proportion of *K. pneumoniae* isolates from COVID-19 patients has been documented to be significantly lower compared to COVID-negative controls, implicating that infection with viral agents may reduce the chance to develop bacteremia, a subject that requires further research (Glass et al., 2022). A summary of the factors detailed in this section is listed in Table 2.

3.4. Treatment

In line with general Gram-negative bacteremia treatment, empiric antimicrobial therapy considers prolonged delivery of broad-spectrum β-lactam antibiotics as the first indication (Evans et al., 2021). However, driven by the extensive use of carbapenems, the growth and spread of carbapenem-resistant bacterial infections has emerged as a major public health concern in recent decades. In fact, *K. pneumoniae* resistance to carbapenems has experienced a 3-fold increase worldwide since 2016 [World Health Organization (WHO), 2022a]. A wise strategy to overcome this new threat is founded on the use of carbapenem antibiotics in combination with β-lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam. Thus, notorious advances in terms of drug efficacy and safety have been made over the last decade with new β-lactam/β-lactamase inhibitor combinations (BLIC) synergistically restoring antimicrobial sensitivity, e.g., aztreonam/avibactam, ceftazidime/avibactam, imipenem/relebactam, meropenem/vaborbactam, and cefepime/zidebactam, among others (Vázquez-Ucha et al., 2020).

A recent study in Taiwan reports a significant restoration of imipenem activity against carbapenem-nonsusceptible *K. pneumoniae* when combined with relebactam, increasing susceptibility from 0.8 to 88.4%, 21.4 to 42.9%, and 30 to 40%, in isolates producing carbapenemases of Ambler classes A, B, and D, respectively (Yang T. Y. et al., 2022). In accordance, regarding the same combination, a study analyzing *K. pneumoniae* isolates from Spain and Portugal reports a major decrease in resistance in Ambler class A carbapenemase-harboring strains, while reporting no resistance to ceftazidime/avibactam combination in isolates producing carbapenemases of Ambler classes A and D (Hernández-García et al., 2022). In fact, experimental combination of ceftazidime/avibactam and aztreonam (Shah et al., 2021) or possibly meropenem (Parruti et al., 2019) has been reported successfully not only in clinical case reports but also in a recent cohort study (adjusted HR = 0.136; Zheng et al., 2021). Additionally, use of fosfomycin in combination with third- and fourth-generation cephalosporin has been thoroughly (in a retrospective cohort study analyzing 104 cases of carbapenem resistant *K. pneumoniae* bacteremia) demonstrated as protective (adjusted OR = 0.07) against mortality by sepsis (Liao et al., 2017). While these promising results constitute the latest evidence on advanced approaches in patients, further evidence from animal models reveals that rifampin alone or in combination with colistin have the strongest effect against carbapenemase-producing *K. pneumoniae* sepsis mortality *in vivo* (Pachón-Ibáñez et al., 2018). Moreover, use of amikacin alone or in combination with fosfomycin significantly reduced circulating bacterial load in a sepsis model using carbapenemase-producing *K. pneumoniae* strains (Cebrero-Cangueiro et al., 2021).

In the quest for new therapies, a recent report on animal *K. pneumoniae* sepsis shows that the use of mushroom-derived β-glucans are able to reduce bacterial load while improving physiological parameters associated to the pathobiology of sepsis (i.e., arterial pO_2, plasma lactate, pulmonary compliance, and arterial alveolar oxygen gradient; Masterson et al., 2020). In line with this, Bergenin monohydrate (a plant extract with immunomodulatory properties) has been described to reduce reactive oxygen species (ROS) production and increase cell viability *in vitro* while increasing levels of superoxide dismutase (SOD) and GSH, and reducing bacterial load, levels of inflammatory cytokines interleukin (IL)-6, IL-1β, prostaglandin E2 (PGE2), and tumor necrosis factor (TNF-α), malondialdehyde (MDA) formation, myeloperoxidase (MPO) content, and number of infiltrating leukocytes in a MAPK/NF-κB–dependent manner in the lungs of septic animals challenged with *K. pneumoniae* (Tang et al., 2021). Similarly, AS101 (an inorganic compound with anti-apoptotic, anti-inflammatory, and immunomodulatory effects; Okun et al., 2007) has been described to reduce bacterial load across the liver, kidney, and spleen, and ultimately increasing mice survival from 0% 30 h after *K. pneumoniae* injection *i.p.* to 75% after 72 h in a mouse model of carbapenem-resistant *K. pneumoniae* sepsis (Yang et al., 2021). Also, TNF-related

apoptosis-inducing ligand (TRAIL) encapsulated to a polypeptide-crosslinked nanogel has been described to significantly reduce bacterial load in blood and increase mice survival from 0% 4.5 days after *K. pneumoniae* instillation *i.t.* to 75% after 12 days in a mouse model of *K. pneumoniae* sepsis (Chen et al., 2019). Likewise, adipose-derived mesenchymal stem cells were described to significantly reduce bacterial load across the lung, blood, liver, and spleen; reduce pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in the lung; and reduce tissue immune infiltration and damage, by downregulating genes related to nicotine, thymine, and uracil degradation, and upregulating genes related to unfolded protein response, sirtuin signaling pathway, and leukocyte adhesion and diapedesis, among others, in a mouse model of *K. pneumoniae* induced pneumosepsis (Perlee et al., 2019).

A cutting-edge approach using red blood cell membrane-coated poly(lactic-co-glycolic acid) (PLGA) nanoparticles (γ3-RBCNPs) to improve antibiotic bioavailability showed an astounding increase in mice survival from 0% 4 days after *K. pneumoniae* instillation *i.t.* to 60% after 7 days of instillation, with a significant and systematic reduction in bacterial load across the lung, blood, liver, spleen, and kidney, and a significant reduction in circulating levels of TNF-α, IL-1β, and IL-6 (Liu et al., 2022). Another cutting-edge approach using novel therapeutic antibodies targeting K1-serotype CPS of hypervirulent *K. pneumoniae* (hvKp) strains to promote bacterial phagocytosis by Kupffer cells in the liver were described to significantly reduce bacterial load across the lung, liver, and spleen, of hvKp challenged mice while increasing their survival from 0% 4 days after hvKp injection *i.p.* to 75% after 15 days in a mouse model of *K. pneumoniae* sepsis (Diago-Navarro et al., 2017). On the edge of knowledge, it has been revealed that exposure to blue light (peak 442 nm) significantly reduces bacterial load from the lung, blood, liver, and spleen, while significantly increasing mice survival from 15% 100 h after *K. pneumoniae* instillation *i.t.* to 62% after 125 h of bacterial challenge (Griepentrog et al., 2020). This was explained by local modulation of MPO activity and neutrophil abundance. Interestingly, there was no significant change in mononuclear abundance in the lung. The effect of blue light was mechanistically elucidated and attributed to a neural circuit signaling through a cholinergic anti-inflammatory pathway that directly controls immune responses in the spleen, enhancing control of the infection, and improving survival (Griepentrog et al., 2020). Thus, the potential therapeutic utility of something as trivial as ambient light in the ICU is strongly underscored. These cutting-edge approaches constitute powerful and promising tools in the search for new avenues for the treatment of sepsis that entails further research and encouragement. Lastly, pushing the edges of knowledge, it has been described that *K. pneumoniae* is able to induce alterations in the gut microbiome and cecal metabolome in a mouse model of sepsis (Wu T. et al., 2020). A significant difference in the richness, diversity, and composition of bacterial communities in mice challenged with *K. pneumoniae*, with fewer Bacteroidetes and Firmicutes but higher levels of Proteobacteria and Verrucomicrobia. At the genus level, mice challenged with *K. pneumoniae* had significantly fewer Bacteroides, Parabacteroides, Bifidobacterium, Clostridium, Coprococcus, and Prevotella, which produce short-chain fatty acids (SCFA). Interestingly, absolute concentration of SCFAs both in cecal contents and in serum were consistently lower in mice challenged with *K. pneumoniae*. Notably, supplementation of SCFAs in drinking water significantly reduced bacterial burden and tissue damage in the lungs, and significantly reduced mortality, while increasing phagocytic capacity of alveolar macrophages and pulmonary levels of IL-6 and TNF-α. Thus, characterization of microbial biomarkers displays a sizable potential for aiding in the diagnosis/prognosis of patients under critical care.

Additional evidence from animal models implicates PTX3 pentraxin (a component of humoral innate immunity involved in resistance to selected pathogens by promoting opsonophagocytosis) as protective factor expressed on the host side promoting bacterial phagocytosis, expression of TNF-α, IL-1β, IL-6, and CXCL-1, limiting MPO levels and leukocyte counts, protecting from tissue hemorrhage and increased mortality in septic mice challenged with *K. pneumoniae* (Asgari et al., 2021). Hypoxia-inducible factor (HIF) has also been implicated in modulating the immune system of the host during sepsis (Otto et al., 2021). Using a Cre/lox system, HIF1α was found instrumental to engage host defense against *K. pneumoniae* pneumosepsis for its significant role in bacterial load reduction both in the lung and distant organs and limiting levels of pro-inflammatory cytokines while enhancing the release of IL-10 in the lung (Otto et al., 2021). This modulation was explained by an energetic dysregulation affecting glucose uptake, leading to a significant reduction in TNF-α production in both alveolar and interstitial macrophages in LysM-cre × $Hif1\alpha^{fl/fl}$ mice during onset *K. pneumoniae i.n.* challenge. Collectively, this frontline evidence shows as the foundation for the establishment of new diagnostic tools and the development of the therapeutics of the future.

4. *Staphylococcus aureus*

Staphylococcus aureus is a facultative Gram-positive organism mostly recognized for its pathogenic potential. Notwithstanding, *S. aureus* is a frequent colonizer in human hosts (Laux et al., 2019), with abundances reported recently as 32% for neonates (Arora et al., 2023), 25–29% for pediatrics (McNeil et al., 2022; Arora et al., 2023), and 30% for adults (Erayil et al., 2022). Although colonization is asymptomatic, it has been deemed a risk factor for developing BSI in the event of pathogenic transformation and bloodstream invasion (Tacconelli et al., 2017). Currently, there is no consensus on what single event leads to pathogenic progression. Rather, virulence mechanisms appear to be related to circumstance, environmental opportunity, and particularly, to host-pathogen interplay (Kwiecinski and Horswill, 2020).

4.1. Epidemiology

Staphylococcus aureus is reported as the leading causative agent for BSI in North America and the second most prevalent for the rest of the world (Diekema et al., 2019). Moreover, a recent study conducted in Australia reported an increased incidence of *S. aureus* bacteremia in older men, especially over 60 years old, almost doubling the incidence reported in the same age group in women (Imam et al., 2019). Furthermore, another study reported that MRSA bacteremia in patients older than 75 years old represents a significant risk factor (OR = 2.4) for 30-day mortality (Bassetti et al., 2018). Similarly, a prospective observational study carried out in Australia and New Zealand found a strong majority of the samples associated to

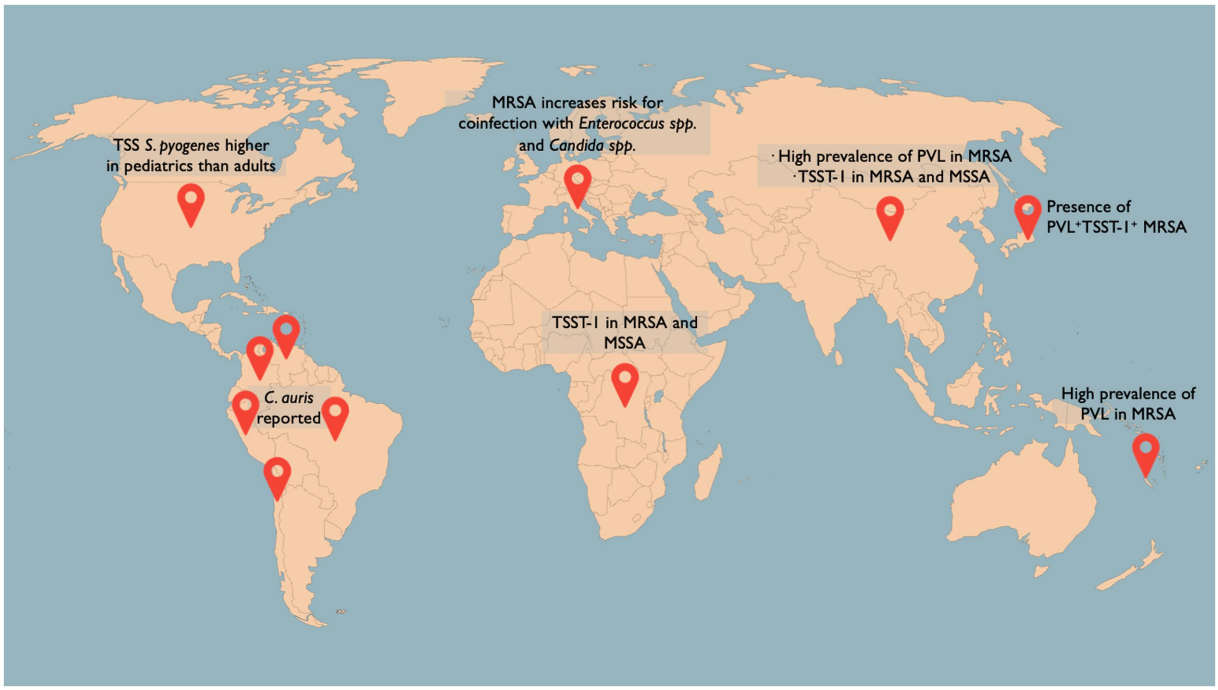

FIGURE 1
Selection of the latest figures on sepsis-associated pathogen epidemiology worldwide. *Streptococcus pyogenes*-associated TSS is reported in the United States with an uneven distribution between adults (22.5%) and pediatrics (7.1%; Meehan et al., 2018). TSST-1 has been identified in 67% of MRSA and 4% of MSSA blood isolates in the Democratic Republic of the Congo (Vandendriessche et al., 2017) and in 23.1% of MRSA and 6.7% of MSSA blood isolates in China (Liu et al., 2018). PVL$^+$TSST-1$^+$ isolates have been found in 0.4% of all MRSA isolates in Japan (Kaneko et al., 2023). A high prevalence of PVL$^+$ MRSA strains in adults has been found in New Caledonia Island (Imauven et al., 2022). An escalating number of countries in Latin America have reported the detection of *Candida auris* (Riera et al., 2022).

advanced age (>70 years-old, 63.1%), male sex (64%), and methicillin susceptibility (75.9%), and multivariate analysis further revealed sepsis as the most influent risk factor for 30-day mortality in patients with *S. aureus* bacteremia (OR = 4.01; Turnidge et al., 2009). Bloodstream coinfection with other pathogenic agents is less frequent, although it has been reported that *S. aureus*/*Candida albicans* coinfection has a catastrophic proportion of 82% for 30-day mortality and a high correlation with indwelling vascular devices (Wu Y. M. et al., 2021). In fact, a retrospective observational study conducted in Italy showed that patients older than 80 years afflicted with MRSA BSI had the highest risk for 30-day mortality and coinfection with *Enterococcus* spp. and *Candida* spp. were significant risk factors for this outcome (Giovannenze et al., 2021; Figure 1). More recently, *S. aureus*/SARS-CoV-2 coinfection has gained more insight, with reported 30-day mortality of 67%, largely attributable to hospital-onset bacteremia in patients mostly under mechanical ventilation (Cusumano et al., 2020).

4.2. Pathogen factors

A recent study comparing the pathogenic mechanisms of fixed clonal complexes of *S. aureus* during sepsis showed only one virulence property that remained consistent (Recker et al., 2017). These were polymorphisms in the *capA* gene, which encodes an enzyme involved in capsule biosynthesis, which is responsible for host immunity protection and acts as a virulence factor during sepsis. Notably, this gene is absent in *S. aureus* clones in North America (Boyle-Vavra et al., 2015), further highlighting the absence of a consistent set of virulence factors and the overall versatility of this pathogen. Another virulence factor of interest is cytotoxin Panton–Valentine Leukocidin (PVL), which is highly prevalent in community-acquired methicillin-resistant *S. aureus* (MRSA) and has been associated with higher risk of developing sepsis (Ahmad et al., 2020; Monecke et al., 2020). A study conducted in China comparing MRSA strains isolated from adult and pediatric patients reported a higher prevalence of PVL in adult MRSA isolates (55.8 vs. 35.3%), in addition to higher antibiotic resistance and higher mortality in adults (Zhao et al., 2022; Table 1). Clone ST5/ST764-MRSA SCC*mec* II was the predominant isolate in adults, whereas clone ST59-MRSA SCC*mec* IV was the predominant isolate in pediatrics (Zhao et al., 2022). Also regarding PVL, another study comparing adult and pediatric isolates from patients admitted to the ICU in New Caledonia Island (Southwest Pacific region) reported a significantly higher prevalence of PVL$^+$ MRSA strains in adults (61 vs. 30%), which carried an implicit higher risk for developing sepsis and a fatal outcome (OR = 4.57; Imauven et al., 2022).

Added to the array of virulence factors of *S. aureus* is toxic shock syndrome toxin-1 (TSST-1), which relates to the rapid development of symptoms consistent with sepsis, including hypotension and organ dysfunction. Although there is an insufficient number of large-scale studies on TSST-1 molecular epidemiology, recent data from the Democratic Republic of the Congo identified TSST-1 in 67% of MRSA and 4% of MSSA blood isolates (Vandendriessche et al., 2017), while

data from China reports TSST-1 in 23.1% of MRSA and 6.7% of MSSA blood isolates (Liu et al., 2018). For their joint virulence and its inherent potential to cause a fatal outcome (Hayakawa et al., 2020), despite representing only 0.4% of all MRSA isolates in a whole-genome analysis carried out in Japan (Kaneko et al., 2023) and 0% of all isolates in a worldwide comparative genomic analysis (Zhou et al., 2021), systematic monitoring of $PVL^{+}TSST\text{-}1^{+}$ isolates becomes a relevant facet of *S. aureus* virulence to survey. A summary of the factors detailed in this section is listed in Table 2.

4.3. Host factors

Host immunity plays a pivotal role in clearing *S. aureus* presence in the bloodstream. Notably, neutrophils isolated from patients undergoing *S. aureus* sepsis exhibit increased formation and release of neutrophil extracellular traps (NET) and pro-inflammatory cytokines TNF-α, IL-1β, and IL-8 (Gupta et al., 2022). Moreover, neutrophil and lymphocyte abundance as a ratio has been demonstrated as an independent predictor for 90-day mortality. Such simple but relatively costly measurement shows potential as a prognostic tool, which if supplemented with appropriate sensitivity and specificity information, may prove useful in orienting the management of septic patients (Greenberg et al., 2018). Likewise, serum levels of C5a and IL-10 were found significantly higher in samples from *S. aureus* bacteremic patients when compared to matched hospitalized and community controls (Eichenberger et al., 2020). Also, in the quest for prognostic predictors during *S. aureus* bacteremia, activity of caspase-1 in neutrophils and monocytes, serum levels of IL-18, and whole blood mRNA levels of inflammasome mediator NOD-like receptor family pyrin domain-containing 3 (NLRP3), were found to display a significant difference between survivors and non-survivors (Rasmussen et al., 2019). Bioinformatic screening of blood samples of *S. aureus* bacteremic patients further reveals direct correlation between the expression of a set of five genes (*COX7C, NDUFA4, ATP5J, NDUFB3*, and *COX7A2*; relating to aerobic respiration, cellular stress response, mitochondrial electron transport, mitochondrial transport, and oxidative phosphorylation) and adverse outcome (Wu H. et al., 2021). Although further testing of the prognostic value of such analysis is required, the high-throughput approach is noteworthy and the potential of a multiplex approach to follow the development of sepsis is promising.

Similarly, meta-analysis of existing databases further corroborates the plausibility for bioinformatics approaches to aid in tackling sepsis from a molecular flank. Analysis of post-translational modifications to mRNA (in particular, methylation of the sixth N atom on the adenine base, m6A) integrated into single nucleotide polymorphisms (SNP) revealed a significant presence of a set of m6A-SNPs during *S. aureus* sepsis (Sun et al., 2020; Table 1). Such set was characterized and associated to DNA repair, vesicle-mediated transport, peptidyl-serine phosphorylation, leukocyte migration, catabolic processes, regulation of endopeptidase activities, phagocytosis, and platelet degranulation. Although no further clinical characterization was reported, the value in diagnostics, prognosis, and therapeutics is yet to be explored.

Host defense against *S. aureus* is not limited to leukocytes, as recently demonstrated in a translational report (Sun et al., 2021). Prompted by an observation of the strong association between thrombocytopenia (without leukopenia or leukocytosis) and patient mortality, authors used an *in vivo* model to demonstrate that the underlying mechanism of platelet-mediated antibacterial activity is impaired by *S. aureus* pore-forming α-toxin (*Hla*). Interaction of α-toxin with platelet chemoreceptor P2Y12 was inhibited by using FDA-approved and commercially available antiplatelet drug ticagrelor, resulting in extended protection from thrombocytopenia, enhanced bacterial clearing, protection from organ damage, and increased survival. Thus, in a single report platelet contribution to host immunity and the repurpose of an available drug to protect against *S. aureus* bacteremia was elegantly demonstrated. Not surprisingly, ulterior reports have extended these findings by corroborating the protective effect of ticagrelor against *S. aureus* bacteremia. In a nationwide observational cohort study carried out in Denmark, ticagrelor use was demonstrated protective not only against *S. aureus* bacteremia but also, in a higher proportion, against sepsis and pneumonia (Butt et al., 2020). Further evidence on ticagrelor repurposed use is described in a recent case report detailing a steep correction in platelet count and complete recovery of a patient afflicted with methicillin-sensitive *S. aureus* (MSSA) bacteremia treated with ticagrelor for 3 months (Ulloa et al., 2021). A summary of the factors detailed in this section is listed in Table 3.

4.4. Treatment

Empiric treatment for *S. aureus* is initiated pending susceptibility tests and is prophylactically directed against MRSA with the use of vancomycin or daptomycin (Liu et al., 2011). Evidence has been presented in favor of the use of initial doses of vancomycin ≥20 mg/kg for a faster resolution of systemic inflammation (Wesolek et al., 2018). Once susceptibility is elucidated, if isolate is MSSA, antibiotic treatment is de-escalated to a β-lactam agent. Use of combination therapy is controversial, with weak evidence found in a recent clinical trial (Pujol et al., 2020) and several cohort studies (Rieg et al., 2017; Davis et al., 2018; Guthridge et al., 2021; Kufel et al., 2023). Appraisal of the therapeutic value of monotherapy was presented in a recent clinical trial carried out in South Korea, in which treatment of MSSA bacteremia with nafcillin was associated with higher Sequential Organ Failure Assessment (SOFA) scores, higher rates of treatment failure, and astoundingly higher mortality figures than treatment with cefazolin (Lee et al., 2018). In fact, odds ratios for treatment with cefazolin over nafcillin were 0.39 for SOFA score ≥ 2, 0.43 for treatment failure, and 0.15 for 90-day mortality. Overall protective effect of treatment of MSSA bacteremia using cefazolin was quantified with an adjusted OR = 0.44. Interestingly, matched propensity scores resulted significant only for the bacteremic group and not for the septic group, highlighting the higher level of complexity in the pathobiology of sepsis. A relevant preoccupation is the increasing resistance to methicillin, where median percentage resistance increased from 16.6% in 2017 to 18.3% in 2020, according to a recent global report (World Health Organization (WHO), 2022a). Other reports on antimicrobial resistance related to sepsis describe *S. aureus* methicillin resistance to be 17.6% in Australia and 15.5% in Europe (Coombs et al., 2022). Management of MRSA by means of either monotherapy or combination therapy of vancomycin or daptomycin, with or

without a β-lactam agent, shows no significant differences in 30-day mortality risk, length of stay, or risk of persistence, as evidenced by a recent meta-analysis (Yi et al., 2021). A fact to be assessed in terms of harms versus benefits when sizing therapeutical value and antibiotic resistance.

5. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an aerobic Gram-negative bacterium distributed ubiquitously in the environment. *P. aeruginosa* infections are frequently nosocomial and opportunistic, as they frequently occur concomitantly to existing physical, phagocytic, or immunologic dysfunctions in host defense, leading to bloodstream, urinary tract, respiratory, and intra-abdominal infection (Tang et al., 2017; Li X. et al., 2023).

5.1. Epidemiology

Recent reports place *P. aeruginosa* as the fourth most common cause of BSI globally (Diekema et al., 2019) and the third most frequent pathogen isolated in catheter-associated urinary tract infection and ventilator-associated pneumonia (Weiner-Lastinger et al., 2020). This is relatively consistent throughout the world, except in North America, where *P. aeruginosa* BSI ranks fifth (Diekema et al., 2019). Despite its relatively low proportion in BSI incidence, a recent study analyzing isolates from the United States reports *P. aeruginosa* as the second most frequent pathogen causing BSI mortality, accounting for nearly a quarter of the cases between 2016 and 2020 (Ohnuma et al., 2023).

5.2. Pathogen factors

Although pathogenesis of *P. aeruginosa* has been extensively studied in models of pneumonia, burn wounds and urinary infection, few studies have explored virulence factors expressing in sepsis-inducing strains. A recent transcriptomic analysis of clinical isolates associated to BSI revealed high-level expression of a novel cell-surface signaling system named Hxu (a response system for sensing extracellular porphyrin rings of haem; Otero-Asman et al., 2019), whereas deletion or overexpression of this pathway resulted in reduced or enhanced BSI, respectively (Yang F. et al., 2022). Several genes related to *P. aeruginosa* metabolism have been associated with the level of virulence of the strain PAO1, extensively used in sepsis models. Deletion of the regulator of α-ketoglutarate transport *mifR* resulted in a significant improvement of survival following pneumonia-induced sepsis in a murine model as well as a reduction in pro-inflammatory cytokines and reduced NLRP3 inflammasome activation (Xiong et al., 2022). In addition, expression of enzymes able to degrade host extracellular matrix components such as heparinase contribute to the virulence of sepsis-inducing strain *P. aeruginosa* PA14. Mutation of the gene encoding for heparinase (*hepP*) impaired bacterial dissemination and prevented mortality in murine models of thermal injury and intraperitoneal PA14 injection, indicating that *hepP* contributes to the pathogenesis of PA14 (Dzvova et al., 2018). A summary of the factors detailed in this section is listed in Table 2.

5.3. Host factors

Recent studies indicate that susceptibility to develop systemic infection is associated with various predisposing host factors. In fact, immune compromised hosts exhibit an adjusted odds ratio of 3.7 for BSI in a multivariate logistic regression model considering age, sex, ethnicity, chronic comorbidities, source of infection, recent ambulatory procedures, urinary catheterization, residence in skilled nursing facilities, chronic hemodialysis, current and recent hospitalization, and prior exposure to β-lactams and fluoroquinolones in the past 90 days (Hammer et al., 2017). Moreover, the latter odds ratio has been reported as high as 13.82 for BSI and 23.1 for 30-day mortality, also in multivariate analyses (Tan et al., 2021). Interestingly, according to a 2019 report on community-onset *P. aeruginosa* urinary infection in elderly people, odds ratio for sepsis were highest for patients who received healthcare (OR = 5.52), who had an indwelling urinary catheter (OR = 3.25), and who were male (OR = 3.16), as determined in a stepwise logistic regression (Esparcia et al., 2019). An interesting instance of host susceptibility is presented by the growing number of cases reported on *P. aeruginosa* sepsis in pediatric hosts with X-linked agammaglobulinemia (XLA, OMIM #300755; Bhardwaj et al., 2017; Biscaye et al., 2017; Birlutiu et al., 2019; Huang H. et al., 2020), an immunodeficiency characterized by failure to produce mature B lymphocytes resulting in clinically undetectable levels of all immunoglobulin isotypes. Pediatric patients with XLA rapidly develop sepsis after *P. aeruginosa* infection and, while specific pathogen identification delays targeted therapy, the appearance of landmark skin lesions (ecthyma gangrenosum) represent an informative sign of the underlying causative agent (*P. aeruginosa*).

An additional useful example that warrants attention is the protective effect observed for hemoglobin levels in *P. aeruginosa* pediatric sepsis as an outcome, with an astounding OR = 0.155 obtained after multivariate logistic regression analyses in a matched case–control study conducted in Taiwan (Kung et al., 2020). Extensive research has characterized the immune responses at the lung and skin in mouse models of acute *P. aeruginosa* infection but the role of both innate and adaptive immune cells during *P. aeruginosa* sepsis is still poorly understood. Bacterial dissemination and sepsis are usually accompanied by increased numbers of phagocytes, but in a model of burn wound infection, neutrophil and monocyte recruitment to the seroma fail to contain *P. aeruginosa* dissemination, promoting the development of sepsis (Brammer et al., 2021). However, modulation of these cells by host-derived molecules may prevent severe sepsis and mortality. Endogenous hydrogen sulfide (H_2S) produced by cystathionine-γ-lyase enhances neutrophil recruitment and their phagocytic activity resulting in reduced mortality in a mouse model of sepsis induced by a multidrug-resistant strain. In addition, H_2S reduces the expression of *P. aeruginosa* quorum sensing genes, favoring pathogen phagocytosis. Moreover, clinical correlates show that patients who survived sepsis had higher levels of circulating H_2S compared to non-survivors, strongly suggesting that H_2S plays a protective role during *P. aeruginosa* sepsis (Renieris et al., 2021).

Host immunosuppressive mechanisms may also play a detrimental role during *P. aeruginosa* sepsis. Animal models of secondary bacteremia following cecal punction ligation exhibit improved survival after a partial deletion of Tregs and a reduction in anti-inflammatory cytokine IL-10 (Hu et al., 2018). In line with this, treatment with the immunoregulatory molecule ethyl pyruvate reduced lung levels of

IL-10 and the expression of *FOXP3* in lung-derived Tregs in a two-hit model of sepsis, reversing *P. aeruginosa* secondary pneumonia (Chen et al., 2017). IL-10 deficient mice are more susceptible to PA14-induced pneumonia but do not display bacteremia (Belo et al., 2021), suggesting that IL-10 helps to control local antimicrobial responses, but high lung levels may favor bacterial dissemination. Therefore, the role of IL-10 and other anti-inflammatory host-derived molecules during *P. aeruginosa* sepsis is poorly understood and requires further research to confirm its role increasing host susceptibility to severe sepsis and mortality. *P. aeruginosa* not only interacts and promotes colonization and coinfection with *S. aureus* (Clancy et al., 2014) and *Stenotrophomonas maltophilia* (McDaniel et al., 2020) but is also a common cause of secondary bacterial infections in patients hospitalized for COVID-19 (Lansbury et al., 2020; Garcia-Vidal et al., 2021), a fact exemplifying its opportunistic nature. *P. aeruginosa* is associated with high in-hospital mortality rates and prolonged lengths of stay (Naylor et al., 2018). A summary of the factors detailed in this section is listed in Table 3.

5.4. Treatment

Clinically, antimicrobial treatment proceeds observing four arms: controlling the source (i.e., infected catheters or ventilator reservoirs), timely initiation of therapy, use of mono/combination therapy, and limiting antibiotic resistance. Extensive and uncontrolled use of antibiotics has contributed to the increase in multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains, which are difficult to treat, especially in cases of severe sepsis. In fact, *P. aeruginosa* is the fourth leading pathogen behind all deaths attributable to antibiotic resistance in high-income countries (Murray et al., 2022). Genetic characterization of *P. aeruginosa* bacteremia at advanced ages indicates a significant presence and association with fatal outcome of resistance genes bla_{GES}, *aadB*, *gyrA* (T83I), *parC* (S87L), virulence gene *exoS*, multilocus sequence typing (MLST) ST235, O-antigen serotype O11 (Recio et al., 2021), strong biofilm producing (di Domenico et al., 2021), and non-motile strains (Gupte et al., 2021). Interestingly, recent prospective study indicates that carbapenem resistance is not significantly related to treatment failure, a fact likely related to the site of infection, host susceptibility, and clinical severity (Lee C. M. et al., 2022). Recent studies in patients have shown inconclusive about the benefit of the combination of antibiotics such as ceftazidime-avibactam against MDR and XDR strains (Corbella et al., 2022). Although ceftolozane/tazobactam is effective against complicated urinary tract infections and complicated intra-abdominal infections, univariate analyses reveal that treatment using this therapeutical combination is significantly associated with a successful management of the clinical presentation of sepsis (Bassetti et al., 2019). Additionally, early use of this combination in neutropenic patients has been shown to control progression of skin infection, suggesting a protective effect on further dissemination with or without sepsis (Coppola et al., 2020).

By translation, a recent *in vivo* study explored the efficacy of different antibiotics in mouse models of sepsis caused by *P. aeruginosa*. Although carbapenem combinations did not show improved efficacy against carbapenemase-producing *P. aeruginosa*, meropenem monotherapy showed promising *in vivo* efficacy against peritoneal sepsis (Herrera-Espejo et al., 2022). Interesting evidence from *in vivo* models reveals that peptidylarginine deiminase (PAD) type 2 deficiency ($Pad2^{-/-}$) significantly improves survival in *P. aeruginosa* pneumonia-induced sepsis by attenuating acute lung injury (Wu Z. et al., 2020). Under the proposed mechanism, PAD2 deficiency enhances bacterial clearance by reducing caspase-1-dependent pyroptosis in bone marrow-derived macrophages. Thus, PAD2 is revealed as a promising molecular target that warrants further research.

Other experimental therapies have been tested alone or in combination with antibiotics in murine models and clinical assays. Because *P. aeruginosa* binds to host epithelial cells via pili and type-IV pili (T4P) are crucial for bacterial twitching, attachment, biofilm formation, and motility (particularly T4P containing PilA subunits; Zahedi bialvaei et al., 2021a), mAb against disulfide turn region of PilA (QA) and PilQ, alongside with clinically available antibiotics levofloxacin, ceftazidime, and gentamicin, showed a synergistic effect in the treatment of a mouse model of *P. aeruginosa* sepsis by preventing bacterial dissemination and increasing overall survival (Zahedi bialvaei et al., 2021b). Similarly, a case describing the use of a combination of antibiotics and a cocktail of two phages (PNM and 14–1) to attend an infection with an XDR strain secondary to liver transplantation in a septic toddler was deemed successful after confirming full clearance of *P. aeruginosa* from the bloodstream (van Nieuwenhuyse et al., 2022). Moreover, *in vitro* studies using the phage PNM have shown a synergistic effect in combination with suboptimal concentrations of colistin, aztreonam, or gentamycin against clinical isolates of *P. aeruginosa* (van Nieuwenhuyse et al., 2022).

6. *Streptococcus pyogenes*

Streptococcus pyogenes is a facultative anaerobe Gram-positive bacterium that strictly infects humans (Gera and McIver, 2013). *Streptococcus pyogenes* infections are highly contagious and range from pharyngitis, and skin or soft tissue (non-necrotizing) infection, to respiratory tract infection, pregnancy-associated infection, necrotizing fasciitis, and bacteremia with toxic shock syndrome (TSS; Walker et al., 2014).

6.1. Epidemiology

Recent analyses report an increasing incidence of *S. pyogenes* infections with a high case fatality in the older population (Shakoor et al., 2017; Meehan et al., 2018; Blagden et al., 2020; Vilhonen et al., 2020; Bläckberg et al., 2022; Thomson et al., 2022). The most prevalent form of clinical presentation of invasive *S. pyogenes* infection is bacteremia (75%), followed by focus without bacteremia (19%) and necrotizing fasciitis (7%; Meehan et al., 2018). Moreover, TSS is reported in 19% of bacteremia cases with an uneven distribution between adults (22.5%) and pediatrics (7.1%; Meehan et al., 2018), the latter figure reported higher in the United States (16.9%; Gaensbauer et al., 2018). Nevertheless, *S. pyogenes* bacteremia is significantly less represented (<8%) when compared to other pathogens (Ferreira et al., 2023; Tabah et al., 2023).

6.2. Pathogen factors

Until recently, the best described virulence factor of invasive *S. pyogenes* infection is the M protein, encoded by the *emm* gene and

expressed on the bacterial surface. A recent Spanish report on *emm* diversity in pediatrics revealed clone *emm1*/ST28 as the most prevalent and the most consistently detected over the 12 years span of the report, followed by clones *emm12*/ST36-ST242 and *emm6*/ST382 (Sánchez-Encinales et al., 2019). This trend was echoed by findings of a retrospective cohort study in Sweden, which included 286 samples from adult patients collected in a 4-year period (Bläckberg et al., 2022) and a prospective German nationwide cohort study, which included 719 isolates from patients of all ages collected in a 6-year period (Imöhl et al., 2017). A different pattern was observed in Finland where, in a recent cohort study spanning 12 years and focusing on women of childbearing age, *emm28* showed as the most frequent type of *S. pyogenes* strain, displaying a significant association with delivery and puerperium-related infections leading to bacteremia (Gröndahl-Yli-Hannuksela et al., 2021). Other proposed virulence factors arising during invasive *S. pyogenes* disease include streptococcal superantigens *speG*, *speH*, *speJ*, and *speK*, which, in the presence of certain underlying comorbidities (i.e., diabetes, chronic skin lesions, liver dysfunction, and respiratory distress), modulate the risk of invasive *S. pyogenes* disease (Imöhl et al., 2017).

Evidence from *in vivo* studies highlight the relevance of non-canonical Tyr-phosphatase M5005_Spy_1476 as a molecular mediator for *S. pyogenes* pathogenesis, an enzyme reported to maintain this pathogen in a virulent state leading to increased subject mortality by modulating its ability for adherence and invasion of host cells, and for *in vitro* biofilm formation in a mouse model of sepsis (Kant and Pancholi, 2021). Similar evidence has been reported for predicted gene *spy1343*, which codes for an inferred 298-amino acid protein that belongs to the LysR family of DNA-binding transcriptional regulators. Interestingly, mice challenged with a mutant strain carrying a deletion of *spy1343* exhibited a significant increase in mortality when compared to their control (WT) counterparts (Sitkiewicz and Musser, 2017). Explanation for this excess of virulence lies in the control of *spy1343* over genes that participate in short-chain fatty acid metabolism, which have been linked to overall bacterial pathogenesis and overall virulence. Additional evidence from *in vivo* studies reveals that *S. pyogenes* virulence may be traced from a clinical standpoint by evaluating the pattern of leukocyte and platelet abundance. Multidimensional scaling of median values of absolute counts of such type of cells was able to discriminate clusters of virulence and disease progression over time with precision, using simple, routinary readouts in experimental infection using a small animal model. Thus, underscoring the potential and unprecedented diagnostic/prognostic value of computer science in human infection progression, particularly in sepsis (Loof et al., 2018; Table 1). A summary of the factors detailed in this section is listed in Table 2.

6.3. Host factors

Host immune responses during *S. pyogenes* sepsis has received modest attention in both *in vivo* and *in vitro* models. It has been proposed that vascular endothelial growth factor (VEGF) expressed in endothelial cells may promote antimicrobial response against *S. pyogenes* infection, as observed *in vitro* with human endothelial cells increasing lysosomal biogenesis and function and overall bacterial xenophagy against *S. pyogenes* through the activation of TFEB and its downstream genes (e.g., *ATPV6* and *LAMP1*), and *in vivo* with VEGF treatment significantly increasing subject survival rate (Lu et al., 2022). Moreover, patients with severe invasive disease (sepsis, bacteremia, necrotizing fasciitis, and TSS) exhibit significantly lower serum levels of VEGF compared to those with non-invasive disease (Lu et al., 2022). Also relating to host immune response, TLR signaling plays an important role in early immune reactions against *S. pyogenes*. Nucleic acid detection by the endosomal receptor TLR13 mediates immune cell activation, expression of pro-inflammatory cytokines, and formation of ROS and reactive nitrogen species (RNS) against *S. pyogenes in vitro*. Mice defective in endosomal signaling (*Unc93b1*$^{-/-}$) exhibited higher bacterial burden at sites of lesion and the spleen, and significantly increased systemic inflammation in a murine model of soft tissue infection. Therefore, endosomal TLR signaling may play an important role in activating antimicrobial responses at local infection sites and prevent systemic disease (Hafner et al., 2019). Several studies have reported a synergistic interaction for infection between *S. pyogenes* and pathogenic viruses of the respiratory tract (Brundage, 2006; Herrera et al., 2016), a fact made evident during the 1918 influenza pandemic (Morens and Fauci, 2007), the 2009 H1N1 influenza pandemic (Jean et al., 2010), and more recently, the 2019 SARS-CoV-2 pandemic (Khaddour et al., 2020). However, data on *S. pyogenes* superinfections are scarce and interactions with additional infective agents warrant further resource allocation and research (Turner, 2022). A summary of the factors detailed in this section is listed in Table 3.

6.4. Treatment

Empiric antimicrobial therapy typically initiates pending culture results to then be tailored accordingly. Penicillin monotherapy in the setting of high inoculum has been associated with treatment failure (Stevens et al., 1994). For this reason, adjunctive use of clindamycin is recommended for its strong association with lower mortality (OR = 0.44; Babiker et al., 2021). An increasing number of isolates with resistance to clindamycin and other macrolides have been consistently identified around the globe [e.g., the United States (DeMuri et al., 2017), Hungary (Gajdács et al., 2020), India (Jayakumar et al., 2022), and China (Lu et al., 2017)] with the surprising exception of Spain, where tetracycline, erythromycin, and clindamycin resistance rates declined between 2007 and 2020 (Villalón et al., 2023).

Owing to its incapacity for horizontal gene transfer, *S. pyogenes* resistance to β-lactam antibiotics is a feature considered rare (Hayes et al., 2020). However, a report by the Active Bacterial Core surveillance (US Centers for Disease Control and Prevention, CDC) has recently made the alarming discovery of a *S. pyogenes* strain (*emm43.4*/PBP2x-T553K) with increased β-lactam resistance (Chochua et al., 2022; Table 1). Similarly, a recent report from active surveillance in Israel described the emergence of an outbreak of a new MDR strain *emm*93.0 responsible for an unusually large number of invasive *S. pyogenes* infections, especially present in the bloodstream (Ron et al., 2022). These unprecedented findings underline the importance of population-based pathogen surveillance programs internationally. Nonetheless, there is consensus that *S. pyogenes* infections—even when invasive—are associated with a low attributable mortality, unless they are invasive and meet the criteria for TSS, for which cases the mortality rate can reach up to 44% (Schmitz et al., 2018).

7. *Candida* species sepsis

Invasive fungal infections are clinical manifestations of fungal infections different from superficial infections proven by culture and isolation from sterile sites such as deep tissue, cerebrospinal fluid, or blood (Donnelly et al., 2019).

7.1. Epidemiology

Candida species are the predominant cause of life-threatening invasive fungal infections in hosts with decreased defenses (e.g., immunocompromised individuals, patients who have endured invasive clinical procedures, or have experienced major trauma; Bongomin et al., 2017) and constitute the most common cause of fungal BSI (Pappas et al., 2016), a clinical condition that has experienced a significant and concerning increase over the last decade, as a retrospective cohort study comprising 465 candidemia episodes in Germany (Mohr et al., 2020) and a retrospective cohort study comprising 170 candidemia episodes in Switzerland (Battistolo et al., 2021) report. Although the latest report by the SENTRY Antifungal Surveillance Program carefully details different forms of invasive fungal infection, it does not present isolated data for fungal BSI (Pfaller et al., 2019). Nevertheless, recent observational data ratify *Candida albicans* as the leading species during fungal BSI and further expand the list with other non-albicans species [in hierarchical order: *Candida parapsilosis*, *Candida glabrata*, and *Candida tropicalis* (Doğan et al., 2020); *Candida tropicalis*, *Candida parapsilosis*, and *Candida glabrata* (Al-Musawi et al., 2021)]. However, in one study from Saudi Arabia (Aldardeer et al., 2020) and another from India (Lamba et al., 2021), *Candida albicans* was relegated to the second place, being *Candida glabrata* and *Candida tropicalis* the leading species for fungal BSI, respectively.

7.2. Pathogen factors

Candida species grow in yeast or filamentous (pseudohyphae and hyphae) morphologies and this morphology strongly correlates to its pathogenicity, revealing fungal programs of invasion, virulence, and overall versatility and capacity for adaptation (Lo et al., 1997; Bartie et al., 2004). For a formidable review on pathogen virulence factors and the immune response during *Candida* sepsis we strongly recommend consulting article by Patricio et al. (2019). One such factor is (1,3)-β-D-Glucan (BDG), a major structural component of the inner cell wall in *Candida* species, which not only serves as the main pathogen-associated molecular pattern that interacts with pattern recognition receptors on the host side but has also been implicated a prognostic value. Although without accuracy or performance test for comparison with other diagnostic methods, BDG^{-} candidemia has been recently identified as an independent protective factor from poor clinical outcome (Agnelli et al., 2019).

7.3. Host factors

Correspondingly, timely removal of central venous catheter has also been identified as an independent protective factor against mortality (Kutlu et al., 2022). Incidentally, delayed removal of central venous catheter during candidemia has been consistently identified as a risk factor for mortality in adults (Huang H. Y. et al., 2020; Lee et al., 2020), pediatrics (Lee W. J. et al., 2022), and neonates (Chen Y. N. et al., 2022). Other risk factors for adult mortality with great representation across quantitative multivariate reports are concomitant bacteremia (Lee et al., 2020; Pieralli et al., 2021; Zhong et al., 2022; Gebremicael et al., 2023), high SOFA score (Bienvenu et al., 2020; Huang H. Y. et al., 2020; Jung et al., 2020; Kutlu et al., 2022), presence of liver cirrhosis (González-Lara et al., 2017; Bartoletti et al., 2021; Battistolo et al., 2021; Meyahnwi et al., 2022), kidney dysfunction (Poissy et al., 2020; Mazzanti et al., 2021; Kutlu et al., 2022), and others summarized in Table 3 (Bassetti et al., 2020; Yoo et al., 2020; Kim et al., 2021). Particularly for early-age groups, additional risk factors for mortality include breakthrough candidemia (Lee W. J. et al., 2022), previous use of antibiotics for >2 weeks, persistent candidemia, and preterm gestation (<32 weeks; Eisi et al., 2022), placing the emphasis in the implementation and follow-up of protocols to properly manage neonatal care.

Chronic comorbidities appraised as compounded scores (i.e., Karnofsky Performance Status <70 and Charlson Comorbidity Index ≥4) were also found to increase the risk of mortality during candidemia (Bassetti et al., 2020; Yoo et al., 2020; Kim et al., 2021; Vázquez-Olvera et al., 2023). Mixed *Candida*/bacterial BSI is referenced to occur in 18–56% of candidemia cases (Bouza et al., 2013; Kim et al., 2013; Chen et al., 2020) and recent articles agree [20.5% of candidemias reported in (Zhong et al., 2020) and 29.7% of candidemias reported in (Lee E. H. et al., 2022)]. The most frequent bacteria isolated from the bloodstream were coagulase-negative *Staphylococcus*, followed by *Klebsiella pneumoniae*, and *Staphylococcus aureus*. As expected, mixed BSI poses an even more complex challenge to host responses and, although a significant increase in mortality would be expected, the only significant increments were seen in length of ICU stay and length of mechanical ventilation use (Zhong et al., 2020). Conversely, *Candida* BSI following admission and treatment for COVID-19 has been found to significantly shift the outcome of patients toward fatality, along increasing the utilization of mechanical ventilation, the need for central venous catheter and parenteral nutrition, and overall length of stay (Rajni et al., 2021; Kayaaslan et al., 2023). Interestingly, admission and treatment for COVID-19 was found to double the incidence rate of developing candidemia, as a retrospective cohort study report (Kayaaslan et al., 2021). Even more interestingly, candidemia was found in a significant portion of patients who underwent surgery during their treatment for COVID-19 and those with a history of corticosteroid use. In fact, prior corticosteroid use was identified as a significant risk factor for mortality with a strength of association (OR = 4.4) comparable to advanced age (≥65 years old, OR = 5.6) and presence of sepsis (OR = 7.6), as demonstrated by multivariate analyses. The detrimental effect of prior corticosteroid use is a novel finding that requires further research for its potential value as a prognostic tool at COVID-19 admission. Other predictors of mortality for COVID-19 patients with candidemia identified through multivariate analyses include increased length of stay, high levels of D-dimer, use of tocilizumab (Rajni et al., 2021), ECMO support (Alessandri et al., 2023), high SOFA score (Omrani et al., 2021), and presence of a central venous catheter (OR = 19.07; Kayaaslan et al., 2023).

Several approaches for prediction and prognosis have proposed different combinations of readouts to match and even surpass current prognostic tools. Of note, accordingly to the era of data science, a machine learning-based algorithm using a myriad of clinical variables as input was able to predict candidemia with an AUC of 87.4% (Ripoli et al., 2020), an extraordinary magnitude that outperforms not only classical stepwise multivariable logistic regressions performed in the same report but also other independent studies proposing different arrangement of variables, i.e., the delta neutrophil index (with an AUC of 80.4%; Park et al., 2020) and a multivariate conditional regression-based risk score (with an AUC of 76.8%; Poissy et al., 2020). Mortality has also been appraised as an outcome, with a composite score (consisting of a combination of the SOFA score and the Charlson Comorbidity Index) reported to outperform (AUC of 79%) the independent performance of its components (AUC of 77% for SOFA score, AUC of 69.7% for Charlson Comorbidity Index; Asai et al., 2021; Table 1). A summary of the factors detailed in this section is listed in Table 3.

7.4. Treatment

Treatment of *Candida* spp. involves prompt initiation of antifungal therapy with echinocandins, azoles, and amphotericin B formulations (Pappas et al., 2016). The echinocandin group includes caspofungin, anidulafungin, and micafungin, all of which are noncompetitive inhibitors of the production of BDG (Denning, 2003). Given their broad-spectrum efficacy against *Candida* spp., echinocandins are frequently used to treat candidemia and invasive candidiasis (Pappas et al., 2016). In fact, initial antifungal therapy with fluconazole has been found an independent predictor of mortality elevating the risk death by roughly 200% in a cohort study in Italy (Pieralli et al., 2021). However, corroboration of this effect was inconclusive in a subsequent independent cohort study conducted in France, which compared echinocandins and azoles as first-line antifungal therapy without reaching significance in multivariate analyses (Bienvenu et al., 2020). Aside from *Candida auris*, antifungal resistance is systematically led by non-*albicans* species in China (Zhang et al., 2020; Liu et al., 2021), South Korea (Kwon et al., 2021), Turkey (Guner Ozenen et al., 2023), Italy (Mazzanti et al., 2021), Thailand (Ngamchokwathana et al., 2021), and Saudi Arabia (Al-Dorzi et al., 2018), out of which, *C. tropicalis* represents the major contributor. Data on resistance/susceptibility would appear to draw a trend of inverse sensitivity between compounds of the echinocandin group and fluconazole, with *C. albicans* displaying higher figures of resistance to echinocandin compounds than non-*albicans* species and lower figures of resistance to azole compounds than non-*albicans* species (Al-Dorzi et al., 2018; Jung et al., 2020; Guner Ozenen et al., 2023). Nevertheless, it has been reported that empirical administration of high-dose liposomal amphotericin B (L-AmB) is associated with better management of fungal invasiveness, less ICU-acquired candidemia, less need for an antifungal agent additional to L-AmB, and ultimately a reduction in ICU mortality, emphasizing the feasibility and relative safety of a preemptive antifungal therapy strategy to combat bloodstream *Candida* colonization (Azoulay et al., 2017). Regardless of the advancements detailed insofar, this review emphasizes the knowledge gap in molecular factors of resistance for *Candida* species, particularly when compared to the bacterial agents of sepsis reviewed above.

7.5. *Candida auris* sepsis

Candia auris is a human pathogenic yeast first isolated in Japan in 2009 (Satoh et al., 2009) that has gained notoriety for its high potential for invasive BSI, high mortality rates, moderate preventability, difficult identification by conventional techniques, and its virtually complete resistance to azoles (Geremia et al., 2023). Prompted by the escalating number of countries worldwide reporting the detection of *C. auris* (Briano et al., 2022; Riera et al., 2022; Figure 1), along with similar concerns for other neglected non-bloodstream fungal infections, the WHO has issued its first global effort to systematically prioritize fungal pathogens, in which *C. auris* ranks as the second most threating fungus to human health [World Health Organization (WHO), 2022b]. For an impeccable and updated review on *C. auris* virulence factors, risk factors, and antifungal resistance, please consult review by Geremia et al. (2023). Notwithstanding the growing number of studies describing its many facets, in pragmatic terms what concerns about *C. auris* sepsis is its often misclassification [as *C. famata*, *C. haemulonii*, or *Rhodotorula glutinis* (Kathuria et al., 2015)] and its pattern of multidrug resistance (Gómez-Gaviria et al., 2023).

Because host response to sepsis is inextricably canonical and *C. auris* virulence is considered intrinsically low (Geremia et al., 2023), the strong points for *C. auris* clinical management are then relegated to source control (Hinrichs et al., 2022) and drug delivery. With environmental source control (Akinbobola et al., 2023) falling partially outside of the scope of clinical management and efforts for nosocomial containment being not dissimilar to other fungal agents, a cardinal condition for source control is then the ability for detection. If the resources for accurate and routinely detection are limited, then emphasis is shifted toward drug delivery. With resistance to current drugs on the rise (Shastri et al., 2020; Briano et al., 2022), measurement of clinical management outcomes is then shifted from hospital discharge toward mortality. With information on specific *C. auris*-case fatality rates being scarce (Geremia et al., 2023), the extent of mortality then falls under the domain of speculation. Thus, we reckon that increasing access to detection and encouraging the appraisal of adverse outcomes are crucial milestones to tackle *C. auris*, particularly in locations where resources and logistics for diagnosis are limited, and consequently supporting the measures taken by the WHO [World Health Organization (WHO), 2022b], which stem from the precautionary principle (Goldstein, 2001). Although considerable steps have been taken very recently toward elucidating its pathobiology using mouse models (Wurster et al., 2022) and improving detection by employing advanced mathematical models (Garcia-Bustos et al., 2020), the knowledge gap about this emerging pathogen is systematic and efforts to contain it require a multidisciplinary approach.

8. Conclusion

Sepsis represents a topic with a high level of complexity that extends beyond patient management and clinical efforts for containment. In this multidisciplinary inter-collaborative scenario, curation of selected evidence is paramount to guide transversal action. We recognize the inherent limitations of *in vitro* studies, the controversy surrounding the verisimilitude of administering LPS (Osuchowski et al., 2018) or using cecal ligation and puncture (CLP) (Deutschman et al., 2022) as animal models of sepsis, as well as the

TABLE 4 Summary of potential therapeutical targets of interest for sepsis detailed in this review.

Approach/target	Mechanism	Reference
E. coli		
Bacterial cell membrane *ompA*	Deletion limits vascular permeability by reducing endothelial apoptosis and VE-Cadherin downregulation.	McHale et al. (2018)
K. pneumoniae		
Mushroom-derived β-glucans	Reduces bacterial load and improves physiological outcomes through dectin-1.	Masterson et al. (2020)
Bergenin monohydrate	Reduces bacterial load and improves physiological outcomes by reducing ROS production, increasing cell viability, increasing levels of SOD and GSH, reducing bacterial load, reducing levels of IL-6, IL-1β, PGE2, and TNF-α, reducing MDA formation, reducing MPO content, and reducing lung leukocyte infiltration.	Tang et al. (2021)
Inorganic compound AS101	Reduces bacterial load and improves survival by modulating apoptosis and inflammation.	Yang et al. (2021)
Membrane receptor TRAIL	Reduces bacterial load and improves survival by modulating apoptosis and inflammation.	Chen et al. (2019)
Adipose-derived mesenchymal stem cells	Reduces bacterial load and improves physiological outcomes by reducing levels of TNF-α, IL-1β, and IL-6, and immune infiltration in the lung.	Perlee et al. (2019)
Red blood cell membrane-coated PLGA nanoparticles	Reduces bacterial load and improves survival by reducing levels of TNF-α, IL-1β, and IL-6.	Liu et al. (2022)
Anti-K1-CPS antibodies	Reduces bacterial load and improves survival by promoting bacterial phagocytosis by Kupffer cells.	Diago-Navarro et al. (2017)
Blue light	Reduces bacterial load and improves survival through activation of neural circuit signaling through a cholinergic anti-inflammatory pathway	Griepentrog et al. (2020)
Supplementation of SCFA	Reduces bacterial load and improves survival by increasing macrophage phagocytic capacity and pulmonary levels of IL-6 and TNF-α.	Wu T. et al. (2020)
Acute phase protein PTX3	Reduces bacterial load and improves survival by inducing expression of TNF-α, IL-1β, IL-6, and CXCL-1, limiting MPO levels and leukocyte counts, and protecting from tissue hemorrhage.	Asgari et al. (2021)
Transcription factor HIF1α	Reduces bacterial load and improves physiological outcomes by limiting levels of pro-inflammatory cytokines while enhancing the release of IL-10 in the lung.	Otto et al. (2021)
S. aureus		
Antiplatelet drug ticagrelor	Reduces bacterial load and improves survival by extending protection from thrombocytopenia and protecting from organ damage.	Sun et al. (2021)
P. aeruginosa		
Regulator of α-ketoglutarate transport *MifR*	Deletion improves survival by limiting leukocyte infiltration, TNF-α, IL-6, and IL-1β production, NLRP3 inflammasome activation, and tissue damage.	Xiong et al. (2022)
Extracellular matrix degrading heparinase *hepP*	Deletion reduces bacterial load and improves survival.	Dzvova et al. (2018)
Endogenous H_2S	Improves survival by enhancing neutrophil recruitment and phagocytic activity.	Renieris et al. (2021)
Peptidylarginine deiminase PAD2	Knockout mice exhibit reduced bacterial load and improved survival by reducing caspase-1-dependent pyroptosis in macrophages.	Wu W. et al. (2020)
Anti-PilQ-PilA DSL antibodies	Reduces bacterial load and improves survival in combination with antibiotics.	Zahedi bialvaei et al. (2021b)
PNM and 14–1 phage	Reduces bacterial load and shows successful in experimental therapy.	van Nieuwenhuyse et al. (2022)
S. pyogenes		
Non-canonical Tyr-phosphatase M5005_Spy_1476	Deletion reduces bacterial load and improves survival.	Kant and Pancholi (2021)
Predicted gene spy1343	Deletion reduces survival.	Sitkiewicz and Musser (2017)
Vascular endothelial growth factor	Administration improves survival.	Lu et al. (2022)
Pattern recognition receptor TLR13	Knockout mice exhibit limited macrophage IL-6 and NO_2 production.	Hafner et al. (2019)

CXCL-1, CXC motif chemokine ligand 1; DSL, C-terminal disulfide loop; GSH, glutathione; HIF1α, hypoxia inducible factor 1 subunit α; IL, interleukin; K1-CPS, K1 serotype capsule polysaccharide; MDA, malondialdehyde; MPO, myeloperoxidase; NLRP3, NOD-like receptor family pyrin domain-containing 3; PAD2, peptidyl arginine deiminase type 2; PGE2, prostaglandin E2; PLGA, poly(lactic-co-glycolic acid); PTX3, pentraxin 3; ROS, reactive oxygen species; SCFA, short-chain fatty acids; SOD, superoxide dismutase; TLR13, toll-like receptor 13; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

impossibility of calculating factual figures of incidence and mortality worldwide (Rudd et al., 2020). Nevertheless, we value them as steps toward an integrated effort to tackle complexity straightforward and comprehensively. Thus, a list of novel approaches and potential therapeutical targets that should be considered for further research is presented in Table 4.

Transversal diversity of microorganism presentation in sepsis, from coinfection with multiple species to coexistence of multiple phylogroups, inextricably limits research conducted under the single-microorganism/single-strain approach right from its inception, for its reductionist nature. Notwithstanding the value in this frequent practice, this review makes clear that results emanating from

integrative studies not only have the upper hand for describing reality but also are one step closer to clinical translation. An example of this is a very recent report identifying patterns of sepsis progression for most of the microorganisms described in this review using state-of-the-art genomics, transcriptomics, proteomics, and metabolomics, to make phylogenetic-oriented descriptions (Mu et al., 2023).

Indubitably, multidisciplinary advancement in basic, translational, and clinical research, is key to make progress filling the gap acknowledged by the World Health Organization (WHO) (2017) and endured yearly by millions around the globe.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and have approved it for publication.

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Conflict of interest

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Glossary

APACHE II	Acute physiology and chronic health evaluation II
AUC	Area under the curve
AUROC	Area under the receiver operating characteristic
BDG	(1,3)-β-D-glucan
BSI	Bloodstream infection
COVID-19	Coronavirus disease 2019
CPS	Capsule polysaccharide
ECMO	Extracorporeal membrane oxygenation
ExPEC	Extraintestinal *E. coli*
UPEC	Uropathogenic *E. coli*
GSH	Glutathione
HR	Hazard ratio
HIF	Hypoxia inducible factor
hvKp	Hypervirulent *K. pneumoniae*
ICU	Intensive care unit
IL	Interleukin
LPS	Lipopolysaccharide
MRSA	Methicillin-resistant *S. aureus*
MSSA	Methicillin-sensitive *S. aureus*
MDR	Multi-drug resistant
NLRP3	NOD-like receptor family pyrin domain-containing 3
OR	Odds ratio
PVL	Panton–valentine leukocidin
PAD2	Peptidyl arginine deiminase type 2
PLGA	Poly (lactic-co-glycolic acid)
PGE2	Prostaglandin E2
ROS	Reactive oxygen species
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SPATE	Serine protease autotransporters of Enterobacteriaceae
SCFA	Short-chain fatty acid
SOD	Superoxide dismutase
SOFA	Sepsis organ failure assessment
TSS	Toxic shock syndrome
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
XDR	Extensively-drug resistant
XLA	X-linked agammaglobulinemia

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Role of *Legionella pneumophila* outer membrane vesicles in host-pathogen interaction

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Legionella pneumophila is an opportunistic intracellular pathogen that inhabits artificial water systems and can be transmitted to human hosts by contaminated aerosols. Upon inhalation, it colonizes and grows inside the alveolar macrophages and causes Legionnaires' disease. To effectively control and manage Legionnaires' disease, a deep understanding of the host-pathogen interaction is crucial. Bacterial extracellular vesicles, particularly outer membrane vesicles (OMVs) have emerged as mediators of intercellular communication between bacteria and host cells. These OMVs carry a diverse cargo, including proteins, toxins, virulence factors, and nucleic acids. OMVs play a pivotal role in disease pathogenesis by helping bacteria in colonization, delivering virulence factors into host cells, and modulating host immune responses. This review highlights the role of OMVs in the context of host-pathogen interaction shedding light on the pathogenesis of *L. pneumophila*. Understanding the functions of OMVs and their cargo provides valuable insights into potential therapeutic targets and interventions for combating Legionnaires' disease.

KEYWORDS

outer membrane vesicles, Legionnaires' disease, *Legionella pneumophila*, host-pathogen interaction, LCV, CAP, HAP

1. Introduction

Lower respiratory infections are the fourth leading cause of death worldwide (WHO, 2020). Pneumonia is the most common type of lower respiratory tract infection clinically represented by pulmonary alveoli inflammation and caused by a diverse class of pathogens including viruses, bacteria, parasites, and fungi. Among many kinds of pneumonia infections, Legionnaires' is a severe manifestation which is caused by the bacteria "*L. pneumophila*." Recent studies have identified *Legionella* spp. among the four most common microbial causes of hospitalizations due to community-acquired pneumonia (CAP) (Stout and Yu, 1997; Woodhead, 2002). Although it is primarily associated with CAP, Legionnaires' disease is also observed in healthcare settings as hospital-acquired pneumonia (HAP) when water systems are poorly managed in hospitals.

Legionella pneumophila, the causative agent of Legionnaires' disease was discovered in 1977 after an outbreak of pneumonia in Philadelphia. Since then it has been linked to many outbreaks caused by improper management of artificial water management. As per the WHO report of September 2022, the overall death rate of Legionnaires' disease is 5–10% among all infected individuals and 40–80% in immunocompromised patients. Proper case management can reduce the mortality rate in immunocompromised to 5–30% (WHO, 2022). Perhaps as a result of a variety of variables such as host risk factors, late diagnosis and poorly maintained artificial water

systems incidences of Legionnaires' disease are increasing every year. To control incidences and improve disease management, understanding the host-pathogen interaction is vital, potentially paving the way for the development of targeted therapeutics and interventions.

Recent research has highlighted bacterial extracellular vesicles as mediators of communication among bacteria and, between bacteria and host cells. These vesicles contribute to the development of disease and regulate the host immune response. Extracellular vesicles are lipid bilayer structures released by living organisms of all domains. The name of these vesicles varies depending on the type of organism and the nature of originating cells. As gram-negative bacteria like *L. pneumophila* contain an outer membrane, vesicles shed by gram-negative bacteria are named outer membrane vesicles (OMVs). Although OMVs were first discovered and visualized by Narayan in 1966, the mechanisms of OMVs regulation and functions are still unclear (Chatterjee and Das, 1967). This review summarizes the pathogenesis of *L. pneumophila*, role of OMVs in host-pathogen interactions, and addresses gaps in the study of *L. pneumophila* OMVs highlighting their importance in the host-pathogen interaction.

2. *Legionella pneumophila*

Intracellular pathogen *L. pneumophila* is an aerobic obligate gram-negative rod that widely inhabits the freshwater environment. This organism is an opportunistic pathogen causing either mild-flu like illness known as Pontiac fever or acute form of pneumonia known as Legionnaires' disease (Hamilton et al., 2018). Typically, the organism is found in freshwater in free form or association with biofilms. The ability of *L. pneumophila* to reproduce within biofilms offers additional defense against environmental stresses like biocides, nutrient depletion, and adverse temperatures. The organism can also infiltrate and persist intracellularly in a variety of protozoans including *Acanthamoeba, Vermamoeba,* and *Naegleria* etc. in both soil and aquatic environments (Newsome et al., 1985; Atlas, 1999; Siddiqui et al., 2021). Since the life cycle of the *L. pneumophila* generally requires endoparasitization and reproduction within eukaryotic protists like amoebae, *L. pneumophila* have also developed the ability to infect human cells, particularly the macrophages.

The typical route of transmission of *L. pneumophila* is through inhalation of contaminated aerosols. Common sources of the spread of *L. pneumophila* in communities include humidifiers, whirlpool spas, air conditioning cooling towers and, hot and cold water systems (Mondino et al., 2020). While in hospitals, infection can occur through the exposure of newborns to infectious aerosols during water deliveries and the aspiration of contaminated water by susceptible hospitalized patients (Muder et al., 1986; Franzin et al., 2001). Direct human-to-human transmission has not yet been documented (Lorry and Rubin, 2008).

Soon after being taken up by lung macrophages, *L. pneumophila* bypasses the airway defense system by evading endocytic maturation pathway, preventing phagosome-lysosome fusion and developing a niche for replication called LCV (*Legionella* containing vacuole). This LCV compartment differs from phago-lysosome compartment since it does not acidify and is formed by recruitment of vesicles from rough endoplasmic reticulum (ER) (Robinson and Roy, 2006). Following the establishment of replication niche, *L. pneumophila* secrete effector proteins to modulate host cell signaling, host membrane trafficking, ubiquitin and autophagy pathways to favor its replication inside host. *L. pneumophila* multiplying inside the LCV is knowns as the replicative phase of the infection cycle. The intracellular replication cycle within the lung cell is completed after depletion of nutrients after which *L. pneumophila* shifts toward transmissive phase by destroying the host cell and release from it. The released bacteria then spread to nearby host cells and starts a new infection cycle (Mondino et al., 2020).

One of the outstanding features of *L. pneumophila* is its ability to reproduce within different hosts. In order to successfully establish infection cycle in multiple hosts series of distinct events are required, many of which are performed by action of one or more of over 300 effector proteins of *L. pneumophila* (Lockwood et al., 2022). Like other bacteria, *L. pneumophila* uses multiple strategies to deliver proteins extracellularly and intracellularly. Particularly, secretion systems are known to facilitate the transportation of proteins and other molecules which play a vital role in their survival, virulence, and interactions with the host. *L. pneumophila* possesses secretion systems I, II, and IV which translocate effector proteins, enzymes, and virulence factors from the bacteria across the bacterial membranes, delivering them into the host cell cytoplasm. Although the T1SS is dispensable for the intracellular life cycle, but it is required for the host cell invasion mechanisms (Fuche et al., 2015). The T2SS and T4SS have received significant attention in *L. pneumophila* research because of their crucial roles in infection (Cianciotto, 2005). During *L. pneumophila* development inside the cell, the T2SS system transports more than 25 effector proteins, which are important for bacterial replication in various hosts (Cianciotto, 2009; Cianciotto, 2013). Additionally, the T2SS may play a role in biofilm formation and the dispersal of *L. pneumophila* from biofilms, which can contribute to bacterial transmission and persistence in water systems (Cianciotto, 2009; Cianciotto, 2013).

The Dot/Icm type IV secretion system significantly contributes to *L. pneumophila* virulence. Both LCV biosynthesis and intracellular replication in human and protozoans hosts require the Dot/Icm T4SS (Andrews et al., 1998; Segal and Shuman, 1999; Nagai and Roy, 2003). The *L. pneumophila* T4SS is situated at the poles of bacteria and polar secretion of effectors is necessary to alter the host endocytic pathway hence promoting bacterial survival in the host (Jeong et al., 2017). Importantly, T4SS secrete more than 330 effector proteins which regulates all intracellular life stages of *L. pneumophila* and target the fundamental cellular functions shared by protozoa and mammals (Kitao et al., 2020). Effectors Sidk, VipD, and PieE participate in *L. pneumophila* uptake and evasion from the endocytic maturation pathway. Many other effector proteins, including SidM, SidD, RaIF, and LseA, facilitate the interaction of the endoplasmic reticulum (ER) and the formation of LCV. Once the LCV is established, the organism secretes many other effectors into the host through the Dot/Icm system to hijack host cell functions including mRNA processing, the ubiquitin pathway, cell signaling, and cell death pathways (Kitao et al., 2020).

Overall, the secretion systems in *L. pneumophila* are involved in many functions like protein synthesis in host cells, the secretion of effectors to create an infectious niche, the transfer of DNA, and the secretion of autotransporters which are related to virulence and pathogenesis of the bacteria (Green and Mecsas, 2016). However,

soluble secretion systems are effective over short distances as they require close physical contact between bacteria and host cells.

Apart from using secretion systems for protein delivery over short distances, *L. pneumophila*, like other gram-negative bacteria, uses outer membrane vesicles (OMVs) as long-distance delivery vehicles for transporting bioactive chemicals from bacteria to environment or host cells.

3. Outer membrane vesicles

3.1. Production

Secretion of extracellular vesicles (EVs) is a conserved mechanism found in all life forms, including bacteria, archaea, fungi, and complex eukaryotes (Deatherage and Cookson, 2012). These small lipid-membrane bounded particles, which range in size from 20–400 nm, are released from cells but lack the ability of self-replication (Maas et al., 2017; Liu et al., 2022). Bacterial vesicles production was first observed in 1960; since then, OMVs have been extensively studied in bacteria including *Escherichia coli*, *Neisseria*, *Vibrio*, *Bacteroides*, *Pseudomonas aeruginosa*, *Campylobacter jejuni* and *Actinobacillus* (Chatterjee and Das, 1967; Devoe and Gilchrist, 1973; Hoekstra et al., 1976; Logan and Trust, 1982; Nowotny et al., 1982; Grenier and Mayrand, 1987; Kadurugamuwa and Beveridge, 1995) (Toyofuku et al., 2019). In gram-positive bacteria, EVs bud from cytoplasmic membrane containing cytoplasmic contents, also known as membrane vesicles (MV) whereas in gram-negative bacteria, EV bleb from the outer membrane contain both periplasmic and cytoplasmic components referred as outer membrane vesicles (OMVs) (Brown et al., 2015; Toyofuku et al., 2019). According to recent research, gram-negative bacteria also release double- and triple-membrane vesicles in addition to OMVs which are hypothesized to be the results of bacterial cell lysis with and without bacteriophages (Toyofuku et al., 2019).

Although OMVs are produced by various bacterial species, the rates of OMV production vary among different bacterial species (Devoe and Gilchrist, 1973; Gankema et al., 1980; Wensink and Witholt, 1981; Ribeiro de Freitas et al., 2022). Moreover, the growth and nutrient conditions also influence the production and composition of OMVs within the same bacterial specie. Several studies have shown that bacteria increase vesicle production after exposure to certain antibiotics (Orench-Rivera and Kuehn, 2016). This increased OMV production can either release more enzymes such as beta-lactamases to destroy antibiotics or capture surrounding antibiotics by acting as decoys to protect bacteria against certain antibiotics. Bacterial vesiculation can also be influenced by the presence of a host. Studies reported differences in OMVs production upon exposure to host components and tissue. Using enterotoxigenic *E. coli* infection mouse model, electron microscopy observations revealed that vesicles were more abundant on ETEC cells recovered post-infection from the mouse small intestine (Ellis and Kuehn, 2010).

3.2. Elements/components of OMVs

Elements of OMVs purified by ultracentrifugation, filtration, or chromatography included components predominantly present in membranes, such as proteins and lipids of periplasm, as well as other cytoplasmic components (Roier et al., 2016). Protein compositions of OMVs identified the presence of outer membrane (OM) proteins, periplasmic proteins, and flagellin (OMVs derived from motile-bacteria). In lipid compositions, lipopolysaccharide (LPS) and lipoproteins were identified. Notably, a significant abundance of LPS component was observed in all OMVs originating from gram-negative bacteria (Toyofuku et al., 2019). Flagellin, LPS, OM proteins, and lipoproteins are also proposed as key players in OMV biogenesis mechanisms (Avila-Calderón et al., 2021). Oligosaccharides, also known as glycans are present in the outer membrane and can be found associated with OMVs. Since OMVs are involved in bacteria-bacteria and bacteria-host interactions, the successful docking of OMVs with other bacterial and host cell surfaces is associated with the presence of adhesive oligosaccharides within the OMVs (Knoke et al., 2020). Furthermore, the composition of oligosaccharides in OMVs varies among different bacterial strains or species. Thus, oligosaccharide present in OMVs composition can serve as a molecular fingerprint of the specific type of OMVs. However, further research and analysis are necessary to fully understand the diversity and functional significance of oligosaccharides within OMVs of different bacterial strains.

Apart from flagellin, oligosaccharide, outer membrane, and periplasmic proteins, OMVs contain a variety of cargos, including nucleic acids such as plasmids, DNA, RNA, and cytosolic proteins including virulence factors (Figure 1). Although various components have been identified from the OMVs of gram-negative bacterial species, several key components remain constant in OMVs, providing us with the advantage of understanding them from the perspective of a shared origin.

4. Outer membrane vesicles in host-pathogen interaction

Previous studies described OMVs production as a by-product of cell lysis but recent studies demonstrate that the OMVs are actively produced by all gram-negative bacteria. These OMVs are enriched in cytoplasmic, periplasmic, virulence proteins, and specific lipids suggesting that bacteria purposefully release OMVs. There could be two reasons for this: first as a means of interaction with other bacteria and host, and secondly, for survival in stressful environments (McBroom et al., 2006).

OMVs play diverse roles in both pathogenic and non-pathogenic bacteria (Figure 2). In non-pathogenic bacteria, OMVs can act as vehicles for intercellular communication among bacterial populations. OMVs carry signaling molecules, quorum sensing factors, or small RNA molecules that facilitate coordination and cooperation among bacteria. This communication helps regulate microbial community dynamics and can contribute to host homeostasis. Further, OMVs can mediate nutrient exchange among bacterial populations by carrying enzymes or nutrient-binding molecules that scavenge and acquire nutrients from the environment. This way OMVs can benefit both the producing bacteria and neighboring microbes. OMVs can also contribute to the formation and maintenance of bacterial biofilms by carrying the extracellular polymeric substances (EPS), including polysaccharides and proteins, which provide structural support to the biofilm. Recently, the beneficial role of OMVs produced by gut microbiota or commensal bacteria has also been highlighted. A study reported that OMVs released from *Akkermansia muciniphila* which colonizes the intestinal mucous layer can restore the gut microbiota balance by specifically stimulating the

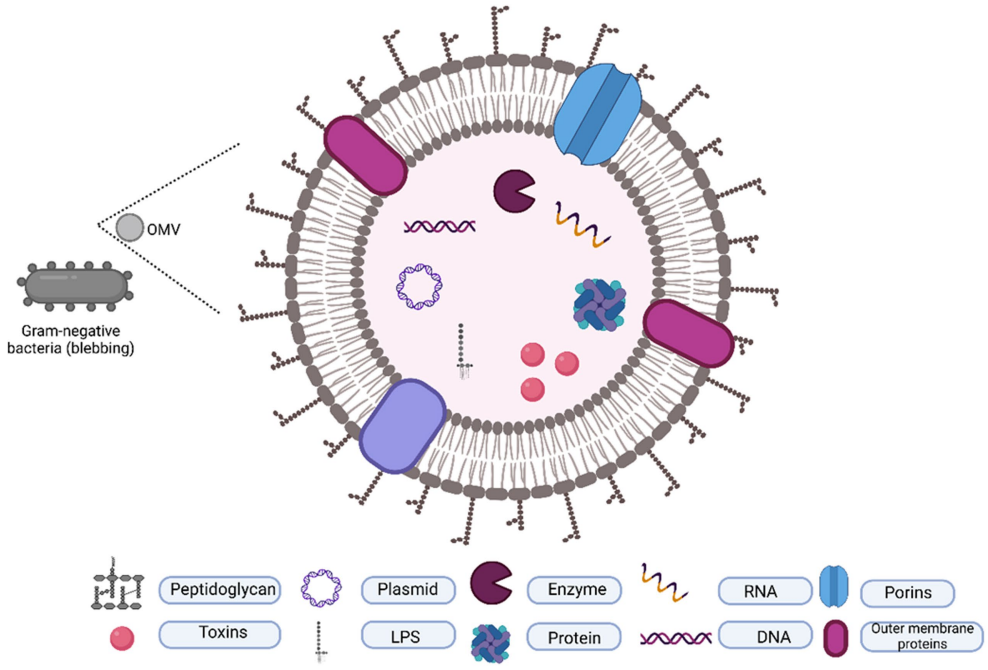

FIGURE 1
Typical composition of OMVs. OMVs are originated by outer membrane blubbing and contain a lipid layer packaged with proteins, toxins, DNA, and RNA.

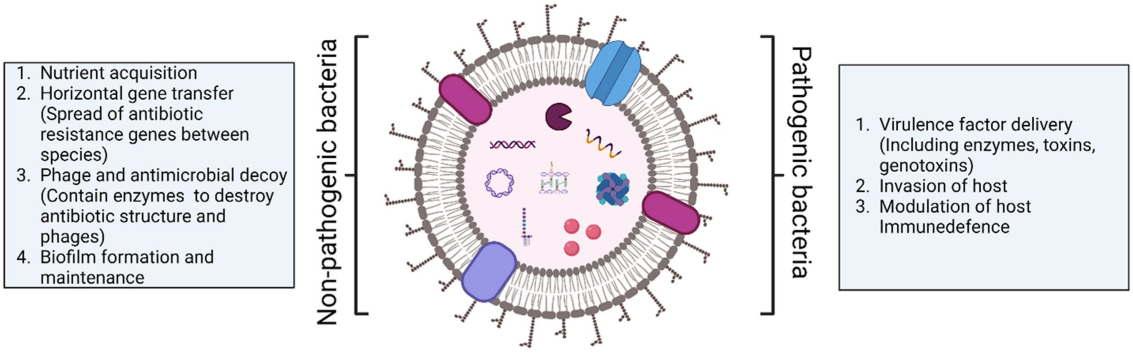

FIGURE 2
Diverse functions of OMVs released by non-pathogenic and pathogenic bacteria.

growth of beneficial bacteria while suppressing the proliferation of opportunistic pathogens. In addition, OMVs also improved immune functions of mucosa by switching IgM to IgA (Wang et al., 2023).

Among pathogenic bacteria, OMVs aid in the delivery of virulence factors/toxins, immune evasion, and modulation of host factors to facilitate bacterial growth. As the focus of this review is to summarize the role of OMVs in host-pathogen interaction with context to *L. pneumophila,* we will not discuss extracellular functions in detail.

4.1. Mechanisms of OMVs interaction with host cells

Although there is considerable evidence that OMVs can enter host cells and release their cargo to modulate host cell functions, the precise mechanisms underlying how OMVs interact with and are taken up by host cells are still not completely known. OMVs from pathogenic bacteria interact with a variety of host cells including immune cells (epithelial cells, macrophages, dendritic cells and neutrophils) and non-immune cells (endothelial cells, osteoblasts and synovial cells) (Schultz et al., 2007; Kaparakis et al., 2010; Maldonado et al., 2011; Kim et al., 2013; Lappann et al., 2013; Jäger et al., 2014; Kaparakis-Liaskos and Ferrero, 2015). Generally, there are five endocytic pathways for OMVs entry to host cells including macropinocytosis, clathrin mediated endocytosis, caveolin mediated endocytosis, lipid-raft mediated endocytosis and direct membrane fusion. Examples exist for each pathway in literature.

Evidence of macropinocytosis or actin mediated endocytosis was found when uptake of *P. aeruginosa* OMVs by airways epithelial cells was reduced after treatment with actin

polymerization inhibitor, a crucial protein for actin-dependent macropinocytosis (Bomberger et al., 2009). Interestingly *P. aeruginosa* OMVs also required lipid-raft mediated endocytosis pathway for its entry to human lung epithelial cells (Bauman and Kuehn, 2009). OMVs of *L. monocytogenes* interacted with Caco-2 cells by acting-mediated endocytosis or macropinocytosis pathway (Karthikeyan et al., 2019). Clathrin-mediated endocytosis was shown to be OMVs entry route for *Brucella abortis* in human monocytes (Pollak et al., 2012), Enterohemorrhagic *E. coli* in human brain microvascular endothelial cells (HBMEC) and Caco-2 cells (Bielaszewska et al., 2013), *Aggregatibacter actinomycetemcomitans* in HeLa cell and human gingival fibroblasts (HGF) (Thay et al., 2014; Vanaja et al., 2016). Finally, OMVs are also able to uptake by host cells by direct membrane fusion which has been demonstrated in *P. aeruginosa*, *A. actinomycetemcomitans* and *L. pneumophila* by labeling OMV membranes with Rhodamine R-18 fluorescent dyes (Bomberger et al., 2009; Rompikuntal et al., 2012; Jäger et al., 2015).

However, studying interaction between OMVs and host cell is complex as the majority of pharmacological inhibitors of endocytic pathways have impact on many mechanisms, making it frequently difficult to identify the uptake process. Additionally, more than one mechanism for OMV uptake can be found within same bacterial species. OMVs content and size can also influence the uptake pathway. Details of OMVs entry mechanisms and factors effecting entry have been published elsewhere in 2020 (Caruana and Walper, 2020) and immunological effects of OMVs interactions with different host cells have also been described before (Kaparakis-Liaskos and Ferrero, 2015). Here, we discuss the interaction of *L. pneumophila* with host cell, and the subsequent immune responses triggered by the host.

5. *Legionella pneumophila* outer membrane vesicles

5.1. *Legionella pneumophila* OMVs production

Research on the biology of *Legionella* and Legionnaires' disease for four decades has provided important insight on bacterial infection strategies. *L. pneumophila* OMVs added a new aspect to the pathogenesis of *L. pneumophila.* Flesher et al. discovered OMVs in *L. pneumophila* for the first time as membrane blebs in 1979 while studying the cell-envelope structure of bacterium using electron microscopy (Flesher et al., 1979). These vesicles were later isolated by ultracentrifugation of bacterial culture supernatants after filtration through 0.22um of the *L. pneumophila* and purity was analysed by negative staining electron microscopy (EM) and atomic force microscopy (AFM) in 2008. In microscope, they ranged in size from 100–200 nm (Galka et al., 2008). AFM and EM are considered standard method to visualize and validate the purity of OMVs fractions (Théry et al., 2018).

L. pneumophila produce OMVs throughout the life cycle including log phase and stationary phase and at different growth conditions including both extracellular and intracellular growth. Jung et al. confirmed intracellular production of OMVs by co-incubating *L. pneumophila* with amoeba *Dictysotelium discoideum* host for 24 h. Thin-section electron microscopy showed blebs from the *L. pneumophila* membrane surface within the *Legionella*-specific phagosome of infected *D. discoideum* host cells (Galka et al., 2008).

5.2. *Legionella pneumophila* OMVs binding to and uptake by host cells

The uptake of OMVs by other bacteria and host cells is a dynamic and complex process involving various pathways of interactions as described earlier. Once internalized, OMVs can deliver their cargo, including proteins, toxins, nucleic acids, antibiotic-resistance enzymes, and several other factors.

So far, the binding of *L. pneumophila* OMVs with human alveolar epithelial cells has been confirmed using confocal laser microscopy by labeling OMVs with green fluorescent anti-LPS antibody (conjugated with Alexa flour 488). After 8 h of OMVs incubation with A549 cells, confocal microscopy revealed acquisition of green color on epithelial cells surface. Further binding of OMVs to host cells also changed the cell morphology toward round shape suggesting OMVs can not only bind to host cells but also trigger significant morphological changes in host cells (Galka et al., 2008). Later *L. pneumophila* OMVs interaction with human macrophages were also observed using same Alexa-flour anti-LPS antibody. Strong green fluorescence signal was detected just after 3 min incubation of OMVs with differentiated human mononuclear cells (MNCs) and this signal intensity increased with time and with increasing OMVs protein concentration. This time and dose-dependent binding of *L. pneumophila* OMVs with human macrophages suggested that OMVs can fuse with host cells and deliver their cargo (Jäger et al., 2015). To study whether *L pneumophila* OMVs can be internalized to host cells by direct membrane fusion, Jäger et al. incubated OMVs with liposomes made up of eukaryotic phospholipids membranes. By using Fourier transform infrared spectroscopy and Förster resonance energy transfer (FRET) they found that OMV membrane material could be incorporated into liposomes model of eukaryotic membrane by direct membrane fusion (Jäger et al., 2015). However, involvement of other OMVs entry pathways is not studied in *L. pneumophila.*

Binding of *L. pneumophila* OMVs with host cells was also confirmed *in-vivo*, when lung tissue explants from healthy donors were incubated with *L. pneumophila* OMVs. Immunostaining revealed the localization of OMVs predominantly on alveolar macrophages after 24 and 48 h (Jäger et al., 2014).

5.3. *Legionella pneumophila* OMVs role in intracellular infection cycle

The key feature of *L. pneumophila* infections is to escape phago-lysosomal degradation and develop replicating niche. By coating latex beads with *L. pneumophila* OMVs Fernandez et al. reported that OMVs can inhibit the fusion of phagosomes containing *L. pneumophila* with lysosomes suggesting OMVs have ability of mediate the pathogenesis (Fernandez-Moreira et al., 2006). Another research found that *L. pneumophila* OMVs pre-treated macrophages had more *Legionella*-containing vacuoles (LCV) and produced less pro-inflammatory cytokines hence OMVs exposed macrophages are

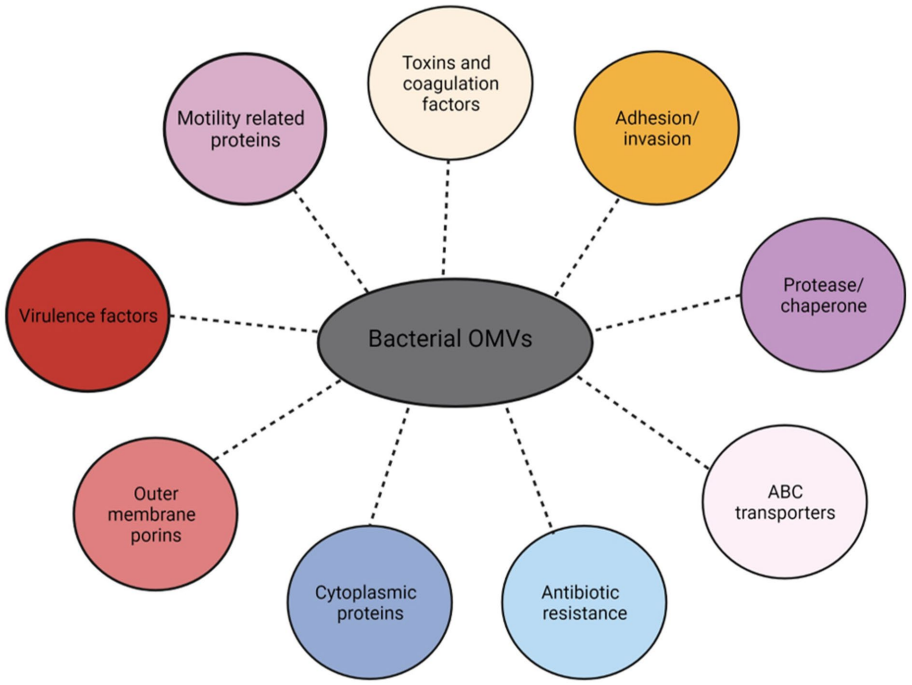

FIGURE 3
Proteins families identified in Outer Membrane Vesicles (OMVs).

more susceptible for bacterial replication than unexposed macrophages (Jung et al., 2016).

5.4. Are *Legionella pneumophila* OMVs cytotoxic to host cells?

The extent of cytotoxicity associated with *L. pneumophila* OMVs remains unclear to date. Studies *in vitro* indicated that *L. pneumophila* OMVs are not cytotoxic to host cells. For example, in one study, the metabolic activity of PMA-differentiated human U937 macrophage cell line was studied using Alamar blue dye reduction. OMVs did not affect cell vitality after incubation with 100 μg mL^{-1} protein concentration of OMVs for 24 h (Jäger et al., 2015). Consistent with these results another research group observed non-significant reduction in growth of H292 alveolar epithelial cells after 72 h incubation with 50 μg mL^{-1} *L. pneumophila* OMVs (Galka et al., 2008). However, *in-vivo* examination of lung tissues explants after treatment with 100 μg mL^{-1} of *L. pneumophila* OMVs revealed that after attachment to alveolar epithelial cells OMVs caused damage in septa and epithelia over time suggesting OMVs can cause damage to host cells during intracellular infection (Jäger et al., 2014). Results were found opposite when *L. pneumophila* OMVs were incubated with amoeba host model *Acanthamoeba castellanii*. Surprisingly, growth of *A. castellanii* was increased after co-incubation with OMVs. As amoeba like *A. castellanii* feeds on bacteria using peptides and amino acids generally and OMVs contain significant protein hence OMVs may serve as a food source for *A. castellanii*.

The cytotoxity of OMVs to host cells may depend on multiple factors including type of host, dose of OMVs, time duration and growth phase (replicative and transmissive) of *L. pneumophila*. Further investigation is required to conclude the cytotoxity potential of *L. pneumophila*.

5.5. The content of *Legionella pneumophila* OMVs- what is known

While the exact mode of OMV-host interaction remains to be elucidated, studies on OMV cargos have highlighted they may contribute to bacterial pathogenesis and host immune modulation. This section highlights the probable contributions of OMV components in host-pathogen interaction and discuss the gaps in *L. pneumophila*.

5.5.1. Proteins

Proteome analysis of gram-negative bacterial OMVs revealed that they contain diverse protein families, which include predominantly outer membrane proteins, antibiotic resistance enzymes, and other proteins (Figure 3) (Uddin et al., 2020). Following OMVs isolation, determining the protein concentration provides an estimation of the amount of OMVs present. Therefore, protein concentration of OMVs is commonly used in dosage studies of OMVs. It helps standardize the OMV dosage across experiments, ensuring consistent and reproducible results and gives direct correlation between the amount of OMVs administered and the biological response observed, such as cytokine induction, immune response, or cellular signaling.

For protein component analysis of *L. pneumophila* OMVs, OMVs were separated from bacterial supernatant fraction by ultracentrifugation (at 150,000 × *g* for 3 h) (Wai et al., 2003). Pellet obtained was suspended in Tris–HCl and termed as OMVs which was

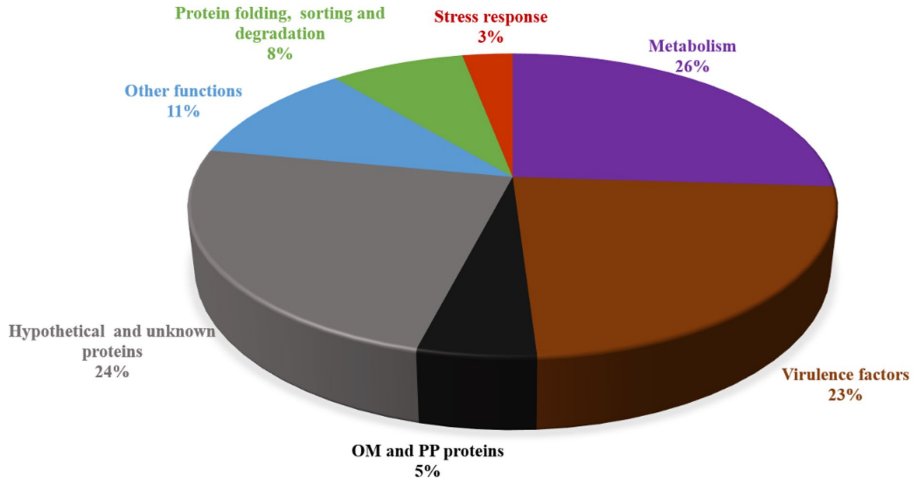

FIGURE 4
Protein families identified by KEGG pathway prediction in *L. pneumophila* OMVs.

visualized by Electron microscopy in order to confirm the absence of non-OMV protein aggregates. The remaining liquid supernatant fraction was termed as SSP fraction. MALDI-TOF mass spectrometry analysis of *L. pneumophila* secretome including both pellet (OMV) and soluble supernatant fraction (SSP) revealed that OMVs contain 33 specific proteins that are not released by any other secretion systems.

Total 74 proteins were found in OMVs fraction of *L. pneumophila*, percentage of each functional group is described in Figure 4 (Galka et al., 2008). Eighteen out of 33 OMV-specific proteins are predicted to contribute bacterial virulence. While 41 proteins were common in both fractions and belonged to carbohydrate metabolism, energy metabolism, amino acid metabolism, and protein sorting (Galka et al., 2008). Table 1 summarizes the known functional proteins of *L. pneumophila* OMVs among the whole secretome studied by Galka et al. (2008). The presence of many virulence factors, metabolic and protein folding proteins within OMVs suggest that *L. pneumophila* may exploit OMV to deliver factors and manipulate host cell functions for its survival. Interestingly, a greater number of virulence associated proteins were found in the OMV fraction as compared to soluble supernatant fraction; out of 25 virulence factors isolated from whole secretome of *L. pneumophila*, 18 were associated with OMVs. This distribution suggests that OMVs are specific carrier of virulence-associated proteins.

Composition and functions of these OMV proteins are not studied yet during intracellular infection of *L. pneumophila*. However, the literature on over all bacterial infection suggests that each set of OMV proteins may play a role in the pathogenesis of bacteria, as mentioned in the following discussion.

5.5.1.1. Membrane associated proteins

Nearly all OMVs are loaded with OM (outer membrane) proteins and PP (periplasmic proteins). OM proteins primarily responsible for OMVs formation may also perform additional functions like adhesion to host cells (Lee et al., 2007). OmpH found in *L. pneumophila* OMVs is a major structural protein associated with membrane phospholipids, essential for outer membrane formation and hence may play role OMV formation (Khemiri et al., 2008). Another protein found in *L. pneumophila* OMVs is com1 which was annotated in 2008 as OM protein because of its similarity to Com1 of *Coxiella burnetii* outer membrane protein, later localization studies in 2011 confirmed that com1 is periplasmic protein and was designated as DsbA2. Literature shows that DsbA2 is responsible for assembly of Dot/Icm T4SS during *L. pneumophila* infection. This 27 kDa protein is also responsible for *L. pneumophila* motility and viability (Hendrix et al., 1993; Jameson-Lee et al., 2011). MOMP (Major outer membrane protein) which is considered as important virulence factor of *L. pneumophila* was also found in OMVs. During infection of *L. pneumophila* MOMP binds with complement proteins and facilitate the bacterial uptake *via* complement receptors. It has also been shown to reduce phagocytosis of macrophage and increase the expression of IL-10, NOD2, MCP-1, and RIP2 (Bellinger-Kawahara and Horwitz, 1990; Yang et al., 2021).

5.5.1.2. Invasion and adhesion factors

OMVs are also enriched with proteins and enzymes that enhance their invasive properties, thereby promoting OMV internalization at the host interface (McMillan et al., 2021). They can be found either localized in outer membrane of OMVs or in lumen of OMVs. *L. pneumophila* OMVs contain phospholipase, chitinase and astacin protease in lumen. Astacin proteases belong to the family of metalloproteinases and are involved in protein degradation. In *L. pneumophila*, astacin proteases are associated with tissue damage and host cell invasion. These proteases can degrade extracellular matrix components, such as collagen and elastin, facilitating tissue penetration and dissemination within the host. They also contribute to the activation of host cell signaling pathways, modulating the host immune response, and promoting bacterial survival (Bond and Beynon, 1995; Banerji et al., 2008). Similarly, phospholipases are enzymes that hydrolyse phospholipids, breaking them down into their constituent parts. In *L. pneumophila,* phospholipases are involved in altering the host cell membrane and promoting both invasion of bacterium in host cell and escape of the bacterium out the Legionella-containing vacuole (LCV) into the host cell cytoplasm. By disrupting the host cell membrane, phospholipases also contribute to the cytotoxic effects of *L. pneumophila* infection (Istivan and Coloe, 2006). Chitinases are enzymes that degrade chitin, a complex polysaccharide found in the protozoa and biofilms.

TABLE 1 *Legionella pneumophila* OMVs protein families identified by 2-DE and *in silico* data analysis.

Proteins families	Gene identity (As defined in genome of *L. pneumophila* Philadelphia-1)	Predicted KEGG function in *L. pneumophila*	Description in other OMVs proteome
Protein folding, sorting or degradation			
Thiol disulphide interchange protein	lpg0123	Protein folding, sorting or degradation	*Escherichia coli* (Aguilera et al., 2014) *Neisseria meningitidis* (Vaughan et al., 2006)
L-lysine dehydrogenase (LDH)	lpg1350	Protein folding, sorting or degradation	No
DnaK chaperone protein, heat shock protein Hsp70	lpg2025	Protein folding, sorting or degradation; stress	*Francisella novicida* (Pierson et al., 2011)
Aminopeptidase	lpg2631	Protein folding, sorting or degradation	*Pseudomonas aeruginosa* (Bauman and Kuehn, 2006)
Peptidylprolyl cis-trans isomerase B (ppiB)	lpg2726	Protein folding, sorting, or degradation	*Pseudomonas aeruginosa* (Choi et al., 2011) *Acinetobacter baumannii* (Yun et al., 2018)
Outer membrane protein and periplasmic protein			
OmpH outer membrane protein	lpg0507	OMV biogenesis/Other functions	*Pasteurella multocida* (Fernández-Rojas et al., 2014) *Fusobacterium necrophorum* (Bista et al., 2023)
27 kDa outer membrane protein (Com1)	lpg1841	OMV biogenesis/Other functions	No
Major outer membrane protein (MOMP)	lpg2960	OMV biogenesis/Other functions	No
Rod shape determining protein (MreB)	lpg0811	Cell envelope	No
Metabolism			
Catalase/hydroperoxidase (KatG)	lpg0194	Energy metabolism; amino acid metabolism	*Francisella novicida* (McCaig et al., 2013)
Acetoacetate decarboxylase	lpg0672	Carbohydrate metabolism; lipid metabolism	No
Acetyl-CoA acetyltransferase (fadA)	lpg1353	Lipid metabolism; amino acid metabolism; xenobiotics biodegradation and metabolism	*Fusobacterium nucleatum* (Liu et al., 2019)
Glutamine synthetase, type I (glnA)	lpg1364	Energy metabolism; amino acid metabolism; peptidoglycan biosynthesis	No
Nucleoside diphosphate kinase (ndk)	lpg1548	Nucleotide metabolism	No
Aconitate hydratase (acnA)	lpg1690	Carbohydrate metabolism; energy metabolism	*Mycobacterium tuberculosis* (Lee et al., 2015) *Bacteroides fragilis* (Zakharzhevskaya et al., 2017)
Long-chain fatty acid transporter	lpg1810	Lipid metabolism	No
S-adenosylmethionine synthetase (metK)	lpg2022	Amino acid metabolism	No
Aspartate semialdehyde dehydrogenase (Asd)	lpg2302	Amino acid metabolism	No
Malate dehydrogenase (mdh)	lpg2352	Carbohydrate metabolism; energy metabolism	*Acinetobacter radioresistens* (Fulsundar et al., 2015) *Bacillus subtilis* (Bergsma et al., 1981) *Escherichia coli* (Aguilera et al., 2014)
GTP cyclohydrolase I (folE2)	lpg2766	Metabolism of cofactors and vitamins	*Pseudomonas syringae* (Chowdhury and Jagannadham, 2013) *Campylobacter jejuni* (Taheri et al., 2019)
Polyribonucleotide nucleotidyltransferase (Pnp)	lpg2768	Nucleotide metabolism	*Francisella novicida* (Pierson et al., 2011) *Neisseria meningitidis* (Williams et al., 2007)
Inosine 5′-monophosphate dehydrogenase	lpg2843	Nucleotide metabolism	*Staphylococcus haemolyticus* (Cavanagh et al., 2019) *Fusobacterium nucleatum* (Munshi, 2020) *Cryptococcus neoformans* (Rodrigues et al., 2008)
ATP synthase subunit A and B	lpg2984	Energy metabolism	No
Fumarylacetoacetate hydrolase	lpg2279	Amino acid metabolism; xenobiotics biodegradation and metabolism	*Moraxella catarrhalis* (Schaar et al., 2011)
Glu/Leu/Phe/Val dehydrogenase	lpg2275	Amino acid metabolism	No
Endo-1,4 beta-glucanase	lpg0482	Metabolism	No
Virulence factors			
Astacin protease (legP)	lpg2999	Virulence/pathogenesis	No
Phospholipase/lecithinase/hemolysin, lysophospholipase A	lpg2837	Virulence/pathogenesis	*Pseudomonas aeruginosa* (Kadurugamuwa and Beveridge, 1995)
IcmX (IcmY)	lpg2689	Virulence/pathogenesis	No[a]
Tail fiber protein (SclB)	lpg2644	Virulence/pathogenesis	No[a]

(Continued)

TABLE 1 (Continued)

Proteins families	Gene identity (As defined in genome of *L. pneumophila* Philadelphia-1)	Predicted KEGG function in *L. pneumophila*	Description in other OMVs proteome
SdeD (LaiF)	lpg2509	Virulence/pathogenesis	No[a]
TPR repeat protein	*lpg2222*	Virulence/pathogenesis	*Porphyromonas gingivalis* (Mantri et al., 2015) *Fibrobacter succinogenes* (Arntzen et al., 2017) *Francisella tularensis* (Klimentova et al., 2019)
LaiE	lpg2154	Virulence/pathogenesis	No[a]
Phospholipase C	lpg1455	Virulence/pathogenesis	*Pseudomonas aeruginosa* (Bomberger et al., 2009) *Acinetobacter baumannii* (Jha et al., 2017)
Flagellin (FliC)	lpg1340	Virulence/pathogenesis	*Pseudomonas aeruginosa* (Bauman and Kuehn, 2006) *Campylobacter jejuni* (Jang et al., 2014)
Major acid phosphatase (Map)	lpg1119	Virulence/pathogenesis	No
Chitinase	lpg1116	Virulence/pathogenesis	*Francisella novicida* (Pierson et al., 2011) *Bacillus thetaiotaomicron* (Elhenawy et al., 2014)
ecto-ATP diphosphohydrolase II	lpg0971	Virulence/pathogenesis	No
Macrophage infectivity potentiator (Mip)	lpg0791	Virulence/pathogenesis	*Neisseria meningitidis* (Williams et al., 2007)
Hsp60, 60 K heat shock protein (HtpB)	lpg0688	Virulence/pathogenesis	*Piscirickettsia salmonis* (Oliver et al., 2016) *Campylobacter jejuni* (Lindmark et al., 2009) *Neisseria meningitidis* (Ferrari et al., 2006)
Icmk (DotH)	lpg0450	Virulence/pathogenesis	No[a]
IcmE (DotG)	lpg0451	Virulence/pathogenesis	No[a]
Phosphatidylcholine hydrolysing phospholipase	lpg0502	Virulence/pathogenesis	No
Zinc metalloprotease (ProA, Msp)	lpg0467	Virulence/pathogenesis	*Vibrio cholerae* (Rompikuntal et al., 2015)
Stress response			
Cold shock protein CspE	lpg2825	Stress	*Klebsiella pneumoniae* (Zhang et al., 2021)
DNA binding stress protein	lpg0689	Stress	*Pseudomonas syringae* (Kulkarni et al., 2014)

[a]Specific effector proteins of *L. pneumophila*.

In *L. pneumophila*, chitinase plays a role in the bacterium's environmental invasion, survival and virulence in protozoa or biofilm matrices (Chen et al., 2020). Other protein found in *L pneumophila* OMVs, the heat shock protein Hsp60 is found crucial for bacterial adhesion and invasion in HeLa cell model (Garduño et al., 1998).

5.5.1.3. ABC transporters and metabolism enzymes

OMV-associated ABC transporters and metabolic enzymes play a crucial role in bacterial survival during nutritional deficiency (Nevot et al., 2006; Lappann et al., 2013). Interestingly, *L. pneumophila* OMVs contain a great percentage of metabolic enzymes. Metabolic enzymes are crucial for energy production and the biosynthesis of essential molecules required for bacterial growth and survival. The TCA cycle, where these enzymes function, is vital for the bacterium's adaptation to various environmental conditions and its ability to replicate within host cells during infection. Acetyl-CoA produced by FadB- FadA (acetyl-CoA acetyltransferase) mediate degradation of fatty acids and feeds directly into the TCA cycle. Asd (aspartate semialdehyde dehydrogenase) is important in the biosynthesis of amino acids and Asd mutant of *L. pneumophila* was unable to survive in amoeba and macrophage (Harb and Kwaik, 1998). Role of OMV-packaged metabolic enzymes in bacterial pathogenies is still unclear. They may influence the metabolic state of neighboring cells, potentially modulating host cell signaling pathway and host responses, scientific evidence of which is missing. Understanding the role of these enzymes in OMVs can provide valuable insights into *L. pneumophila* physiology, virulence, and their interactions with the host.

5.5.1.4. Virulence factors

Many virulence factors are associated with bacterial membrane vesicles of pathogenic bacteria which either damage host cells directly or modulate host immune defence (Grenier and Mayrand, 1987; Kadurugamuwa and Beveridge, 1995; Kolling and Matthews, 1999; Horstman and Kuehn, 2000; Haurat et al., 2011; Prados-Rosales et al., 2011; Coelho et al., 2019). *Several virulence factors were found in L. pneumophila OMVs.* A virulence protein of *L. pneumophila* KatG detoxifies antibacterial reactive oxygen compounds produced by host macrophages (Manca et al., 1999). Another major virulence protein Mip, is a stable homodimer. Mip can bind to collagen IV based on its PPIase activity and therefore enables *L. pneumophila* to transmigrate over tissue barriers of lung epithelial cells (Wagner et al., 2007). It can also promote proliferation of bacteria in LCV by inhibiting the acidification of phagosome consequently reducing the phagocytosis of macrophages (Shen et al., 2022). IcmX protein plays role in the establishment of LCV and pore formation in macrophage cell membrane (Shevchuk et al., 2011). Another virulence factor Zinc metalloprotease or Msp have been reported to cause destruction in lung tissue explants by collagen IV degradation (Scheithauer et al., 2021). Intracellular studies are needed to confirm the contributions of these virulence factors.

5.5.1.5. Protein degradation and stress response

Function of cold-shock and DNA binding stress proteins are not well studied in *L. pneumophila*. But literature on other bacteria shows that cold-shock proteins can sense and respond to temperature changes and other environmental stresses and allows bacteria to thrive

in various challenges (Keto-Timonen et al., 2016). Many proteins were identified in *L. pneumophila* OMVs which participate in protein folding, sorting and degradation. As these protein folding chaperons are involved in the regulation of membrane dynamics and curvature, they might get packed inside during biogenesis of OMVs. However, their exact functions in OMVs are still uncertain. OMVs can also serve to remove toxic compounds such as misfolded protein by this function group of proteins under environmental stresses (Rollauer et al., 2015; Schwechheimer and Kuehn, 2015).

5.5.2. Nucleic acids associated with OMVs

Diverse genetic materials have been found in association with OMVs including; chromosomal DNA, Plasmid DNA, phage/viral DNA, mRNA, rRNA, sRNA and tRNA (Yaron et al., 2000; Biller et al., 2014; Ho et al., 2015; Blenkiron et al., 2016).

5.5.2.1. RNA cargo

OMVs contain variety of RNAs including mRNA, rRNA, sRNA and tRNA (Domingues and Nielsen, 2017). Interestingly, while the function of OMVs-associated DNA in disease pathogenesis remains uncertain, OMVs-associated RNA plays a substantially significant role during disease pathogenesis. Bacterial RNA can be packaged and transferred to other bacteria and host *via* OMVs (Tsatsaronis et al., 2018; Lee, 2019). Table 2 summarizes the studies on association of RNA with OMVs and their functions including *L. pneumophila*. Among all groups of RNA, sRNA has been found more significant in host-pathogen interaction which range in size from 20 to 200 nucleotide. Although there are largely unknown about packing, delivery stability, and host selection they have shown many regulatory mechanisms by binding to protein targets and modify their functions (Lalaouna et al., 2013; Koeppen et al., 2016). This binding can have a wide range of negative effects on the cell's metabolic, apoptotic, and immunomodulatory processes. These sRNA of bacteria are similar to miRNA and small interfering RNAs (SiRNA) of eukaryotes in function. Hence during host-pathogen interaction they mimic host miRNA, which plays key role in gene expression regulation, and modulate host cell functions including immune responses.

So far, two studies have been done on *L. pneumophila* OMV-associated small RNAs. One comprehensive study identified OMV-associated very small RNAs (vsRNAs <16 nt) in 5 different bacterial species including *L. pneumophila*. RNA-seq of vsRNA revealed their abundance within OMVs along with thermodynamically stable tRFs (transfer RNA fragment). Presence of tRFs and their bioinformatic analysis by BLASTN, RNA hybrid, DIANA-microT suggest that they are eukaryotic miRNA analogues and target human mRNAs. Gene function analysis on tRFs targets by PANTHER described that they have diverse targets like cell differentiation, B cell chemotaxis, metal ion binding, and, MAP kinase activity and, regulation of cellular response to stress and macrophage colony-stimulating factor production etc. (Diallo et al., 2022). This phenomenon suggests that small RNAs packaged in *L. pneumophila* OMVs can influence the transcriptome profile of neighboring host cells during infection. Another RNA-seq study revealed that *L. pneumophila* translocate small RNAs (sRNA) by OMVs which are eukaryotic analogous. These sRNAs target host defence signaling pathways by binding to the UTR of RIG-I, IRAK1 and cReI and finally downregulating the IFNβ production (Sahr et al., 2022). Thus, a noteworthy aspect of communication between *L. pneumophila* pathogen and host is direct miRNA-like regulation of the expression of the innate immune response. Surprisingly, the discovered "bacterial miRNAs" serve two purposes: as trans-kingdom signaling molecules as well as being crucial for bacterial own survival. For example, regulatory RNA called RsmY is believed to control the life cycle *L. pneumophila*, and tRNA-Phe to be involved in protein production. It is important to find out how the sRNAs of *L. pneumophila* and other bacteria contained in OMVs affect eukaryotic cells as well as whether they share any common strategies for modulating the host immune response. Further research on the delivery of sRNAs through OMVs and their influence on host-pathogen interactions is anticipated. Due to their immunomodulatory abilities, OMVs may be the ideal vehicle for the delivery of sRNAs that target certain host genes. These numerous variables and processes are still difficult to understand and are leading to a new understanding of host-pathogen interactions.

5.6. The content of *Legionella pneumophila* OMVs- what needs to be known

5.6.1. Lipids

The lipid composition of OMVs is found consistent across gram-negative bacterial species except some geometric changes (Silhavy et al., 2010; Gnopo et al., 2020). They serve two functions. First, they participate in the biogenesis of OMVs. Deformation of the bacterial outer membrane is necessary for OMV production and this deformation is controlled by regulating the concentration and structure of individual phospholipid and lipid A molecules (McMahon and Gallop, 2005). Second, lipids of OMVs play a role in immune response regulation. Lipid A, an endotoxin that contributes to the amphipathic base structure of LPS, is a microbe-associated molecular pattern (MAMP) which is recognized by eukaryotic pattern recognition receptors (PRRs). In response to contacts with gram-negative bacteria, PRRs regulate inflammatory reactions including host immunity, and cell death (Loppnow et al., 1989; Gioannini et al., 2004; Simpson and Trent, 2019). In addition to lipid A, cardiolipins are essential outer membrane components. These tetra-acylated di-phosphatidylglycerols also engage TL4/MD-2 receptor to activate or modulate host immune response (Mileykovskaya and Dowhan, 2009; Chandler and Ernst, 2017).

The lipidome profile of *L. pneumophila* OMVs has not been characterized yet. However, cell envelope of *L. pneumophila* is described in detail. Like other gram-negative bacteria, *L. pneumophila* also contain outer membrane made up of inner phospholipids leaflet and outer lipopolysaccharides (LPS) leaflet. Outer membrane has embedded proteins that play a variety of roles in virulence, including attachment and uptake into host cells. Lipids are composed of dimethylphosphatidylethanolamine, phosphatidylethanolamine, phosphatidylglycerol, cardiolipin, and phosphatidylcholine (Finnerty et al., 1979). These phospholipids in bacterial cell envelop is considered as permeability barrier but intriguingly, the removal of phosphatidylcholine from *L. pneumophila's* envelope led to decreased cytotoxicity and reduced bacterial yield within macrophages (Conover et al., 2008). Moreover, absence of this lipid effected binding efficiency of bacteria to macrophages. Sawada et al. reported that *L. pneumophila* LPS specifically binds

TABLE 2 Summary of published articles reporting RNA association with OMVs.

Organism	OMV-associated RNA studied	Study approach	Function studied	Reference
E. coli strain 536	Complete RNA profile (rRNA, tRNAs, small RNAs, and mRNA)	RNA-sequencing	OMV RNA uptake by the bladder epithelial cells	Blenkiron et al. (2016)
P. aeruginosa	Short RNA (sRNA)	RNA-sequencing	One candidate (sRNA52320) 1. Reduced IL-8 secretion in primary human airway epithelial cells 2. Modulated OMV-induced KC cytokine secretion and neutrophil infiltration in mouse lung	Koeppen et al. (2016)
S. enterica serovar *Typhimurium*	Complete RNA profile (OMVs were enriched in mRNA and non-coding RNA)	High throughput sequencing	Not assessed	Malabirade et al. (2018)
Actinomycetemcomitans	msRNAs (miRNA-size, small RNAs)	Northern blot and RT-qPCR	msRNA (A.A_20050) decreases IL-15, IL-13 and IL-5 secretion in Jurkat T-cells	Choi et al. (2017)
P. gingivalis	msRNA (miRNA-size, small RNAs)	Northern blot and RT-qPCR	msRNA (P.G_45033) decreases IL-15, IL-13 and IL-5 secretion in Jurkat T-cells	Choi et al. (2017)
Treponema denticola	msRNA (miRNA-size, small RNAs)	Northern blot and RT-qPCR	msRNA (T.D_2161) decreases IL-15, IL-13 and IL-5 secretion in Jurkat T-cells	Choi et al. (2017)
Vibrio cholerae	Small non-coding RNA gene- vrrA	Mutation analysis	*vrrA* mutant 1. Overproduces OmpA porin leading to increased OMV production 2. increased ability of bacteria to colonize the intestines of infant mice	Song et al. (2008)
L. pneumophila	The top 20 enriched sRNA in OMVs were identified (2 further studied) 1. RsmY is analog to host miRNA144 (it regulates RIG-I expression) 2. 2. tRNA-Phe targets the UTR of the *irak1*	RNA-sequencing	1. RsmY reduced the RIG-I and IFN-β response expression in the host. 2. tRNA-Phe downregulated IRAK1 expression	Sahr et al. (2022)

with pulmonary surfactant protein of lungs which play important role in innate immunity. This interaction leads to localization of *L. pneumophila* in lysosome and subsequently inhibition of bacterial intracellular growth (Sawada et al., 2010).

In conclusion, as outer membrane lipids participate in OMVs biogenesis, virulence mechanism and activation of host receptors by PRRs, studying *L. pneumophila* OMVs lipid is equally important than any other cargo to understand the contribution of OMVs in host-pathogen interaction.

5.6.2. DNA cargo

In *L. pneumophila*, no research has been done yet on the OMV-associated DNA. Several investigations have reported the presence of DNA in OMVs fraction. Although the actual route through which DNA incorporates into OMVs is still not clear [37–39], literature shows that these OMV-associated DNA serves important role in horizontal DNA transfer and transfer of several functions in bacterial communities including antibiotic resistance, virulence, degradation (Supplementary Table S1) (Klieve et al., 2005; Rumbo et al., 2011; Velimirov and Hagemann, 2011; Fulsundar et al., 2014; Kulkarni et al., 2015; Chatterjee et al., 2017; Qiao et al., 2021). Studies on extracellular bacterial OMVs confirmed the presence of virulence genes in the DNA profile of OMVs also found transfer and expression of these genes in recipient bacteria (Yaron et al., 2000). However, such transfer between pathogenic bacteria and eukaryotic host is understudied. To date, there is only one study on *P. aeruginosa* which describes that OMVs have potential to deliver DNA to eukaryotic cells (Bitto et al., 2017). Regarding the direct influence of OMV-packaged DNA in host-pathogen there are few studies and needs to be further elucidated. As bacterial RNA and DNA are recognized by host endosomal nucleic acid receptors TLR7, 8, and 9 respectively (Jurk et al., 2002; Latz et al., 2004; Eigenbrod and Dalpke, 2015), so OMV associated DNA activate immune response by inducing Toll-like receptor 9 (TLR9) signaling (Perez Vidakovics et al., 2010; Bitto et al., 2021). A study in 2021 reported that *S. aureus* membrane vesicles were able to induce TLR9 suggesting that OMVs may contain immunostimulatory DNA.

6. *Legionella pneumophila* OMVs and host immune response

It has been demonstrated that OMVs of pathogenic bacteria promote the development of infection and host inflammation (Craven et al., 1980; Fiocca et al., 1999; Ren et al., 2012; Jung et al., 2016). OMVs can interact with a wide variety of immune cells and induce immunological responses (Kaparakis-Liaskos and Ferrero, 2015). Despite the fact that OMVs can cause inflammation in a variety of host tissues, the underlying processes are unclear. During infection, *L. pneumophila* interact with lung epithelial cells and lung macrophages. The epithelial surface containing resident immune cells, is the first line of defence. The interaction of OMVs with epithelial cells and macrophages not only induces cytokines but also stimulates PRR signaling. This is because OMVs contain numerous microorganism-associated molecular patterns (MAMPs), including RNA, DNA, LPS, peptidoglycan, and lipoproteins. MAMPS engage host PRRR and start the pro-inflammatory signaling chain. Since OMVs from different bacterial species change in their composition

and content, so do the processes by which they trigger PRR signaling. For example, the LPS content of *E. coli* OMVs interacts with toll-like receptor 4 (TLR4) of human epithelial cells and drives TH4-dependent CXCL8 production (Soderblom et al., 2005), while *L. pneumophila* LPS poorly recognize TLR4 as lipid A of *L. pneumophila* contains unusual long, branched-chain fatty acids (Zähringer et al., 1995). Instead in *L. pneumophila* peptidoglycan-associated lipoproteins (PAL) are recognized by TLR2 (Liu and Shin, 2019).

Pneumonia induced by *L. pneumophila* is characterized by acute lung damage and severe hypoxemia. Patients exhibit elevated levels of inflammatory cytokines, including TNF, IFN-, IL-12, IL-6, IL-8, and granulocyte-colony stimulating factor, and disease severity is closely associated with the intensity of these inflammatory responses. To investigate the role of *L. pneumophila* OMVs in the inflammatory responses during bacterial infection, Galka et al. conducted a study where they examined the cytokine profiles of alveolar macrophages after a 15-h incubation with *L. pneumophila* OMVs (Galka et al., 2008). In comparison to the cytokine profiles observed during *L. pneumophila* infection, which included the induction of IL-2, IL-4, IL-6, IL-8, IL-17, IL-1β, INF-γ, MCP-1, TNF-α, and G-CSF, the OMVs were found to upregulate CCL2, CXCL8, G-CSF, IFNβ, IL6, IL7, and IL13. This highlights that IL-7 and IL-13 is a common secretion (Schmeck et al., 2007). The cytokine response induced by OMVs was found to be both dose-dependent and time-dependent in certain studies. In investigations involving *L. pneumophila* OMVs and a macrophage cell line called THP1, an increase in cytokine induction, including IL-8, IL-6, IL-10, TNF-α, and IL-1β, was observed as the concentration of OMVs increased. Additionally, this cytokine secretion was significantly higher at 48 h compared to 24 h. Using murine BMDM macrophages, similar time and dose dependent increase was observed for the CXCL1 cytokine (Jung et al., 2016).

Taken together, *L. pneumophila* OMVs have shown immunomodulatory potential when studied in epithelial cells A549, macrophage cell line THP-1, and murine BMDM suggesting the contribution of OMVs in patients' inflammatory profile. However, the exact mechanism, and responsible cargos behind these immune modulations are largely unknown.

7. Potential applications of OMVs

7.1. OMV-based vaccine

OMVs are highly desirable as candidate vaccines due to their numerous inherent qualities. One of the key advantages is their exceptional stability even when exposed to different temperatures and treatments. Additionally, OMVs contain a variety of immunogenic membrane-associated and cytoplasmic components of their originating bacterium, are non-replicative, and thus safe (Arigita et al., 2004). Furthermore, the particulate nature of OMVs allows them to stimulate the innate immune system, resulting in their intrinsic adjuvant activity. This quality enables OMVs to enhance T-cell and antibody responses to antigens, making them even more effective (Etchart et al., 2006; Nieves et al., 2011; Reza Aghasadeghi et al., 2011). Finally, OMVs may be bioengineered to express any desired antigen and they can be modified to reduce their endotoxicity (Sanders and Feavers, 2011; Baker et al., 2014). This versatility makes OMVs particularly useful.

Despite of having good vaccination potential, OMV-based vaccines have been under development for more than two decades. However, significant progress has been made in the control of meningococcal serogroup B infection (Oster et al., 2005). This discovery of a vaccine with wide protective effectiveness against several *N. meningitidis* serogroup B isolates led to substantial advancement in the field. To date, 3 meningococcal vaccines are developed and commercialized. The VA-MENGOC-BC vaccine, developed and tested by the Finlay Institute in Cuba during an outbreak between 1987 and 1989, demonstrated 83% effectiveness after 16 months in young adults (Sierra-González, 2019). Similarly, the MenBvac vaccine, developed and evaluated by the Norwegian Institute of Public Health during an outbreak from 1988 to 1991, showed 87% effectiveness after ten months (Bjune et al., 1991). Another vaccine, MeNZB, was developed between 2004 and 2008 through collaboration between various institutions and was proven to be 73% effective in young adults. The most recent OMV-based vaccine, Bexsero, manufactured by Novartis and approved by the European Medicines Agency, comprises three recombinant antigens and detergent-extracted OMVs from the New Zealand strain (Semchenko et al., 2019). For use against gram-negative infections, more OMV-based vaccines are presently being created, although none of them have reached the clinical trial stage.

7.2. OMV based therapeutics – drug delivery

Early in the 1890s, cancer patients were treated with weakened bacteria as they stimulate anti-tumor cytokines CXCL10 and interferon-gamma. However, the safety concerns of using bacterial components have limited the clinical application of bacteria-mediated cancer therapy (Patyar et al., 2010; Hirayama and Nakao, 2020). Deletion of the msbB gene in *Salmonella* led to the reduction of immunotherapy's side effects. The use of msbB-mutant *Salmonella* can protect the animal from septic shock caused by lipid A-stimulated tumor necrosis factor (TNFα) (Low et al., 1999). Although an impressive antitumor impact was seen in an animal investigation, *Salmonella*-mediated antitumor was unsuccessful in phase I clinical trial because it did not sufficiently reduce tumor growth (Mi et al., 2019). These studies provide solid ground for the use of modified OMVs in cancer immunotherapy. Recently, it was demonstrated that OMVs originating from attenuated *E. coli* strains may effectively prevent tumor growth and be employed as therapeutic agents to treat cancer. To reduce the negative effects of bacterial endotoxin lipopolysaccharide, the lipid A acyltransferase - (*msbB*) gene in *E. coli* was mutated. 12 h after the delivery of attenuated OMVs, a significant fluorescence signal was seen at the tumor location (Kim et al., 2017). As OMVs have an innate potential to protect their cargo, recent studies have reported effective drug delivery by OMVs. Successful drug loading into the OMV lumen has been achieved using electroporation. Allan and Beveridge in 2003 demonstrated that gentamicin loaded *P. aeruginosa* (PAO1 strain) OMVs hold the potential for treating Cepacia syndrome caused by *Burkholderia cepacia* (Allan and Beveridge, 2003).

8. Conclusion

The knowledge about function and fate of *L. pneumophila* OMVs inside host is still in the early stages, but available literature suggests its functional role in disease pathogenesis through OMVs attachment and uptake by host cells. However, there is a need for reliable and standardized methods to analyse the mechanisms by which OMVs enter and behave inside host cells. Furthermore, OMVs carry a variety of virulence factors which provides an intriguing hypothesis regarding the delivery of these factors to host cells. To gain more insights into this process, further research is needed, particularly through intracellular infection studies. Additionally, the variety of cargo carried by OMVs highlights the importance of studying individual components to understand their contributions to disease development. While some studies have been conducted on protein and RNA components in *L. pneumophila*, limited research has focused on other components, which leaves their role in pathogenesis uncertain. Lastly, understanding the potential benefits of *L. pneumophila* OMVs is crucial, as they may have ability to play a dual role in the intracellular infection cycle. On one hand, they can help bacteria by delivering virulence factors, thereby promoting their virulence. However, on the other hand, these OMVs also carry immunomodulatory properties that could be beneficial to the host by potentially triggering a protective immune response. In first case scenario, by targeting specific components within the OMVs that are involved in their interaction with human lung cells or macrophages, novel treatment approaches for this infection may be developed. For the second case scenario, immunomodulatory properties of OMVs could be utilized for the development of vaccines for protecting patients from bacterial infections.

In summary, the discovery of OMVs has significantly advanced our understanding of bacterial physiology. Despite the existing challenges, we anticipate that future research including the characterization of *L. pneumophila* OMVs produced during infection and their interactions with the host, along with investigations of the mechanisms of vesicle secretion, will deepen our knowledge about bacterial strategies and host defence responses. Finally, exploring *L. pneumophila* OMVs may guide us in the development of innovative immunization approaches and predictive biomarkers for treatment and vaccine efficiency.

Author contributions

AA: Conceptualization, Writing – original draft. FC: Conceptualization, Visualization, Writing – review & editing. PL: Funding acquisition, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1270123/full#supplementary-material

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Emerging evidence of microbial infection in causing systematic immune vasculitis in Kawasaki disease

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Kawasaki disease (KD) is a systematic vasculitis that is often complicated by coronary artery lesions and is a leading cause of acquired heart disease in developed countries. Previous studies have suggested that genetic susceptibility, together with an inducing infectious agent, could be involved in KD pathogenesis; however, the precise causative agent of this disease remains unknown. Moreover, there are still debates concerning whether KD is an infectious disease or an autoimmune disease, although many studies have begun to show that various pathogens functioning as critical inducers could activate different kinds of immune cells, consequently leading to the dysfunction of endothelial cells and systematic vasculitis. Here in this review, we attempt to summarize all the available evidence concerning pathogen infections associated with KD pathogenesis. We also discuss the related mechanisms, present a future perspective, and identify the open questions that remain to be investigated, thereby providing a comprehensive description of pathogen infections and their correlations with the host immune system in leading to KD.

KEYWORDS

bacteria, fungi, Kawasaki disease, vasculitis, virus

1 Introduction

Pathogen infectious diseases have posed a great challenge to human health worldwide (Baldari et al., 2023). Currently, various pathogens have been suggested as critical triggers in inducing systematic vasculitis in children with Kawasaki disease (KD), which is a leading cause of acquired heart disease in developed countries (McCrindle et al., 2017). High-dose intravenous immunoglobulin (IVIG) infusion and aspirin can subdue KD symptoms and partially reduce the occurrence of coronary artery lesions (CALs); however, approximately 10%–20% of affected children develop recrudescent or persistent fever even after IVIG infusion, and those patients have a higher risk of CAL (Li et al., 2018; Nadig et al., 2023). Critically, if this disease is not untreated in a timely manner, sudden death may occur due to coronary artery aneurysms (Shulman and Rowley, 2015; McCrindle et al., 2017; Sosa et al., 2019). Although genetic background (Chang et al., 2014), urban industrialization, environmental factors (Chang et al., 2020; Corinaldesi

et al., 2020), and regional winds together with large-scale atmospheric circulation (Rodo et al., 2011, 2014), have been suggested to correlate with KD, these theories fail to explain the seasonal epidemics of this illness, and also fail to explain why Kawasaki disease does not broadly recur. Nevertheless, an increasing number of epidemiological and clinical data all point to KD having an infectious etiology. For example, epidemiological data from multiple centers worldwide demonstrate that KD has a significant seasonal epidemic (Valtuille et al., 2023), frequent occurrence, and low recurrence characteristics in young children (Nakamura et al., 2008, 2012; Burns et al., 2013; Lin et al., 2015; Ozeki et al., 2017, 2018; Kido et al., 2019; Kim et al., 2020; Xie et al., 2020). Notably, several studies have shown that both the immune repertoire (Kuo et al., 2019) and the heterogeneous host immune response including the autoantibody responses in KD children resemble those observed in patients with bacterial or viral infections (Lindquist and Hicar, 2019; Jackson et al., 2021; Ghosh et al., 2022), lending further support of an infectious disease cause of KD.

Additionally, serum KD-specific molecules which were mostly derived from biofilms possessed molecular structures common to MAMPs (microbe-associated molecular pattern) from *Bacillus cereus*, *B. subtilis*, *Yersinia pseudotuberculosis* (*Y. pstb*), and *Staphylococcus aureus* (Kusuda et al., 2014), implicating a possible relationship between MAMPs and the etiological mechanism of KD vasculitis. Recently, at least 14 types of viruses have been suggested to correlate with KD based on serological and polymerase chain reaction (PCR) analysis of clinical samples (Principi et al., 2013). However, another study showed that at least 15 types of viruses were related to KD because the isolation rates of various viruses in KD patients were significantly higher than those in the control group (Huang et al., 2015; Jackson et al., 2021). Viral infections can cause vascular damage either through direct invasion of the vascular endothelium or provoking a rapid cell-damaging event (Hara et al., 2021). This in turn results in a larger release of proinflammatory cellular components from damaged endothelial cells, pyroptosis, or proinflammatory cell death (Mohandas et al., 2023), hence making various kinds of innate immune cells infiltrate the coronary arteries of KD subjects (Kuijpers et al., 1999; Takahashi et al., 2010a). These data thus suggest that different kinds of microbes are implicated in the pathogenesis of KD, but which microbes are the key inducers and the underlying mechanisms remain unclear. In this review, to better understand the comprehensive profiles between microbial infection and KD pathogenesis, we summarized the major features of our current understanding with respect to various pathogens related to KD. We also discuss the state of this field in KD with respect to the relationship and/or mechanisms concerning the abnormal immune response triggered by various infectious agents, and the open questions that remain to be investigated.

Abbreviations: BCG, Bacillus Calmette-Guérin; CAA, Coronary artery aneurysm; CAWS, Candida albicans water soluble fraction; DAMP, Damaged-associated molecular patterns; ELISA, Enzyme-linked immunosorbent assay; KD, Kawasaki disease; LCWE, Lactobacillus casei cell wall extract; MAMP, Microbe associated molecular pattern; PAMP, Pathogen associated molecular pattern; PCR, Polymerase chain reaction; PRR, Pattern recognition receptor; SEB, Staphylococcal enterotoxin B; SEC, Staphylococcal enterotoxin C; SPE, Streptococcal pyrogenic exotoxins; TCR, T-cell receptor; TSS, Toxic shock syndrome; TSST-1, Toxic shock syndrome toxin-1.

2 Involvement of pathogens during KD pathogenesis

2.1 Viral infection and KD

2.1.1 DNA viruses

Several DNA viruses, including Epstein–Barr virus (EBV), human adenovirus, human parvovirus B19, torque teno virus, herpes family virus, varicella zoster virus, bocaparvo virus, and cytomegalovirus have been identified to be associated with KD pathogenesis.

2.1.1.1 Human adenovirus

Adenovirus type 2 was first isolated from a patient with fatal Kawasaki disease (Embil et al., 1985), while another case report showed that human adenovirus infection can be found in monozygotic twin boys who developed KD (Fukuda et al., 2017). Among the adenovirus-infected cohort, the overall incidence of KD was 5.29 times higher than that of the non-adenovirus-infected control subjects (adjusted HR 5.29, 95% CI: 2.48–11.3), as shown by a population-based cohort study (Huang et al., 2020), suggesting a correlation between adenovirus infection and KD pathogenesis. Notably, there are also studies showing a lack of association between adenovirus infection and KD, suggesting that more intense research is needed to explore the relationships between adenovirus infections and KD (Okano et al., 1990; Shike et al., 2005).

2.1.1.2 Human parvovirus B19

Human parvovirus B19 (HPV-B19) is a single-stranded DNA virus that may have a pathogenic role in the development of KD with other predisposing factors because it can cause symptoms resembling those observed in KD patients (Nigro et al., 1994; Holm et al., 1995). Importantly, HPV-B19 infection should be considered in the differential diagnosis of KD patients who show atypical clinical symptoms during the erythema infectiosum epidemic stage (Oura et al., 2022).

2.1.1.3 Torque teno virus

The torque teno virus (TTV), which is a single-stranded circular DNA virus, was first found in the lymph node of a KD patient (Katano et al., 2012). For instance, a high viral load of torque teno virus 7 (TTV7) was identified in KD patients (Thissen et al., 2018; Spezia et al., 2023a), and the viral load of TTV positively correlated with the level of total bilirubin and aspartate aminotransferase in KD patients (Spezia et al., 2023b), suggesting that TTV might play a critical role in the pathophysiology of patients with KD.

2.1.1.4 Herpes simplex virus

Herpes simplex virus (HSV) consists of multiple subtypes (Rowley et al., 2011), and its family members, including EBV, HHV-6 and varicella-zoster, were all found to be involved in KD. For instance, a previous study showed that the DNA sequence of EBV can be detected in KD patients (Kikuta et al., 1988), and there are many cases of KD-like lesions, specifically coronary artery aneurysms (CAAs), that were suggested to be caused by EBV infection (Kikuta et al., 1993; Rosenfeld et al., 2020; Xiao et al., 2020). However, EBV might not be the direct causative agent of KD, as shown by another study (Kikuta et al., 1990). Notably, a case of Kawasaki disease triggered by EBV virus infection was found to be complicated with familial Mediterranean fever (Maggio et al., 2019). Moreover, the prevalence

of EBV in KD children was significantly lower during the early stage (van Stijn et al., 2020), and deoxyuridine 5′-triphosphate nucleotide hydrolase (dUTPase), a pathogen nonstructural protein encoded by EBV, can stimulate monocyte-derived macrophages through Toll-like receptor 2-dependent signaling transduction (Ariza et al., 2009), suggesting that DUTPase could be used as a potential target for drug development against EBV infection and KD treatment.

In addition to EBV, certain KD patients also have concomitant varicella zoster virus or coxsackievirus A4 infection (Turkay et al., 2006; Toprak et al., 2015). Given that the features of HHV6-infected patients resemble those symptoms observed in KD children (Kakisaka et al., 2012; Alramadhan et al., 2020), HHV-6B was thus suggested to be a critical mediator during the pathogenesis of KD, and HHV-6B infection was also suggested to be responsible for the increased number of KD patients during the SARS-CoV-2 pandemic (Dursun and Temiz, 2020).

2.1.1.5 Bocavirus

Human bocavirus (HboV) is a single-stranded DNA etiologic agent that has been suggested as a cause of acute respiratory tract infection in children (Schildgen et al., 2008). This virus was first identified in nasopharyngeal, serum or stool samples, and was thus suggested to play a pathogenic role in some cases of Kawasaki disease (Catalano-Pons et al., 2007). Late, this work was verified by the results from another group showing that HboV can indeed be detected in nasopharyngeal secretions of KD patients, demonstrating a coincidental or possible etiological association between HboV infection and KD pathogenesis (Santos et al., 2011). Furthermore, a significant correlation between HboV infection and KD incidence was identified based on epidemiological data (Kim et al., 2014; Lim et al., 2021), whereas some investigators have proposed that there is little correlation between HboV infection and KD based on the serological test (Lehmann et al., 2009). Cytomegalovirus was also suggested to be involved in the development of atypical KD and coronary aneurysms (Catalano-Pons et al., 2005; Guc et al., 2008). Taken together, more intense researches is needed to elucidate the precise mechanism concerning DNA viruses associated with KD pathogenesis.

2.1.2 RNA viruses associated with KD

Apart from the DNA viruses mentioned above, a total of nine types of RNA viruses have been suggested to correlate with KD pathogenesis, including coxsackie virus, enterovirus, human coronavirus NL63 (HCoV-NL63), influenza virus, measles virus, SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2), feline virus, influenza A virus H1N1 and human immunodeficiency virus, as discussed below.

2.1.2.1 Coxsackie virus

The coxsackie virus, which belongs to enteroviruses of small RNA viridine, has been identified as the main cause of viral myocarditis in humans since 1955 (Dalldorf, 1955). Both coxsackie virus B3 (CVB3) and coxsackie virus A4 were identified to correlate with KD (Rigante et al., 2012; Ueda et al., 2015), and this type of virus can induce neonatal symptoms similar to viral myocarditis observed in KD (Verma et al., 2009).

2.1.2.2 Enterovirus

It has been demonstrated that the KD incidence in the enterovirus (EV)-infected cohort was significantly higher than that in the non-EV-infected cohort (Weng et al., 2018), thereby indicating a high correlation between EV infection and KD. In addition, a decreased incidence of severe enterovirus infection cases is simultaneously correlated with decreased KD hospitalizations during the SARS-CoV-2 epidemic (Guo et al., 2022), thus suggesting that enterovirus might function as a critical mediator during the pathogenesis of KD.

2.1.2.3 HCoV-NL63 virus

Although HCoV-NL63 was once identified in several KD patients (Dominguez et al., 2006), most data later do not support an association between HCoV-NL63 infection and KD (Baker et al., 2006; Chang et al., 2006; Lehmann et al., 2009). In fact, only 1 (2%) of 48 patients with KD was found to be positive for HCoV-NL63/NH (Shimizu et al., 2005), although HCoV-229E was also suggested to be involved in KD (Lehmann et al., 2009; Shirato et al., 2014).

2.1.2.4 Influenza virus

Influenza viruses have been revealed to positively correlate with the monthly KD incidence (Kim et al., 2014). For instance, influenza A H1N1/09 virus has been shown to be associated with the pathogenesis of KD by several groups (Joshi et al., 2011; Wang et al., 2019; Banday et al., 2021). Additionally, Parainfluenza type 3 virus (PIV-3) was also found to correlate with KD (Schnaar and Bell, 1982; Karron et al., 1993), suggesting that influenza virus infection has etiological importance in the development of KD. However, given that concomitant influenza infection affects the clinical manifestations of KD and impacts the laboratory test results of the disease (Huang et al., 2015), it remains to be determined regarding influenza infection and KD pathogenesis.

2.1.2.5 Measles virus

The measles virus (MeV), which is an enveloped RNA virus, frequently causes acute febrile illness accompanied by a rash (Takemoto et al., 2022). This virus can be isolated from KD children, and the symptoms caused by MeV infection partially resemble those observed in KD patients (Whitby et al., 1991; Kuijpers et al., 2000).

2.1.2.6 SARS-CoV-2

The RNA respiratory virus SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) can induce multisystem inflammatory syndrome in children (also called MIS-C), including multifocal endovascular dermatitis, thrombosis, and systemic thrombotic microangiopathy, which resemble certain features observed in KD (Ackermann et al., 2020; Consiglio et al., 2020; Loomba et al., 2020; Bukulmez, 2021; Cherqaoui et al., 2021; Sancho-Shimizu et al., 2021; Sokolovsky et al., 2021; Zhang et al., 2021).

Moreover, SARS-CoV-2 can be detected in certain KD patients, and a host of SARS-CoV-2-positive patients exhibit KD-like syndrome (Consiglio et al., 2020; Jones et al., 2020; Toubiana et al., 2020; Sharma et al., 2021). However, although high titers of anti-SARS-CoV-2 antibodies have been detected both in KD and multisystem inflammatory syndrome patients (Kabeerdoss et al., 2021), the two diseases are different because of the differential T-cell subsets, interleukin (IL)-17A, and biomarkers associated with arterial damage (Consiglio et al., 2020). On the other hand, global studies have reported that the incidence of KD declined during the COVID-19 pandemic, suggesting a potential KD pathogenesis involving transmission among children (Ae et al., 2022). However, several earlier studies showed that

the KD incidence has increased during the pandemic (Ouldali et al., 2020; Roe, 2020; Stower, 2020; Viner and Whittaker, 2020), supporting the hypothesis that KD might be caused by an unknown RNA virus that may function as the main trigger in inducing abnormal immune responses in genetically susceptible individuals.

2.1.2.7 Other types of RNA viruses

In addition to the RNA viruses mentioned above, several other types of RNA viruses were also found to be involved in KD. For example, both a novel feline virus (Moynahan, 1987) and the influenza A virus (Wang et al., 2019) were suggested to be related to KD symptoms. Notably, HIV patients also show symptoms similar to those observed in KD patients (Johnson et al., 2016). The intracytoplasmic inclusion bodies induced by viruses can be isolated from KD patients, suggesting that the infectious etiologic agent of KD might be associated with an unknown novel RNA virus (Rowley et al., 2011). In addition, dengue virus was also identified in the serum of certain KD patients in southern Thailand, and mosquitoes were hypothesized to work with the dengue virus to spread the KD pathogen, thus inducing cell proliferation and morphological changes in endothelial cells and coronary arteritis lesions in KD patients (Sopontammarak et al., 2008). Moreover, regions with the highest reported arboviral infections in Venezuela simultaneously have the highest incidence of KD (Paniz-Mondolfi et al., 2020), demonstrating the critical roles of viral infections in mediating the pathogenesis of KD.

2.2 Bacterial infection associated with KD

Regarding bacterial infection, the superantigens produced by gut bacteria may be involved in the onset of KD. Until recently, there were five *Streptococcus* spp. (*S. pneumonia*, *pseudopneumoniae, oralis, gordonii, and sanguinis*) were found to increase during the acute phase in KD patients based on metagenomic sequencing, indicating that Streptococci are involved in the pathogenesis of KD disease (Kinumaki et al., 2015). Furthermore, the stool of KD patients contains higher numbers of gram-positive bacteria, including *Streptococcus*, *Staphylococcus*, *Eubacterium*, and *Peptostreptococcus* genera, Hsp60-producing gram-negative bacteria, and a lower number of lactobacilli, when compared with those from healthy control children (Yamashiro et al., 1996; Takeshita et al., 2002; Nagata et al., 2009). Specifically, three pathogens, *S. pyogenes* (Leahy et al., 2012), *S. mitis* Nm-65 (Tabata et al., 2021), and *S. sanguis* (Tsurumizu et al., 1991), have been identified in the pleural fluid, tooth surface or blood of KD patients. Additionally, serum IgM antibodies against superantigens of *S. aureus* and *S. pyogenes* have been identified in KD patients (Matsubara et al., 2006), and these two pathogens together can produce 19 different superantigens (Llewelyn and Cohen, 2002). Mechanistically, *S. aureus* isolated from the rectum or pharynx of KD patients can secrete toxic shock syndrome toxin 1 (TSST-1) and staphylococcal protein A, which in turn stimulate $V\beta2^+$ lymphocyte amplification and are thus involved in the abnormal immune responses of KD patients (Leung et al., 1993; Wann et al., 1999; Leung et al., 2002).

Regarding *Yersinia pseudotuberculosis* (Konishi et al., 1997), the *Propionibacterium acnes* strain and its products cytopathogenic proteins (CPPs; Kato et al., 1983; Tomita et al., 1987) can all be isolated from KD patients, suggesting a causative role of bacterial infection in mediating the pathogenesis of KD. Moreover, several recent studies suggest that *Y. pstb* infection is closely related to KD pathogenesis (Kato et al., 2019; Kamura et al., 2020; Miyata et al., 2022; Ohnishi et al., 2022), and the antibody titers of *Y. pstb* were significantly elevated in both Chinese and Japanese KD patients (Chou et al., 2005; Tahara et al., 2006). In contrast, a recent study showed that the positive rate of *Y. pstb* infection is much lower in KD patients (Horinouchi et al., 2015; Hayashi et al., 2023), but when the population is exposed to a higher risk of *Y. pstb* infection, the incidence of KD is much higher (Vincent et al., 2007).

2.3 *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* hypothesis related to KD

In addition to the microbes mentioned above, *M. pneumoniae* infection was identified in an important proportion of KD patients (Umezawa et al., 1989; Ebrahim et al., 2011; Lee et al., 2011; Tang et al., 2016; Wang et al., 2021; Huang et al., 2022). For instance, the *M. pneumoniae* infection-positive rate in KD patients was significantly higher than that in non-KD patients during the SARS-CoV-2 epidemic (Ding et al., 2021), and certain KD patients were found to be coinfected with *M. pneumoniae* and Epstein–Barr virus (Huang et al., 2012).

Additionally, the positive rate of serum *Chlamydia pneumoniae* IgM antibody in KD children was significantly higher than that in the control group (Numazaki and Chiba, 1996); however, another study showed that the link between *C. pneumoniae* infection and KD pathogenesis or coronary artery lesions remains to be clarified (Chua et al., 2000; Strigl et al., 2000), suggesting that more intense research is needed to confirm the correlations between *M. pneumoniae* or *C. pneumoniae* infection and KD pathogenesis.

2.4 Rickettsia infection and KD

Rickettsia-like organisms were also found in biopsies of the skin and lymph nodes of KD patients (Tasaka and Hamashima, 1978). However, in most cases, only *Coxiella burnetiid* but not *Rickettsia conorii*, *R. typhi*, *Coxiella burnetii* or *Ehrlichia phagocytophila* was suggested to cause KD-like symptoms in young children (Kafetzis et al., 2001), suggesting its specific causative roles in KD pathogenesis.

2.5 Pathogen infection evidenced from experimental studies with a murine model

Given that the fungus *Candida albicans* can be isolated from KD patients, and its extract, the *Candida albicans* water soluble fraction (CAWS) intraperitoneally injected in mice could induce symptoms resembling those observed in KD patients (Murata, 1979; Martinez et al., 2012; Yoshikane et al., 2015; Stock et al., 2016; Noval Rivas and Arditi, 2020). Furthermore, β-glucan, which is the major component of CAWS, is also increased in KD patients (Ishibashi et al., 2014). Mechanistically, the mannoprotein-β-glucan complex of *C. albicans* can affect the functions of leukocytes, endothelial cells, and platelets *in vitro* (Kurihara et al., 2003). The systematic vasculitis induced by CAWS in mice can be alleviated after administration of human immunoglobulin or etanercept (Takahashi et al., 2010b; Ohashi et al.,

TABLE 1 Microbial etiology demonstrated as critical triggers of Kawasaki disease.

Pathogens	Related to KD	References
Virus		
Epstein–barr Virus (EBV)	EBV infection is associated with recurrence of KD	Kikuta et al. (1990)
	EBV infection is associated with the development of coronary aneurysms in KD	Kikuta et al. (1993)
	EBV infection was first demonstrated in KD cases by PCR	Rosenfeld et al. (2020)
Adenovirus	The cause of KD was not proved to be adenovirus by TaqMan PCR test	Shike et al. (2005)
	The specific immune response to HADV-3 plays a key role in the occurrence of KD	Fukuda et al. (2017)
Human parvovirus B19 (HPV-B19)	HPV-B19 can cause some symptoms resembles to those observed in KD	Nigro et al. (1994)
	Infection with HPV-B19 is closely associated with KD and collagen diseases	Holm et al. (1995) and Oura et al. (2022)
	HPV-B19 DNA was identified in the blood and pathological tissues of adult KD patients	Flossdorf et al. (2020)
Torque Teno virus 7	A low copy number torque teno virus 7 was detected in cervical lymph nodes of a KD case by using real-time PCR	Katano et al. (2012)
	TTV7 variants were detected by metagenomic sequencing and PCR method in two KD patients	Thissen et al. (2018)
Herpes virus	Patients with KD and HHV6 infection had similar skin changes at the BCG vaccination site	Kakisaka et al. (2012)
	The number of KD patients increased significantly due to HHV-6 infection during the SARS-CoV-2 epidemic	Dursun and Temiz (2020)
	A child with incomplete KD complicated with HHV-6B infection developed aseptic meningitis	Alramadhan et al. (2020)
Varicella Zoster Virus	A case of KD patient was found to infect with EB virus and varicella-zoster virus	Turkay et al. (2006)
	A case of KD patient was found to complicate with varicella-zoster virus infection	Toprak et al. (2015)
Human boca virus (HboV)	Certain KD patients were found to infect with HboV by using PCR method	Catalano-Pons et al. (2007)
	The serological data shows no association between HBoV infection and KD occurrence	Lehmann et al. (2009)
	Human boca virus DNA was identified in the nasopharyngeal secretions of a male child with KD	Santos et al. (2011)
	KD was significantly correlated with the monthly incidence of human boca virus	Kim et al. (2014)
Cytomegalovirus	Two infants with cytomegalovirus infection developed atypical KD and coronary aneurysm	Catalano-Pons et al. (2005)
	A case of atypical KD was found to infect with cytomegalovirus	Guc et al. (2008)
Dengue virus	The dengue virus titer is positive in certain KD children	Sopontammarak et al. (2008)
Coxsackie virus	The *coxsackie virus* infection was found in two cases of KD by using ELISA method, and the CVB3 antibody was detected by complement binding assay	Rigante et al. (2012)
	The antibody titer to coxsackie virus A4 was significantly higher than those in an adult KD case	Ueda et al. (2015)
Enterovirus	The cumulative incidence of KD in enterovirus-infected cohort was significantly higher than that in non-EV-infected cohort	Weng et al. (2018)
	The decrease in the number of KD hospitalizations was positively correlated with the decrease in the number of severe enterovirus infections	Guo et al. (2022)
HCoV-NL63	A lack of evidence proving human coronavirus NL63 infection associated with KD induction	Shimizu et al. (2005)
	The infection rate of HCoV-NL63 in KD patients is very low	Dominguez et al. (2006)
	Lack of association between infection with HCoV-NL63 virus and KD	Chang et al. (2006)
	Serological data showed no association with HCoV-NL63 infection in KD children	Lehmann et al. (2009)
	Serological data support that HCoV-NL63 is not involved in KD, but suggest that HCoV-229E may be involved in KD	Shirato et al. (2014)
Parainfluenza type3 virus	The parainfluenza type 3 virus infection was suggested to associate with KD occurrence	Schnaar and Bell (1982)
	The parainfluenza virus type 3 infection was found to associate with KD in one patient	Johnson and Azimi (1985)
Measles-virus	The measles-virus infection was suggested to associate with KD occurrence	Whitby et al. (1991)

(Continued)

TABLE 1 (Continued)

Pathogens	Related to KD	References
SARS-CoV-2	The KD incidence has increased during the SARS-CoV-2 pandemic	Ouldali et al. (2020), Sandhaus et al. (2020), and Stower (2020)
	The cases of SARS-CoV-2 infection children have a higher frequency of myocarditis or pericarditis than the classic KD	Ventura et al. (2020)
	The KD incidence is increased during the pandemic of SARS-CoV-2 or influenza A H1N1 in western counties	Kam et al. (2020)
	The SARS-CoV-2 cases show a highly active proinflammatory cytokine response similar to KD	Choi (2020)
	Asymptomatic children with SARS-CoV-2 infection shows a hyperinflammatory syndrome similar to KD shock syndrome	Rehman et al. (2020)
	Several concurrent incomplete KD cases with SARS-CoV-2 infection were identified	Rivera-Figueroa et al. (2020) and Raut et al. (2021)
Feline virus	KD was suggested to correlate with a new feline virus transmitted by fleas	Moynahan (1987)
H1N1	The H1N1 virus infection was identified in the cases of incomplete KD patients	Wang et al. (2019)
Human immunodeficiency virus	The inflammatory characteristics of pediatric KD resembles the symptoms of adult immunodeficiency virus syndrome	Johnson et al. (2016)
Virus-like particles	The virus-like particles were found in the circulating peripheral blood of KD patients	Lin et al. (1992)
	The accumulation of virus-like particles (VLP) in lung tissue of KD patients, and the intracytoplasmic inclusion bodies of skin cells in the ciliated bronchial wall of KD patients were suggested to be induced by virus-like particles	Rowley et al. (2011)
Bacteria		
Staphylococcus aureus	The amplification of T cells in KD patients may be caused by a new clone of TSST-producing *S. aureus*	Leung et al. (1993)
	High levels of extracellular SpA secreted locally by *S. aureus* in the gastrointestinal tract may lead to KD-like symptoms	Wann et al. (1999)
	The value of *S. aureus* in larynx and rectum mucosa was higher in KD patients	Abe et al. (2003)
	Staphylococcus superantigens is associated with KD pathogenesis	Matsubara and Fukaya (2007)
	Multiple superantigens are involved in KD by using serum IgG and IgM antibodies against all the superantigens	Matsubara et al. (2006)
Yersinia pseudotuberculosis (*Y. pstb*)	*Y. pseudotuberculosis* was isolated from the stool of a KD patient	Konishi et al. (1997)
	Some studies have shown that superantigen (YPM) is produced *in vivo* and plays an important role in the pathogenesis of pseudomonas tuberculosis infection	Abe et al. (1997)
	KD was significantly associated with myocarditis and the increase of yersinia antibody titer	Chou et al. (2005)
	The incidence of coronary artery lesions in *Y. pseudotuberculosis* positive group was significantly higher than that in Yersinia negative group in KD patients	Tahara et al. (2006)
	The KD incidence is higher when the population is exposed to the risk of *Y. pseudotuberculosis* infection	Vincent et al. (2007)
	Specific molecules in the serum samples of KD share a common molecular structure with the microbe associated molecular pattern (MAMP) of *Y. pseudotuberculosis*	Kusuda et al. (2014)
	KD Patients associated with *Y. pseudotuberculosis* infection had significantly more frequent cardiac sequelae (CS)	Horinouchi et al. (2015)
	A KD-like patient who was positive for yersinia tuberculosis was diagnosed with far east scarlet fever	Ocho et al. (2018)
	LOOP-mediated isothermal amplification method identifies *Y. pseudotuberculosis* infection in KD patient	Kato et al. (2019)
	The symptom caused by pseudomeric *mycobacterium tuberculosis* infection resembles o the features observed in KD	Kamura et al. (2020)
Propionibacterium acnes	The levels of anti-cytopathic protein (CPP) antibodies in serum of KD patients are increased during the acute phase	Tomita et al. (1987)
	The variant strain of *P. acnes* may have a causative role in KD and house-dust mites a role as vectors	Kato et al. (1983)
Rickettsia-Lick organism	The clostridium bursteni is associated with KD instead of other rickettsiae pathogen	Kafetzis et al. (2001)
Streptococcus sanguis	Streptococcus hemorrhage can be isolated from KD patients in acute stage	Tsurumizu et al. (1991)
Bacillus cereus	KD specific molecules in serum share a common molecular structure with the MAMP of *B. cereus*.	Kusuda et al. (2014)

(Continued)

TABLE 1 (Continued)

Pathogens	Related to KD	References
Lactobacillus casei	The asymmetric inflammatory coronary inflammation was detected in the LCWE-induced mouse model	Lehman et al. (1988)
	The macrophage dectin-1/Syk-mediated pathway is involved in LCWE-induced CALs and production of IL-6 and MCP-1	Lin et al. (2013)
	$CD8^+$ T cells functionally contributed to the development of KD vasculitis in LCWE-induced mouse model	Noval Rivas et al. (2017)
	The endothelial progenitor cell Notch4 signaling pathway was identified in the LCWE-induced mouse model	Wang et al. (2016)
	The adrenergic stimulation after KD vasculitis can cause myocardial hypertrophy and bridging fibrosis in the LCWE-induced mouse model	Matundan et al. (2019)
	The electrophysiological abnormalities and cardiac neuronal remodeling were observed in the LCWE-induced mouse model	Abe et al. (2020)
	The coronary artery stenosis with severe coronary vasculitis and elastin degradation was detected in the LCWE-induced mouse model	Suganuma et al. (2020)
	Nonpathogenic LCWE-specific T-cell combinations are related to KD occurrence	Hsieh et al. (2021)
Mycobacterium SSP	Atypical mycobacterium infection enhances autoimmunity leading to coronary arteritis after vaccination with BCG	Nakamura et al. (2007)
	The development of tuberculid in the two infants might be associated with the remnant immune activation of KD	Yamada et al. (2016)
Streptococcus	Group A streptococcus was not detected in an adult Japanese female with KD	Hattori et al. (2005)
	The superantigens of *S. pyogenes* are involved in KD pathogenesis based on the IgM antibodies test	Matsubara et al. (2006)
	KD is associated with many streptococcal superantigens	Matsubara and Fukaya (2007)
	The first case of incomplete KD complicated with *S. pyogenes* pneumonia was reported	Leahy et al. (2012)
	The complete genome sequence of *Streptococcus* nM-65 can be isolated from KD patient	Tabata et al. (2021)
Fungi		
Candida albicans	The fungi *C. albicans* isolated from KD patients can produce coronary arteritis in mice	Murata (1979)
	CAWS strongly inhibits leukocyte function *in vitro*	Kurihara et al. (2003)
	The adeno-associated virus vector encoding IL-10 improves CAWS-induced cardiac dysfunction and lethality in mouse	Nakamura et al. (2018)
	The genetic background of CAWS immune response is related to the occurrence of coronary arteritis	Nagi-Miura et al. (2004)
	The genetic control of susceptibility to induction of vasculitis by the *candida albicans* extract is dependent on the mouse strains, but is not linked to the histocompatibility-2 loci	Takahashi et al. (2004)
	Severe stenosis of the aorta and coronary arteries, and fibrinoid necrosis in the vessel walls were observed in the CAWS-induced DBA/2 mouse strain	Hirata et al. (2006)
	Most CAWS strains can induce vasculitis	Nagi-Miura et al. (2008)
	Candida cell wall mannan might contribute to coronary arteritis and acute shock, and that an alteration of mannan structure could be responsible for Candida pathogenicity	Tada et al. (2008)
	The human immunoglobulin suppresses development of murine systemic vasculitis induced by CAWS	Takahashi et al. (2010b)
	The important role of CCR2 involved in the pathogenesis of CAWS-induced mouse model	Martinez et al. (2012)
	Etanercept is effective in inhibiting CAWS-induced vasculitis and may be a new therapeutic drug for KD	Ohashi et al. (2013)
	The preformed toxins and the *Candida* species were identified as the dominant fungus leading to KD	Rodo et al. (2014)
	The α-mannan contained in *C. albicans* extract could induce coronary arteritis and acute shock	Tada et al. (2014)
	KD patients have a higher titer of β -glucan (BG) antibody against candida cell wall	Ishibashi et al. (2014)
	Granulocyte/macrophage colony stimulating factor was found in the CAWS-induced cardiac inflammation site of KD mice	Stock et al. (2016)
	The CAWS-induced mouse model showed inflammatory cell infiltration, destruction of elastic lamellae, loss of medial smooth muscle cells and intimal thickening, whose features resembles the vascular lesions of KD patients	Yoshikane et al. (2015)
	The recognition of A-mannan by A-mannan receptor dectin-2 plays an important role in the pathogenesis of vasculitis in KD mice induced by *C. albicans* cell wall polysaccharide.	Oharaseki et al. (2020)
	The mannoprotein fractions of clinically isolated Candida species can induce vasculitis in mice	Tanaka et al. (2020)
	The cell wall mannoprotein of *C. krusei* could induce coronary vasculitis in mouse model	Yanai et al. (2020)

(Continued)

TABLE 1 (Continued)

Pathogens	Related to KD	References
Mycoplasma		
Mycoplasma pneumoniae	The pulmonary symptoms of KD were suggested to be associated with pneumococcal infection	Lee et al. (2011)
	Incomplete KD patients were found to be related with acute *M. pneumoniae* infection	Ebrahim et al. (2011)
	Many cases of KD simultaneously infected with Epstein–barr virus and *M. pneumoniae* were identified	Huang et al. (2012)
	MP infection occurs in the elderly population, and the respiratory tract involvement rate is higher in KD patients	Tang et al. (2016)
	The MP infection rate in KD patients was significantly higher than that those observed in non-KD patients	Ding et al. (2021)
	M. pneumoniae infection may be associated with a reduced incidence of small CAA in KD patients	Wang et al. (2021)
	The serological test for *M. pneumoniae* infection was positive in one case of acute KD patients	Huang et al. (2022)
	A lack of evidence showing association between *M. pneumoniae* infection and KD induction	Strigl et al. (2000)
Chlamydia		
	The positive rate of serum *C. pneumoniae* IgM antibody in KD was higher than those in control group	Numazaki and Chiba (1996)
	A deficiency evidence of *C. pneumoniae* infection associated with KD occurrence	Chua et al. (2000)

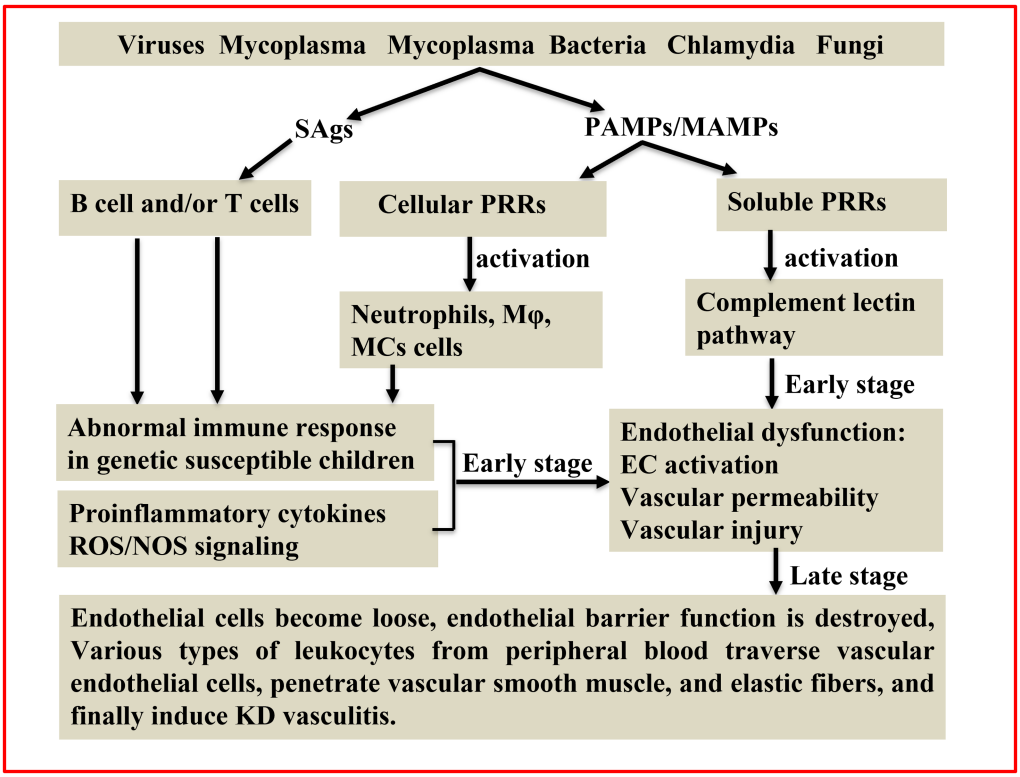

FIGURE 1
Schematic illustrating the pathogenic mechanisms of KD. The superantigens (SAgs) hypothesis and different infectious agents produce pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) were all proposed to be involved in KD pathogenesis. SAgs non-specifically activate T cells and/or B cells. PAMPs/MAMPs also stimulate immune cells [e.g., macrophages (Mφ), dendritic cells (DCs), monocytes (MCs)] and endothelial cells (ECs) through cellular pattern recognition receptors (PRRs; e.g., TLRs, NOD1, and Dectin-1/-2). Additionally, PAMPs/MAMPs can activate the complement lectin pathway through soluble PRRs (e.g., ficolin-1 and mannose binding lectin-2). Activated complement pathways can induce inflammatory vascular damage through recruitment of innate inflammatory cells and direct injury to ECs. This cross-talk among different cells augments the production of proinflammatory cytokines/chemokines and reactive oxygen/nitrogen species (ROS/NOS), hence leading to a systemic inflammatory reaction in KD.

2013). Together, these findings imply that infectious agents might play critical roles in triggering this disease.

Another major KD-like murine coronary arteritis model involves induction by *L. casei* cell wall extract (LCWE), which is widely used to mimic systematic vasculitis in KD patients (Lehman et al., 1988; Abe et al., 2020). In the LCWE-induced mouse model, the TLR2 and ILβ-dependent signaling pathways were suggested to play important roles during its pathogenesis (Rosenkranz et al., 2005; Lee et al., 2012; Matundan et al., 2019). Additionally, the dectin-1/Syk signaling pathway in macrophages (Lin et al., 2013) and the Notch4 signaling

pathway in endothelial progenitor cells are also involved in LCWE-induced coronary artery disease, thereby contributing to the development of KD pathogenesis (Wang et al., 2016). Moreover, LCWE was likewise suggested to function as immunogenic for proinflammatory T helper (Th) 1, Th17, and $CD8^+$ T cells and inducible regulatory T cells (iTreg) (Noval Rivas et al., 2017; Hsieh et al., 2021). Taken together, the systematic vasculitis induced by CAWS or LCWE in mice resembles pathological features observed in KD patients, demonstrating the causative roles of etiological agent infection and related PAMP/MAMP signaling activation in inducing KD vasculitis (Table 1).

3 Summary and perspective

Taken together, various pathogens identified in KD were all suggested to be the critical triggers in causing systematic vasculitis, and these pathogens were demonstrated to work independently or synergistically to potentiate abnormal immune responses by inducing pyroptosis and/or proinflammatory cell death, hence leading to systematic vasculitis in KD (Figure 1). However, whether these pathogens are direct causes or merely the accompanying pathogens after KD induction remains elusive. Additionally, the causative agent of KD remains ambiguous, and several questions remain to be clarified. First, those pathogens suggested to be involved in the pathogenesis of KD largely rely on PCR and serological methods using a relatively small sample size. Second, the differences in timing of obtaining the blood sample and constraints of the study design used to measure pathogens in KD patients by different investigators could make pathogen identification inconsistent. Third, whether KD is caused by a single pathogen or is the combined result of more than one agent remains to be investigated. Consequently, the relationship between pathogen infection and KD vasculitis is far more complex than currently appreciated. Caution should be exercised in the clinic when considering the possible agents merely based on the symptom similarities between KD and other infectious diseases. Importantly, given that the recognition of the infectious origin of KD is a critical prerequisite to understanding its pathogenetic mechanism, more intense research using artificial intelligence, metagenomic sequencing and culturing specific pathogens isolated from KD patients from multiple centers and then verifying each of them in animal models could help uncover the underlying mechanisms of pathogen infections involved and thus facilitate the development of novel intervention strategies for Kawasaki disease.

Author contributions

WW: Writing – original draft, Data curation. LZ: Conceptualization, Funding acquisition, Investigation, Writing – original draft. XL: Writing – original draft, Data curation, Project administration. ZL: Data curation, Resources, Writing – original draft. HL: Conceptualization, Investigation, Supervision, Writing – review & editing. GQ: Conceptualization, Funding acquisition, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Roles of Lipolytic enzymes in *Mycobacterium tuberculosis* pathogenesis

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Mycobacterium tuberculosis (Mtb) is a bacterial pathogen that can endure for long periods in an infected patient, without causing disease. There are a number of virulence factors that increase its ability to invade the host. One of these factors is lipolytic enzymes, which play an important role in the pathogenic mechanism of Mtb. Bacterial lipolytic enzymes hydrolyze lipids in host cells, thereby releasing free fatty acids that are used as energy sources and building blocks for the synthesis of cell envelopes, in addition to regulating host immune responses. This review summarizes the relevant recent studies that used *in vitro* and *in vivo* models of infection, with particular emphasis on the virulence profile of lipolytic enzymes in Mtb. A better understanding of these enzymes will aid the development of new treatment strategies for TB. The recent work done that explored mycobacterial lipolytic enzymes and their involvement in virulence and pathogenicity was highlighted in this study. Lipolytic enzymes are expected to control Mtb and other intracellular pathogenic bacteria by targeting lipid metabolism. They are also potential candidates for the development of novel therapeutic agents.

KEYWORDS

***Mycobacterium tuberculosis*, lipolytic enzymes, virulence factor, pathogenicity, therapeutic targets**

1 Introduction

Tuberculosis (TB), which is caused by infection with *Mycobacterium tuberculosis* (Mtb), is identified as the one of the earliest human illnesses that continue to be among the most lethal infectious diseases. Tuberculosis (TB) was the second most common infectious illness killer globally after COVID-19 in 2022. It was also the primary cause of death for those who tested positive for HIV and a major factor in deaths linked to antibiotic resistance. In 2022, there were 10.6 million cases of tuberculosis reported globally. Between 2020 and 2022, the incidence rate of tuberculosis (TB) climbed by 3.9% per 100,000 people annually, reversing a two-decade trend of annual declines of about 2% (WHO, 2023). Despite the various interventions that have been used to prevent and treat TB, the cure for the disease is yet to be found. The main reason for this is that Mtb is an incredibly complex and unique pathogen that can evade the immune system (Bullen et al., 2023; Sengupta et al., 2023; Shariq et al., 2023). The primary form of TB is usually found in macrophages, where the bacteria can survive and replicate (Dey and Bishai, 2014). However, the exact pathogenicity mechanism remains unclear (Li et al., 2019).

To treat this disease and approach a world free of tuberculosis, new vaccines and medications should take Mtb virulence characteristics into account. Studies on the molecular mechanisms underlying the pathogenicity, virulence, and persistence of mycobacteria made significant strides in recent years. The discovery of crucial mycobacterial virulence genes has been one of the noteworthy achievements. The majority of these virulence genes encode regulators, cell surface proteins, lipid pathway enzymes, and proteins that are involved in signal transmission.

This review focuses on Mtb lipolytic enzymes whose inactivation results in a significant reduction in the levels of pathogenicity or virulence. Forrellad et al. (2013) reported that the virulence determinants were categorized into the following groups based on their function. We ranked them in order of relevance to the role of lipolytic enzymes: (1) lipid and fatty acid metabolism, including the catabolism of cholesterol; (2) macrophage-inhibiting proteins, such as those involved in response to nitrosative and oxidative stress, phagosome arresting, and inhibition of apoptosis; (3) cell envelope proteins, such as lipoproteins, cell wall proteins, and secretion systems; (4) proteases, including metalloproteases; (5) protein kinases; (6) importer and exporter proteins for metal transport; (7) proteins with unknown functions, such as the PE and PE-PGRS families, PE are distinguished by around 100 amino acid conserved N-terminal domains (Cole et al., 1998). The characteristic motif Pro (P) - Glu (E) is where the name PE originates. The largest subfamily of PE is called PE-PGRS, and it is distinguished by PE N-terminal domain and PGRS (Polymorphic GC-rich Repetitive Sequences) in C-terminal domain; (8) transcriptional regulators, such as sigma factors and two component systems; (9) additional virulence pr oteins.

Mtb differs from pathogenic bacteria in that it has a wide range of intricate lipids and lipoglycans on its cell envelope. From the inside out, the components of the Mtb cell envelope are as follows: (i) plasma membrane; (ii) cell wall made up of different non-covalently linked proteins, lipids and carbohydrates, as well as three covalently linked macromolecules (mycolic acids, arabinogalactan and peptidoglycan) and (iii) a capsule composed of lipids, proteins and polysaccharides (Brennan, 2003). Pathogenic mycobacteria are distinguished by their unique cell envelope, which comprises various lipids esterified with structurally related long-chain multi-methyl-branched fatty acids. It has long been believed that these lipids are crucial for both the virulence and structure of the tubercle bacillus cell envelope. It is known that Mtb has 250 kinds of genes encoding putative enzymes involved in lipid metabolism from *in silico* analysis (Cole et al., 1998), while only 50 such genes encoding for these enzymes in *Escherichia coli*, which has a similar genome size (Neyrolles and Guilhot, 2011). The lipolytic enzymes involved in lipid metabolism are mainly hydrolyzing carboxyl ester to fatty acids and glycerol, which are utilized in colonization, persistence, virulence and as an energy source. Based on the specificity of their respective substrates, enzymes related to lipid metabolism can be divided into four main categories: esterases, lipases, phospholipases, and cutinases, detailed information on these enzymes are below.

2 The classification of lipolytic enzymes in *Mycobacterium tuberculosis*

Lipolytic enzymes such as lipases, esterases, phosphlipases, and cutinases are significantly involved in the pathological processes that enhance their survival. However, more of their characteristics still need to be studied in detail. Studies on mycobacteria infection revealed the role played by lipolytic enzymes in pathogenicity. Lipolytic enzymes share the GXSXG pentapeptide sequence, which is a feature of the α/β hydrolase fold family of proteins (Johnson, 2017). In general, four types of lipolytic enzymes can be identified, based on the characteristics and degree of specificity of the relevant substrates (Delorme et al., 2012; Dedieu et al., 2013). These four classes of lipolytic enzymes include: (1) lipases, with the consensus sequence GXSXG, which hydrolyze water-insoluble long-chain carboxylesters like long-chain triglycerides (TAG); (2) esterases (or carboxylesterases), with the consensus sequence GXSXG, which hydrolyze small and partially water-soluble carboxylesters; (3) phospholipases, with the consensus sequence G-X_1-S-X_2-G, which are sub-classified into four groups (PLA1, PLA2, PLC and PLD) cleaving the different bond position of phospholipids; (4) cutinases, with the consensus sequence G-[YF]-S-[QL]-G, which break down all types of carboxylesters, including TAG, and phospholipids, as well as cutin. Cole et al. (1998) annotated 24 putative lipase/esterase gene from the Mtb genome, known as the "Lip family." However this classification does not differentiate between lipases and esterases. This review is mainly introduced the lipolytic enzymes in the following four categories: Lip family, other lipase/esterase, phospholipases and cutinases. Creating bioactive compounds and substrates that serve as the carbon and energy sources depend on bacterial lipolytic enzymes. Additionally, the bacterial lipolytic enzymes play a significant role in controlling the host's protective immunological responses and signal transduction cascades. Due to space limitation, Table 1 only summarizes some representative well-characterized lipolytic enzymes that are currently known. Others are in Supplementary Table S1.

2.1 Lip family

From the genome annotation, it has been shown that 24 genes (C to Z, excluding A and B) may encode lipolytic enzymes known as "Lip family" (Table 1 and Supplementary Table S1). The consensus sequence GXSXG, which is a feature of members of the α/β hydrolase fold family, is the only factor used to categorize these proteins. This classification does not differentiate between lipases and esterases. Genome sequence analysis cannot be used as the single criterion for categorizing the proteins. The only method for differentiating between lipase and esterase enzymes is based on biochemical characterization. Lipases hydrolyze water-insoluble long-chain carboxylesters like TAG, while esterases hydrolyze small and partially water-soluble carboxylesterases. The "Lip family" is made up of both lipase and esterase enzymes (Cole et al., 1998; Camus et al., 2002; Canaan et al., 2004; Delorme et al., 2012; Shen et al., 2012; Li et al., 2017; Yang et al., 2019). The LipC (Rv0220) (Shen et al., 2012), LipD (Rv1923) (Singh et al., 2014), LipE (Rv3775) (Yang et al., 2019), LipF (Rv3487c) (Delorme et al., 2012), LipH (Rv1399c) (Canaan et al., 2004), LipJ (Rv1900c) (Kumari and Kaur, 2021), LipK (Rv2385) (Chownk et al., 2018), LipL (Rv1497) (Dey et al., 2022), LipN (Rv2970c) (Jadeja et al., 2016), LipR (Rv3084) (Zhang et al., 2019), LipS (Rv3176c) (Chownk et al., 2017), LipU (Rv1076) (Li et al., 2017), LipW (Rv0217c) (Delorme et al., 2012), and LipX (Rv1169c) (Singh et al., 2016) are functionally characterized as esterases. LipD (Rv1923) (Singh et al., 2014), LipQ (Rv2485c) (Kumar et al., 2017a), LipT (Rv2045c) (Singh et al., 2010), and LipY (Rv3097c) (Singh et al., 2014) are functionally

TABLE 1 Lipolytic enzymes of *M. tuberculosis* H37Rv and their function.

Enzyme classification	Gene Product	Subcellular localization	Enzymatic activity	Function	References
Lip family	LipX (PE11, Rv1169c)	**Cell wall	**PE family **Esterase **Hydrolysis of *p*-NP acetate Specific activities: 1215 mU mg^{-1} with Tween 20	**LipX modifies lipid content and cell wall architecture **Stimulates cytokines like IL-10 and IL-4 to create an environment that is primarily of the Th2-type **Mtb ΔLipX had poorer survival in activated THP-1 macrophages	Cascioferro et al. (2007), Deng et al. (2015), Singh et al. (2016), Rastogi et al. (2017)
	LipY (Rv3097c)	**Cytoplasmic **Cell envelop	**Lipase **Hydrolysis of TAG **PE-PGRS family Specific activities: 41 nmol mg^{-1} min^{-1} with triolein K_m: 7.57 mmol L^{-1} and V_{max} of 653.3 nmol mg^{-1} min^{-1}	**LipY inhibits Th1 and Th17 responses and stimulates Treg cell induction **LipY participates in the metabolism of lipids	Deb et al. (2006), Mishra et al. (2008), Daleke et al. (2011), and Singh et al. (2014)
Other esterase/lipase	Rv2224c (MT2282; Hip1; CaeA)	**Cell wall	**Serine protease **Carboxyesterase	**Reduces proinflammatory response, inhibits antigen presentation and T cell responses **It is required for virulence of Mtb	Lun and Bishai (2007), Rengarajan et al. (2008), Madan-Lala et al. (2011, 2014), and Naffin-Olivos et al. (2014)
	Rv0183	**Cell wall	**Lipase **Hydrolysis of MAG **Phospholipase Specific activities: 27 U mg^{-1}	*Possible function in the lipid metabolism of the host cell membrane **Alveolar macrophages involved in tuberculosis physiology exhibit inflammatory markers as IL-6, NF-$_k$B, TLR2, TLR6, TNF-α and MyD88 when Rv0183 is present	Côtes et al. (2007), Xu et al. (2010), and Liu et al. (2018)
Phospholipase	PLC	**Cell wall **Membrane	**Phospholipases **Hydrolysis of *p*-NP choline (*p*-NPC) Specific activities (μ mol min^{-1} mg^{-1}): PLC-A: 10.5, PLC-B: 10.1, PLC-C: 9.3, PLC-D: 9	**Help mycobacteria adapt to the iron-limited intracellular environment **Hydrolytic activity on the phospholipids in the host cell membrane **PLC-encoding genes were strongly upregulated under phosphate starvation	Bacon et al. (2007), Bakala N'goma et al. (2010), and Le Chevalier et al. (2015)
Cutinase	Culp6/ Cut6 (Rv3802c)	**Cell wall	**Phospholipase A **Thioesterase **Lipase With *p*-NPB Specific activity: 40 pmol min^{-1} mg^{-1} V_{max}: 1.62 mol min^{-1} mg^{-1} k_{cat}: 8.81 S^{-1} K_m: 23.52 mmol L^{-1}	**Cut6 participates in the production of mycolic acid **Essential for *in vitro* growth of the bacilli **Cut6 promotes the production of IFN-γ by Th1-type T cells	Sassetti et al. (2003), Mattow et al. (2007), West et al. (2008), 2009, Parker et al. (2009), Crellin et al. (2010), and Shanahan et al. (2010)

*Bioinformatics prediction.
**Experimentally defined.

characterized as lipases. Among 24 lipolytic enzymes in Lip family, there are 12 proteins (LipC, LipF, LipH, LipI, LipM, LipN, LipO, LipQ, LipR, LipU, LipW, LipY) homologous to the human Hormone Sensitive Lipase (hHSL). The epinephrine-sensitive lipolytic enzyme hormone-sensitive lipase (HSL) was originally discovered in adipose tissue (Vaughan et al., 1964). This enzyme family, also known as the "Lip-HSL" family, is essential for the release of free fatty acid from TAG that is kept in adipocytes (Lafontan and Langin, 2009; Lampidonis et al., 2011). The conserved GXSXG and HGGG motifs, which include the catalytic serine and oxyanion hole, respectively, are present in the core α/β hydrolase domain of HSL.

2.2 Other lipases/esterases

In recent years, more enzymes have been identified as lipases/esterases through experiments. A series of enzymes containing Rv3091, Rv0183, Rv1592c, Rv2037c, and Rv1683 are functionally characterized as lipases. Rv0774c, Rv1075c, Rv3036c, Rv0045c, Rv1430, and Rv3539 are functionally characterized as esterases. Rv2224c and Rv0519c, with the catalytic triad that is found in esterases, lipases, and proteases, are identified as lipases/esterases by experiments (Ferre and Clote, 2005; Srivastava et al., 2008).

With the development of bioinformatics, comparative proteomics studies are increasingly being undertaken to find new virulence factors, like therapeutic targets and vaccine candidates (Cole et al., 1998). Nearly 40% of open reading frames in the genome of Mtb have been classified as hypothetical proteins (Mazandu and Mulder, 2012). Determining the biological functions of these hypothetical proteins would undoubtedly improve comprehension of the Mtb life cycle. The hypothetical lipases/esterases such as Rv2030c, Rv1367c, Rv1922, Rv1063c and Rv3728, summarized in Table 2 and Supplementary Table S2, are predicted to be important in the life cycle in Mtb according to their subcellular localization and similarity to the lipolytic enzymes that have important roles in the Mtb.

2.3 Phospholipases

The phospholipase A1 (PLA1), A2 (PLA2), C (PLC), and D (PLD) in the Mtb H37Rv phospholipase family hydrolyze phospholipids at various locations (Raynaud et al., 2002). Similar to the *Pseudomonas aeruginosa plc* genes, there are three adjacently positioned phospholipase C genes [*plc-a* (*rv2351c*), *plc-b* (*rv2350c*), *and plc-c* (*rv2349c*)] and a fourth truncated gene *plc-d* (*rv1755c*) that is situated elsewhere on the Mtb genome (Kong et al., 2005). The overall amino acid identity of PLC-A, PLC-B, and PLC-C is approximately 69%, while the amino acid identity of their C-terminal region is approximately 70% with PLC-D. The 227 amino acids in the N-terminal domain of PLC-D are absent in Mtb H37Rv. Furthermore, PLC-ABC exhibits between 30 and 40% amino acid sequence identity with PLC-H (hemolytic PLC) and PLC-N (nonhaemolytic PLC) from *Paeruginosa aeruginosa*. PLC-H and PLC-N have functions in the virulence of this pathogen *P. aeruginosa* (Ostroff et al., 1990; Guest et al., 2023).

PLC has been identified as a pathogenic component in many bacteria, including *Bacillus cereus* (Gilmore et al., 1989), *Clostridium perfringens* (Titball et al., 1989; Logan et al., 1991), *Listeria monocytogenes* (Boland et al., 1992) and *Pseudomonas aeruginosa* (Berka et al., 1981). All the recombinant PLC hydrolyze *p*-Nitrophenyl (NP) caproate: with the maximum specific activities (μmol min^{-1} mg^{-1}) of 10.5 for PLC-A, 10.1 for PLC-B, 9.3 for PLC-C and 9 for PLC-D, respectively. Compared to PLCs of *B. cereus* and *Clostridium perfringens*, these specific activities of Mtb PLCs were 100 times lower (Johansen et al., 1996).

Parker et al. (2009) described mycobacterial phospholipase A activity (PLA), which was also a possible mycobacterial cutinase. The pathophysiology of disease and inflammatory states in humans are significantly influenced by PLAs, which hydrolyze phospholipids to fatty acids. PLA activity in Mtb has been demonstrated, and it has been discovered that this activity is connected to the cell wall and membrane fractions (Parker et al. 2009). These enzymes, which are most likely excreted from the Mtb cell wall, may cause the release of FA by hydrolyzing the phospholipids in the host cell membrane, supplying a carbon source and aiding in the activities involved in cell growth. It has been discovered that PLD activities occur in Mtb in addition to PLC and PLA activity. Although this enzyme may not be directly involved in virulence, because it is found in numerous species, including both pathogenic and non-pathogenic strains, it may nevertheless play a significant biological role in this genus of mycobacteria (Johansen et al., 1996; Gomez et al., 2001).

Rv0183 was identified as a lysophospholipase, which did not hydrolyze lysophospholipid substrates lysophosphatidylcholine but hydrolyzed monoacylglycerol substrates preferentially (Côtes et al., 2007). LipF (Rv3487c), a cell wall phospholipase C in Mtb hydrolyzes the phosphatidylcholine substrate.

2.4 Cutinases

Cutinases (EC 3.1.1.74), also known as cutinase-like proteins (CULPs) are serine esterases with Ser-His-Asp catalytic triad. Since they exhibit various characteristics shared by lipases and esterases, they are frequently identified as intermediaries between the two enzymes. Cutinases lack a hydrophobic "cap" or secondary structure covering their active site, in contrast to lipases However, a certain degree of flexible "mini-cap" present in the active site of cutinases (Longhi et al., 1997; Martínez and Maicas, 2021). These characteristics may allow the active site to adapt to varied substrates, including phospholipids and TAG, as well as big substrates like cutin.

There are at least seven genes called *cut* (1 to 7) which encode for the cutinase family in the Mtb genome. Although mycobacteria's CULP members lack cutinase activity, they may be crucial in controlling various pathogenic processes (West et al., 2009). It has been discovered that Cut7 (Rv1984c) hydrolyzes medium-chain carboxylic esters, monoacylglycerols, and preferential phospholipids (Cole et al., 1998), whereas Cut4 (Rv3452) acts like phospholipase A2 (Schué et al., 2010).

3 The function of lipolytic enzymes in *Mycobacterium tuberculosis*

Mtb has devoted a sizable amount of proteins to improving its survival, which is not surprising, given that it is an obligate

TABLE 2 Hypothetical lipolytic enzymes of *M. tuberculosis* H37Rv and their predicted function.

Enzyme classification	Gene product	Predicted subcellular localization	Predicted function	Comments	References
Lipase	Rv1922	Extracellular	May contribute to loss of virulence	Similar to Mtb hypothetical proteins Rv1497(LipP), Rv2463(LipE), Rv3775(LipF), *M. bovis* Mb1957, etc. Contains PS00013 Prokaryotic membrane lipoprotein lipid attachment site	Dogra et al. (2015)
Esterase	Rv1062	Cytoplasmic	May contribute to loss of virulence	Similar to lipase and phospholipase	Ortega et al. (2016)
	Rv2565	Extracellular	Potential vaccine or drug targets Phosphatidylcholine metabolic process	Similar to *M. bovis* Mb2594	Knapp and Mcdonough (2014), Kumar et al. (2017c), and Johnson et al. (2020)
	Rv3728	Membrane protein	Involved in efflux system Lipid metabolic process Response to antibiotic Tetracycline transport; Cell wall and cell processes	Similar to *M. bovis* Mb3755	Knapp and Mcdonough (2014) and Kanji et al. (2018)
	Rv2030c	Extracellular	Response to antibiotic; Transferase activity; Nucleoside metabolic process; Predicted possible vaccine candidate	Similarity to *M. bovis* Mb2055c, Mb2056c	Mushtaq et al. (2015)
	Rv1367c	Extracellular	β-lactamase; Carboxylesterase; Possibly involved in cell wall biosynthesis	Some similarity to penicillin binding proteins, e.g., penicillin-binding protein 4 from *Bacillus subtili*; Similar to *M. bovis* Mb1402c	No information available

intracellular pathogen. The current review focuses on Mtb lipolytic enzymes which significantly lower pathogenicity or virulence levels upon inactivation. Lipolytic enzymes promote mycobacterial survival by mediating lipid and fatty acid metabolism, disrupting phagosomes function, and regulating cytokine production (Figure 1).

3.1 Lipid and fatty acid metabolism

Global TB control efforts are significantly impacted by understanding the pathogen-host interaction in active tuberculosis. The role of exosomes in facilitating soluble mediator exchange and cell-to-cell interaction is increasingly acknowledged. These exosomes, released from the bacillus and infected host cells, contain lipids and proteins derived from both the host and Mtb. Lipidomics-based research has examined the lipid contents of exosomes from patients with active TB and healthy controls. The findings revealed the presence of triacylglycerols (TAG), free fatty acids, cholesterylesters (CE), phosphatidylcholines, phosphatidylinositols, and sphingomyelins (Sun et al., 2021; Biadglegne et al., 2022). TLC separation of extracted lipids from caseous granulomas, compared to healthy tissue, showed increased levels of CE, TAG, and cholesterol in lung biopsies from TB patients (Kim et al., 2010). A granuloma, formed when macrophages, lymphocytes, and dendritic cells aggregate at the infection site, is a major histopathological feature of TB. Macrophages within these granulomatous structures, in both experimental animal models and human disease, are termed foamy macrophages (FMs) (Ridley and Ridley, 1987; Cardona et al., 2000). It is demonstrated that FMs, laden with lipid bodies predominantly composed of TAG and CE, are crucial to the pathophysiology of TB (Peyron et al., 2008).

The extracellular lipolytic enzymes (LEs) of Mtb hydrolyze host lipids into fatty acids (FAs). These extracellular LEs break down extracellular host TAG into monoacylglycerol (MAG) and diacylglycerol (DAG) at varied rates, resulting in the release of free FAs (Côtes et al., 2008). FAs are imported into Mtb through a multiprotein complex known as the mammalian cell entry (Mce) system and lipid uptake coordinator A (LucA) (Nazarova et al., 2017; Wilburn et al., 2018). These free FAs are utilized by five enzymes, located at key points in the TAG metabolic pathway as depicted in Figure 1, to enhance the *de novo* synthesis of Mtb TAG as intracellular lipid inclusions (ILI). These enzymes include fatty acyl-coenzyme A (FA-CoA), glycerol phosphate acyltransferase (GPAT), acylglycerol-phosphate acyltransferase (AGPAT), phosphatidic acid phosphatase (PAP), and Triacylglycerol synthase 1 (Tgs1) (Low et al., 2010; Daniel et al., 2011). The accumulated Mtb TAG can be hydrolyzed by mycobacterial intracellular lipolytic enzymes during the phases of intracellular persistence and reactivation. The lipolytic enzyme LipY is capable of releasing fats from the accumulated TAG of Mtb for use during starvation (Deb et al., 2006). These fatty acids can then enter the β-oxidation pathway for energy production and be synthesized into mycolic acids, the major components of the Mtb cell envelope (Boshoff and Barry, 2005; Kremer et al., 2005; Dhouib et al., 2010; Singh et al., 2010; Hsieh et al., 2012; Lee et al., 2013). Overall, lipolytic enzymes are crucial for maintaining the pathophysiology of the bacteria, as illustrated in Figure 1.

LipY is capable of hydrolyzing ILI-containing TAGs (Deb et al., 2006; Mishra et al., 2008). Under nutrient-deficient conditions in

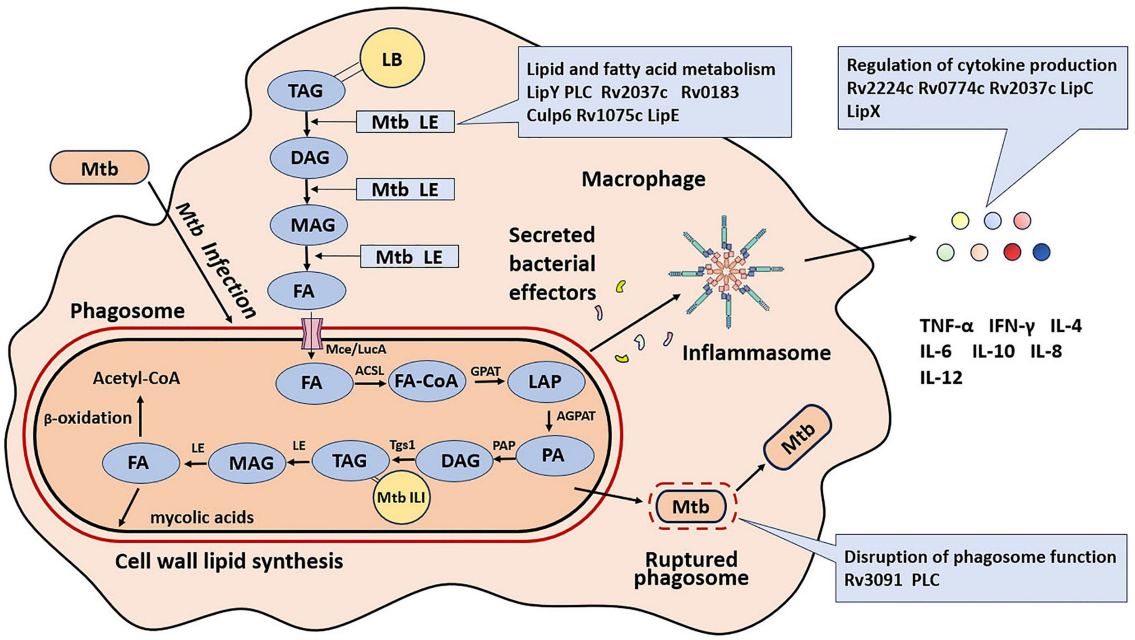

FIGURE 1
Roles of lipolytic enzymes in the pathogenesis of *M. tuberculosis*. LB, lipid inclusion body; TAG, triglyceride; FA, free fatty acid; Tgs1, triacylglycerol synthase 1; ILI, intracellular lipid inclusion; LE, lipolytic enzyme; DAG, diacylglycerol; MAG, monoacylglycerol; FA-CoA, free fatty acid acyl-coenzyme; GPAT, glycerol phosphate acyltransferase; AGPAT, acylglycerol-phosphate acyltransferase; PAP, phosphatidic acid phosphatase; Mce, multiprotein complex termed; LucA, lipid uptake coordinator A. LPA, lyso-phosphatidic acid; PA, phosphatidic acid.

Mtb, LipY expression is strongly induced, allowing the efficient utilization of accumulated TAGs without requiring an additional carbon source (Daniel et al., 2011). Among other members of the "Lip family," such as LipC, LipL, LipK, and LipX, LipY demonstrates the highest specificity in degrading long-chain TAGs (Deb et al., 2006). LipY, following its solubilization from inclusion bodies, was purified. It exhibits optimal activity in hydrolyzing triolein, with K_m of 7.57 mmol L^{-1} and V_{max} of 653.3 nmol mg^{-1} min^{-1}. The N-terminal region of LipY shows sequence homology with the proline-glutamic acid (PE) family protein, featuring polymorphic GC-rich repetitive sequences (Mishra et al., 2008). The C-terminal region of LipY is homologous to the HSL family and contains the conserved active-site motif GDSAG (Deb et al., 2006). LipY is a dual-cellular localization protein, found either intracytoplasmically or associated with the cell envelope. The PE N-terminal domain is cleaved by the ESX-5 secretion system. In the ΔLipY mutant, the capability to hydrolyze TAG was significantly reduced, highlighting this enzyme's potential role in utilizing TAG during the dormancy and reactivation phases of Mtb (Deb et al., 2006). During active TB infection, the expression of LipY can be inferred from the detection of LipY-specific antibodies in patients (Daleke et al., 2011). LipY expression in macrophages reaches its peak within 24 hours following Mtb infection. In comparison with the wild-type H37Rv strain, mice infected with LipY-overexpressing H37Rv strain exhibited increased bacillary loads, exacerbated pathological conditions, weight loss, and higher mortality rates. Conversely, mice vaccinated with the recombinant LipY antigen demonstrated increased resistance to infection when challenged with the LipY-overexpressing strain. In these animal models, not only was there a decrease in Th1 and Th17 immune responses, but there was also an observed increase in the levels of regulatory T (T-reg) cells. These findings suggest that LipY plays a role in diminishing host defense mechanisms and augmenting the pathogenicity of Mtb (Singh et al., 2014).

Mtb possesses phospholipases C (PLC), which are critical for its pathogenicity in mice. PLC might perform several functions related to virulence, such as releasing fatty acids from host phospholipids. The upregulation of Mtb plc genes upon entry into phagocytic cells (Raynaud et al., 2002) and the comparatively high phospholipase activity in mycobacteria isolated from host tissues (Wheeler and Ratledge, 1991, 1992) align with this role. Several studies supporting this virulence-related function are as follows: Raynaud et al. (2002) found that triple and quadruple plc-knock-out mutants of Mtb were attenuated during later infection stages in mice, underscoring the role of PLC in mycobacterial persistence within the host. Furthermore, PLC-overexpressing Mtb strains showed increased survival compared to the PLC mutants (ΔPLC) when phosphatidylcholine was the sole nutrient source (Le Chevalier et al., 2015). Additionally, the hydrolytic action of the four Mtb PLCs on the host cell's membrane phospholipids proved detrimental to mice macrophages (Bakala N'goma et al., 2010).

Phospholipase D (Rv2037c) utilizes lipids such as TAG, glycerol, and phosphatidylcholine from the host as an internal energy source to sustain infection and intracellular survival (Kumari et al., 2020). Rv2037c is a conserved transmembrane lipolytic enzyme characterized by a conserved pentapeptide (GXSXG motif). However, sequence alignment did not reveal the putative Ser-His-Asp triad typical of lipases; instead, only a Ser-Asp catalytic dyad was identified, a feature characteristic of

PLD (da Mata Madeira et al., 2016). Cell wall modifications may be associated with enhanced resistance of the Rv2037c-overexpressing *M. smegmatis* strain to various stressors, including lysozyme, SDS, nutrient deprivation, acidic environments, and anti-TB medications (Kumari et al., 2020). The increased lipid content in the Rv2037c-overexpressing *M. smegmatis* strain, combined with damage to the outer membrane of macrophages and degradation of macrophage lipids by Rv2037c, collectively suggest its potential role in infection and intracellular survival.

Rv0183, localized at the cell wall, is involved in the hydrolysis of host cell lipids, as demonstrated by immunolocalization studies (Xu et al., 2010). It shares 36% and 34% amino acid sequence identity with rat and human monoglyceride lipases, respectively (Côtes et al., 2007). Homologues of Rv0183 have been identified in *M. leprae* (Ml2603, 79% sequence identity), *M. smegmatis* (MSMEG_0220, 68% sequence identity), and *M. bovis* (Mb0189, 100% sequence identity) (Rameshwaram et al., 2018). Rv0183, exhibiting a significant preference for monoacylglycerols, may act as a monoglyceride lipase, producing fatty acids for mycobacteria (Côtes et al., 2007). Using a disrupted mutant of the Rv0183 ortholog in *M. smegmatis*, MSMEG_0220, Dhouib et al. demonstrated Rv0183's role in remodeling the mycobacterial cell wall. This mutant displayed a more homogenous culture with reduced cell clumping and a different colony morphology compared to the wild-type strain.

Culp6 (Rv3802c) is implicated in the production of mycolic acids, unique α-branched lipids present in the cell walls of mycobacteria. The bilayered cell wall of mycobacteria is integrated with these mycolic acids, which are essential for Mtb survival and play a significant role in cell signaling and evasion of host defenses, including granuloma formation (Parker et al., 2009). All mycobacterium genomes encode the cutinase family member Culp6, demonstrated to be vital for bacilli survival *in vitro* through transposon mutant construction (Sassetti et al., 2003). Parker et al. (2009) showed that Culp6, with thioesterase and phospholipase A (PLA) activity, hydrolyzes the phospholipid phosphatidylinositol mannoside (PIM). Since mycolic acid biosynthesis involves multiple ester and thioester bonds, these phospholipase and thioesterase activities align with its role in mycolic acid production. Furthermore, it has been observed that THL, an inhibitor of the human fatty acid synthase thioesterase (FASTE) domain similar to Rv3802c, reduces mycolic acid formation, leading to defects in the mycobacterial cell wall (Ravindran et al., 2014). However, the circumstantial evidence necessitates more definitive proof to confirm or refute Rv3802's involvement in mycolic acid biosynthesis.

Rv1075c may play a role in lipid and fatty acid metabolism, providing carbon and energy when Mtb resides within host cells (Yang et al., 2019). Located at the cell wall and cell membrane of Mtb, Rv1075c is the only GDSL lipase reported in Mtb. It features a "GDSL" motif at the N-terminus, exhibits regiospecific activities, and demonstrates multifunctional substrate specificity (Akoh et al., 2004). The GDSL family is characterized by an active Ser-Asp/Glu-His site, with the active serine residue located at the N-terminus (Brick et al., 1995; Upton and Buckley, 1995). TesA of *Pseudomonas aeruginosa* PAO1, another GDSL-like lipase/acylhydrolase, shares 24.16% identity with Rv1075c (Kovacic et al., 2013). The transcriptional expression of rv1075c is enhanced at a lower pH (4.5), mimicking the acidic phagosome environment of macrophages. A rv1075c transposon insertion mutant strain showed reduced Mtb infection in mice, and bacterial growth in human peripheral blood mononuclear cell-derived macrophages and THP-1 cells was also dramatically decreased. Further research into Rv1075c's role in lipid metabolism and Mtb's intracellular survival could provide insights into how the bacterium utilizes host lipids/esters during *in vivo* infection and identify potential vulnerabilities for drug discovery.

LipE (Rv3775) is potentially involved in TAG metabolism in Mtb and crucial for intracellular survival. Yang et al. (2019) demonstrated that *lipE* expression is induced under stress conditions mimicking the intracellular environment of Mtb. Deb et al. (2006) found that a 12-day hypoxic growth of Mtb led to triglycerol accumulation, subsequently resulting in *lipE* upregulation. *LipE* can hydrolyze medium-chain triglycerol glyceryl trioctanoate. Additionally, Mtb ΔLipE showed a reduced bacterial burden in THP-1 cells, macrophages derived from human peripheral blood mononuclear cells, and mice infected with Mtb.

3.2 Disruption of phagosome function

Degradation of invasive Mtb by macrophage phagosomes is a crucial defense mechanism against Mtb infection. By interfering with the normal process of phagosome maturation, inhibiting acidification, and preventing their fusion with lysosomes, Mtb primarily infects macrophages and creates a replicative niche within these cells (Koul et al., 2004; Härtlova et al., 2018). The role of lipolytic enzymes in disruption of phagosome function is degrading the phagosomal membrane and modifying its permeability.

Several Mtb proteins are involved in disrupting proper functioning of the phagosome. Recently, Rv3091 was shown to permit an avirulent bacterium (*M. smegmatis*) to escape from a phagosome (Cui et al., 2020). The extracellular PLA Rv3091, which belongs to the patatin-like family in mycobacterium, displays the distinctive hydrolase α/β fold. Patatin was first discovered in potato tubers (Vancanneyt et al., 1989). The patatin domain includes an active site with a Ser-Asp catalytic dyad and an oxyanion hole stabilizing the enzyme-substrate transition state (Rydel et al., 2003). The nucleophilic serine in the patatin domain is situated in a tight turn between an α-heli and a β-sheet in a well-conserved β-β-α-β core structure, which it shares with mammalian lipases in a conserved core module (Schneider et al., 2006). Overexpressing *rv3091* in the surrogate *M. smegmatis* improves its capacity to survive, and the extracellular activity of Rv3091 promoted Mtb escape from the phagosome from macrophage phagosomes. *In vivo* experiments on mice demonstrated that the Rv3091 is involved in the pathogenicity of mycobacterium. The bacterial burden and damage to the lungs of infected mice were dramatically increased by the recombinant *M. smegmatis* strain that overexpressed the *rv3091*. Therefore, The PLA activity of Rv3091 enhances mycobacteria's intracellular survival in macrophages, in addition to conferring phagosomal resistance. This protein also assisted the bacteria to use different lipids as the carbon source for their growth. Thus, Rv3091 protein could act as a potential target for the development of novel TB treatments (Cui et al., 2020).

Phospholipases C may also play the role of disrupting phagosome function by altering the permeability and degradation of the

TABLE 3 The role of cytokines in *M. tuberculosis*.

Cytokine	Receptor/signal	Roles	References
TNF-α	TNFR1, TNFR2 JNK, p38, NFκB	Positive: Essential for survival following Mtb infection. Initiation of innate cytokine and chemokine response and phagocyte activation. Negative: Mediator of tissue damage.	Wajant et al. (2003) and Domingo-Gonzalez et al. (2016)
IFN-γ	IFNGR1, IFNGR2 JAK/STAT	Positive: Essential for survival following Mtb infection. Expressed by antigen-speciic T cells. Coordinates and maintains mononuclear inflammation. Negative: Potentially pathogenic.	Schroder et al. (2004) and Domingo-Gonzalez et al. (2016)
IL-4	IL-4R	Positive: Enhance B cell proliferation, differentiation and isotransformation. Negative: Inhibit macrophage and IFN-γ function	Nelms et al. (1999)
IL-6	IL-6R, gp130 JAK, STAT3, MAPK	Positive: Potentiates early immunity-nonessential unless a high-dose infection.	Heinrich et al. (2003) and Domingo-Gonzalez et al. (2016)
IL-10	IL-10R, TLR, MyD88	Negative: Inhibit the activation of macrophages, neutrophil B cells, mast cells, eosinophils, the production of Th1 cytokine.	Redford et al. (2011)
IL-8	CXCR1 CXCR2	Positive: Expressed on neutrophils mediates accumulation.	Yoshimura, 2015 and Domingo-Gonzalez et al. (2016)
IL-12	12Rβ1, IL-12Rβ2 JAK2, TYK2, STAT4	Positive: Essential for survival following Mtb infection. Mediate early T-cell activation, polarization, and survival. Negative: Overexpression of IL-12 is toxic during Mtb infection.	Vignali and Kuchroo (2012) and Domingo-Gonzalez et al. (2016)

phagosomal membrane. However, Cavalier et al. (2020) showed that Mtb PLCs had no effect on virulence in the macrophages and mouse infection model, and that PLCs were not required for phagosomal rupture. The explanation is because mycobacterial phospholipases PLC are not released into the culture media, in contrast to other pathogenic bacteria (Marquis et al., 1995). Instead, they stay attached to the cell membrane. The location of these enzymes seems to contradict with their function in degradating phagosomal membrane. According to Wheeler and Ratledge (1992), this arrangement suggests that mycobacterial phospholipases have a non-aggressive role. This could eventually lead to the controlled release of fatty acids from the host, enabling intracellular mycobacteria to get nutrients without seriously harming the host. Chronic disease-causing mycobacterial agents might benefit from this characteristic.

3.3 Regulation of cytokine production

Mtb can fine-tune the innate immune response of the host to increase its virulence by generating inflammatory cytokines (Domingo-Gonzalez et al., 2016) (Figure 1). Table 3 summerizes some cytokines related to lipolytic enzymes, their receptors and respective roles in Mtb.

Rv2224c (Hip1, CaeA) is a serine hydrolase located at the cell surface that prevents dendritic cells from producing a number of pro-inflammatory cytokines such as IL-12, IL-6 and TNF-α (Madan-Lala et al., 2014). Rv2224c has the catalytic triad S228-D463-H490 that is found in esterases, lipases, and proteases (Ferre and Clote, 2005). Rv2224c, as an esterase/lipase, is preferentially hydrolyzing ester bonds of substrates with about 3 to 7 carbon atoms chain length (Lun and Bishai, 2007). It has been identified as a critical immunomodulatory protein that inhibits robust macrophage activation after Mtb infection. It regulates the initiation and intensity of pro-inflammatory responses (Rengarajan et al., 2008; Madan-Lala et al., 2011; Naffin-Olivos et al., 2014). The pathogen is expected to benefit from suppressing early pro-inflammatory responses because it will be able to evade immune identification (Vandal et al., 2009). Rv2224c and its orthologue from *M. smegmatis* are crucial for preserving the integrity of the cell envelope and conferring resilience to stressors. Furthermore, the GroEL2 protein, which is an immunomodulatory protein, is a substrate of Rv2224c (Naffin-Olivos et al., 2014). GroEL2 encodes a chaperone-like protein and is cleavaged to a monomeric form from a multimeric form. Even though GroEL2 remains uncleaved in the Rv2224c mutant strain, ectopic synthesis of cleaved GroEL2 monomers in this strain restores wild type levels of cytokine responses in infected macrophages. It is suggested that Rv2224c-dependent proteolysis substrate is a unique regulatory mechanism in Mtb as it enables the pathogen to quickly adapt to shifting immunological settings in the host during infection (Naffin-Olivos et al., 2014). The role of Rv2224c in the virulence of Mtb due to its role as esterase/lipase are needed to further study.

Rv0774c may contribute to mycobacterium's ability to avoid the extremely harsh environment in the macrophages by inhibiting host's protective response and remodeling the cell wall lipid. The expression of Rv0774c in *M. smegmatis* led to substantial upregulation of the TLR2 receptor (Toll-Like Receptor) and IL-10 cytokine production. However, the production of pro-inflammatory cytokines such IL-12, TNF-α, IFN-γ and MCP-1 were reduced (Kumar et al., 2017b). Rv0774c may be involved in the surface mycolation of trehalose monomycolate to create trehalose dimycolate via mycolylmannosylphosphorylheptaprenol. The function of Rv0774c involved in this cell wall lipid remodeling conferred altered morphology and increased streptomycin resistance. Overall, Rv0774c expression alters the survival of *M. smegmatis* in macrophages while also changing the associated immunological response (Kumar et al., 2017b). An effective therapeutic target for the treatment of tuberculosis may be the heightened anti-inflammatory response, which could be one of the causes of bacterial persistence inside macrophages. However, the interactions between various pathways in Mtb and *M. smegmatis* are different. Therefore, gene knock out and animal studies could be used to confirm the importance of Rv0774c in enhancing the virulence of Mtb in the future.

Many lipolytic enzymes have other functions related to the pathogenicity and virulence of Mtb in addition to those mentioned above. Phospholipase Rv2037c causes BALB/c mice, a kind of immunodeficient mouse widely used in the study of oncology, physiology, immunology, to produce pro-inflammatory cytokines such IL-8, IL-12 and TNF-α, thereby suggesting its role in immune-modulation. Rv0183 dramatically increased apoptosis and inflammatory markers like IL-6, NF-B, TLR2, TLR6, TNF-γ and MyD88 when it was ectopically expressed in murine macrophages (Xu et al., 2010). LipC (Rv0220), a cell surface esterase, has immunogenicity and can induce the production of proinflammatory cytokines and chemokines such as IL-8, IL-12, TNF-α and MCP-1 in macrophages and lung epithelial cells (Shen et al., 2012).

LipX regulates the secretion of macrophage IL-6 and ultimately contributes to the cell death of the macrophage (Deng et al., 2015; Singh et al., 2016; Rastogi et al., 2017). LipX (Rv1169c) is a member of the PE family, which is specific to pathogenic mycobacteria (including Mtb and *M. bovis*) but absent in non-pathogenic *M. smegmatis*. LipX is located in the cell wall of Mtb and is surface-exposed, which may play a role in the hydrolysis of host lipids (Cascioferro et al., 2007). Anti-LipX antibodies are observed in TB patients (Narayana et al., 2007) and human lung granulomas (Sampson, 2011). This suggests that LipX may be overexpressed during an active Mtb infection. Additionally, the expression levels of LipX increased under conditions of acidic stress, adaptation to stationary phase, starvation, and in hypoxic lipid-loaded macrophages (Schnappinger et al., 2003; Voskuil et al., 2004; Daniel et al., 2011). Mtb ΔLipX exhibited lower survival in activated THP-1 macrophages compared to the wild type Mtb strain (Rastogi et al., 2017). According to all of these investigations, LipX is crucial for mycobacterial pathogenicity.

4 Lipolytic enzymes as biomarkers, drug targets, and vaccine candidates

The rapid emergence of antibiotic resistance in bacteria calls for the development of new, powerful antibiotics for treating infections. Lipolytic enzymes are now emerging as potential targets for new generation of treatments. This is due to their crucial roles in virulence and survival, particularly in the case of Mtb (Kim and Shin, 2023). Lipolytic enzymes play a significant role in hydrolyzing host lipids into fatty acids that provides energy to the bacilli, serves as a major nutrition source during dormancy and reactivation phases, as well as serves as the only source of precursors for the synthesis of the cell wall. In addition to their potential as therapeutic agents, lipolytic enzymes of Mtb could also serve as biomarkers in the serodiagnosis of active tuberculosis (Brust et al., 2011).

According to a research by Low et al., TAGs are extensively accumulated and degraded in bacilli as they enter and exit hypoxia-induced dormancy, respectively (Low et al., 2009). Additionally, these actions are accompanied by the dynamic emergence and disappearance of TAG lipid particles inside cells (Dhouib et al., 2011). Regrowing bacilli exhibit a notable correlation between reduced TAG levels and elevated cellular TAG lipase activity, suggesting that TAG usage plays a crucial role in mycobacteria's ability to proliferate again after emerging from the non-replicating stage (Low et al., 2009). As a result, lipolytic enzymes like LipY, which are primarily generated and produced during reactivation conditions and are not expressed under normal growth settings, may serve as helpful biomarkers to identify reactivated forms of tuberculosis (Mishra et al., 2008). Lipolytic enzymes Rv0183, Rv1984c and Rv3452 cause TB patients to have strong humoral reactions. In particular, Rv3452 showed excellent serodiagnostic qualities in both populations, indicating that this marker will be highly valuable for diagnostic purposes in the future.

Inhibitors that target lipolytic enzymes appear to be potential therapeutics against Mtb. Tetrahydrolipstatin is a pancreatic lipase inhibitor pharmacophore that was used to develop chemical inhibitors against cell wall lipase Rv3802c. These inhibitors exhibited antibacterial activity *in vitro*, indicating that Rv3802c may be a promising therapeutic target for Mtb (West et al., 2011). Kumari et al. (2020) indicated that Rv2037c is a phospholipase that can be potentially used for the creation of innovative drugs for TB treatment (Kumari et al., 2020). Rv0183 is a monoacylglycerol lipase (MGL) that has potential as a druggable target. Thiadiazole carbamate compound lalistat is a particular inhibitor of human lysosomal acid lipase. It has been shown to hinder the *in vitro* development of Mtb. Lipolytic enzymes such as LipI, G, M, N, and O were found to be the targets of lalistat (Rameshwaram et al., 2018). LipX is speculated to act on the ultimate hydrolyzed product of TAG to release free fatty acids that serve as the building blocks for maintaining and modifying the cell wall of Mtb in a hostile environment (Singh et al., 2016). LipX has been determined as essential for mycobacterial pathogenicity and it enhances resistance to various environmental stresses that bacteria experience in the phagosome (Singh et al., 2016). Therefore, LipX function-inhibiting small molecules may be clinically valuable in controlling Mtb infections.

Culp1(Rv1984c), Culp2 (Rv2301) and Culp6 (Rv3802c) are thought to be novel targets for the TB vaccine since they induce IFN-γproduction (Shanahan et al., 2010). Compared to Culp1and Culp6 alone, Culp1-6 fusion protein showed an higher level of protection against infection (Shanahan et al., 2010). The localization of Rv3097c has the potential for creating recombinant mycobacteria expressing heterologous antigens on their surface in order to product vaccine (Cascioferro et al., 2007). Other surface lipolytic enzymes mentioned in this review have the potential as vaccines.

5 Discussion

One of the main factors contributing to Mtb pathogenesis is capability of this bacteria to elude immune destruction and endure in macrophages, where it eventually results in chronic infection. However, the pathogenesis mechanism of Mtb is still unknown, making TB diagnosis and treatment difficult. Understanding the molecular mechanism of pathogenicity, virulence, and persistence has advanced significantly in recent years. The discovery of crucial proteins responsible for mycobacterial pathogenicity has been one important contribution. The majority of these virulence proteins are involved in lipid metabolism and signal transduction pathways (Forrellad et al., 2013).

Mtb depends on lipids for growth and virulence expression. Foamy macrophages gather lipids in granulomas during Mtb infection, giving Mtb metabolic adaption and survival strategies against various challenges. Antibiotics that target the bacterial cell wall or transcription may become less effective against drug-tolerant Mtb due to the involvement of host-derived lipid molecules, such as cholesterol and triacylglycerol. The metabolism of lipids is significantly influenced by lipolytic enzymes.

In this review, We have classified lipolytic enzymes in to four kinds: lipases, esterases,cutinases and phospholipases, but still there are other kinds, such as β-lactamases enzymes, PE/PEE family and HSL family. β-lactamases (EC 3.5.2.6), with the consensus sequence S-X(T)-X(S)-K, are responsible for their resistance to β-lactam antibiotics such as cephalosporin, penicillin, carbapenem(ertapenem) and cephamycin. A four-atom ring known as a beta-lactam ring unites the molecular structures of these antibiotics. The ring is broken by the lactamase enzyme, rendering antibacterial effects of β-lactam antibiotics inactive (Hugonnet et al., 2009). LipD, LipE, LipL, and LipP exhibit β-lactamases activity (Supplementary Table S1).

The existence of two multigene families that combined account for about 10% of the chromosomal coding potential is one of the Mtb genome's most intriguing features. The conserved N-terminal regions of these two protein families, known as PE and PPE, are approximately 100 and 180 amino acids long, respectively (Cole et al., 1998). While PPE stands for the Pro-Pro-Glu motif, which is often found in the first 10 amino acids of these proteins, PE is named after the characteristic motif Pro-Glu. Of these two families, PE is the biggest. PE-PGRS with a C-terminal PGRS (Polymorphic GC-rich Repetitive Sequences) domain is the the largest subfamily of PE. LipX, also known as PE11, belongs to the PE family. LipY belongs to the PE-PGRS family. It has been shown that PE and PPE increase bacterial survival and alter human immunity, cell death, and metabolism (Yan et al., 2020).

Lipolytic enzymes are extremely flexible weapons that mycobacteria can use. There is growing evidence for their functions in (i) lipid and fatty acid metabolism, (ii) disruption of phagosome function, and (iii) regulation of cytokine production. Actually, we also can discover that many lipolytic enzymes have more than one roles in the virulence and pathogenicity of Mtb. PLC performs a number of virulence-related roles. First, PLC may release fatty acids from host phospholipids, which could supply the bacteria with nutrients. Second, PLC has the potential to completely destroy the phagosomal membrane or alter its permeability. Lastly, PLC may alter the host immune responses by interfering with signal transduction processes in infected cells through the activation of the arachidonic acid cascade. In addition to lipid metabolism, many lipolytic enzymes can induce immune responses from the host but the detailed mechanism still needs to be explored.

This review has taken into account bioinformatics prediction data, however it primarily focuses on lipolytic enzymes whose particular roles in virulence have been established. There are still a large number of hypothesized lipolytic enzyme genes that need to be studied for physiological properties and functions (Supplementary Table S2). There are other functions of this enzymes that we have not introduce certainly because of the less resports, such as LipX, It has been reported to induce necrosis in the host with unknown mechanism (Deng et al., 2015; Rastogi et al., 2017).

In conclusion, Despite lipolytic enzymes have important roles in in the virulence and pathogenicity of Mtb, there are only a limited number of accessible in-depth studies. Current research has largely focused on a small number of well-established lipolytic enzyme family members, such as Lip family. Through more research in the future, a deeper understanding of the functions of these fascinating mycobacterial lipolytic enzymes might be achieved. More studies on how they relate to Mtb-host interaction, Mtb survival, and Mtb pathogenesis should also be undertaken. Given the reported roles of certain well-known lipolytic enzymes so far, we predict the discovery of more effector lipolytic enzymes in the future.

Author contributions

HL: Writing – original draft. JX: Writing – review & editing. HW: Writing – review & editing. SW: Writing – review & editing. RF: Writing – review & editing. XL: Writing – review & editing. ZL: Writing – review & editing. NS: Writing – review & editing.

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Conflict of interest

ZL was employed by the SAFE Pharmaceutical Technology Co. Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2024.1329715/full#supplementary-material

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Microbiota changes: the unseen players in cervical cancer progression

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Cervical cancer ranks among the most prevalent cancers globally with high-risk human papillomaviruses implicated in nearly 99% of cases. However, hidden players such as changes in the microbiota are now being examined as potential markers in the progression of this disease. Researchers suggest that changes in the vaginal microbiota might correlate with cervical cancer. This review provides a comprehensive look at the microbiota changes linked with the advancement of cervical cancer. It also scrutinizes the databases from past studies on the microbiota during healthy and cancerous stages, drawing connections between prior findings concerning the role of the microbiota in the progression of cervical cancer. Preliminary findings identify *Fusobacterium* spp., *Peptostreptococcus* spp., *Campylobacter* spp., and *Haemophilus* spp., as potential biomarkers for cervical cancer progression. *Alloscardovia* spp., *Eubacterium* spp., and *Mycoplasma* spp. were identified as potential biomarkers for HPVs (+), while *Methylobacterium* spp. may be indicative of HPV (–). However, the study's limitations, including potential biases and methodological constraints, underscore the need for further research to validate these findings and delve deeper into the microbiota's role in HPV development. Despite these limitations, the review provides valuable insights into microbiota trends during cervical cancer progression, offering direction for future research. The review summarizes key findings from previous studies on microbiota during healthy and cancerous stages, as well as other conditions such as CIN, SIL, HPV (+), and HPV (–), indicating a promising area for further investigation. The consistent presence of HPV across all reported cervical abnormalities, along with the identification of distinct bacterial genera between cancerous and control samples, suggests a potential link that merits further exploration. In conclusion, a more profound understanding of the microbial landscape could elucidate the pathogenesis of cervical diseases and inform future strategies for diagnosis, prevention, and treatment.

KEYWORDS

microbiota changes, cervical cancer, progression, biomarkers, vaginal microbiota

1 Introduction

Cervical cancer (CAN) is a significant health concern for women worldwide, ranking as one of the most common cancers (Arbyn et al., 2020; WHO, 2020; Aobchey et al., 2022; Wang et al., 2022). As per the World Health Organization (WHO), in 2018, approximately 570,000 women were diagnosed with cervical cancer globally, resulting in roughly 311,000 fatalities

(WHO, 2020; Wickramasinghe et al., 2021; Zhang et al., 2022). Furthermore, the WHO projected in 2020 that the annual incidence of new cases of this disease could rise from 570,000 to 700,000 between 2018 and 2030 (WHO, 2020).

The prevalence of this disease is notably higher in low-to middle-income countries, nonetheless, it affects women globally. The number of deaths in low and middle-income countries accounted for an estimated 90% of the 311,000 global fatalities. Consequently, age-standardized incidence rates fluctuate from 75 per 100,000 women in the highest-risk countries to fewer than 10 per 100,000 women in the lowest-risk countries (Bray et al., 2018; WHO, 2020). This disparity underscores the urgency to address cervical cancer, particularly in regions with higher risk factors.

Human papillomavirus (HPV) infection is pivotal in the development of cervical cancer, with nearly 99% of cases associated with high-risk HPV strains (WHO, 2020; Jiang and Wang, 2022). However, other contributing factors such as tobacco use, immunosuppression, malnutrition, and low socioeconomic status are also implicated (ACCP, 2004; Ghebre et al., 2017; Zhang et al., 2020). Persistent infection with various types of HPV is acknowledged as a contributing factor in the progression of cervical intraepithelial neoplasia (CIN) and invasive cervical cancer (ICC). Nevertheless, the complete involvement of HPV in the entire tumorigenic process remains a topic of ongoing debate due to insufficient data (Muñoz, 2000; Castellsagué, 2008; Wheeler, 2013; Kori and Arga, 2018; So et al., 2020; Kang et al., 2021).

Recent literature posits an intriguing hypothesis: microorganisms may play a significant role in malignancies. This theory suggests that there could be unexplored mechanisms during infections where these microscopic entities take a leading role (Parkin, 2006; Godoy-Vitorino et al., 2018).

This perspective necessitates a broader understanding of the microbial world and its potential influence on disease processes. The interplay between HPV and other microorganisms could add another layer of complexity to the etiology of cervical cancer, warranting further investigation. This new viewpoint not only challenges our current knowledge but also paves the way for innovative research directions in cervical cancer pathogenesis.

The detection of microbial diversity, first accomplished in 1677 by Van Leeuwenhoek through microscopic observation, has evolved significantly over time (Wei et al., 2021). In cervical cancer diagnostics, the Papanicolaou smear, a microscopic biopsy image analysis, has traditionally been the primary modality (Long et al., 2017; Kori and Arga, 2018). However, its reliability is debatable due to its dependence on human interpretation (Long et al., 2017). Despite several alternate cervical cancer screening methods proposed over the years such as cytological testing alone, standalone hrHPV testing, and cytological + hrHPV combination testing (co-testing) (Curry et al., 2018; Kim et al., 2018; Terasawa et al., 2022), the 5 years survival rate remains a dismal 66% (Long et al., 2017; Basic et al., 2021; Qu et al., 2021; Hou et al., 2022).

Treatment strategies for cervical cancer, such as surgical resection, radiotherapy, and chemotherapy, are frequently challenged by tumor metastasis and recurrence, complicating disease management (Mallmann and Mallmann, 2016; Vordermark, 2016; Koh et al., 2019; Li et al., 2021). Further, patients often suffer from side effects related to these treatments. This highlights a significant problem: the urgent need for novel, reliable diagnostic methods for cervical cancer that can improve early detection and thereby enhance survival rates (Zhu et al., 2016; Long et al., 2017; Liu et al., 2018; Koh et al., 2019; Han et al., 2021).

The exploration of microbial diversity has been significantly enhanced by advancements in culture technologies. However, due to inherent challenges associated with laboratory culturing procedures, our understanding is not yet exhaustive (Wei et al., 2021). In response to this, techniques centered around molecular sample analysis have emerged within the field of omics, paving the way for a more detailed investigation of microbial diversity (Wei et al., 2021).

The advent of laboratory automation has facilitated the deployment of high-throughput-omics technologies. These sophisticated methodologies enable an in-depth characterization of samples collected from both patients and healthy individuals, thereby expanding our knowledge of microbial ecosystems. One such transformative innovation is next-generation sequencing (NGS). This technique has unlocked the potential to delineate the intricate complexity of microbial communities and human microbiota, providing valuable insights into the influence of the microbiome on human health and disease pathologies (Peterson et al., 2009).

Among the various omic approaches, metagenomics stands out for its ability to divulge specific information about the genomes and genes within a microbial community. It serves as an essential first step in microbiome studies (Marchesi and Ravel, 2015; Aguiar-Pulido et al., 2016). The primary goal of metagenomics is to determine the taxonomic profile of a microbial community, typically involving NGS post-DNA extraction from samples, followed by assembly or mapping to a reference database, and subsequent annotation (Marchesi and Ravel, 2015; Aguiar-Pulido et al., 2016). This method has become particularly prevalent in investigating the microbial composition within the vaginal environment.

The female genital tract serves as a critical ecological niche for human microbiota (Gao et al., 2013), housing *Lactobacillus* species that contribute to metabolic processes, immunological responses, and overall gynecological health (Kang et al., 2021). Known for probiotic benefits, *Lactobacillus* species help combat vaginal dysbiosis (Machado et al., 2022; Pacha-Herrera et al., 2022; Rodríguez-Arias et al., 2022). Detailed insights follow in this review's upcoming sections. There is an emerging body of literature suggesting that alterations in the vaginal microbiota may be linked to cervical cancer (Klein et al., 2020b; Norenhag et al., 2020; So et al., 2020; Tango et al., 2020; Kang et al., 2021; Sims et al., 2021; Wu et al., 2021; Zhou et al., 2021). Furthermore, numerous studies propose that the vaginal microbiota could play a crucial role in defending women against infections such as HPV, vulvovaginal candidiasis, and other sexually transmitted diseases (Liu et al., 2013; Lewis et al., 2017; Arroyo Mühr et al., 2021; Kang et al., 2021). As such, the cervical microbiota could potentially serve as a biomarker for assessing the risk of cancer progression (Mitra et al., 2016a,b; Curty et al., 2019; Arroyo Mühr et al., 2021).

The exploration of the human microbiome has been an exciting journey, with techniques evolving from 16S sequencing (Audirac-Chalifour et al., 2016; Dareng et al., 2016; di Paola et al., 2017; Klein et al., 2020b; Norenhag et al., 2020; So et al., 2020; Tango et al., 2020; Sims et al., 2021; Wu et al., 2021; Zhou et al., 2021), PCR (Norenhag et al., 2020), and microarray (Borgdorff et al., 2014; Norenhag et al., 2020) to cutting-edge methods like RNA-seq (Kori and Arga, 2018; Klein et al., 2020a; Chang et al., 2021) and Whole Genome Shotgun (WGS) (Klein et al., 2020a; Wei et al., 2021). A significant milestone

in this journey was the commencement of the Human Microbiome Project (HMP) in 2008, which aimed to map the microbial landscape across various body parts, including the lower genital tract of healthy individuals (Castanheira et al., 2021). From this wealth of research, a startling revelation has emerged: approximately 20% of all fatal cancers are microbially induced (Godoy-Vitorino et al., 2018). Moreover, numerous studies have drawn significant correlations between alterations in the microbiome and cancer phenotypes (Elinav et al., 2019; Poore et al., 2020; Banavar et al., 2021). This underlines the potential of the microbiota as a treasure trove of biomarkers that could revolutionize clinical diagnostics and disease management.

This review is an ambitious endeavor to chart the intricate relationship between the microbiota and cervical cancer progression. We delve into the diverse universe of microorganisms implicated in cervicovaginal dysbiosis, providing an authoritative synthesis of prior research on both CONTROL (healthy) samples and CAN stage. Our goal is to offer an updated perspective on the role of microbiota in cervical cancer progression, thereby filling a crucial gap in the existing literature.

While our analysis provides a comprehensive overview based on the data available at the time of our research, it's crucial to recognize the fluidity and rapid evolution of scientific knowledge. As such, newer developments may not have been captured. This underscores the need for ongoing research in this field. Therefore, we strongly advocate for broader studies using metagenomics and metatranscriptomics, as these techniques hold immense promise in untangling the intricate role of microbiota in cervical cancer progression. By deepening our understanding of this critical issue, we can pave the way for innovative therapeutic interventions, heralding a new era in women's health management.

2 Cervical cancer and HPV

According to the World Health Organization (WHO, 2020), the principal instigator of cervical pre-cancer and squamous cervical cancer is the asymptomatic, persistent or chronic infection with one or more high-risk HPV types. While over 100 HPV types have been identified, only a fraction are associated with cervical cancer. Indeed, two specific types, HPV 16 and 18, are implicated in approximately 70% of all reported cervical cancer cases (Pappa et al., 2018; Cohen et al., 2019; Lin et al., 2019; WHO, 2020). Other high-risk HPV types, such as 31, 33, 45, and 58, are less frequently linked to cervical cancer, with prevalence varying by geographic location. Additionally, low-risk HPV types 6 and 11, although not contributing to cervical cancer, are responsible for most genital warts or condylomas (WHO, 2014).

The role of genetic variation in cervical cancer has been underscored by genome-wide association studies (GWAS). Lin et al. (2019), reported that cervical cancer harbors genetic variations across multiple susceptibility loci (Bahrami et al., 2018; Lin et al., 2019). The viral oncoproteins E6 and E7 appear to play a pivotal role in HPV-infected cervical cancers. Integration of the viral genome into the host DNA results in the upregulation of E6 and E7, leading to the deregulation of key proteins within cellular signaling pathways, including the inhibition of two vital tumor suppressor proteins, p53 and pRb (Oyervides-Muñoz et al., 2018; Lin et al., 2019). The combined effect of E6 and E7 viral proteins triggers the process of immortalization in HPV-infected cells. This precedes the malignant metamorphosis of these cells (Da Silva et al., 2021).

Furthermore, Lau et al. (2015) revealed that DNA tumor virus oncogenes, including E7, can bind to and suppress the cGAS-STING DNA-sensing pathway (Lau et al., 2015; Lin et al., 2019). However, it's worth noting that not all integrations necessarily rely on the expression of the E6 and E7 oncogenes (Groves and Coleman, 2015; Lin et al., 2019). In addition to these findings, several reports have identified driver mutations in cervical cancer, such as PIK3CA (phosphatidylinositide 3-kinases catalytic subunit α), a central protein in the PI3K pathway, KRAS (Kirsten rat sarcoma viral oncogene homolog), and EGFR (epidermal growth factor receptor) (Lin et al., 2019).

3 Vaginal microbiota

The vaginal microbiota is a critical component of women's health (Wu et al., 2021). This complex ecosystem, which operates in harmony with the host, provides protective mechanisms against dysbiosis and infection (Klein et al., 2020a). The function of the vaginal mucosa as a barrier against pathogens is facilitated by the interaction of epithelial cells, the immune system, and various microorganisms (Borgdorff et al., 2016; Taddei et al., 2018; Castanheira et al., 2021).

Dominating this ecosystem are *Lactobacillus* species, which play a significant role in maintaining vaginal health. By producing lactic acid, these bacteria sustain a low pH environment in the cervicovaginal setting, thereby preventing the colonization of harmful opportunistic pathogens, preserving the cervical epithelial barrier, and impeding mucin degradation (Amabebe and Anumba, 2018; Klein et al., 2020a; Norenhag et al., 2020; Salinas et al., 2020; So et al., 2020; Kang et al., 2021; Wu et al., 2021).

However, the composition of the vaginal microbiota is not static. It can be influenced by numerous factors such as genetics, diet, lifestyle, hygiene practices, ethnicity, reproductive age, infections, male factor, usage of antibiotics and contraceptives, sexual activity, physiological status, pregnancy and estrogen levels (Mitra et al., 2016b; Kwasniewski et al., 2018; Wu et al., 2021; Zhou et al., 2021; Baud et al., 2023).

Thanks to new molecular techniques, over 50 microbial species have been identified within the vaginal microbiota, with *Lactobacillus* spp. being the most prevalent (Norenhag et al., 2020; Wu et al., 2021). Among them, *L. crispatus*, *L. gasseri*, *L. inners*, and *L. jensenii* are the most commonly found (Wu et al., 2021).

Further research about the vaginal microbiota in healthy women from different ethnic groups (White, Black, Hispanic and Asian) by Ravel et al. (2011) led to the classification of vaginal bacterial communities into five distinct "community state types" (CST). In this classification, *Lactobacillus* species dominated groups I, II, III, and V. Group IV, on the other hand, was characterized by a diverse set of anaerobic bacteria, including bacteria like *Prevotella* spp., *Streptococcus* spp., *Dialister* spp., *Fannyhessea* spp. (previously known as *Atopobium*), *Gardnerella* spp., *Megasphaera* spp., *Peptoniphilus* spp., *Sneathia* spp., *Eggerthella* spp., *Aerococcus* spp., *Finegoldia* spp., and *Mobiluncus* spp. These findings were consistent with previous research employing 16S rRNA genes (Srinivasan and Fredricks, 2008; Zhou et al., 2010).

Despite the diversity in bacterial species, a commonality across all CST groups was the presence of lactic acid-producing bacteria, suggesting a conserved function throughout these communities.

4 Vaginal microbiota and cervical cancer

The human body is a dynamic ecosystem for a myriad of microbes, collectively known as the microbiome. This microbiome plays a pivotal role in maintaining normal bodily functions, including immune modulation and overall protection (Wei et al., 2021; Zhou et al., 2021). Over time, evidence has emerged highlighting the connection between the microbiome, inflammation, and the development and progression of cancer. According to Zhou et al. (2021) and Wei et al. (2021), disturbances in microbial homeostasis can trigger a cascade of immune responses. Chronic inflammation, a byproduct of such disruptions, is a known carcinogenic factor, heightening the host's susceptibility to cancer (Zhou et al., 2021).

Given the profound implications of the microbiome on health and disease, specifically cervical cancer, advanced research is warranted. The advent of laboratory automation and high-throughput technologies has revolutionized our understanding of microbiome diversity and its potential impacts (Wei et al., 2021). There is mounting scientific evidence pointing towards a correlation between microbiota and cervical cancer (Castanheira et al., 2021; Kang et al., 2021; Wei et al., 2021; Wu et al., 2021; Zhou et al., 2021).

Cervicitis, or inflammation of the cervix, can stem from various conditions, including microbial infections. Chronic cervicitis has been linked to the development of cervical cancer. Pelvic inflammatory disease (PID) in women is typically triggered by ascending bacterial infections from the cervix to the uterus and fallopian tubes. Bacterial vaginosis (BV), a condition characterized by dysbiosis of cervicovaginal bacteria, is also associated with cervicitis. Notably, the microenvironment fostered by BV is reported to facilitate persistent HPV infection, a known precursor to cervical cancer (Castanheira et al., 2021; Zhou et al., 2021).

Various microorganisms, including *Fusobacterium* spp., *Mycoplasma genitalium*, *Chlamydia trachomatis*, *Sneathia* spp., *Anaerococcus* spp., *Peptostreptococcus* spp., *Gardnerella* spp., *Prevotella* spp., *Fannyhessea* spp., *Streptococcus* spp., *Dialister* spp., *Megasphaera* spp., *Peptoniphilus* spp., *Finegoldia* spp., *Mobiluncus* spp. and *Lactobacillus iners* have been implicated in the onset of cervical cancer. Interestingly, *L. iners* is found more frequently in infected women compared to their healthy counterparts. Table 1 provides a detailed overview of these microorganisms and their association with cervical cancer.

As illustrated in Table 1, microorganisms such as *Fusobacterium* spp., *Sneathia* spp., *Anaerococcus* spp., *Peptostreptococcus* spp., *Gardnerella* spp., *Prevotella* spp., *Dialister* spp., and *Megasphaera* spp. have been identified as biological markers for cervical cancer (CAN), high-grade squamous intraepithelial lesions (HSIL), and cervical intraepithelial neoplasia (CIN). Conversely, *Lactobacillus crispatus*, *Lactobacillus gasseri*, and *Lactobacillus jensenii* are associated with a decreased risk of infections, including HPV, CIN, and CAN. For an organized overview of this information, please refer to Table 2. This table presents a clear view of the microorganisms found at different disease stages in the vagina. These stages include squamous intraepithelial lesions (SIL)—further divided into low-grade (LSIL) and high-grade (HSIL), cervical intraepithelial neoplasia (CIN), invasive cervical cancer (ICC) or cervical cancer (CAN), and other infections such as pelvic inflammatory disease (PID), HPV, and bacterial vaginitis (BV). Additionally, a column has been included to indicate the microorganisms found in healthy controls (HC). Figure 1 provides a visual representation of the microorganisms present during vaginal inflammation, offering insight into the microbial landscape under these conditions.

5 Microbial markers and cervical cancer

Advancements in microbiome research have unveiled new avenues for understanding the root causes of various diseases, including cancer. With the advent of high-throughput technologies such as genomics, transcriptomics, metagenomics, and metatranscriptomics, researchers can now generate an enormous amount of data (Wei et al., 2021). When it comes to cervical cancer, this vast repository of data is meticulously scrutinized to identify potential biomarkers that could transform its diagnosis and prognosis (Norenhag et al., 2020; Han et al., 2021; Kang et al., 2021; Wei et al., 2021; Zhou et al., 2021).

Researchers propose that certain microorganisms serve as beneficial diagnostic markers for cervical cancer or as indicators of infection severity. From the host's perspective, diverse types of biomarkers (prognostic, predictive, and diagnostic) are being explored to enhance the management of cervical cancer.

High-throughput technologies have paved the way for a deeper exploration of the complex relationship between microbiota and cancer. The capacity to examine the entire microbiome and its intricate micro-ecosystems has led to the identification of specific microbial entities as predictive markers of cancer (Wei et al., 2021). At present, research is primarily centered around four main areas: characterizing microbial diversity and composition, conducting microbial functional analyses, predicting biomarkers, and investigating potential therapeutic applications. However, these areas are still nascent and need to be solidified in clinical practice (Wei et al., 2021).

To fully unravel the correlation between the microbiome and cancer, the consistent use of high-throughput methodologies is deemed necessary. Various studies conducted on the microbiota associated with cervical cancer or cervical disease have reiterated the urgent need for reliable biomarkers to improve the diagnosis of cervical cancer or prevent it. There is a pressing need to devise novel diagnostic strategies incorporating microbiological markers for early detection of cervical cancer in patients (Kang et al., 2021; Sims et al., 2021; Wei et al., 2021; Wu et al., 2021; Zhou et al., 2021).

Several microorganisms, including *Fusobacterium* spp., *Sneathia* spp. (*S. amnii*), *Anaerococcus* spp., *Peptostreptococcus* spp., *Gardnerella* spp. (*G. vaginalis*), *Prevotella* spp., *Dialister* spp., *Fannyhessea* spp. (*F. vaginae*), *Streptococcus* spp., *Megasphaera* spp., *L. crispatus*, and *L. gasseri* (Kori and Arga, 2018; Klein et al., 2020b; Tango et al., 2020), have been suggested as microbiological markers for cervical cancer. The profound implications of these findings for the future of cancer diagnostics underscore the importance of continued research in this promising field.

6 Data exploration

In an effort to gain deeper insights from the literature, a rigorous process of search was carried out. This process targeted papers that

TABLE 1 Association between microorganisms and cervical cancer.

Microorganisms	Relationship with cervical cancer	References
Fusobacterium spp.	- *Fusobacterium* spp., inclusive of *Sneathia* spp., is implicated in creating an immunosuppressive microenvironment characterized by anti-inflammatory cytokines. - It plays a significant role in the development of cervical cancer.	Audirac-Chalifour et al. (2016) and Zhou et al. (2021)
	- Identified as a microbial biomarker for HPV infection.	Audirac-Chalifour et al. (2016) and Zhou et al. (2021)
	- *Sneathia* spp., from the *Fusobacterium* genus, has associations with HPV, cervical intraepithelial neoplasia (CIN), and cervical cancer (CAN). - Produces FadA, a virulence factor disrupting the cervical cancer signaling pathway. - Overexpression of FadA gene is observed in CAN patients.	Audirac-Chalifour et al. (2016), Mitra et al. (2016b) and Wu et al. (2021)
	- Increased presence of *Fusobacterium* spp., may lead to local immunosuppression, promoting HPV immune evasion and disease progression.	Mitra et al. (2016b)
	- Distinctly higher levels of *Fusobacterium* spp. in the CAN group. - Identified as a marker for both CAN and high-grade squamous intraepithelial lesions (HSIL) groups. - May contribute to CAN pathogenesis through chronic inflammation, antiapoptotic activity, or production of carcinogenic substances.	Norenhag et al. (2020), Sims et al. (2021) and Wu et al. (2021)
	- The presence of *F. necrophorum* is specifically reported in CAN cases	Audirac-Chalifour et al. (2016), So et al. (2020) and Castanheira et al. (2021)
Mycoplasma genitalium	- *M. genitalium* is an independent pathogenic microorganism causing a series of intracellular infections. - It disrupts tight junctions from epithelial cells, which can lead to bacterial vaginosis (BV) and cervicitis. - It increases the incidence of cervical lesions. - Some studies suggest that *M. genitalium* can induce chromosomal damage in cells, potentially leading to the formation of cancerous cells. - Both *M. genitalium* and *M. hominis* are common mycoplasmas found in female tract infections. - These mycoplasmas have been identified in patients with cervicitis and BV. - There is a documented association between *M. genitalium* and BV.	Klein et al. (2020a) and Zhou et al. (2021)
Chlamydia trachomatis	- *C. trachomatis* has been identified as a co-factor for cervical cancer (CAN) development in epidemiologic studies.	Audirac-Chalifour et al. (2016)
	- This microorganism can damage the cervical mucosal barrier, facilitating high-risk HPV (hr-HPV) infection of the cervical epithelium. - It may induce chronic inflammation and influence local and cellular immunity of the cervix, inhibiting HPV clearance. - The persistence of HPV infection, facilitated by *C. trachomatis*, could lead to cervical cancer. - Some research suggests that non-bacterial components might affect CAN progression, but there is a lack of extensive studies on this topic.	Zhou et al. (2021)
	- It has been associated with cervicitis, the persistence of HPV infection, and BV. - Meta-analyses have reported a positive correlation between cervical HPV infection and BV. - HPV is considered a primary factor responsible for CAN development.	Kwasniewski et al. (2018) and Klein et al. (2020a)
Sneathia spp.	- *Sneathia* spp. is a potential microbiological marker of HPV infection.	Mitra et al. (2016b) and Zhou et al. (2021)
	- It has a significant association with Bacterial Vaginosis (BV) infection.	Mitra et al. (2016b)
	- Reports indicate its presence in cervical intraepithelial neoplasia (CIN) stages 1, 2, and 3.	So et al. (2020)
	- Identified as a marker genus of high-grade squamous intraepithelial lesions (HSIL) group.	Wu et al. (2021)

(Continued)

TABLE 1 (Continued)

Microorganisms	Relationship with cervical cancer	References
	- *S. sanguinegens* is associated with high-grade CIN, whereas *S. amnii* (previously named *Leptotrichia amnionii*) has been linked with cervical cancer, but not HPV infection or CIN.	Mitra et al. (2016b)
	- A documented correlation exists between colonization with *S. amnii* and cervical cancer in HPV-positive subjects. - *S. amnii* has also been reported as a reliable predictor of BV.	Audirac-Chalifour et al. (2016)
Gardnerella spp.	- *Gardnerella* spp., particularly *G. vaginalis*, is proposed as a molecular marker due to its role in biofilm formation, which may contribute to the persistence of HPV infection. - *Gardnerella* spp. and *Streptococcus* spp. may serve as biomarkers to potentially distinguish invasive cervical cancer (ICC) from cervical intraepithelial neoplasia (CIN), indicating possible disease progression. - *Gardnerella* spp. has been associated with bacterial vaginosis (BV) and HPV infection. - It is a representative genus in the CIN group and is reported as a biomarker to differentiate patients with CIN from healthy individuals. - The presence of *Gardnerella* spp. has been reported in patients from high-grade squamous intraepithelial lesions (HSIL + HPV +) and Low-grade squamous intraepithelial lesions (LSIL) groups. - An enrichment of anaerobic bacteria like *G. vaginalis* has been described in women with CIN and cervical cancer (CAN). This bacterium, a gram-variable facultative anaerobe, becomes more abundant during BV. - *G. vaginalis* has been significantly associated with the risk for HSIL, high-grade CIN, and CAN. It was identified as a high risk for developing CIN2, CIN3, and CAN. - High levels of *Gardnerella* spp. are common among women persistently infected with hr-HPV for 1 year. Specifically, *G. vaginalis* has been associated with CIN. - Another study reported a higher prevalence of *G. vaginalis* in HPV-negative women with non-cervical lesions (NCL), but its presence decreased across the HPV-positive, SIL, and CAN groups.	Audirac-Chalifour et al. (2016), Mitra et al. (2016b), Kwasniewski et al. (2018), Klein et al. (2020a), Norenhag et al. (2020), Castanheira et al. (2021), Kang et al. (2021) and Zhou et al. (2021)
Dialister spp.	- *Dialister* spp., along with *Prevotella* spp., has been reported as marker genera of the cervical cancer (CAN) group. These are opportunistic pathogens whose activities are influenced by or influence *Lactobacillus* spp. - *D. invisus*, a Gram-negative coccobacillus, has been linked to new HPV-type infections within a year in women with typical cytological results. - Notably, *D. invisus* has been significantly associated with high-grade squamous intraepithelial neoplasia and an increased risk of CAN	So et al. (2020) and Wu et al. (2021)
Eggerthella spp.	- *Eggerthella* spp. has been mentioned only once in relation to cervical cancer. - There is no available information that directly associates or disassociates it with cervical cancer. - It is included in the IV CST (community state type), a classification system for vaginal microbial communities.	Ravel et al. (2011)
Prevotella spp.	- The abundance of *Prevotella* spp. is associated with HPV persistence and is inversely related to the quantity of *Lactobacillus*. This bacterium may cause infections like bacterial vaginosis (BV) and has been linked with HPV persistence. Notably, *P. bivia*, *P. amnii*, and *P. timonensis* have been reported in HPV positive samples. - *Prevotella* spp. and *Lactobacillus* spp. reportedly play an antagonistic role in the progression of squamous intraepithelial lesions (SIL) and cervical cancer (CAN) through NLRs signaling and other pathways. They are reported as marker genera of the CAN group. It's speculated that these bacteria might drive chronic inflammation and antiapoptotic activity. - *Prevotella* spp. is abundant in the cervical intraepithelial neoplasia (CIN) group. Furthermore, three potential biomarkers have been identified: *Lactobacillus* spp., *Gardnerella* spp., and *Prevotella* spp., which can robustly predict and distinguish patients with CIN from healthy individuals. - Specifically, *P. buccalis* and *P. timonensis* have been significantly associated with the risk for high-grade squamous intraepithelial lesions (HSIL) and CAN. These species have been reported in the CIN1 group, while *P. disiens* has been reported in the CIN2 or CIN3 groups.	Amabebe and Anumba (2018), So et al. (2020) and Wu et al. (2021)

(Continued)

TABLE 1 (Continued)

Microorganisms	Relationship with cervical cancer	References
Fannyhessea spp.	- A high abundance of *Fannyhessea* spp. in the cervix vaginal microflora may serve as a critical marker for cervical lesions. - The dominance of *A. vaginae*, similar to *G. vaginalis*, is particularly noted in cases of bacterial vaginosis and significantly contributes to the risk of developing cervical neoplasia. - *F. vaginae* has been reported in cases of cervical intraepithelial neoplasia 2 or 3 (CIN2 or CIN3). Additionally, infection with this bacterium is significantly associated with the risk of developing CIN2, CIN3, and cervical cancer (CAN). - Both *G. vaginalis* and *F. vaginae* have been proposed as molecular markers due to their ability to form a biofilm that may contribute to viral persistence.	So et al. (2020) and Kang et al. (2021)
Streptococcus spp.	- *Streptococcus* spp. species have been related to bacterial vaginosis (BV). They have also been identified in cases of high-grade squamous intraepithelial lesion (HSIL), low-grade squamous intraepithelial lesion (LSIL), and normal controls. - Related to aerobic vaginitis (AV), another factor possibly related to CIN. - -*Streptococcus* spp. has been reported as a representative genus in the cervical cancer (CAN) group. A study suggested that it could serve as a potential biomarker for distinguishing CAN, possibly through the activation of multiple inflammatory cytokines, and may affect human vaginal and cervical epithelial cells. - *Gardnerella* spp., *Streptococcus* spp., *Finegoldia* spp., *Anaerococcus* spp., and *Lactobacillus* spp. are considered the most impactful factors to differentiate CAN from cervical intraepithelial neoplasia (CIN). However, when it comes to distinguishing invasive cervical carcinoma (ICC) from CIN, *Gardnerella* spp. or *Streptococcus* spp. have been reported as potential biomarkers. - Specifically, *S. dysgalactiae* has been reported in cases of CAN.	Kwasniewski et al. (2018), Amabebe and Anumba (2020), So et al. (2020), Plisko et al. (2021) and Kang et al. (2021)
Mobiluncus spp.	- *Mobiluncus* spp. has been reported as one of the organisms causing bacterial vaginosis (BV). This condition occurs when there's an imbalance in the natural bacteria levels in the vagina, leading to discomfort and pain. - There are mainly two species of *Mobiluncus* spp. that have been identified: *M. mulieris* and *M. curtisii*.	Mitra et al. (2016a), Amabebe and Anumba (2018), Kwasniewski et al. (2018) and Klein et al. (2020a)
Megasphaera spp.	- Marker genera of the CAN (community state type anaerobe non-dominated) group. - Mentioned in relation with the SIL (squamous intraepithelial lesion) group, particularly noted for its relative abundance of *M. elsdenii* and presence in the CAN group. - *Sneathia* spp., *M. elsdenii*, and *Shuttleworthia satelles* are most representative according to the SIL group. - *M. elsdenii* was reported for the first time in women with SIL. - *Megasphaera* spp. and *Sneathia amnionii* are considered predictors of bacterial vaginosis (BV).	Audirac-Chalifour et al. (2016) and Wu et al. (2021)
Peptoniphilus spp.	- Reported as a marker genera of the CAN (community state type anaerobe non-dominated) group. - Part of the IV CST (community state type), a classification system for vaginal microbial communities.	Ravel et al. (2011), So et al. (2020) and Wu et al. (2021)
Aerococcus spp.	- *Aerococcus* spp. has been reported in relation to HPV clearance in CONTROL samples. - It is included in the IV CST (community state type), a classification system for vaginal microbial communities.	Ravel et al. (2011)
Finegoldia spp.	- Reported as a significant factor in distinguishing CAN (community state type anaerobe non-dominated) from CIN (cervical intraepithelial neoplasia). - *F. magna* is associated with high-grade squamous intraepithelial neoplasia and CAN risk. - It has a significant relationship with the risk of developing CIN2 or CIN3, and CAN. - *F. magna* typically appears on the skin and mucous membranes. - It is associated with vaginosis.	So et al. (2020) and Kang et al. (2021)
Lactobacillus jensenii	- Depletion of specific *Lactobacilli* species—*L. crispatus*, *L. gasseri* or *L. jensenii*-is associated with a predisposition towards bacterial vaginosis and other proinflammatory states. - This depletion can lead to DNA cell damage and potentially carcinogenic changes. - In 20% of CAN (community state type anaerobe non-dominated) cases, there were low levels of *L. jensenii*, which were related to severe lesions. - Women with high-grade CIN (cervical intraepithelial neoplasia) had lower levels of *L. jensenii* than those with low-grade CIN. - *L. jensenii* and *L. vaginalis* were found only in samples from women with NCL (no cervical lesion).	Audirac-Chalifour et al. (2016), Mitra et al. (2016a), Castanheira et al. (2021) and Sims et al. (2021)

(Continued)

TABLE 1 (Continued)

Microorganisms	Relationship with cervical cancer	References
Lactobacillus gasseri	- *L. gasseri* is reported to potentially be associated with the most rapid clearance of acute HPV infection. - It has been proposed as a potential therapeutic species for maintaining cervical health. - PCR-based techniques have shown that *L. gasseri* is negatively associated with *L. iners* and *F. vaginae* species, which often co-associate and are suggested to pose an intermediate and high risk for the development of CIN (cervical intraepithelial neoplasia). - Depletion of specific *Lactobacilli* species—*L. crispatus*, *L. gasseri* or *L. jensenii*-is associated with a predisposition towards bacterial vaginosis and other proinflammatory states. - This depletion can lead to DNA cell damage and potentially carcinogenic changes.	Mitra et al. (2016a), Castanheira et al. (2021) and Sims et al. (2021)
Lactobacillus crispatus	- The vaginal epithelial mucus layer's protective function is enhanced, and autophagy activity is observed when *L. crispatus* dominates the vaginal microbiota. - Vaginal microbiota dominated by *L. crispatus* is associated with a lower risk of HPV, CIN (cervical intraepithelial neoplasia), and CAN (community state type anaerobe non-dominated) infection. - *L. crispatus* is related to maintaining the integrity of the protective mucosal surface layer and poses a lower risk of opportunistic bacterial and viral urogenital infections. - The presence of *L. crispatus* has been negatively correlated with CIN. - A marked decrease of *L. crispatus* was found in the CIN1, CIN2, CIN3, and CAN groups. - *L. crispatus* has been reported as the most protective microorganism against HPV and HIV due to its antimicrobial compound production. - Depletion of *L. crispatus* and increased abundance of anaerobic bacteria such as *Gardnerella vaginalis*, *Peptostreptococcus anaerobius*, and *Porphyromonas venonis* is significantly more common in women with CIN and CAN. - *L. crispatus* has been reported as the most effective microorganism in preventing bacterial dysbiosis compared to *L. iners*. - Evidence suggests that *L. iners* is associated with disease. - H_2O_2 is thought to be produced by *L. crispatus* rather than *L. iners*. - *L. crispatus* produces both D-lactic acid and L-lactic acid.	Audirac-Chalifour et al. (2016), Amabebe and Anumba (2018), Norenhag et al. (2020), So et al. (2020), Castanheira et al. (2021), Sims et al. (2021) and Zhou et al. (2021)
Lactobacillus iners	- *L. iners* is the most commonly reported *Lactobacillus*-dominated CST (community state type) detected in women diagnosed with CIN (cervical intraepithelial neoplasia). - It has a small genome, indicative of a symbiotic or parasitic lifestyle. - Some researchers suggest that *L. iners* may have clonal variants that promote health in some cases and are associated with dysbiosis and disease predisposition in others. - Microbiomes dominated by *L. iners* are less protective against cervicovaginal infections and exhibit higher rates of HPV infection and cervical dysplasia. - *L. iners* does not appear to share many protective mechanisms with other *Lactobacillus* species and is considered intermediate in its ability to prevent cervical disease. - Compared to other *Lactobacillus* species, *L. iners* may be less capable of inhibiting the colonization of strict anaerobes and pathobionts. - *L. iners* appears more capable of surviving and adapting to a wide range of pH and other metabolic stress-related conditions due to the constitutive and inducible expression of genes not seen in other Lactobacilli. - *L. iners*-dominated microbiota is usually associated with dysbiosis and appears less stable and more prone to transition. - *L. iners* was more frequently detected in co-infected women than healthy ones. - It has been reported in high proportion in women with HSIL (high-grade squamous intraepithelial lesions) and LSIL (low-grade squamous intraepithelial lesions) along with *L. acidophilus* and *L. crispatus*. - *L. iners* has been found in women with HIV, HPV, HSV-2 (herpes simplex virus, type 2), CIN, and CAN (community state type anaerobe non-dominated). - The presence of *L. iners* has been proposed as a higher risk of HPV, SIL (squamous intraepithelial lesions), and CAN. - There's an association between *L. iners* and CIN or even CAN.	Audirac-Chalifour et al. (2016), Mitra et al. (2016a), Amabebe and Anumba (2018), Kwasniewski et al. (2018), Norenhag et al. (2020), So et al. (2020), Castanheira et al. (2021), Sims et al. (2021) and Zhou et al. (2021)

HC, healthy controls; SIL, squamous intraepithelial lesions; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; CIN, cervical intraepithelial neoplasia; ICC, invasive cervical cancer; CAN, cervical cancer; HPV, human papillomavirus; BV, bacterial vaginitis; PID, pelvic inflammatory disease.

TABLE 2 Microorganisms linked to various conditions in the vaginal environment.

HC	SIL		CIN	ICC/CAN	Other conditions (PID, HPV, BV)
	LSIL	HSIL			
L. crispatus (Ravel et al., 2011; Mitra et al., 2016b; Amabebe and Anumba, 2018) *L. jensenii* (Audirac-Chalifour et al., 2016) *L. gasseri* (Mitra et al., 2016b) Kwasniewski et al. (2018) reported the next list about *Streptococcus* species: *S. agalactiae*, *S. alactolyticus*, *S. anginosus*, *S. australis*, *S. bovis*, *S. cristatus*, *S. fryi*, *S. gallinaceus*, *S. gordonii*, *S. infantis*, *S. intermedius*, *S. macedonicus*, *S. milleri*, *S. mutants*, *S. oligofermentans*, *S. oralis*, *S. oligofermentans*, *S. oralis*, *S. orisratti*, *S. parasanguinis*, *S. pasteuri*, *S. pseudopneumoniae*, *S. sanguinis*, *S. thermophiles*, *S. tigurinus*, *S. vestibularis*	*Fusobacterium* spp. (Audirac-Chalifour et al., 2016; Castanheira et al., 2021) *Sneathia* (Audirac-Chalifour et al., 2016; Castanheira et al., 2021) *G. vaginalis* (Kwasniewski et al., 2018) Kwasniewski et al. (2018), reported the next list about *Streptococcus* species: *S. agalactiae*, *S. alactolyticus*, *S. anginosus*, *S. australis*, *S. bovis*, *S. cristatus*, *S. fryi*, *S. gallinaceus*, *S. gordonii*, *S. infantis*, *S. intermedius*, *S. macedonicus*, *S. milleri*, *S. mutants*, *S. oligofermentans*, *S. oralis*, *S. oligofermentans*, *S. oralis*, *S. orisratti*, *S. parasanguinis*, *S. pasteuri*, *S. pseudopneumoniae*, *S. sanguinis*, *S. thermophiles*, *S. tigurinus*, *S. vestibularis*	*Fusobacterium* spp. (Audirac-Chalifour et al., 2016; Castanheira et al., 2021; Wu et al., 2021) *Sneathia* (Audirac-Chalifour et al., 2016; Castanheira et al., 2021; Wu et al., 2021) *Gardnerella* (Kwasniewski et al., 2018) *G. vaginalis* (Kwasniewski et al., 2018; So et al., 2020; Castanheira et al., 2021) *Prevotella* (Wu et al., 2021) *P. buccalis*, *P. timonensis* (So et al., 2020) *F. vaginae* (So et al., 2020) *Dialister* (So et al., 2020) *Megasphaera* (Audirac-Chalifour et al., 2016; Wu et al., 2021) Kwasniewski et al. (2018), reported the next list about *Streptococcus* species: *S. agalactiae*, *S. alactolyticus*, *S. anginosus*, *S. australis*, *S. bovis*, *S. cristatus*, *S. fryi*, *S. gallinaceus*, *S. gordonii*, *S. infantis*, *S. intermedius*, *S. macedonicus*, *S. milleri*, *S. mutants*, *S. oligofermentans*, *S. oralis*, *S. oligofermentans*, *S. oralis*, *S. orisratti*, *S. parasanguinis*, *S. pasteuri*, *S. pseudopneumoniae*, *S. sanguinis*, *S. thermophiles*, *S. tigurinus*, *S. vestibularis*	*Fusobacterium* (Audirac-Chalifour et al., 2016) *Sneathia* (Mitra et al., 2016b; Sims et al., 2021) *Sneathia sanguinegens* (Mitra et al., 2016a; So et al., 2020) *Mycoplasma* (Klein et al., 2020a) *Chlamydia trachomatis* (Zhou et al., 2021) *Anaerococcus tetradius* (Mitra et al., 2016b) *Peptostreptococcus anaerobius* (Mitra et al., 2016b; So et al., 2020) *Gardnerella* (Kang et al., 2021) *Gardnerella vaginalis* (So et al., 2020; Zhou et al., 2021) *P. bucalis*, *P. timonensis*, *P. disiens* (So et al., 2020) *Fannyhessea vaginae* (Audirac-Chalifour et al., 2016; So et al., 2020) *Streptococcus* (Kang et al., 2021) *Finegoldia magna* (So et al., 2020)	*Fusobacterium* (Sims et al., 2021; Wu et al., 2021) *Fusobacterium necrophorum* (Audirac-Chalifour et al., 2016; So et al., 2020; Castanheira et al., 2021) *Sneathia* spp. (Mitra et al., 2016b) *Sneathia amnii* (Audirac-Chalifour et al., 2016; Mitra et al., 2016b) *Mycoplasma* (Wu et al., 2021) *Anaerococcus* (Kang et al., 2021; Wu et al., 2021) *Peptostreptococcus* (Wu et al., 2021) *P. anaerobius* (So et al., 2020) *G. vaginalis* (Mitra et al., 2016b; So et al., 2020) *Prevotella* (Wu et al., 2021) *P. bucalis*, *P. timonensis* (So et al., 2020) *Fannyhessea vaginae* (So et al., 2020) *Streptococcus* (Audirac-Chalifour et al., 2016; Kang et al., 2021) *Dialister* (Wu et al., 2021) *D. invisus* (So et al., 2020) *Megasphaera* (Audirac-Chalifour et al., 2016; Wu et al., 2021) *Peptoniphilus* (So et al., 2020; Wu et al., 2021) *Finegoldia magna* (So et al., 2020)	**PID:** *P. anaerobius* (So et al., 2020) **HPV:** *Sneathia* spp. (Mitra et al., 2016a,b; Zhou et al., 2021), *Fusobacterium* (Audirac-Chalifour et al., 2016), *Chlamidia trachomatis* (Klein et al., 2020a; Zhou et al., 2021) *Anaerococcus* (Wu et al., 2021), *Gardnerella vaginalis* (Zhou et al., 2021), *Prevotella* (So et al., 2020; Wu et al., 2021), *Streptococcus* (Kang et al., 2021), *Peptoniphilus* (Kang et al., 2021) **BV:** *Fusobacterium* (Amabebe and Anumba, 2018), *Fannyhessea* (Amabebe and Anumba, 2018), (Kwasniewski et al., 2018; Klein et al., 2020a; Castanheira et al., 2021; Zhou et al., 2021), *Mycoplasma* (Amabebe and Anumba, 2018; Kwasniewski et al., 2018; Klein et al., 2020a), *M. genitalium*, *M. hominis* (Klein et al., 2020a; Zhou et al., 2021), *S. sanguinegens* (Klein et al., 2020a), *S. amnii* (Audirac-Chalifour et al., 2016), *Peptostreptococcus* (Mitra et al., 2016b; So et al., 2020), *Gardnerella* (Mitra et al., 2016b), *G. vaginalis* (Klein et al., 2020a; Castanheira et al., 2021), *Prevotella* (Ravel et al., 2011; Amabebe and Anumba, 2018; Kwasniewski et al., 2018; Castanheira et al., 2021; Wu et al., 2021), *Streptococcus* (Amabebe and Anumba, 2018), *Dialister* (Amabebe and Anumba, 2018), *Megasphaera* (Amabebe and Anumba, 2018; Klein et al., 2020a; Castanheira et al., 2021), *Mobiluncus* (Mitra et al., 2016b; Amabebe and Anumba, 2018; Kwasniewski et al., 2018), *M. mulieris*, *M. curtisii* (Klein et al., 2020a)

HC, healthy controls; SIL, squamous intraepithelial lesions; LSIL, low-grade squamous intraepithelial lesions; HSIL, high-grade squamous intraepithelial lesions; CIN, cervical intraepithelial neoplasia; ICC, invasive cervical cancer; CAN, cervical cancer; HPV, human papillomavirus; BV, bacterial vaginitis; PID, pelvic inflammatory disease.

provided accessible information in the NCBI databases. Initially, six potential articles were identified, each providing specific details on vaginal microbiota and cervical health conditions, as illustrated in Table 3. However, upon further exploration for raw data within the NCBI, only four of these articles—marked with asterisk—offered the required information.

Despite the heterogeneity inherent in each database, we undertook a data exploration process to confirm if earlier published data (Ravel et al., 2011; Audirac-Chalifour et al., 2016) aligns with recent publications (So et al., 2020; Kang et al., 2021). It is worth noting that such analyses invariably encounter limitations rooted in the data source, standardization of metadata information, and the procedures employed for sequencing results, among other factors. Nevertheless, our investigation focused on deciphering microbiota patterns across various cervical health conditions.

Regarding the fourth study under scrutiny (Kang et al., 2021), the absence of raw data within the manuscript necessitated a

comprehensive reanalysis of the samples provided, guided solely by accession numbers for sequences in the NCBI database. This re-evaluation was executed employing the QIIME-2022.8 pipeline, strictly adhering to the author's guidelines delineated within their paper. Denoising was performed utilizing DADA2 (Divisive Amplicon Denoising Algorithm 2) (Callahan et al., 2016), and despite Kang et al. (2021) usage of the Silva v138 database, we elected to use the Silva (16S/18S rRNA) (Quast et al., 2013; Yilmaz et al., 2014) database v132. Similar to the authors, the sequences among the reanalyzed samples were rarefied to a sequencing depth of 6,919 reads.

Subsequent to the successful acquisition of data from each of the four papers, the next step was to distill this data, as showed in Table 4. All data abundances were normalized to values ranging from 0 to 1. We successfully compiled a total of 496 samples, encapsulating information pertaining to the type of cervical health condition (CAN, CIN, SIL, and control), the respective study reported, and HPV presence (restricted to CAN and Control samples). The statistical analysis and graphic representation were executed using the R 4.2.1 version.

It is important to highlight that our analysis was conducted within certain constraints. The availability of raw data posed a significant limitation, necessitating the reanalysis of samples in specific instances. Additionally, the low number of articles utilized for our analysis, owing to our commitment to use only freely accessible information, may have affected the comprehensiveness of our study. Despite these challenges, we remained committed to conducting a meticulous and robust exploration of the available data.

According to the previous Table 1, where is presented the microorganisms proposed as microbiological markers in cervical cancer or cervix inflammation, the common bacteria genera found in each one of the four articles that were also mentioned in this table (as potential biomarkers) were: *Fusobaterium* spp., *Sneathia* spp., *Streptococcus* spp., *Gardnerella* spp., *Dialister* spp., *Megasphaera* spp., *Peptostreptococcus* spp., *Peptoniphilus* spp., *Prevotella* spp., *Anaerococcus* spp., and *Lactobacillus* spp.

Our findings, as outlined in Table 4, consistently demonstrate the presence of HPV in all documented cases of cervical abnormalities (CAN). To gain a deeper understanding of the bacterial profile associated with CAN, we employed a Venn diagram, as illustrated in Figure 2A. This visualization not only highlights the commonly identified bacteria in CAN cases, but also those observed in control samples. In the context of CAN, our analysis revealed 17 frequently reported bacterial genera: *Fusobacterium* spp., *Sneathia* spp., *Streptococcus* spp., *Gardnerella* spp., *Dialister* spp., *Megasphaera* spp., *Peptostreptococcus* spp., *Peptoniphilus* spp., *Staphylococcus* spp., *Campylobacter* spp., *Parvimonas* spp., *Prevotella* spp., *Haemophilus* spp., *Porphyromonas* spp., *Anaerococcus* spp., *Lactobacillus* spp., *Ureaplasma* spp.

To further elucidate the bacterial landscape, an additional Venn diagram was constructed to identify common bacterial genera in control samples from the studies examined (Figure 2A). We observed that 16 genera were common in these samples: *Sneathia* spp., *Streptococcus* spp., *Gardnerella* spp., *Dialister* spp., *Megasphaera* spp., *Peptoniphilus* spp., *Staphylococcus* spp., *Parvimonas* spp., *Prevotella* spp., *Porphyromonas* spp., *Anaerococcus* spp., *Lactobacillus* spp., *Ureaplasma* spp., *Aerococcus* spp., *Finegoldia* spp., *Enterococcus* spp.

In reference to Table 1, which proposes certain microorganisms as potential microbiological markers for cervical cancer or cervix inflammation, we noted that the following genera were shared between our four selected articles and those suggested as potential biomarkers: *Fusobacterium* spp., *Sneathia* spp., *Streptococcus* spp., *Gardnerella* spp., *Dialister* spp., *Megasphaera* spp., *Peptostreptococcus* spp., *Peptoniphilus* spp., *Prevotella* spp., *Anaerococcus* spp., and *Lactobacillus* spp. This overlap may indicate a significant link between these bacterial genera and cervical health disorders.

Simpson's diversity index, a standard tool for determining alpha diversity, gauges the prevalence of dominant species and inversely correlates with species diversity (Sagar and Sharma, 2012). As illustrated in Figure 2B, our analyses calculated this alpha diversity. The data suggests that as the cervical health condition transitions from Control to SIL, CIN, and CAN, there is an observable increase in microbiota, corroborating previous literature (Klein et al., 2020a; Norenhag et al., 2020; So et al., 2020; Tango et al., 2020; Kang et al., 2021; Sims et al., 2021; Wu et al., 2021; Zhou et al., 2021). Figure 2C presents a similar pattern for HPV (−) samples, where bacterial diversity is comparatively lower than HPV (+) samples. The median value for boxes representing HPV (−) samples is less than 0.25, contrasting with those representing HPV (+) samples. Furthermore, we compiled a list of the top 50 bacterial genera present in HPV (+) and HPV (−) samples across the four studied papers. As depicted in Figure 2D, these bacteria are displayed accordingly.

It is crucial to emphasize that our exploration of the data did not follow the strict guidelines of a meta-analysis or systematic review. This absence of a structured approach may introduce a potential bias in our findings, as we might have overlooked certain studies or emphasized others disproportionately. Also, because we focused on information that's freely available, there may be some limits to the scope and depth of our analysis. Despite these limitations, we see our work as an initial step in understanding the overall trends in microbiota composition during cervical cancer progression. Our findings should be interpreted with caution, considering the potential biases and methodological constraints. However, we believe our research provides valuable insights that can pave the way for future, more thorough investigations in this crucial area.

6.1 Microbiota and HPV

Based on the distinctive microorganisms identified solely in HPV (+) and HPV (−) samples (Figure 2D), existing literature has associated *Methylobacterium* spp. as a predominant bacteria in the ovary (Amabebe and Anumba, 2020) and ovarian cancer (Peric et al., 2019). Our data, as presented in Figure 2D, identified *Methylobacterium* spp. within the HPV (−) groups. Notably, this microorganism emerged as the sole differential entity when compared to HPV (+) samples. However, given the inherent limitations of a review paper, such as data heterogeneity, further research is required to substantiate these findings.

Contrarily, *Alloscardovia* spp., *Eubacterium* spp., and *Mycoplasma* spp. were exclusively detected in HPV (+) samples. Previous reports have also documented *Alloscardovia* spp. in HPV (+) samples (Gao et al., 2013), and a 2019 case study associated this microorganism with preterm premature rupture of membranes (PPROM) (Cardona-Benavides et al., 2019). *Eubacterium* spp., meanwhile, has been reported more frequently in HPV (+) patients than in HPV (−) patients (Carrillo-Ng et al., 2021) and is also associated with BV cases

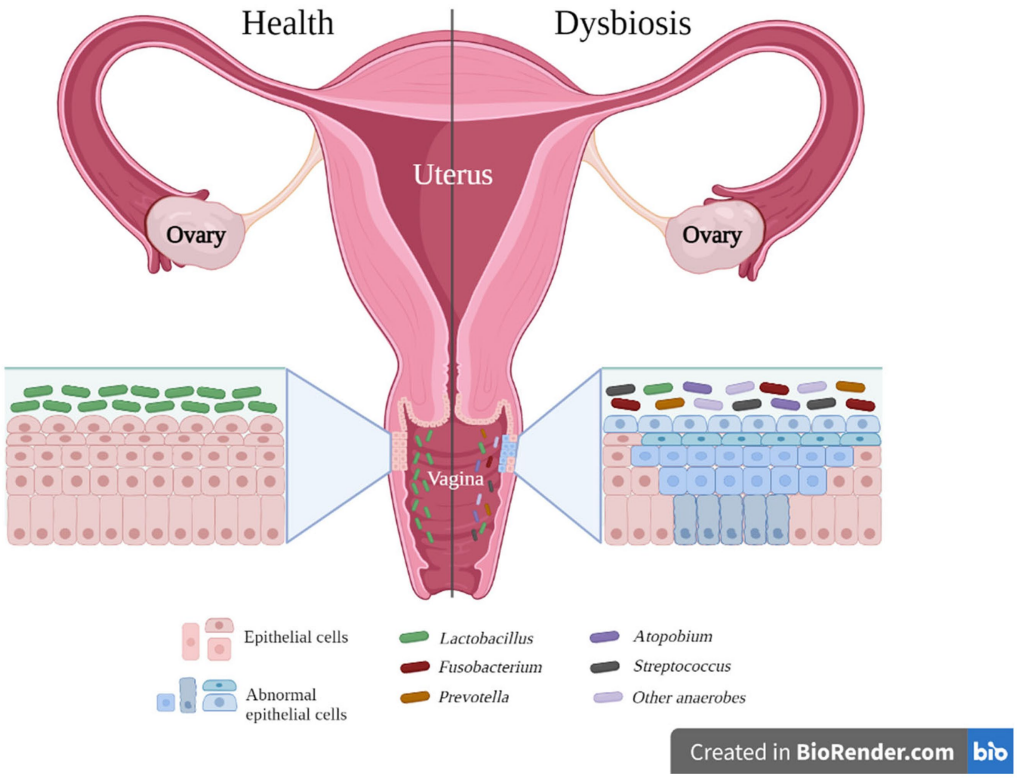

FIGURE 1
Vaginal microorganisms in a healthy vagina versus a dysbiosis stage in the vagina. Source of image: Authors and adapted from Zhou et al. (2021). The figure reveals the transformation that the vaginal microbiota undergoes between healthy and dysbiotic conditions. In a healthy state, the environment is primarily characterized by the presence of *Lactobacillus* species. On the other hand, in a state of dysbiosis, the environment is largely dominated by a variety of other bacteria, including *Prevotella* spp., the bacterium now known as *Fannyhessea* spp. (formerly *Atopobium* spp.), *Streptococcus* spp., and other anaerobic bacteria.

(Fredricks et al., 2005; Srinivasan and Fredricks, 2008). Moreover, *Mycoplasma* spp. has been found to be prevalent among women with HPV (+) (Klein et al., 2020b), with its abundance noted to increase in women with cervical lesions (Pacha-Herrera et al., 2022). *Mycoplasma* spp. has also been implicated as a potential cause of persistent HPV infection (Zhou et al., 2021) and has been identified in BV infections (Ferris et al., 2004; Verhelst et al., 2004; Kwasniewski et al., 2018).

Our review suggests that *Alloscardovia* spp., *Eubacterium* spp., and *Mycoplasma* spp. could potentially serve as biomarkers for HPV (+), while *Methylobacterium* spp. might be a marker for HPV (−). However, it's important to note that due to the limitations inherent in this review and the data evaluated, these observations remain tentative. There is a clear need for continued research to further explore the role of the microbiota in the development of HPV, as this could provide valuable insights that may aid in the fight against this condition.

6.2 Microbiota and cervical cancer progression

Based on the findings outlined in Section 6, Table 5 encapsulates the shared bacteria identified in the intersection of the Venn diagram depicted in Figure 2A. This table effectively illustrates the genera of bacteria that appear to be prevalent as cervical cancer progresses, as well as in healthy controls.

Focusing first on the CONTROL samples, *Fusobacterium* spp., *Peptostreptococcus* spp., *Campylobacter* spp., and *Haemophilus* spp. were conspicuously absent from the bacterial genera identified. Drawing from existing literature, *Fusobacterium* spp. has been exclusively reported in CAN or CIN samples, which may account for the numerous propositions of *Fusobacterium* spp. as a potential marker of CAN (Audirac-Chalifour et al., 2016; Norenhag et al., 2020; So et al., 2020; Castanheira et al., 2021; Sims et al., 2021; Wu et al., 2021; Zhou et al., 2021). The potential of *Fusobacterium* spp. as an oncogenic entity and a promoter of dysplasia development has also been deliberated (Norenhag et al., 2020). Additional characteristics of this microorganism are detailed in Table 1. Thus, Table 5 in alignment with the literature, indicates that *Fusobacterium* spp. is only present in CIN, SIL, and CAN samples.

Peptostreptococcus spp., a bacterial genus not detected in control samples (refer to Table 5), is associated with cervical conditions such as cervical intraepithelial neoplasia (CIN) and cervical cancer (CAN) (Mitra et al., 2016b; So et al., 2020). This bacterium also plays a role in female genital tract infections like bacterial vaginosis and pelvic inflammatory disease (So et al., 2020) and is considered a distinctive marker for the CAN group (Wu et al., 2021).

TABLE 3 Potential articles containing information on vaginal microbiota and cervical health conditions.

Objective	Type of analysis	Technique	Reference
To assess the alteration in vaginal microbiota during the progression of cervical cancer in women infected with high-risk HPV	Metagenomics: 16S rRNA genes	Next-generation sequencing	So et al. (2020)*
To define the changes in the cervical microbiome in women of reproductive age during the transition from squamous intraepithelial lesions (SIL) to cervical cancer (CAN)	Metagenomics: 16S rRNA genes	Whole Genome Sequencing	Wu et al. (2021)
To examine the correlation between infections in the cervix and vagina and the development of pre-cancerous cervical lesions	Metagenomics: 16S rRNA genes	Next-generation sequencing	Kwasniewski et al. (2018)
To delve into the possible connection between the composition of vaginal microbes and CAN, presenting its diagnostic value in predicting, classifying, and tracking CAN progression. This also includes differentiating samples from diseased individuals from those of healthy controls	Metagenomics: 16S rRNA genes	High-throughput sequencing	Kang et al. (2021)*
To foster a comprehensive and precise comprehension of the structure and ecology of the vaginal microbial ecosystem in women without symptoms, with a focus on understanding the purpose and fundamental operation of the vaginal microbiota	Metagenomics: 16S rRNA genes	Pyrosequencing	Ravel et al. (2011)*
To investigate the relationship between the diversity and composition of cervical microbiota, as per a histopathological diagnosis at each stage of CAN's natural history, and the expression levels of cytokines in the cervix	Metagenomics: 16S rRNA genes	Sanger sequencing	Audirac-Chalifour et al. (2016)*

*Articles with raw data freely available for analysis.

TABLE 4 Data derived from databases on NCBI with accessible raw information (Ravel et al., 2011; Audirac-Chalifour et al., 2016; So et al., 2020; Kang et al., 2021).

Type	*n*	Study	*n*	HPV	
				Positive	Negative
CAN	26	Audirac-Chalifour et al. (2016)	8	8	N/A
		Kang et al. (2021)	8	8	N/A
		So et al. (2020)	10	10	N/A
CIN	28	Kang et al. (2021)	8	N/A	N/A
		So et al. (2020)	20	N/A	N/A
SIL	4	Audirac-Chalifour et al. (2016)	4	N/A	N/A
Control	438	Audirac-Chalifour et al. (2016)	17	10	7
		Kang et al. (2021)	7	N/A	7
		So et al. (2020)	20	10	10
		Ravel et al. (2011)	394	N/A	394
Total	496				

n, number of samples.

Campylobacter spp., identified as a CAN marker (Wu et al., 2021), was first reported in CIN cases in 2018 (Zhang et al., 2018). This review's analysis revealed the presence of this bacterium exclusively in CAN samples (Table 5).

Haemophilus spp. is another bacterial genus absent in control samples. It is believed to contribute to trichomoniasis as a colonizing microorganism (Kwasniewski et al., 2018) and has been reported only in CAN samples (So et al., 2020), explaining its absence in the control group (Table 5).

Three bacterial genera—*Finegoldia* spp., *Enterococcus* spp., and *Aerococcus* spp.—were found solely in control samples, contrasting with CAN samples (Table 5). *Finegoldia*'s spp. exclusive presence in control samples might be due to its higher abundance compared to other conditions, as analyses focused on the top 50 most abundant bacteria from each studied paper. More information about *Finegoldia* spp. can be found in Table 1.

Enterococcus spp., commonly found in healthy samples and associated with HPV clearance (Verhelst et al., 2004; Zhou et al., 2007; Ravel et al., 2011; Borgdorff et al., 2014; di Paola et al., 2017), aligns with the results observed in Table 5.

Aerococcus spp., another genus found exclusively in control samples, corroborates previous literature (Verhelst et al., 2004; Zhou

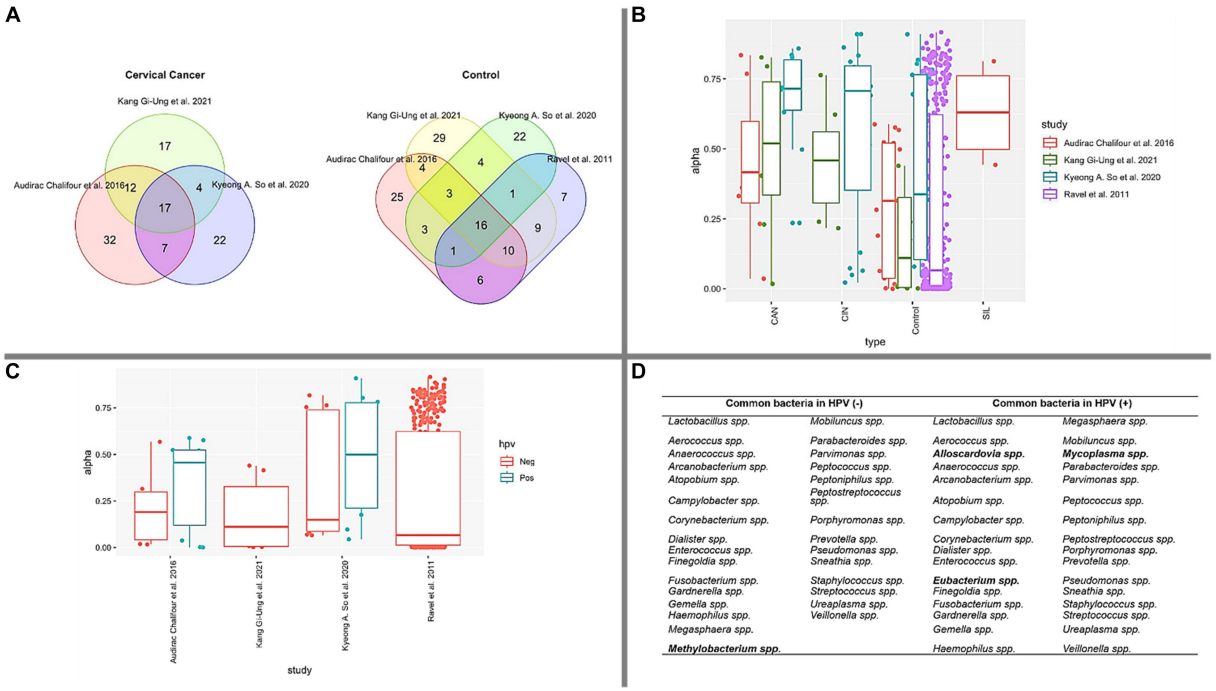

FIGURE 2
Bacterial composition and diversity. **(A)** The Venn diagram depicts the overlap of common bacterial types identified in CAN and CONTROL groups, based on the 50 most abundant bacteria in each study. **(B)** Simpson's index measures genera diversity across various cervical health conditions. Notably, CONTROL samples exhibit lower diversity compared to CAN, CIN, and SIL conditions. The Kruskal-Wallis test yielded a *p*-value <0.05, signifying significant differences in diversity between CONTROL vs. CAN and CONTROL vs. CIN. **(C)** A box plot illustrating the prevalence of HPV infection in control samples across the studied papers. **(D)** Identification of common bacterial types in HPV (–) and HPV (+) groups in each paper, with unique bacteria within each group highlighted in bold.

et al., 2007; Srinivasan and Fredricks, 2008; Ravel et al., 2011). Additional information about *Aerococcus* spp. is available in Table 1.

Sneathia spp., *Streptococcus* spp., *Gardnerella* spp., *Dialister* spp., *Megasphaera* spp., *Peptoniphilus* spp., *Staphylococcus* spp., *Parvimonas* spp., *Prevotella* spp., *Porphyromonas* spp., *Anaerococcus* spp., *Lactobacillus* spp., and *Ureaplasma* spp. were found in both CAN and control samples.

Sneathia spp. has been reported in CIN samples (So et al., 2020), bacterial vaginosis (BV) cases (Srinivasan and Fredricks, 2008; Liu et al., 2013; Borgdorff et al., 2014; Klein et al., 2020a; Zhou et al., 2021), squamous intraepithelial lesion (SIL) samples (Audirac-Chalifour et al., 2016; Wu et al., 2021), HPV infections (Audirac-Chalifour et al., 2016; Dareng et al., 2016; di Paola et al., 2017; Castanheira et al., 2021; Zhou et al., 2021) and as a biomarker of cervical neoplasia (Godoy-Vitorino et al., 2018). However, it is also a common member of the vaginal community (Verhelst et al., 2004; Verstraelen et al., 2004; Kang et al., 2021), suggesting it should not be considered a biomarker. More information about *Sneathia* spp. is provided in Table 1.

Streptococcus spp. has been observed in CAN samples (So et al., 2020; Tango et al., 2020; Wu et al., 2021), cervical disease cases (So et al., 2020), and CIN patients (Tango et al., 2020; Arroyo Mühr et al., 2021). Still, it was also identified as part of the vaginal composition (Verhelst et al., 2004; Zhou et al., 2007; Srinivasan and Fredricks, 2008; Gao et al., 2013; Audirac-Chalifour et al., 2016; Arroyo Mühr et al., 2021; Kang et al., 2021; Wu et al., 2021; Zhou et al., 2021) and associated with HPV clearance (di Paola et al., 2017), which aligns with our findings that this microorganism appears in both groups. Further information about Streptococcus can be found in Table 1.

Gardnerella spp., a bacterium identified in both disease and control samples, is classified as an anaerobic carcinogen (Zhou et al., 2021). It has been found in cervical intraepithelial neoplasia (CIN) samples (Mitra et al., 2016b; So et al., 2020; Sims et al., 2021), bacterial vaginosis (BV) cases (Borgdorff et al., 2016; Mitra et al., 2016b; Amabebe and Anumba, 2018; Klein et al., 2020a; Castanheira et al., 2021), HPV persistent infections (Norenhag et al., 2020), and control samples (Audirac-Chalifour et al., 2016; Tango et al., 2020). This aligns with our paper analysis results. Additional information about Gardnerella can be found in Table 1.

Dialister spp., another genus present in both groups (cervical cancer and control), is an opportunistic pathogen influenced by *Lactobacillus* spp. (Wu et al., 2021). It has been reported as a marker genus in cervical cancer (So et al., 2020; Sims et al., 2021; Wu et al., 2021), and found in CIN samples (So et al., 2020), BV (van de Wijgert et al., 2014; di Paola et al., 2017; Amabebe and Anumba, 2020), and HPV cases (Gao et al., 2013; Audirac-Chalifour et al., 2016; Dareng et al., 2016). However, it's also been observed in control samples (Ravel et al., 2011; So et al., 2020), which concurs with our findings. More details about *Dialister* spp. are provided in Table 1.

Megasphaera spp., found in both disease and normal samples, has been reported in squamous intraepithelial lesion (SIL) and cervical cancer samples (Wu et al., 2021), CIN cases (Mitra et al., 2016b; Sims et al., 2021), BV patients (Amabebe and Anumba, 2018; Klein et al.,

TABLE 5 Microbiota and its presence according to the health condition: CAN or CONTROL.

Bacteria	CAN*	CONTROL*
Fusobacterium spp.	X	
Sneathia spp.	X	X
Streptococcus spp.	X	X
Gardnerella spp.	X	X
Dialister spp.	X	X
Megasphaera spp.	X	X
Peptostreptococcus spp.	X	
Peptoniphilus spp.	X	X
Staphylococcus spp.	X	X
Campylobacter spp.	X	
Parvimonas spp.	X	X
Prevotella spp.	X	X
Haemophilus spp.	X	
Porphyromonas spp.	X	X
Anaerococcus spp.	X	X
Lactobacillus spp.	X	X
Ureaplasma spp.	X	X
Aerococcus spp.		X
Finegoldia spp.		X
Enterococcus spp.		X

*Data according to Figure 2.

2020a; Castanheira et al., 2021) and normal samples (Ravel et al., 2011; Arroyo Mühr et al., 2021). Table 1 provides more details on this bacterium.

Peptoniphilus spp., another common bacterium, has been reported as a cervical cancer marker (So et al., 2020; Wu et al., 2021), found in BV samples (Fredricks et al., 2005; Srinivasan and Fredricks, 2008; van de Wijgert et al., 2014), HPV infections (Shannon et al., 2017; Kang et al., 2021), and control samples (Srinivasan and Fredricks, 2008; Ravel et al., 2011). Further details about *Peptoniphilus* spp. can be found in Table 1.

Staphylococcus spp., although common in control samples (Tango et al., 2020), has also been observed in conditions such as cervical cancer (Tango et al., 2020; Arroyo Mühr et al., 2021), aerobic vaginitis (di Paola et al., 2017), and SIL (Klein et al., 2020a; Arroyo Mühr et al., 2021; Sims et al., 2021; Wu et al., 2021).

Parvimonas spp. is another bacterium observed in both cervical cancer and control samples. It has been reported in control samples (Shannon et al., 2017), cervical diseases (Godoy-Vitorino et al., 2018; So et al., 2020), and BV patients (van de Wijgert et al., 2014).

Prevotella spp. has been found in control samples (Lewis et al., 2017; Zhou et al., 2021) and other conditions like HPV infections (Norenhag et al., 2020), CIN (Mitra et al., 2016b; Godoy-Vitorino et al., 2018; Tango et al., 2020; Sims et al., 2021), CAN (Wu et al., 2021), and BV (di Paola et al., 2017; Amabebe and Anumba, 2018; Kwasniewski et al., 2018; Castanheira et al., 2021). This aligns with the results summarized in Table 5 and detailed in Table 1.

Porphyromonas spp., also present in both disease and control cases (Table 5), has been observed in CAN (Sims et al., 2019, 2021; Wu et al., 2021), BV samples (Fredricks et al., 2005; Srinivasan and Fredricks, 2008; van de Wijgert et al., 2014), and control cases (Verhelst et al., 2004; Srinivasan and Fredricks, 2008).

Anaerococcus spp. has been reported in control samples (Verhelst et al., 2004; Zhou et al., 2007; Srinivasan and Fredricks, 2008; Arroyo Mühr et al., 2021), and diseases such as CAN (Wu et al., 2021), SIL (Mitra et al., 2015, 2016b), and CIN cases (Mitra et al., 2016b; Godoy-Vitorino et al., 2018).

Lactobacillus spp., another common bacterium (Table 5), is seen in various conditions depending on the species—either as a disease marker or a health biomarker in the vaginal composition. For instance, it has been reported in conditions like CAN (Castanheira et al., 2021), CIN (Mitra et al., 2016b; Norenhag et al., 2020; Sims et al., 2021), SIL (Norenhag et al., 2020), HPV clearance (Mitra et al., 2016b; Norenhag et al., 2020), cervical dysplasia (Norenhag et al., 2020), and control samples (Mitra et al., 2016b; Amabebe and Anumba, 2018; Klein et al., 2020a; Norenhag et al., 2020; Castanheira et al., 2021; Sims et al., 2021; Zhou et al., 2021). More information about different *Lactobacillus* species (*L. jensenii*, *L. gasseri*, *L. crispatus*, *L. inners*) can be found in Table 1.

Lastly, *Ureaplasma* spp. has been reported in both CAN (Tango et al., 2020) and control samples (Verhelst et al., 2004; Srinivasan and Fredricks, 2008; Wu et al., 2021), as well as in CIN (Tango et al., 2020), and BV patients (Fredricks et al., 2005; van de Wijgert et al., 2014; Amabebe and Anumba, 2018; Klein et al., 2020a).

The exclusive presence of specific microorganisms in cervical adenocarcinoma cases such as *Fusobacterium* spp., *Peptostreptococcus* spp., *Campylobacter* spp., and *Haemophilus* spp., underscores their potential significance in the pathology of this disease. These unique bacteria could play important roles in the onset and progression of CAN, and their further investigation may provide valuable insights for the development of new diagnostic markers or therapeutic strategies. Understanding the specific functions and influences of these bacteria in CAN is a crucial step toward improving our ability to prevent and treat this form of cervical cancer.

7 Discussion

The evolution of sequencing methodologies has paved the way for revolutionary advancements in our understanding of microbiomes and associated diseases, including cervical cancer. This progression can be traced through the various technological tools utilized in the quest for comprehension.

In this context, innovative technologies like NextSeq500 (by Illumina) have been employed for parallel DNA and RNA sequencing to comprehensively detect detectable and actively transcribed DNA and RNA microbes in cervical specimens. The results from such studies suggest that the choice of approach (RNA-Seq, DNA-Seq) can influence the number of transcripts obtained. The focus of current research endeavors is to maximize sequence retention in order to amass a wealth of data that could prove beneficial for multiple investigations reliant on database information.

These technological advancements present an exciting opportunity to delve deeper into the intricacies of cervical cancer and its relationship with the microbiota. The wealth of information that these technologies can provide will undoubtedly fuel further research, and potentially lead to breakthroughs in diagnostic and therapeutic strategies.

The analyses conducted herein strongly advocate for continued biomarker exploration and the need for up-to-date data to inform the development of new strategies to combat cervical cancer.

Our review provides a comprehensive overview of the microbiota associated with the progression of cervical cancer and enumerates several microorganisms implicated in cervicovaginal dysbiosis.

Furthermore, we outline the principal discoveries of past research related to the microbiota present during the CONTROL (healthy) and CAN stages, as well as other conditions such as CIN, SIL, HPV (+), and HPV (−). This analysis allowed us to identify promising bacteria frequently reported as biomarkers, suggesting that biomarker identification is a compelling field with potential for numerous research projects.

A consistent presence of HPV was demonstrated in all reported cases of cervical abnormalities. We have identified noteworthy bacterial genera that differ between both CAN and control samples through our investigation. The intersection of these identified bacteria with those suggested as microbiological indicators for cervical health issues in existing research implies a potential connection that needs additional exploration. This understanding of the microbial landscape may provide valuable insights into the pathogenesis of cervical diseases and potentially guide future diagnostic and prevention strategies and treatment plans.

The advent of sequencing techniques has shed new light on our understanding of microbial biomarkers. The rise of this technology holds the promise of facilitating more in-depth studies examining the relationship between cancer and the microbiome. Nevertheless, there is a pressing need for additional research and the standardization of methods for metadata acquisition. This will enhance the scalability of results, ultimately aiming to positively impact the health and wellness of women worldwide.

Author contributions

WF: Conceptualization, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. PA: Conceptualization, Project administration, Supervision, Visualization, Writing – review & editing. LV: Visualization, Writing – review & editing. FM: Investigation, Methodology, Project administration, Supervision, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Animal models of *Klebsiella pneumoniae* mucosal infections

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Klebsiella pneumoniae is among the most relevant pathogens worldwide, causing high morbidity and mortality, which is worsened by the increasing rates of antibiotic resistance. It is a constituent of the host microbiota of different mucosa, that can invade and cause infections in many different sites. The development of new treatments and prophylaxis against this pathogen rely on animal models to identify potential targets and evaluate the efficacy and possible side effects of therapeutic agents or vaccines. However, the validity of data generated is highly dependable on choosing models that can adequately reproduce the hallmarks of human diseases. The present review summarizes the current knowledge on animal models used to investigate *K. pneumoniae* infections, with a focus on mucosal sites. The advantages and limitations of each model are discussed and compared; the applications, extrapolations to human subjects and future modifications that can improve the current techniques are also presented. While mice are the most widely used species in *K. pneumoniae* animal studies, they present limitations such as the natural resistance to the pathogen and difficulties in reproducing the main steps of human mucosal infections. Other models, such as *Drosophila melanogaster* (fruit fly), *Caenorhabditis elegans, Galleria mellonella* and *Danio rerio* (zebrafish), contribute to understanding specific aspects of the infection process, such as bacterial lethality and colonization and innate immune system response, however, they but do not present the immunological complexity of mammals. In conclusion, the choice of the animal model of *K. pneumoniae* infection will depend mainly on the questions being addressed by the study, while a better understanding of the interplay between bacterial virulence factors and animal host responses will provide a deeper comprehension of the disease process and aid in the development of effective preventive/therapeutic strategies.

KEYWORDS

***Klebsiella pneumoniae*, animal models, disease pathogenesis, mucosal infection, pre-clinical**

Introduction

Klebsiella pneumoniae is a widely distributed bacterium that colonizes the human skin, mouth, respiratory and gastrointestinal (GI) tracts asymptomatically. It is also one of the main causative agents of hospital infections such as urinary tract infections, pneumonia, liver abscesses, meningitis, and sepsis, being considered an opportunistic pathogen (Conlan et al., 2012; Gorrie et al., 2017; Joseph et al., 2021; Osbelt et al., 2021). In addition, *K. pneumoniae* is among the most relevant strains when considering the increase in antibiotic resistance

worldwide, being classified by the World Health Organization (WHO) as a priority pathogen for which new drugs are needed (WHO, 2017). However, relying on the discovery of new drugs alone may not be enough to suppress the advance of these infections, especially when caused by multidrug-resistant isolates, so new strategies are fundamental to stop the steady increase of cases.

Infections caused by multi-drug resistant bacteria (including *K. pneumoniae*) pose a great burden to healthcare systems worldwide, from the hundreds of thousands of deaths, to the reduced life expectancy and disabilities, along with the cost of the treatment. *K. pneumoniae* is among the four main causes of death by antibiotic resistance bacteria, which are responsible for 929,000 annual deaths according to a study published in 2022 (Antimicrobial Resistance Collaborators, 2022).

K. pneumoniae strains can be classified in two major categories, namely classical (or common) and hypervirulent strains, based on traits such as the hypermucoviscosity (HMV) phenotype and increased expression of siderophores and fimbriae (Chang et al., 2021; Dai and Hu, 2022). There are a few differences in the profile of the infections caused by those types of *K. pneumoniae*. Usually, most cases occur within healthcare environments and are caused by classical strains. Such strains are commonly multi-drug resistant, especially to beta-lactams, including carbapenems. The most common infections caused by the classical strains are urinary tract infections, pneumonia and bacteremia (Paczosa and Mecsas, 2016; Dai and Hu, 2022).

Hypervirulent strains, on the other hand, are acquired in the community and are more invasive, being able to colonize additional sites and cause further damage, when compared to the classical strains. Another important difference between classical and hypervirulent strains is the historically higher susceptibility of the hypervirulent strains to antimicrobials, which is becoming less prevalent in hospital-acquired *K. pneumoniae* in many regions, especially in lower- or middle-income countries (Dai and Hu, 2022; Pulingam et al., 2022). Nevertheless, a very worrying development is the increase in reports of resistant hypervirulent strains leading to more severe, often fatal infections (Choby et al., 2020; Dai and Hu, 2022). A compilation of the most common infections caused by classical and hypervirulent strains of *K. pneumoniae* is shown in Figure 1.

Overall, when considering the diversity of host niches that *K. pneumoniae* can infect and the overall disease burden, animal models are an important tool to elucidate infection mechanisms and develop new, safer therapeutic and prophylactic strategies against this pathogen. The present review focused on the *in vivo* platforms deployed to evaluate the pathogenicity of *K. pneumoniae* during infection of the main mucosal sites (gastrointestinal, respiratory and genitourinary). We explored the methodologies used to establish infection and the results achieved in each animal model. Complex vertebrates with anatomical features and immune response similar to ours, like rodents and primates, were compared with simpler models, such as invertebrates and zebrafish, considering specific disease outcomes, comparative virulence, and host defense mechanisms (Figure 2). The different techniques were compared regarding complexity, requirement for specific equipment, relevance of the results and applicability to humans, and the more robust techniques were highlighted in each case.

Finally, we reviewed the current models used to evaluate the host-pathogen interactions during disease, and the efficacy of therapeutic agents and/or potential vaccine candidates against infections by *K. pneumoniae*.

This review includes papers written in English, chosen using the keywords correlated to the topics "animal models," "*Klebsiella pneumoniae*" and "mucosal infection," with an emphasis in articles published within the last 10 years in journals indexed in the PubMed database. Approval by animal research ethics committee was also used as a selection criterium.

Animal models of infection by *Klebsiella pneumoniae*

Animal models of *Klebsiella pneumoniae* respiratory infections

Hospital or community-acquired *K. pneumoniae* respiratory infections represent a serious public health threat, causing high mortality due to the common development into bacteremia (Antimicrobial Resistance Collaborators, 2022; Chen et al., 2022). *K. pneumoniae* is one of the main causes of pneumonia worldwide as a result from widespread colonization (Martin and Bachman, 2018; Chen et al., 2022). In some countries, the reports of *K. pneumoniae* community-acquired pneumonia cases which progressed to bacteremia, has surpassed those caused by *Streptococcus pneumoniae*—the leading cause of bacterial pneumonia (Lin et al., 2010).

The main models used to investigate the hallmarks of respiratory infections by *K pneumoniae* are mouse and rat, with a few studies being conducted in other mammals such as rabbits and monkeys. The different models of respiratory infections (summarized in Table 1) will be discussed individually in the next sections.

Mouse infection models

In mice, the main method to induce pneumonia is the direct instillation of bacteria in the mice nasopharynx through the nasal cavity (Wieland et al., 2011; Geller et al., 2018). This model has the advantages of the absence of any surgical procedure, in addition to the easy and quick manipulation of the animal. An important factor to consider in this type of infection is the volume of bacteria applied to the mouse. Lower volumes such as 5–10 μL can carry little to none of the inoculum into the lungs and usually promote only local nasopharyngeal colonization. To cause pneumonia, volumes between 25 and 50 μL are necessary, coupled with anesthesia to allow aspiration into the lungs. Interestingly, higher volumes do not appear to enhance lung delivery (Southam et al., 2002) and increase the risk of death by suffocation. Several different mouse strains have been tested using this inoculation model, including BALB/c (Kumar et al., 2020), C57BL/6J (Wolff et al., 2021), CD-1 (Russo et al., 2015), Swiss (Jain et al., 2015), ICR (Yoshida et al., 2001), C3H/HeN and C3H/HeJ (parental toll-like receptor 4 deficient strain) (Branger et al., 2004), MF1 (Mabrook et al., 2022) and Kunming mice (Li-Juan et al., 2022). Similarly, there was also a great variety in the methods of anesthesia, which included, halothane (Lavender et al., 2005), isoflurane (Meijer et al., 2021), ketamine (Dong et al., 2021), ketamine and medetomidine (Otto et al., 2021) or xylazine mix (Zhang et al., 2021). A pneumonia model using direct bacterial instillation was established to evaluate the yersiniabactin receptor FyuA as a recombinant vaccine candidate against respiratory *K pneumoniae* infections in BALB/c mice. In the

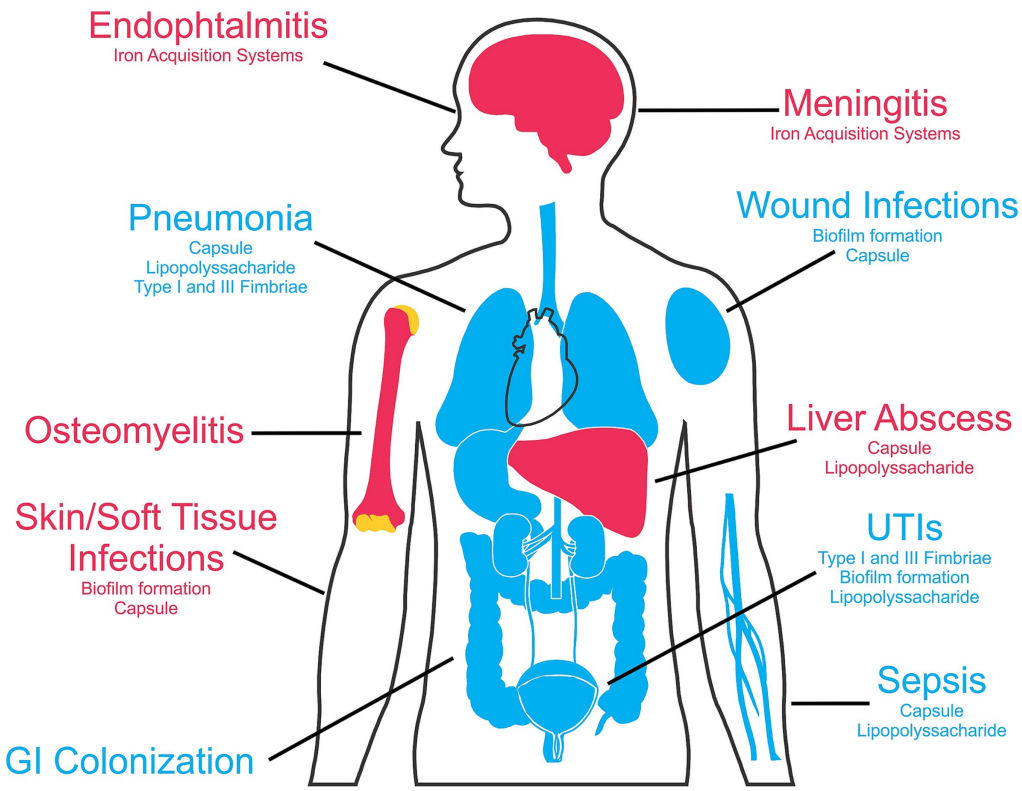

FIGURE 1
Klebsiella pneumoniae human infection sites and main associated virulence factors. The blue color indicates the infections caused by classical *K. pneumoniae* strains, while diseases commonly associated with hypervirulent strains are marked in red. This figure was partly generated using templates from Servier Medical Art (Servier), and SlidesGo (Freepik), licensed under a Creative Commons Attribution 3.0 unported license.

study, the authors used 10^3 colony forming units (CFU) of a K2 isolate, American Type Culture Collection (ATCC) 43816 diluted in 50 µL. The control group showed an increased bacterial burden in the lungs, 48 h following infection, as well as higher levels of the inflammatory cytokines interleukin 17 (IL-17), tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β), oxidative stress markers myeloperoxidase (MPO) and nitrous oxide (NO), and histological damage, while recombinant FyuA immunized mice exhibited increased levels of interleukin 6 (IL-6) and interleukin 12 (IL-12), with a reduced bacterial burden in the lungs, 48 h following infection (Kumar et al., 2020). A similar methodology was applied to C57BL6/J mice, using the same bacterial strain (ATCC 43816) to analyze changes in the lung and gut microbiome following respiratory infection by *K. pneumoniae* and *Streptococci*. An early production of TNF-α was detected in the mouse lungs, followed by lung colonization at 12 h and dissemination to the bloodstream at 18 h post infection. These changes were accompanied by a shift in bacterial distribution in the lungs, with an increase in *Klebsiella* and decrease in *Streptococci*.

Another method of inoculation of bacteria is directly in the trachea, via a small surgery, whereas an incision exposing the trachea allows the inoculum, with volume ranging from 25 to 30 µL, to be delivered with aid of a 26- or 30-gage needle (Zeng et al., 2003; Xu et al., 2014). The mouse lineage most frequently used is C57BL/6 background (Zeng et al., 2003, 2005; Barletta et al., 2012; Xu et al., 2014; Vieira et al., 2016; Morinaga et al., 2019), although NIH/Swiss (Fagundes et al., 2012) and BALB/c mice have also been tested (Deng et al., 2004; Xu et al., 2014; Liu J. et al., 2022). Anesthesia was performed either with a xylazine-ketamine mix (Zeng et al., 2003, 2005; Barletta et al., 2012; Fagundes et al., 2012; Xu et al., 2014; Vieira et al., 2016) or isoflurane (Morinaga et al., 2019). Following the procedure, the incision is closed with surgical staples or Vetbond (Rosen et al., 2015). One example of pneumonia induced by intratracheal bacteria inoculation is the work conducted by Zeng and colleagues (Zeng et al., 2003), in which infection was caused by intratracheal delivery of 10^3 CFU of the ATCC 43816 strain in 30 µL of final volume, through a 26-gage needle. The authors compared the immune response in control mice versus those previously injected with a recombinant, Ad5 human adenovirus-based platform expressing the macrophage inflammatory protein-1 alpha complementary DNA. The latter exhibited recruitment of neutrophils and activated NK cells to the lungs, as well as increased expression of gamma interferon (IFN-γ), which correlated with reduced bacterial counts in the lungs and bloodstream (Zeng et al., 2003). However, tracheal inoculation is possible without the need of surgical procedures. The suspension can be given straight to the lungs of the anesthetized animals by placing a metallic cannula with an inner tube, at the opening of the left bronchus, with 20 µL of volume injected with a micro-injection syringe. The bacteria are suspended in melt agar medium, which serves as an infection enhancer, providing a protective milieu for the inoculum. This method allowed the stable colonization of mice and rat lungs by multiple pathogens, including *K. pneumoniae* (Hoover et al., 2017). However, the authors conclude that this model

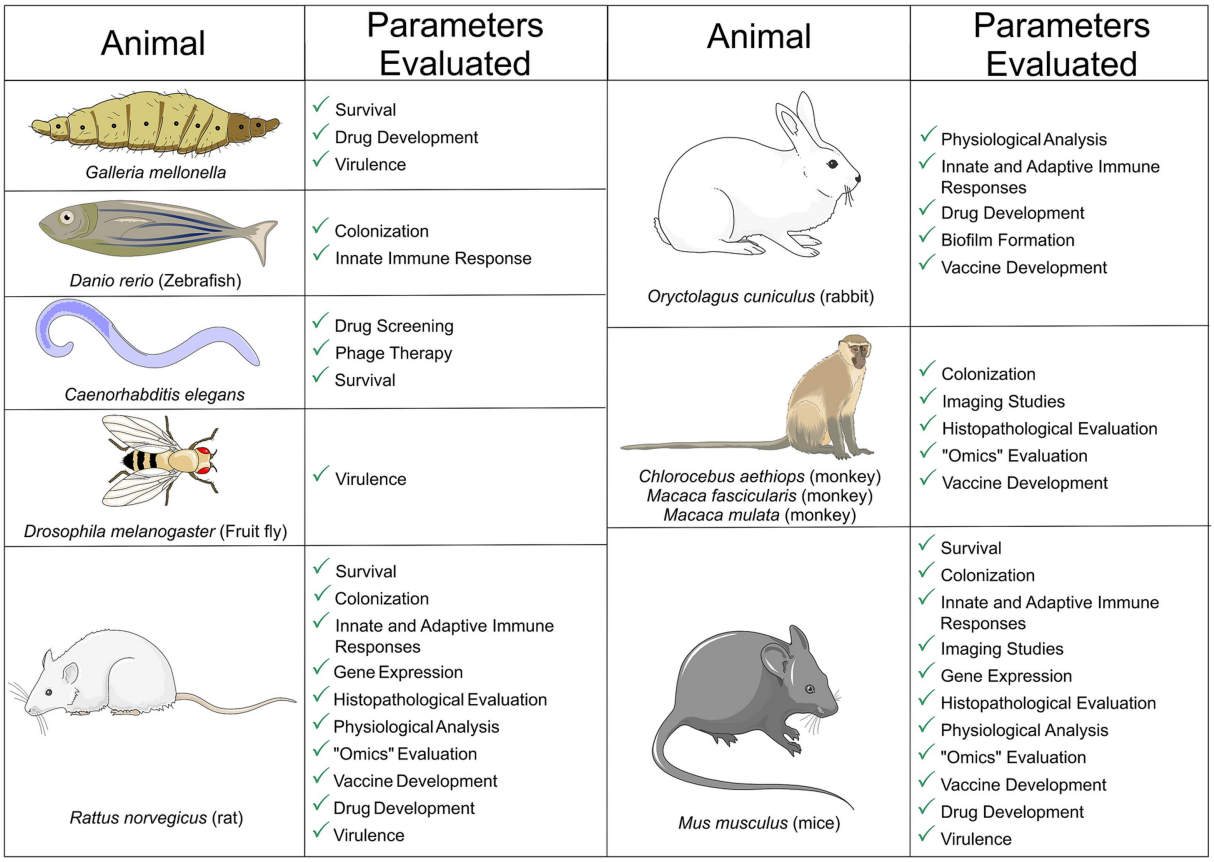

FIGURE 2
Animal models of *Klebsiella pneumoniae* mucosal infections. The main infection outcomes are shown for each model. The figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license. This figure was partly generated using templates from Servier Medical Art (Servier), licensed under a Creative Commons Attribution unported license.

is not suitable for evaluating survival as an endpoint, but rather should be used to determine CFU counts in the lungs. Intratracheal spraying was also performed to induce pneumonia in BALB/c mice anesthetized with pentobarbital. The mice were given 25 μL of *K. pneumoniae* 700603, at a concentration of 3×10^9 (Gou et al., 2021). Similarly, *K. pneumoniae* NTUH-K2044 (hypervirulent K1 strain, 2×10^4 CFU) was aerosolized and given in the tracheal bifurcation of female C57BL/6Cnc mice anesthetized with pentobarbital (Zheng et al., 2022). This infection model using the same hypervirulent strain was lethal for the mice within 48 h of challenge. The transcriptome of the infected animals showed an increased expression of genes related to acute inflammatory responses after 12 h of infection, with migration of granulocytes, monocytes and macrophages (Zheng et al., 2022).

K. pneumoniae-induced pneumonia can also be achieved with retropharyngeal inoculation. In this model, the inoculum was administered in C57BL/6 J mice, after isoflurane anesthesia (Bachman et al., 2015; Holden et al., 2016). This pneumonia model was used to evaluate the contribution of siderophore secretion for lung inflammation and bacteria dissemination during infection. A mutant Δ*tonB* strain that secretes, but does not import the siderophores, induced the release of IL-6 and the chemokines CXCL1 and CXCL2 in lung homogenates, colonized the lungs and spread to the spleen. Comparatively, mutant strains lacking different combinations of siderophores (enterobactin, salmochelin, and yersiniabactin), showed mixed results. Overall, the authors propose that siderophores play an essential role in the pathogenicity of *K. pneumoniae* respiratory infections, via interference with the immune system (Holden et al., 2016).

Another delivery mechanism is via laryngoscopy. Following pentobarbital anesthesia, the animal is positioned in a 45-degree angle and with aid of a laryngoscope blade, the trachea is exposed, and the bacteria are injected with a soft-end needle (Gu et al., 2021). Using the method developed by Spoelstra et al. (2007), a vaccine based on inactivated *Acinetobacter baumanii* was able to cross-protect against *K. pneumoniae* pneumonia, following intranasal challenge. Using the same pneumonia model, an inactivated, whole cell vaccine based on a *K. pneumoniae* isolate also reduced the death toll in the immunized mice (Gu et al., 2021).

A gavage or feeding needle can also be used to inoculate and establish the infection. This model was carried out in ICR:CD-1 (Swiss background) and Swiss mice, where 10^6 to 10^7 CFU of *K. pneumoniae* was given in 30–50 μL of volume directly in the trachea, through the blunt-end needle, in anesthetized animals (Cortés et al., 2002; Rouse et al., 2006). Following the inoculation of 10^7 CFU of an extended-spectrum beta lactamase (ESBL) and non-ESBL producing *K. pneumoniae* strains, the animals were treated with different cephalosporins and the ability of the drugs to prevent colonization was evaluated. In the group challenged with the ESBL-producing strain,

TABLE 1 *In vivo* models of respiratory infection by *Klebsiella pneumoniae*.

Inoculation method	Endpoint	Other analysis	*K. pneumoniae* strains	Type of animal model used	Animal strains	References
Intranasal inoculation	Survival; Bacterial burden in lungs and blood	Cytokine expression; Histological evaluation	Clinical isolates (common and hypervirulent type), ATCC 43816, ATCC 10031	Mice	BALB/C, C57BL/6J, CD-1, Swiss, ICR, C3H/HeJ, MF1, Kunming	Geller et al. (2018), Wieland et al. (2011), Kumar et al. (2020), Wolff et al. (2021), Russo et al. (2015), Jain et al. (2015), Yoshida et al. (2001), Branger et al. (2004), Mabrook et al. (2022), Li-Juan et al. (2022), Lavender et al. (2005), Meijer et al. (2021), Dong et al. (2021), Otto et al. (2021), Zhang et al. (2021)
Intratracheal inoculation (surgical incision)	Survival; Bacterial burden in the lungs and blood	Cytokine and chemokine expression; NK, T and neutrophil cells migration; Changes in the respiratory microbiota	Clinical isolate (common type), ATCC 43816, ATCC 27736	Mice	C57BL/6, NIH/Swiss, BALB/C	Zeng et al. (2003), Xu et al. (2014), Zeng et al. (2005), Vieira et al. (2016), Barletta et al. (2012), Morinaga et al. (2019), Fagundes et al. (2012), Deng et al. (2004), Liu J. et al. (2022)
Intratracheal inoculation (without surgery)	Survival Bacterial load in the lungs, blood, liver, spleen	Cytokine expression; Inflammatory response levels; Cell recruitment; Histological evaluation; Bioluminescent imaging; Cytological evaluation	Clinical isolates (common and hypervirulent), ATCC 43816, Xen 39	Mice	C57BL6/J (WT and mutants with C57/BL6J background), ICR	Zhao et al. (2015), Griepentrog et al. (2020), Olonisakin et al. (2016), Zhang J. et al. (2019) Nikouee et al. (2021), Hu et al. (2020)
Intratracheal spraying	Survival; Bacterial burden in the lungs and liver	Inflammatory response-related gene expression; Cytokine levels	700,603, NTUH-K2044	Mice	C57BL/6Cnc, BALB/C	Gou et al. (2021), Zheng et al. (2022)
Retropharyngeal inoculation	Bacterial burden in the lungs and spleen	Cytokine and chemokine expression; Fitness gene expression during infection	KPPR1 (Rifampin-Resistant Mutant of ATCC 43816)	Mice	C57BL/6, Mutants generated from a C57BL/6 background	Bachman et al. (2015), Holden et al. (2016)
Laryngoscopy	Survival	Cross-protection with an inactivated whole-cell *A. baumanii* vaccine against *K. pneumoniae* challenge	Clinical isolate	Mice	C57BL/6 (WT, Rag1$^{-/-}$ and Tlr4$^{-/-}$)	Gu et al. (2021)
Gavaging	Number of animals infected; Bacterial burden in lungs		Clinical isolates	Mice	ICR:CD-1, Swiss	Cortés et al. (2002), Rouse et al. (2006)
Direct inoculation with a cannula following intubation	Survival; Bacterial burden in lungs and blood	Body temperature and weight	EMC2003 (ESBL variant of ATCC 43816), EMC2014 (KPC variant of ATCC 43816)	Rat	Sprague–Dawley, RP–AEur–RijHsd	van der Weide et al. (2020a,b)
Intubation	Survival	Histological evaluation; Cytokine and chemokine expression	46,114	Rat	Sprague–Dawley	Mei et al. (2017)
Inoculation with a catheter	Bacterial burden in the lungs	Histological evaluation; Cytokine and chemokine expression		Rat	Sprague–Dawley	Gu et al. (2022)

(Continued)

TABLE 1 (Continued)

Inoculation method	Endpoint	Other analysis	*K. pneumoniae* strains	Type of animal model used	Animal strains	References
Intranasal inoculation	Bacterial burden in the lungs	Cytokine expression; Histological evaluation; Physiological signs; Transmission electron microscopy of the lung tissues	ATCC 1705	Rat	Wistar, Sprague–Dawley	Wang et al. (2022), Aljohani et al. (2022)
Left side-only pneumoniae by intubation	Survival; Bacterial burden in the lung and blood	Blood extravasation; Histological evaluation; Tissue weight	ATCC 43816	Rat	RP–AEur–RijHsd	Schiffelers et al. (2000, 2001b), Bakker-Woudenberg et al. (2001)
Inoculation with a catheter	Bacterial burden in the lungs and blood	Histological evaluation; Leukocyte recruitment; Inflammatory response-related gene expression; Inflammatory response markers	NCTC 5055, Clinical isolate	Rat	Wistar, Sprague–Dawley	Chhibber et al. (2004), Chhibber et al. (2003), Sun et al. (2006)
Intrabronchial instillation with a bronchoscope	Lung bacterial burden	Radiographical score; Histopathological score; Host transcriptome	Clinical Isolate (ST258)	Non-rodent animal model	Cynomolgus macaques (*Macaca fascicularis*)	Malachowa et al. (2019)
Direct inoculation in the pleural space	Empyema positive cultures	Gas, pH, glucose and lactic acid levels; Leukocyte count	Clinical isolate	Non-rodent animal model	White New Zealand rabbits	Shohet et al. (1987)
Pre-colonized endotracheal tubes	Hyperthermia in biofilm formation		Clinical isolates	Non-rodent animal model	White New Zealand rabbits	Palau et al. (2023)

neither of the antimicrobials were capable to prevent colonization, while in the non-ESBL group, antibiotic treatment blocked colonization in some animals (Rouse et al., 2006).

It is also possible to deliver bacteria directly in the trachea by positioning the pipette tip above the mouse vocal cords (Zhao et al., 2015; Olonisakin et al., 2016; Griepentrog et al., 2020). The authors used a 200 µL tip to deliver an inoculum of 10^3 CFU of ATCC 43816 in the colonization experiments (10^4 in the lethal model) in wild-type C57BL6/J and the isogenic, thrombospondin-1 negative mutants. The work demonstrated that in the mutant group, the animals survived longer, with a reduced bacterial burden in the lungs and spleen and had a lower pulmonary histopathology score. Cytokine and MPO expression levels were also reduced (Zhao et al., 2015).

Similarly, the tongue pull technique can be used to reach the trachea. With aid of a forceps, the tongue is pulled out and the bacteria are inoculated in the trachea of the anesthetized animal (Zhang G. et al., 2019). This technique was used to deliver 50 µL of the ATCC 43816 strain to C57BL6/J mice. The animals treated with imipenem in combination with andrographolide sulfonate (an anti-inflammatory agent), showed 100% survival rate after challenge, while also reducing the CFU count in the lungs, with controlled inflammation and reduced lung tissue damage parameters (Zhang G. et al., 2019).

An otoscope can also be used to deliver the bacteria into the lungs (Nikouee et al., 2021). This is an interesting technique, since other methods such as tracheostomy can be stressful and induce bleeding and inflammation in the animal (Thomas et al., 2014). With the aid of the otoscope, a catheter was positioned in the trachea to deliver the inoculum to anesthetized ICR mice under immunosuppression with cyclophosphamide. The study intended to conduct a real-time monitoring of infection using a bioluminescent *K. pneumoniae* strain and found that the group treated with the highest immunosuppressor dosage presented increased bioluminescence in the lungs and tissue damage (Hu et al., 2020).

This method was also applied to male C57BL6/J mice, using 3×10^7 CFU of the ATCC 43816 strain to evaluate the role of Beclin-1, an autophagy initiation factor, in pneumosepsis. Overexpression of Beclin-1 resulted in increased autophagy activation and reduced the burden, inflammation and tissue damage during infection, in comparison with the wild-type animals (Nikouee et al., 2021).

Although some of the endpoints varied among the studies, the results suggest that different methods of inoculation promote lung disease with inflammatory infiltrates and increased bacterial loads, and therefore may be used to investigate *K. pneumoniae* infection. An important aspect to be considered is bacterial dissemination from the lungs to other sites—a situation that is frequently observed in humans and is associated with poor prognosis. When considering the reviewed studies, invasiveness is majorly associated with the virulence and dose of the strain used to cause infection, and less a result of the inoculation method. Hypervirulent strains possessing multiple virulence factors, such as KPPR1-derived strains (including ATCC 43816) are highly lethal to mice and provide an interesting platform for investigating disseminated disease. However, since they kill the mice fast, these

strains are usually not ideal for evaluating adaptive immune responses. Classical strains, on the other hand, are rapidly cleared from mice and are not suitable for survival studies (Russo et al., 2018, 2021).

A limitation common to all mouse studies is that mice do not die of bacterial pneumonia, but from disseminated infection with extremely high bacterial loads in the blood and major organs. Human patients, on the other hand, may present much lower bacterial counts and perish from the intense inflammatory responses associated with infection.

Rat infection models

In rats, pneumonia models were established either bilaterally or on the left side-only. For the induction of pneumonia in both lungs, Sprague–Dawley or RP–AEur–RijHsd rats were used. The animals were anesthetized with either isoflurane or medetomidine, intubated, immobilized vertically, and inoculated with 60 µL of ESBL and carbapenemase-producing *K. pneumoniae* isolates (KPC) (van der Weide et al., 2020a,b) to test the efficacy of antibiotic treatment. The use of tigecycline prevented the death of all animals against KPC-induced pneumonia/septicemia, while the group treated with meropenem was not protected. Meanwhile, in the ESBL group, all animals survived when treated with meropenem (Van der Weide et al., 2020b). Another study used tracheal instillation with 2.4×10^8 CFU of strain 46,114 of *K. pneumoniae* to induce pneumonia in Sprague–Dawley rats anesthetized with chloral hydrate. By employing this inoculation method, the effects of the Dusuqing granules, a compound based on an herb used in Chinese traditional medicine, was evaluated in the lung inflammatory process caused by *K. pneumoniae* infection. The treatment was able to reduce the tissue inflammation and damage. Cytokine and chemokine levels were reduced in the lungs of the treated animals, as were the leukocytes in the blood and the bronchoalveolar lavage fluid The authors hypothesize that the anti-inflammatory modulation is related to the downregulation of the NF-κB/MAPK signaling pathway (Mei et al., 2017).

An alternative delivery method in rats is the use of a 22-gage catheter intranasally, through the trachea, in which 50 µL of the *K. pneumoniae* CMCC (B) 46,117 suspension was given to Sprague–Dawley rats (Gu et al., 2022). Using the catheter to deliver the bacterial load, the pneumonia model was employed to investigate the effects of the coadministration of azithromycin with a traditional Chinese medicine formulation. The antibiotic mix improved the bacterial clearance and reduced inflammation parameters (Gu et al., 2022) Direct instillation of the bacterial suspension is also possible in rats. To establish an exudative pneumonia model of *K. pneumoniae*, Wistar male rats were given 50 µL of ATCC 1705 intranasally, in combination with lipopolysaccharide (LPS) administered intraperitoneally daily, for 5 days. This model was used to evaluate the effects of a traditional Chinese medicine formulation in the prophylaxis or treatment of lung infection. While the compound did not show direct antibacterial activity, it reduced the CFU counts in the bronchoalveolar lavage fluid (BALF) and histological damage in the lungs, while improving physiological parameters. Seric IL-6 and alveolar lavage IL-1β cytokine expression was also reduced in the treated group. While the treatment did induce some enhancement, the pre-treatment showed no effect in the pneumonia establishment (Wang et al., 2022). Similarly, female Sprague–Dawley rats anesthetized with isoflurane received 50 µL of an inoculum containing 10^7 CFU of a multi-drug resistant clinical strain with similar volumes in both nares. The authors used a zinc oxide nanoparticle in combination with sulphadiazine, delivered through aerosolization, to evaluate protection against pneumonia. After 4 days, the bacterial counts in the lungs were reduced and the histopathological signs of inflammation tissue damage were diminished (Aljohani et al., 2022).

Left side pneumonia models were established in female RP–AEur–RijHsd rats, where the animals were anesthetized with fluanisone and fentanyl, then pentobarbital. After the procedure, the left bronchus was intubated, and a bacterial load of 10^6 CFU of *K. pneumoniae* strain 43,816 were administered to the left lung, diluted in 200 µL of saline (Schiffelers et al., 2000, 2001a,b; Bakker-Woudenberg et al., 2001). The model was used to demonstrate the kinetics of the deposition and the effect of polyethylene glycol coating of liposomes in the target sites. Lung inflammation induced by *K. pneumoniae* infection resulted in enhanced liposomal deposition, possibly through increased capillary permeability in the inflamed tissue (Schiffelers et al., 2000).

Lobar pneumonia has also been successfully induced in rats using a catheter surgically inserted through the trachea. In female Wistar rats, 10^7 CFU of *K. pneumoniae* National Collection of Type Cultures (NCTC) 5055 (K2/O1 strain) in 1 mL of final volume was given straight to the lungs, following an incision in the rat's trachea, while the animal was anesthetized with pentobarbital, and immobilized in a supinated position (Chhibber et al., 2003, 2004). One of the applications of this model was the use of LPS, or liposomes with a LPS coating, as a vaccine against *K. pneumoniae* pneumonia. Both the pure LPS and the LPS-coated liposomes were able to reduce the bacterial burden in the lungs (Chhibber et al., 2004). A similar model was applied in Sprague–Dawley rats, whereas 200 µL of the inoculum (1.3×10^8 CFU of an ESBL-producing, clinical isolate) was administered with a 26-gage needle, following the exposal of the trachea. The anesthesia was induced with ketamine. This model was used to demonstrate the effects of the exposure of the animals to different concentrations of nitric oxide and oxygen in the treatment of *K. pneumonia*-induced pneumonia. Varied combinations of nitric oxide and lowered oxygen concentration were shown to reduce the total bacterial count in the lungs and blood, while diminishing the expression of the proinflammatory cytokines TNF-α and intercellular adhesion molecule 1 (ICAM-1) expression levels (Sun et al., 2006). In summary, rats have been used as a model for *K. pneumoniae* pneumonia mainly to evaluate the efficacy of antibiotic treatment and experimental vaccines. One advantage of rats is the possibility of inducing lobar pneumonia, as observed in many human patients, especially in community-acquired respiratory infections. Also, the larger size compared to mice facilitates direct access to the lower respiratory tract through surgery or cannulation, allowing the direct delivery of the inoculum. Larger size and increased weight may also be more suitable for evaluating adaptive immune responses in studies using survival as an endpoint, since rats tend to survive longer periods than mice after challenge. Nevertheless, rats require more space and increased maintenance costs when compared to mice and have fewer advocated methodologies for their study in *K. pneumoniae* infections.

Other animal models

Besides mice and rat models, pulmonary *K. pneumoniae* infection was also induced in cynomolgus macaques. In that study, 10^8 and 10^{10} CFU of a carbapenem-resistant ST258 strain, were inoculated in 8–10 years old female primates with aid of a bronchoscope, delivering

the bacterial load directly into the lungs. In both infection groups, the *K. pneumoniae* load was able to induce pneumonia, which was then used to test the potential protective effect of *K. pneumoniae* capsular polysaccharide (CPS) against respiratory infection. The CPS formulation reduced the bacterial burden in the lungs, inducing antibodies that promoted opsonophagocytic killing by polymorphonuclear leukocytes (PMN) *in vitro*. The advantage in the use of primates as a study subject is the closer similarities to humans when compared to rodents, sharing anatomical and immune features, while also maintaining the susceptibility to infection, which is considerably higher in mice (Malachowa et al., 2019). However, the manipulation and maintenance of the animals are more laborious and demanding when compared to small rodents. Only a few specialized animal facilities are equipped to house this type of animal.

An empyema model was established using male and female white New Zealand rabbits. Following the artificial creation of pneumothorax and pleural effusion, 10^9 CFU of a *K. pneumoniae* clinical isolate in 1 mL, was given through a 16-gage cannula. This model was used to analyze the effects of gentamicin and oxygen administration in empyema, which showed that higher O_2 levels can improve the infection prognosis (Shohet et al., 1987).

Another study used male, white New Zealand rabbits to investigate biofilm formation *in vivo*. The model consisted in the introduction of an endotracheal tube previously colonized with different biofilm-producing strains of *K. pneumoniae* or *P. aeruginosa* in the trachea of the rabbits, with a hyperthermia device. The goal was to evaluate the biofilm formation in a fever state, and with temporary 42°C pulses, biofilm formation was greatly, though not completely inhibited (Palau et al., 2023).

Rabbit models of respiratory infections can be an interesting approach, depending on the desired outcomes. In an FDA-backed study to validate new animal models, *Pseudomonas aeruginosa* was used to induce pneumonia and it was found to reproduce many of the typical hallmarks found in humans, such as tissue damage and inflammation, changes in gasometry values, blood pressure, and white-blood cell counts. Eventual infection metastasis was also observed, in some cases leading to death (Nguyen et al., 2021; Gras et al., 2023). However, there is still very limited research using rabbits as a model for *K. pneumoniae* infection, and further studies are needed to accurately determine how robust the model is in terms of reproducibility, evaluation of adaptive immune responses during infection, duration of colonization and survival.

In summary, different animal models have been used to successfully reproduce the hallmarks of *Klebsiella pneumoniae* respiratory infections, with mice being the most used species. However, most of the infection protocols still rely on artificial inoculation routes, involving tracheal incisions or catheters to deliver the bacteria. While these may replicate some of the infections occurring in hospital settings, they do not mimic the natural infection routes in the community.

When considering mouse models in respiratory infections, the direct intranasal inoculation of bacteria appears to be the most advantageous technique to study community acquired infections, since it can: (i) provide data on the different stages of disease pathogenesis as well as the contribution of specific virulence factors; including acquisition, transition to the lungs, establishment and dissemination; (ii) evaluate specific components of the adaptive immune response; (iii) provide reproducible results in survival/colonization without the need for highly specialized training and equipment; (iv) adequately assess the protective efficacy of therapeutic agents or vaccine candidates.

Animal models of *Klebsiella pneumoniae* oral/gastrointestinal infections

Gastrointestinal infections are highly prevalent diseases worldwide, where the most frequent causative agents in these infections are Gram-negative bacteria that reside in the human intestine (Joseph et al., 2021), especially Enterobacteriaceae, including *K. pneumoniae* (Osbelt et al., 2021). Its prevalence in hospitalized patients ranges from 3 to 18% (Gorrie et al., 2017; Joseph et al., 2021), while colonization in healthy individuals varies from approximately 6% in Europe to 20% in Africa (WHO, 2017).

Analysis of rectal and throat swabs from patients admitted in intensive care units found that 6% of these patients were colonized with *K. pneumoniae*. Gut colonization on admission was significantly associated with subsequent infections, with 49% of *K. pneumoniae* infections being caused by a strain found in the patients' microbiota (Gorrie et al., 2017), and possibly transmitted through the fecal-oral route (Young et al., 2020). Furthermore, antibiotic treatment reduces microbial diversity in the GI tract, causing dysbiosis which favors subsequent colonization with *K. pneumoniae* (Martin and Bachman, 2018; Chen et al., 2022). *K. pneumoniae* dysbiosis in the GI correlated with the establishment of systemic infections, such as pneumonia (Wu et al., 2020; Jiang et al., 2022) and liver abscess (Zheng et al., 2021), and it also contributes to inflammatory bowel diseases (Khorsand et al., 2022).

Colonization of GI tract by *K. pneumoniae* is a necessary step for infection (Buffie and Pamer, 2013; Gorrie et al., 2017). Therefore, developing models that mimic this route of colonization and infection is of great importance for understanding disease pathogenesis and for the development of effective therapeutic/preventive strategies. Most animal studies investigating *K. pneumoniae* GI colonization used mice, since they are small, easy to breed and maintain, and reproduce infection hallmarks observed in humans (Ferreira et al., 2018; Muggeo et al., 2018; Cassini et al., 2019; Young et al., 2020; Joseph et al., 2021), as discussed in the next section and compiled in Table 2.

Mouse infection models

The best described methods of inoculation are through gavaging (intragastric administration) or direct oral administration. When the bacterial suspension is given orally, pretreatment with a large-spectrum antibiotic (or a cocktail) is usually performed in the days or weeks preceding challenge to deplete the mouse gut microbiota (Maroncle et al., 2006; Lau et al., 2008; Hennequin and Forestier, 2009). The described volumes of the bacterial suspension vary between 100 and 200 µL. BALB/c (Chiang et al., 2021), C57BL/6 (Nakamoto et al., 2019; Sequeira et al., 2020; Liu J. Y. et al., 2022), C57BL6/J (Young et al., 2020), C57BL/6 N (WT and Rag2 $^{-/-}$) and C57BL/6NTac (Osbelt et al., 2021), MRL/MpJ (Kamata et al., 2020) and CFW1 (Boll et al., 2012) mouse strains have been used in colonization experiments.

Young and colleagues have described a mouse model of GI infection where the animals received the bacteria orally, diluted in a 2% sucrose solution, delivered through a pipette tip, without previous

antibiotic treatment. The authors also induced the infection via gavage, with a 20-gage feeding needle (Young et al., 2020). Administration of at least 10^5 CFU of *K. pneumoniae* was enough to colonize the GI tract and oropharynx, regardless of the infection route. This model can be applied as an alternative to the standard infection protocols where the administration of antibiotics prior the colonization leads to a disruption on the native microbiota allowing *K. pneumoniae* to stablish colonization (Young et al., 2020). It could also be used to study community acquired *K. pneumoniae* infections.

The use of a gavage needle allows direct instillation in the lower GI tract; however, to neutralize the stomachal acidity, a sodium bicarbonate treatment (0.2 M in 200 µL) is performed 5 min prior to the inoculation, resulting in a successful colonization with 100 µL of the bacterial suspension (Calderon-Gonzalez et al., 2023). This model was applied in different mice strains, including C57BL/6 (Calderon-Gonzalez et al., 2023), C57BL/6 J (Lau et al., 2008; Yuan et al., 2019; Young et al., 2020; Kienesberger et al., 2022), OF1 (Maroncle et al., 2006; Hennequin and Forestier, 2009), BALB/c (Hsieh et al., 2010), BALB/cByL (Hsu et al., 2019), CF1 (Perez et al., 2011) and 129 × 1/SvJ (Han et al., 2023).

A work published by Lagrafeuille and colleagues investigated the use of probiotic bacteria as treatment against *K. pneumoniae* infections. They found that the addition of the cell-free supernatant from *Lactobacillus plantarum* impaired biofilm formation by *K. pneumoniae in vitro*. However, when tested in mice, the group infected with both bacteria presented a longer persistence of *K. pneumoniae* when compared with the control group (infected with *K. pneumoniae* only) (Lagrafeuille et al., 2018). This result reinforces the importance of having *in vivo* models to confirm the *in vitro* findings.

Another technique employed to colonize the gut microbiota of mice is through "contamination" of the drinking water with the intended bacteria. In the works describing the method, the animals received preventive antibiotic treatment (also in the water) with either clindamycin or streptomycin, followed by administration of water containing the bacteria (Favre-Bonté et al., 1999; Le Guern et al., 2019). Aiming to demonstrate the dynamics between GI infection and antibiotic administration, male C57BL/6 mice received 10^7/mL of a New Delhi metallo-beta-lactamase-1 (NDM-1) positive strain in the water, after antibiotic treatment. The authors propose a protocol for the use of the antimicrobial where, based on the timing of the colonization process, clindamycin treatment a week before the inoculation showed the best results in the colonization model. When clindamycin was given 2 or 3 weeks before or after the inoculation, gut colonization was not as successful (Le Guern et al., 2019).

Perez and colleagues used a similar model to determine the effect of the antibiotic treatment on GI colonization, establishment, and elimination by *K. pneumoniae*. The animals received antibiotic treatment by subcutaneous injection every day during 8 days; on day 3, they were infected with *K. pneumoniae* and the occurrence of colonization was monitored through fecal analysis (Perez et al., 2011). The levels of *K. pneumoniae* in the feces remained high during antibiotic treatment, but fell gradually afterwords until clearance, suggesting that the reduction of the commensal microbiota by antibiotic therapy favors GI colonization by *K. pneumoniae* (Perez et al., 2011). In humans, although many normal microbiota are not resistant to *K. pneumoniae* colonization, antibiotic treatment can further contribute to *Klebsiella pneumoniae* infection, which often occurs at the hospital environment. The inhibition of exogenous colonization can be attributed either to competition among the microbes or the interaction of the microbiota with mucosal immune defenses, intensifying its response against invaders (Buffie and Pamer, 2013).

A study by Osbelt et al. using germ-free mice transplanted with a human microbiota evaluated stool samples from healthy individuals (adults and kids) and applied an *in vitro* screening to identify the microbiota composition looking for microorganisms present in the healthy donors that could eliminate *K. pneumoniae* colonization. They identified the commensal bacterium *Klebsiella oxytoca* as able to strongly reduce colonization by *K. pneumoniae* (Osbelt et al., 2021). *K. oxytoca* was shown to cooperate with other commensal bacteria to displace *K. pneumoniae* from the GI tract, which could potentially be applied as a probiotic treatment to prevent *K. pneumoniae* infections during hospitalization.

Rat infection models

In female Wistar rats, the GI tract colonization was obtained with an intragastric tubing, inserted surgically through the esophagus, following chloral hydrate anesthesia, with an inoculum of 1 mL containing a mixture of neomycin-resistant *Escherichia coli* and *K. pneumoniae*. In the co-colonization model, the infection lasted longer in animals that received the neomycin treatment when compared to the group who received the bacteria and the irrigation fluid only, without the antimicrobial agent (Ruijs and van der Waaij, 1986). A different instillation approach of the bacteria is via gavage, where 2×10^9 CFU of bacteria diluted in 1 mL was administered to male Sprague–Dawley rats, following treatment with an antibiotic cocktail. Both the bacterial load and the antimicrobials were administered through gavage. *K. pneumoniae* was recovered from stool samples up to 16 days after challenge. The study described how the use of different types of antimicrobials can influence in the transmission of resistance genes via plasmids in the rat GI tract (Ye et al., 2019).

Very few studies have focused on animal models to investigate oral mucosal infections by *K. pneumoniae*. A screening of *K. pneumoniae* strains displaying the HMV phenotype was conducted in African green monkeys, rhesus and cynomolgus macaques, tested via polymerase chain reaction (PCR) of oropharyngeal and rectal swabs. Most of the *K. pneumoniae*-positive cultures were negative for the HMV phenotype, while 19 of the 307 animals tested positive for HMV-positive strains. The work demonstrated that *K. pneumoniae* is able to colonize the oral microbiota in non-human primates, most likely through the fecal-oral route (Burke et al., 2009).

Another study investigated the protective potential of Dentavax, a formulation based on inactivated *K. pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Candida albicans*, and *Lactobacillus acidophilus* strains. Chinchillas were immunized and challenged with 200 µL (5×10^9 CFU/mL) of bacterial suspensions containing all the bacterial species in the formulation, delivered with injections made with a tuberculin syringe, in six different points of the oral cavity. The immunized animals showed a faster recovery, with fewer inflammatory histological signs. In the peripheral blood of the immunized animals, increased phagocytic activity of polymorphonuclear leukocytes were observed, while the sera showed immunoglobulin G (IgG) production against the bacteria included in the vaccine. Also, specific secretory

TABLE 2 *In vivo* models or oral and gastrointestinal infections by *Klebsiella pneumoniae*.

Inoculation method	Endpoint	Other analysis	*K. pneumoniae* Strains	Type of animal model used	Animal strains	References
Water inoculation	GI tract colonization	Histological evaluation	Clinical isolates	Mice	C57BL/6J	Lau et al. (2008)
Intranasal inoculation	GI tract colonization		Clinical isolate	Mice	Swiss-Webster	Lau et al. (2008)
Oral administration	GI tract colonization; Oropharyngeal colonization; Fecal CFU count; Survival; Burden in the kidneys, liver and spleen	Histopathological evaluation; Cell proliferation and differentiation, Cytokine expression; Microbiome diversity; Serum LPS, bilirubin and alkaline phosphatase levels; Genotyping and expression levels; Body weight, Metabolomic analysis	Clinical isolates, B5055	Mice	BALB/c, C57BL/6, C57BL6/J, C57BL/6N, C57BL/6NTac, MRL/MpJ, CFW1	Osbelt et al. (2021), Young et al. (2020), Chiang et al. (2021), Liu J. Y. et al. (2022), Nakamoto et al. (2019), Kamata et al. (2020), Boll et al. (2012)
Gavaging	Survival; GI tract colonization; Burden in the spleen, liver and lungs; Fecal CFU count	Body weight; Histopathological evaluation; Gene expression; Microbiome diversity; Serum level of aspartate transaminase, alanine transaminase, triglycerides, thiobarbituric acid-reactive substances; Cell proliferation and differentiation; Echocardiographic parameters; Competition during colonization	Clinical isolates, ATCC BAA-2146, ATCC 10031, NTUH-K2044	Mice	C57BL/6, C57BL/6J, OF1, BALB/c, BALB/cByL, CF1, 129×1/SvJ	Young et al. (2020), Lau et al. (2008), Hennequin and Forestier (2009), Maroncle et al. (2006), Calderon-Gonzalez et al. (2023), Yuan et al. (2019), Kienesberger et al. (2022), Hsieh et al. (2010), Hsu et al. (2019), Perez et al. (2011), Han et al. (2023), Lagrafeuille et al. (2018)
Contamination of the water	GI tract colonization; Fecal CFU count	Microbiome diversity; *In situ* hybridization	Clinical isolates	Mice	C57BL/6	Favre-Bonté et al. (1999), Le Guern et al. (2019)
Nasogastric tube insertion	Fecal CFU count		Clinical isolate	Rat	Wistar	Ruijs and van der Waaij (1986)
Gavaging	Fecal CFU count	Microbiome diversity; Genotyping	Clinical isolate	Rat	Sprague–Dawley	Ye et al. (2019)
Contamination of the water	Burden in the heart, intestine, spleen and liver	Neutrophil recruitment; Macrophage phagocytic ability	Clinical isolates	Non-rodent animal model	Zebrafish	Zhang X. et al. (2019)
Injection in different sites of the mouth cavity	Abscess and inflammatory infiltration	Histopathological evaluation; S-IgA and IgG secretion in serum and feces	Clinical isolate	Non-rodent animal model	Chinchilla rabbits	Marinova et al. (2000)

IgA (S-IgA) production was detected in the feces (Marinova et al., 2000).

As mentioned for respiratory infection models, rodents are the preferred animals to evaluate *K. pneumoniae* GI infections. Several inoculation techniques have been employed to successfully establish colonization. However, an important issue regarding GI models is the presence of a natural microbiota that greatly impacts the infection outcomes. An alternative to surpass this limitation is the use of animals with a humanized microbiota, that allows the evaluation of bacterial interactions during infection, mimicking the conditions

found in the human host. Another important aspect of GI infections is the effect of previous antibiotic treatment on dysbiosis, that leads to *K. pneumoniae* infections in humans; this can also be evaluated using mice with a humanized microbiota, providing important insights for future antibiotic treatment.

In summary, considering the differences between the mouse GI microbiome—which is naturally resistant to *K. pneumoniae* colonization—and the human microbiome, a more permissive environment for *K. pneumoniae*, an ideal model would employ animals transplanted with human bacteria. The oral route of administration is preferable because it better mimics the natural acquisition of the pathogen and allows the investigation of host defense mechanisms activated during the bacterial transition from the oral cavity to the lower GI tract. However, when considering specific infections such as liver abscess, a more artificial route, such as intraperitoneal injection may be used (Wu et al., 2022). Though, depending on the invasiveness of the strain, oral inoculation can also lead to infection in other sites as liver and spleen (Hsieh et al., 2012).

Animal models of *Klebsiella pneumoniae* urinary tract infections

Urinary tract infections (UTI) are among the most common infections in the community and in hospital settings. An UTI can progress to pyelonephritis, kidney damage and sepsis. Bacteria are the main causative agents of UTI, including Gram-positive and Gram-negative pathogens, with the most prevalent being *Escherichia coli* and in second position, *Klebsiella pneumoniae*. Both are commonly identified in infections of variable severity (Flores-Mireles et al., 2015). A prediction using statistical models showed that just in the year of 2019, around 65,000 deaths were credited to urinary infections caused by multi-drug resistant bacteria. With another 200,000 deaths indirectly related to such infections (Li et al., 2022). Another reason of concern is the increasing rates of antimicrobial resistance in UTIs, which limit the therapeutic choices and worsen the disease burden, reinforcing the urgent need for new therapies and prophylactic strategies (Flores-Mireles et al., 2015).

In that sense, animal models are important for studying the host-pathogen interactions during urinary tract infections, and for testing new antibiotics/vaccines. As described for other mucosal infection models, most animal studies evaluating UTI are performed in mice, which are a better option than rats, as they have a greater number of glycolipid receptors in the urethral tissue, thus promoting better adhesion. Furthermore, the bladders of mice and humans share conserved proteins named uroplakins, which play a role in fimbriae-mediated bacterial adhesion (Murray et al., 2021).

The next section includes the animal models used to study UTIs. These studies are summarized in Table 3.

Mouse infection models

The most described method of bladder inoculation is via a catheter inserted directly through the urethra. Anesthetized animals are placed horizontally and catheterized. After the insertion, with the aid of a syringe, the bacterial suspension or treatment is inoculated into the lower urinary tract (bladder) of female mice (Thai et al., 2010).

Many different mouse strains have been used to study *K. pneumoniae* urinary infections, including C3H/HeN (Rosen et al., 2008a,b), C3H/HeJ (Rosen et al., 2008c), BALB/c (Gomes et al., 2020) and CBA/J (Saenkham et al., 2020; Mason et al., 2023). Anesthesia is usually performed using methoxyflurane (Rosen et al., 2008a,b,c) or a ketamine and xylazine mixture (Gomes et al., 2020). A study evaluating the association between diabetes and urinary tract infections inoculated C3H/HeN, C3H/HeJ and C57BL/6 mice with different bacterial strains, including a cystitis isolate of *Klebsiella pneumoniae* TOP52 1721, using a transurethral catheter. After the infection period, the bladder and kidneys were aseptically removed for bacterial counts. The TOP52 1721 strain was able to cause infection in the bladder and kidneys of mice with diabetes at higher titers than in healthy mice, indicating an increased susceptibility to UTIs in that group (Rosen et al., 2008c).

A study characterized the molecular difference of FimH of a uropathogenic *E. coli* (UPEC) isolate UTI89 and *Klebsiella pneumoniae* cystitis isolate TOP52. Strains used for the urinary tract infection model were UTI89 cystitis isolate UPEC; UTI89 Δ*fimH* (mutant strain lacking the adhesin FimH); TOP52 1721, a *K. pneumoniae* cystitis isolate; and TOP52 Δ*fimK* (mutant strain lacking the fimbriae regulator FimK). The infection was induced by inoculating 50 µL of a 1 to 2×10^7 CFU suspension the strains via the urethra, into 8-week-old female C3H/HeN mice, and to perform the quantification of bacteria present in the animals' tissues, the bladder and kidneys were collected aseptically after 6 h, 1 and 14 days, and plated. The bacterial titers in the bladder were higher at all timepoints for *E. coli* UTI89 strain (which had fimbriae) when compared to UTI89 Δ*fimH* (FimH mutant), which was also seen between the wild-type *K. pneumoniae* TOP52 strain, in comparison to the FimH-negative mutant. The bladder CFU counts of the UPEC group was higher than the *K. pneumoniae* group. However, 14 days post-infection, the recovered CFU was similar. Colonization of the kidneys was initially higher in the UPEC group but became similar to *K. pneumoniae* in later data points. Wild type and mutant *K. pneumoniae* strains showed equal titers in all measured times. The study demonstrated that for *K. pneumoniae*, FimH did not have a critical role in the initial steps of bladder infection, however it became required in later stages. For *E. coli* UTI89, FimH appears to play a more prominent role. Overall, the study shows the role that FimH in the bladder colonization and invasion, infection persistence and development of intracellular bacterial communities (IBCs) in both strains (Rosen et al., 2008b).

Using the same infection model, Rosen et al. evaluated the role of the FimK regulator in the urinary tract infection process. *K. pneumoniae* lacking *fimK* showed an increased type I fimbriae expression, which reflected in the increased CFU count in the bladder and kidneys, and IBC formation in the animal group infected with the mutant strain, when compared to the wild type. The authors propose that the downregulated type I fimbriae expression could partially explain how UTIs caused by *E. coli* infections are more common when compared to *K. pneumoniae* (Rosen et al., 2008a).

A study by Gomes et al., describing the transcriptional regulator of the *kpfR* gene cluster demonstrated that the regulator plays an important role in *K. pneumoniae* pathogenicity in urinary tract of mice. In the study, female BALB/c mice were inoculated via the transurethral route with a K2 clinical strain isolated from a patient diagnosed with UTI or its isogenic KpfR-negative mutant. The mutant, which exhibited a hyper fimbriated phenotype, displayed reduced ability to colonize the mouse bladder and was cleared faster than the wild-type strain. The authors suggest that overexpression of fimbriae

TABLE 3 *In vivo* models of urinary tract infections by *Klebsiella pneumoniae*.

Inoculation method	Endpoint	Other analysis	*K. pneumoniae* strains	Type of animal model used	Animal strains	References
Transurethral catheterization	Burden in the bladder, kidneys, spleen and liver; Urine CFU count	Histopathological evaluation; IBC quantification; Colonization competition; Body weight	Clinical isolate, KPPR1, NTUH-K2044	Mice	C3H/HeN, C3H/HeJ, BALB/c, CBA/J	Rosen et al. (2008a,b,c), Gomes et al. (2020), Saenkham et al. (2020), Mason et al. (2023)
Transurethral inoculation with urethral tubing	Burden in the bladder	CFU count of the catheter	Clinical isolate	Mice	C57BL/6NCr	Murphy et al. (2013)
Transurethral catheterization	Burden in the bladder and kidneys		Clinical isolate	Rat	Sprague–Dawley	Reid et al. (1985)
Transurethral catheterization	Burden in the bladder, kidneys; Urine CFU count		Clinical isolates, ATCC 10031	Rat	Wistar (CFHB)	Camprubí et al. (1993), Regué et al. (2004)

in the mutant promotes a more robust immune response that leads to quick bacterial elimination by the host (Gomes et al., 2020).

A similar infection model used a silicone tube attached to a needle, which was inserted in the bladder through the urethra opening of C57BL/6NCr mice under isoflurane anesthesia. In this study, 50 µL of the 10^7 CFU bacterial suspension were inoculated. Using this model, the role of type I and III *K. pneumoniae* fimbriae in the colonization of the urinary tract was evaluated in the presence (or absence) of the silicone implant in the bladder, which was used to simulate a urinary catheter. In the presence of the implant, colonization of the bladder was augmented, especially when the insertion had been performed 24 h prior to the inoculation. *K. pneumoniae* strains lacking the type I and type III fimbriae demonstrated a reduced ability to colonize the bladder in both the catheterized and un-catheterized groups at the 6- and 48-h endpoints. Finally, the number of bacteria recovered from the implants was lower in the group inoculated with the mutant lacking type III fimbriae, suggesting a bigger impact of this fimbriae in the colonization of the abiotic surface (Murphy et al., 2013).

The murine model of urinary tract infection was also used for assessing the impact of hyperglycosuria on bacterial colonization by *Klebsiella pneumoniae*. The excess glucose excretion was induced using dapagliflozin, a drug for controlling diabetes. The *K. pneumoniae strain* used was KPPR1 and the experiments were conducted on adult (4–6 weeks) female CBA/J mice. The infection was induced by inoculation of 10^8 CFU of the KPPR1 strain into the bladder via transurethral route. A syringe pump connected to a polyethylene tube, with a constant, low flow, was used to prevent leakage of the inoculum due to urinary reflux. Urine was collected at 6, 24, 48 h, or 7 days, and plated for bacterial count; the bladder, spleen and liver were removed aseptically and plated as well. A higher bacterial load was evident in both urine and bladder of the mice with hyperglycosuria. This group also exhibited a greater systemic spread as other organs, such as spleen and liver showed a higher bacterial load, indicating that high glucose levels promote UTI by *K. pneumoniae* (Saenkham et al., 2020).

In conclusion, mice have been extensively used as models of *K. pneumoniae* UTI, including local bladder infection and ascending pyelonephritis. In that regard, the increased vesical-ureteric reflux presented by the C3H/HeJ strain—a retrograde urine flow resulting from a congenital anomaly of the urinary tract (Murawski et al., 2010)—makes it an ideal model to investigate more complicated, ascending infections, like those observed in human patients with the same condition.

An important aspect that remains to be explored is the gender-related differences in UTI by *K. pneumoniae*. There are protocols available to induce infection in male mice using transurethral instillation (Zychlinsky Scharff et al., 2017) or surgically through an abdominal incision followed by direct bladder inoculation using a needle (Olson et al., 2016). Studies with *E. coli* have demonstrated that male mice develop more severe, chronic infections that are influenced by androgen exposure (Olson et al., 2016). Therefore, UTI studies using male mice are necessary to evaluate gender-related differences in *K. pneumoniae* infections.

Rat infection model

A few studies have used rats to investigate UTI by *Klebsiella pneumoniae*. In female Sprague–Dawley rats anesthetized with pentobarbital, bladder colonization was achieved with a catheter, in which, 50 µL of a 5×10^9 bacterial suspension incorporated in agar beads were inoculated to the animals. Using this urinary infection model, the effectiveness of a treatment with *Lactobacillus casei* prior to the infection was evaluated. *L. casei* was also incorporated into beads and while it is not able to colonize the kidneys of the animals, the colonization of the urinary tract with *L. casei* prevented the installment of UTI and pyelonephritis in the animals who received the lactobacilli-coated beads (Reid et al., 1985). A similar method was used in Wistar (CFHB) rats, in which following anesthesia the abdominal area was massaged to expel the urine. Then, a urinary cannula was used to introduce the inoculum, in a final volume of 1 mL. When the inoculation step was finished, a clamp was used in the urethral meatus to prevent bacterial leakage and taken out after 10 min

(Camprubí et al., 1993; Regué et al., 2004). This protocol was used to evaluate the role of the O-antigen and CPS in the UTI development. Strains lacking the O-antigen showed a substantial decrease in the ability to colonize the rat's kidneys and bladder, whereas, in the infection model tested, the K-antigen did not appear to bear the same level of importance (Camprubí et al., 1993).

Other UTI animal models

An *ex vivo* porcine model was employed to induce a catheter-associated UTI. The model is based on a modified Foley catheter, which was introduced in the urethral tract of a euthanized female pig. With a silicone tube, the catheter was placed, then inflated to stay in place throughout the experiment. The catheter contained pre-formed 24 h bacterial biofilms, including a carbapenem-resistant *K. pneumoniae* strain. Following the introduction, the apparatus was irrigated with a combination of antibiotic solutions, then segmented for bacterial quantification. The animal's urethra and bladder were also analyzed (Vargas-Cruz et al., 2019). The model reproduced the parameters of catheter associated infections in human patients, while showing that the antibiotic irrigation was able to reduce bacterial colonization of the urinary tract.

As discussed for respiratory and GI infections, mice have been used in most of the studies investigating UTI by *K. pneumoniae*, with transurethral injection using catheters being the main technique for bacterial inoculation. This inoculation route results in direct delivery of bacteria in the bladder, but it has a few limitations: the size and position of the mouse urethra make it hard to access; urine flow may contribute to bacterial leaking during the procedure, resulting in variations in the number of CFU inoculated. Despite those limitations, local infections in the urinary tract are an important tool to investigate the contribution of *K. pneumoniae* virulence factors to disease and develop new therapeutics/vaccines to control UTIs.

Transurethral inoculation of *K. pneumoniae* may also be employed to evaluate ascending UTIs—an important feature in urinary tract infections in humans. It is also a simpler technique that requires some level of expertise, but the animal manipulation does not demand specific equipment or local surgery, which includes the recovery process as a potential issue. Therefore, it should be considered as the gold standard technique for UTI studies. Conversely, given the prevalence of catheter-induced UTIs in hospital dwellings, models that mimic this type of device should be considered when evaluating nosocomial infections.

Non-mammal disease models in *Klebsiella pneumoniae*

Very few models were described for *K. pneumoniae* infections using animals other than rodents. A recent work by Zhang and colleagues have used zebrafish (*Danio rerio*) to investigate the variations in infection by different *K. pneumoniae* strains, and the innate immune responses mounted against these bacteria. The fish were infected by immersion in a bacterial suspension for 8 or 24 h, followed by organ collection and bacterial counting. The intestines were the organs with the highest bacterial loads (Zhang X. et al., 2019).

The zebrafish model is also suitable to analyze neutrophil and macrophage migration during *K. pneumoniae* infection, since they harbor a mammalian-like innate immune system; however, for evaluation of adaptive responses, a mammalian model is required (Zhang X. et al., 2019). Another limitation of this model is the lack of control in the bacterial loads infecting each animal since they are immersed in the suspension. In the same work, the authors used a *Galleria mellonella* infection model. This is a convenient model to determine the virulence of a bacterial strain or even to compare two or more strains' behavior during infection, which was the case in the cited study. The authors were able to screen among different strains and select the one with the highest potential of causing infection after colonization, which was achieved via injections with 1×10^6 CFU, in 10 µL, of a *K. pneumoniae* suspension, using a Hamilton syringe into the haemocoel of the larvae proleg (Zhang X. et al., 2019). The signs of a successful infection included changes in pigmentation, from the typical clear color to a darker tone, combined with the lack of motility are signs of lethality in the larvae (Harding et al., 2013).

Another *in vivo*, non-vertebrate model platform, used to evaluate therapies against *K. pneumoniae* infections is the nematode *Caenorhabditis elegans*. In a phage therapy assay, *K. pneumoniae*, *E. coli* and *Enterobacter cloacae* strains were used in a liquid-phase infection model, in which, either the prophylactic or therapeutic use of the bacteriophages were able to increase the survival of *C. elegans* against the pathogens alone or in combination (Manohar et al., 2022). Antimicrobial screening studies were also conducted in *C. elegans*; using different concentrations of multiple antimicrobials in a lethality assay against carbapenem-resistant *K. pneumoniae* isolates. The lethality model showed comparable results to the usual Kirby-Bauer disk diffusion resistance test, for most of the drugs. Employing the same survival model, dose-dependent drug toxicity was also analyzed and provided data consistent with preconized protocols. Based on the screening results, a therapy protocol was selected and used in two hospitalized patients (Yao et al., 2022).

Comparative virulence of *K. pneumoniae* isolates was assessed in a *Drosophila melanogaster* (fruit fly) model. Multirresistant isolates with matte and mucoid phenotypes were tested in colonization/lethality assay. The animals (3–5 days old) were injected with increasing bacterial loads using a 10-µm needle. The isolates only killed a small number of flies, however, the CFU counts in the mucoid phenotype group was higher when compared to the non-mucoid strains. It is unclear if the lethality was not achieved due to the type of bacterial strain, or the fly model is not adequate for this type of analysis (Lee et al., 2018).

Overall, animal models of *K. pneumoniae* employing non-mammalian species are still scarce, and future studies are required to assess the potential applicability of the data to human infections. Nevertheless, the current results demonstrate that these models could contribute to our understanding of *K. pneumoniae* pathogenesis, more specifically in drug screening or comparative virulence assays.

Limitations and shortcomings of animal models for the study of *Klebsiella pneumoniae* infections

As described throughout this review, mice studies represent the most used model to investigate *K. pneumoniae* infections in different mucosal sites. However, this model has important limitations such as a high intrinsic resistance to *K. pneumoniae* strains, including clinical

strains, and differences in *K. pneumoniae* lethality in different mice lineages (Mizgerd and Skerrett, 2008). Meanwhile, primates appear to be more susceptible to *K. pneumoniae* infections, which makes sense when considering the high anatomical and immunological similarities between humans and primates. However, they are extremely expensive and require specialized animal facilities, which greatly limits their use. There is also an ethical concern regarding the use of highly sentient beings in experimentation when other options are available. Therefore, improving the rodent models and combining *in vitro* techniques will provide more applicable results.

A particularity of *K. pneumoniae* is the differences between the classical and hypervirulent strains. Often, hypervirulent strains are lethal in mouse studies, however, that may not be the intention of the study (colonization studies, for example). There is a lack of well described bacterial strains leading to non-lethal infections. Unlike infection models for better stablished pathogens, such as *Streptococcus pneumoniae* (Chiavolini et al., 2008), the choice of the bacterial strains ends up being difficult, since there are few correlates of virulence or lethality in mice. Recent studies investigating the genetic diversity of *K. pneumoniae* clinical isolates (Martin et al., 2023) and correlation of certain virulence factors with disease potential in mice (Russo et al., 2021) provide valuable insights in the pathogenesis of *K. pneumoniae*. However, the data are from systemic infections resulting from subcutaneous or intraperitoneal challenges; it remains to be investigated whether the same is true for mucosal infections. Even when looking at survival as an endpoint, the current animal models may not represent the natural stages of disease development seen in patients infected with *K. pneumoniae,* which limit the extrapolation of the results to clinical scenarios.

As an option to replace the use of animals in the basic research of UTI treatments, an *in vitro* model was developed, using real urine, simulating the physiological conditions of the urinary tract with a Foley catheter passing through the cap of a conical tube, surrounded by tryptone soy agar. The bacterial growth was evaluated by analyzing the bacterial counts in the catheter (Gaonkar et al., 2003).

Techniques for animal replacement involve the use of artificial environments that mimic human niches, as in 3D cell culture or microfluidic systems, exemplified by lab- or organ-on-a-chip technologies. The tridimensional cell culture models resemble natural tissue structures, resulting in functional organoids maintained in culture conditions, allowing an increase in the development speed in the pre-clinical stages of research. Though the technique is in constant evolution, there are still limitations and questions about its reproducibility and biological significance (Shah et al., 2023). Similarly, the microfluid chip models are also a promising alternative, being able to reproduce physiological events such as the physical forces and cell organization (Feaugas and Sauvonnet, 2021). However, there are also drawbacks in its application: the systems are still in validation process, without well-established standard procedures, and the complexity of the human systems make it almost impossible to replicate identically (Leung et al., 2022). Therefore, although animal replacement techniques have important limitations, especially considering systemic studies (like for instance, in vaccine development), there is definitely a huge potential for development of novel therapies.

Another option is the substitution of the typical vertebrate models by invertebrates, such as *C. elegans*, *D. melanogaster*, or *Galleria mellonella*. Just like any *in vivo* model, there are advantages and disadvantages. Invertebrates are cheaper, often easy to manipulate and are less complex when compared to vertebrate mammals. Nonetheless, there are also limitations in the application of those models. In the *G. mellonella* case in particular, the larvae has an innate immune system somewhat similar to ours, but it lacks an adaptative response (Ménard et al., 2021). In *K. pneumoniae* infection models, *G. mellonella* has been used with vastly different goals, such as to evaluate phage-mediated survival (Feng et al., 2023), synergistic activity of antimicrobials (Ribeiro et al., 2023) and pathogenicity (Liu et al., 2023). However, when used to compare hypervirulent and common *K. pneumoniae* strains, the model did not reproduce the same differences observed in outbred mice (Russo and MacDonald, 2020).

Similarly, *C. elegans* has been used as a platform to study *K. pneumoniae* infection, antimicrobial drugs activity (Yao et al., 2022) and bacteriophage therapy efficacy (Manohar et al., 2022). Survival studies and analysis of *K. pneumoniae* virulence can also be achieved using *D. melanogaster* (Lee et al., 2018). There is a description of colonization and bacterial metastasis in zebrafish, using different clinical *K. pneumoniae* strains isolated from patients' gut microbiota (Zhang X. et al., 2019). All those studies represent interesting approaches to investigate some traits of *K. pneumoniae* infections, but they do not allow a detailed analysis of the host immune responses during infection, nor the mechanisms responsible for increased survival after vaccination/treatment. A recent review in vaccine development against *K. pneumoniae* can be found in Assoni et al. (2021).

Conclusion

Mammals, and specifically, rodents like mice and rats, are the most widely models for studying *K. pneumoniae* infection. The anatomical similarities and comparable immune responses, combined with the easy handling and availability of a variety of strains make these animals the gold standard in *K. pneumoniae* research *in vivo*. However, they present important limitations, instigating the development of more physiological approaches, which mimic the hallmarks of human infections and allow a better understanding of the host-pathogen interactions during disease. Transgenic animals with humanized immune systems, and/or those with humanized microbiota may represent a more physiological platform to study *K. pneumoniae* infections. Additionally, inoculation procedures that closely emulate the natural pathogenic process may provide a better understanding of the different infection stages.

The choice of the most appropriate animal model must also consider the anatomical and immunological specificities of a particular strain, which greatly impact the outcome of the study. The use of neutropenic mice, for instance, usually provides increased bacterial burden, while anatomical differences in the urinary tract may favor more severe diseases. These traits, as well as the use of animals with specific mutations, could provide reproducible and stable infections, allowing the evaluation of intended outcomes, which could be further correlated with human patients.

Given the ability of *K. pneumoniae* to colonize different host niches, causing diseases of variable severity, it is important to develop models that replicate the conditions (from the pathogen and the host) associated with bacterial persistence in each host tissue. In that sense, whole transcriptome and proteome profiling may help identify the

virulence factors involved in different stages of disease and how these factors interact to promote bacterial persistence in different sites within the host. Furthermore, the use microbial communities (co-infection) may better represent the natural environment in which *K. pneumoniae* colonizes the host, since different microbes are known to cooperate or compete for the same host niche. Finally, since *K. pneumoniae* is known to form biofilms *in vitro* and *in vivo*, the use of biofilm bacteria instead of planktonic may help unveil disease mechanisms which are unique to this bacterial phenotype. This has already been demonstrated during colonization of the bladder and may also be important in other tissues such as the lungs.

Author contributions

LA: Writing – review & editing, Writing – original draft. AC: Writing – original draft. BV: Writing – original draft. BM: Writing – original draft. AL: Writing – original draft. TC: Writing – review & editing, Supervision, Conceptualization. MD: Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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